Analysis of Conditional Knock-out of Calpain Small Subunit, *capns1*, in Central Nervous System Development and Function

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Abstract

Calpains, a highly conserved family of calcium-dependent cysteine proteases, are divided in two groups; classical and non-conventional calpains. Calpain-1 and calpain-2, the classical ones, are ubiquitously expressed and abundant in the CNS. Findings through different experimental approaches, predominantly pharmacological calpain inhibitors, proposed the necessity of the proteases for the modulation of various biological events particularly in the CNS, or a functional link between calpain and neurodegeneration. Significant functions associated with calpain activity are neuronal proliferation/differentiation, signal transduction, apoptosis, and synaptic plasticity; or neuronal death in Alzheimer's disease, Huntington's disease, Parkinson's disease, and ischemic stroke. However, due to limited insights of the approaches taken, such as non-specificity of the inhibitors, the exact roles of calpains in the CNS and the key mechanisms underlying them remain controversial. Calpain-1/calpain-2 germline knock-out are embryonic lethal at a very early stage hindering the use of these lines as mouse models for CNS studies. Accordingly, this thesis research introduced a unique brain-specific calpain-1/calpain-2 knock-out and explored the role of the proteases in brain development/function and in neuronal death. The first set of analyses examined how the elimination of calpain-1/calpain-2 activities in mouse brain impacts CNS development in general and synaptic plasticity in CA1 neurons of hippocampus. CNS-specific elimination of CAPNS1, the common small subunit, abolished calpain-1/calpain-2 activities in mouse brain. In contrast to Calpain-1/calpain-2 germ line knock-outs, the brain-specific knock-outs are viable and the general development of mouse brain is normal. However, morphology of dendrites in pyramidal neurons of the hippocampal

CA1 region showed significantly decreased dendritic branching complexity and spine density. Consistent with dendrite morphological abnormalities, electrophysiological analyses revealed a significant decrease in field excitatory postsynaptic potentials, long term potentiation, and learning and memory in the hippocampal CA1 neurons of the mutants. In the second part of this research we investigated the direct role of the calpains in neuronal death and their potential downstream targets in *in vitro* models of PD and ischemic stroke. Our findings indicated that ablation of calpains activity improves survival of different types of neurons against mitochondrial toxin 1-methyl-4-phenylpyridinium (MPP+), glutamate, and hypoxia. Importantly, we demonstrated an increase in p35-cleavage to p25, a cyclin dependent kinase 5 (Cdk5) activator, and that restoration of p25 significantly suppresses the neuronal survival associated with calpain deficiency. Taken together, this work unequivocally establishes two central roles of calpain-1/calpain-2 in CNS function in plasticity and neuronal death.

Dedication

For the most loving ones in my life

My beloved parents,

For the person that I am today

My sisters Atoosa, Maryam and Mina,

For their love and support through my life

My love Adrian and my sweet angel Hannah Eliza,

For their unconditional love and devotion

Table of contents

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Thank you…..

List of table and figures

List of abbreviations

AADC	Aromatic amino acid decarboxylase
AAV	Adeno-associated virus
ACSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AMPA	α-mino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid Receptor
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
Bak	Bcl-2 homologous antagonist/killer
Bax	BCL2-associated X protein
BDNF	Brain-derived neurotrophic factor
Bcl2	B-cell lymphoma 2
Bcl-Xs	B-cell lymphoma-extra large
Bid	BH3 interacting-domain death agonist
BrdU	Bromodeoxyuridine
Ca^{2+}	Calcium
CA1	Cornu ammonis 1
Calpain	Calpain calcium-activated papain-like
CaMK	Ca2+/calmodulin-dependent protein kinase
cAMP	cyclic adenosine monophosphate
CAPN	Calpain
CAPNS	Calpain small subunit
Caspase	Cysteine-aspartic proteases
Cdk5	Cyclin dependent kinase 5
cDNA	Complementary DNA
CGN	Cerebellar granule neurons
cKO	Conditional knock-out
CNS	Central nervous system
DA	Dopamine
DG	Dentate gyrus

DIG	Digoxigenin
DIV	Days *in vitro*
DNA	Deoxyribonucleic acid
EC	Entorhinal cortex
EPSC	Excitatory postsynaptic current
eEPSC	Evoked EPSC
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetra acetic acid
EPSP	Excitatory postsynaptic potential
ER	Endoplasmic reticulum
ETC	Electron transport chain
fEPSP	Field EPSP
g	Gram
G1	Growth phase
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GluA	AMPA receptor subunit
GluN	NMDA receptor subunit
GTP	Guanosine-5'-triphosphate
HD	Huntington’s disease
h	Hour
K^+	Potassium
KO	Knock-out
LB	Lewy body
LGMD2A	Limb-girdle muscular dystrophy-2A
LRRK2	Leucine-rich repeat kinase 2
LTP	Long term potentiation
M	Mitosis phase
MAO-B	Monoamine oxidase B
MAP	Microtubule-associated protein
MAPK	Mitogen-activated protein kinase
MCAO	Middle cerebral artery occlusion
MEF	Mouse embryonic fibroblasts
MEF2	Myocyte enhancer factor-2

mEPSC	Miniature EPSC
MF	Mossy fiber
Mg^{2+}	Manyasium
mGluR	Metabotropic glutamate receptors
MPP+	1-methyl-4-phenylpyridinium
MPPP	1-Methyl-4-phenyl-4-propionoxypiperidine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Multiple sclerosis
MT	Microtubule
MWM	Morris water maze
Na^{+}	Sodium
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Neuronal growth factors
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
OGD	Oxygen glucose deprivation
6-OHDA	6-hydroxydopamine
p	probability
PC	Pyramidal Cell
PCD	Programmed cell death
PCR	Polymerase chain reaction
PD	Parkinson’s disease
PINK1	PTEN induced putative kinase 1
PKA	Protein kinase A
PKC	Protein kinase C
PP	Perforant pathway
PSD	Post-synaptic density
PVDF	Polyvinylidene fluoride
Q	Glutamine
R	Arginine
ROS	Reactive oxygen species
RVG	Rabies-virus-glycoprotein
S	Synthesis phase
SAP97	Synapse-associated protein 97

SBDP	Spectrin breakdown product
SC	Schaffer collateral
SCOP	Suprachiasmatic nucleus circadian oscillatory protein
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Ser	Serine
SER	Smooth endoplasmic reticulum
siRNA	Small interfering Ribonucleic acid
SNc	Substantia nigra pars compacta
SVZ	Subventricular zone
TBS	Theta burst stimulation
TEVP	Tobacco etch virus protease
TH	Tyrosine hydroxylase
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
4-VO	4 Vessel occlusion
VZ	Ventricular zone

List of publications

Mandana Amini, Chun-lei Ma, Rasoul Farazifard, Yi Zhang, Jacqueline Vanderluit, Joanna Susie Zoltewicz, Fadi Hage, Joseph M. Savitt, Diane Lagace, Ruth S Slack, Jean Claude Beique, Peter A Greer, Richard Bergeron, David S Park. (2013) Conditional disruption of calpain in the CNS alters dendrite morphology, impairs LTP, and promotes neuronal survival following injury. **The Journal of Neuroscience, 33(13):5773-84.**

Mount MP, Zhang Y, **Amini M**, Callaghan S, Kulczycki, Mao Z, Slack RS, Anisman H, Park DS. (2013) Perturbation of transcription factor Nur77 expression mediated by myocyte enhancer factor 2D (MEF2D) regulates dopaminergic neuron loss in response to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). **The Journal of Biological Chemistry, 288(20):14362-71.**

Thesis format

The thesis presented here consists of a series of manuscripts, in agreement with the guidelines set forth by the Department of Cellular and Molecular Medicine, Neuroscience program; Faculty of Medicine, University of Ottawa. It is composed of four chapters: a general introduction, followed by two manuscripts and a general discussion of the thesis findings.

Chapter one is a general introduction summarizing calpain's characteristics as a cysteine protease and reviews research in the physiological role of calpain in CNS development and plasticity, as well as the role of calpain in neuronal death in Parkinson's disease and Stroke. The final part of this chapter refers to the supporting/opposing arguments in the field and how we have addressed them.

Chapter two presents a manuscript entitled "Conditional disruption of calpain in the CNS alters dendrite morphology and impairs LTP and learning and memory". This work was published as a part of the article entitled "Conditional disruption of calpain in the CNS alters dendrite morphology, impairs LTP, and promotes neuronal survival following injury" on the Journal of Neuroscience in 2013.

Chapter three presents a manuscript entitled "Classical calpain promotes neuronal death by cleavage of p35, a cdk5 co-activator, to p25 in Parkinson's and ischemic related injuries". This work was published as the second part of the article entitled "Conditional disruption of calpain in the CNS alters dendrite morphology, impairs LTP, and promotes neuronal survival following injury" on the Journal of Neuroscience in 2013.

Chapter 4 presents an overview of my major findings, the significance of the data and future directions they could take.

General Introduction

1.1. Calpain System

Calpains are a highly conserved family of cysteine proteases expressed in almost all eukaryotes (Carafoli and Molinari, 1998; Sorimachi et al., 2011a). They are non-lysosomal calcium-dependent enzymes mainly localized in cytoplasm and optimally activated in neutral pH (Goll et al., 2003). The protein was first discovered by Gordon Guroff in 1963 in rat brain as a Calcium-Activated Neutral Proteinase, CAPN (Guroff, 1964), named calpain later. There are so far 15 human calpain genes called *capn1-3, 5-16* as well as two genes for small subunits (regulatory subunits), *capns1* and *capns2* (Dear and Boehm, 2001; Croall and Ersfeld, 2007). The calpain family is divided into subfamilies according to different characteristics. They have been previously categorized as ubiquitous calpains includes CAPN1-2, CAPN5, CAPN7, CAPN10, CAPN13 and CAPN15-16; and tissue specific calpains comprising CAPN3, CAPN6, CAPN8-9, CAPN11-12 (Croall and Ersfeld, 2007; Sorimachi et al., 2011a). These are also referred as 'conventional' and 'non-conventional' calpains respectively. Recent studies classify the members into 'classical' or 'typical' and 'non-classical' or 'atypical' calpains according to their domain structures. Typical calpains are characterized by presenting penta-EF-hand motifs in their C-terminal calcium binding domain while atypical calpains lack this region **(Table 1)** (Croall and Ersfeld, 2007).

Calpains are among the most studied proteases since their discovery. A number of calpain isoforms have already been linked to physiological functions and pathological events in the cell (Goll et al., 2003; Croall and Ersfeld, 2007; Sorimachi et al., 2011a). Genetic studies have linked some calpains like CAPN3, CAPN8-9, and CAPN10 to limb-girdle muscular dystrophy type 2A (LGMD2A) (Richard et al., 2000; Huang and Wang,

2001; Sorimachi et al., 2011a), gastric mucosal defense (Hata et al., 2010; Sorimachi et al., 2011a), and type 2 diabetes (Horikawa et al., 2000) respectively. The ubiquitous calpains, calpain-1 and calpain-2, are the most extensively studied isoforms. They were the first identified calpains and are traditionally called μ-calpain and m-calpain respectively, due to their requirement for intracellular calcium concentration (Goll et al., 2003). They are the predominant calpains in central nervous system (CNS) and are ubiquitously expressed in the brain, localized in soma, axon, and dendrites (Bevers and Neumar, 2008). A broad range of proteins in the CNS are considered to be calpain-1/calpain-2 substrates. Calpain-1/calpain-2 are referred to as the modulator proteases since they cleave a limited number of sites in substrates that transforms the substrates structure and activities, rather than degrading them. Thus, calpain-1/calpain-2 are suggested to be related to a variety of essential cellular functions and significant signal transduction pathways in life and death in the CNS, such as development (Arthur et al., 2000; Gil-Parrado et al., 2002; Li et al., 2009), cell signaling (Sato and Kawashima, 2001), apoptosis (Gil-Parrado et al., 2002), synaptic plasticity (Lynch and Baudry, 1984, 1987; Denny et al., 1990; Liu et al., 2008; Zadran et al., 2010a); and neuronal injuries in Parkinson's disease (PD) (Mouatt-Prigent et al., 1996; Crocker et al., 2003), Huntington disease (HD) (Gafni and Ellerby, 2002; Vosler et al., 2008), Alzheimer's disease (AD) (Di Rosa et al., 2002; Raynaud and Marcilhac, 2006), and ischemic stroke (Bevers and Neumar, 2008). In this research study, the term 'calpain' refers to calpain-1 and calpain-2.

Table 1. Calpain Family. There are 15 catalytic and 2 regulatory genes for calpain homologous in mammals. The family is classified by expression pattern or the domain, mainly domain IV, structure. (Modified from http://calpain.org/images/fig/table1.png)

	Gene	Name in number	Expression	5-EF-hand
The large subunits	*CAPN 1*	Calpain 1	Ubiquitous	+
	CAPN 2	Calpain 2	Ubiquitous	+
	CAPN 3	Calpain 3a-c	Skeletal muscle, lens retina	+
	CAPN 5	Calpain 5	Ubiquitous? (testis, brain)	-
	CAPN 6	Calpain 6	Placenta, Embryonic muscle	-
	CAPN 7	Calpain 7	Ubiquitous	-
	CAPN 8	Calpain 8a Calpain 8b	Stomach	+ -
	CAPN 9	Calpain 9	Digestive tracts	+
	CAPN 10	Calpain 10a-h	Ubiquitous	-
	CAPN 11	Calpain 11	Testis	+
	CAPN 12	Calpain12a-c	Hair follicle	+
	CAPN 13	Calpain 13	Ubiquitous	+
	CAPN 14	Calpain 14	Ubiquitous?	+
	CAPN 15	Calpain 15	Ubiquitous	-
	CAPN16	Calpain 16	Ubiquitous?	-
The small subunits	*CAPNS1*	CAPNS1	Ubiquitous	+
	CAPNS2	CAPNS2	Ubiquitous	+

1.2. Calpain Structure

Calpain was first purified to homogenous state by Imahori's group in 1978 (Ishiura et al., 1978). Since then, a variety of calpain subunits and their homologous have been identified by cDNA cloning and their structures studied with major focus on calpain-1 and calpain-2 structures.

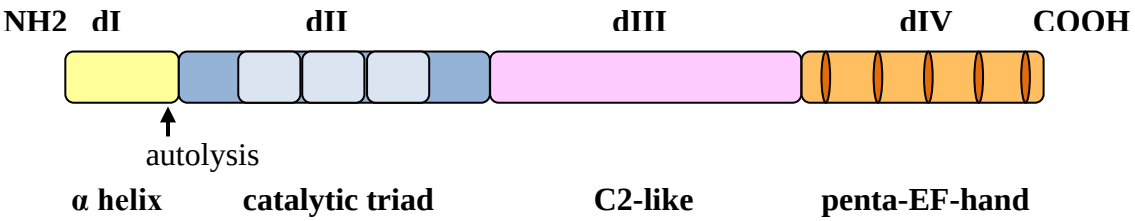
Calpain-1 and calpain-2 are heterodimers formed by distinct 80 kDa large subunits called CAPN1 and CAPN2 respectively and a 30 kDa small subunit, CAPNS1, common to both. The large subunits have 55-65% sequence homology and consist of four domains, dI-dIV. CAPNS1 has two domains dV-dVI **(Fig. 1.1)** (Goll et al., 2003). The N-terminal domain of the large subunits, domain I, comprises a single α helix anchored to domain VI of the small subunit and is proposed to stabilize the circular domain arrangement of the protein. The domain also contains the autolysis site cleaved upon the activation of calpain (Suzuki et al., 1981; Khorchid and Ikura, 2002; Sorimachi et al., 2011a). Domain II contains two core domains, dIIa and dIIb, which form a single functional domain after binding to calcium. This domain has the protease activity and Cys-His-Asn, the catalytic triad residues, is located in it. The domain also contains two non-EF-hand Ca^{2+} binding sites which have significant role in calpain activity (Hosfield et al., 1999; Sorimachi and Suzuki, 2001; Moldoveanu et al., 2002; Sorimachi et al., 2011a). Domain III of ubiquitous calpain is a C2-like domain. The function of this domain is unknown but it may cause the translocation of calpain to the cell membrane by binding to phospholipids and thereby regulating its activity (Sorimachi and Suzuki, 2001; Goll et al., 2003; Sorimachi et al., 2011a). The C-terminus of the large subunit has slight similarity to calmodulin and contains five helix-loop-helix structural domains, EF-hand Ca^{2+}-binding motifs which

bind to calcium. This domain interacts with dV of the small subunit and contributes to heterodimers formation (Blanchard et al., 1997; Lin et al., 1997).

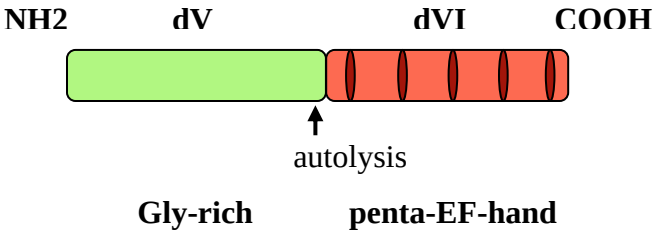
The 30 kDa common small subunit of ubiquitous calpain, CAPNS1, is formed by two domains, domain V and domain VI. The N-terminus of the subunit contains Gly-clusters, called Gly-rich domain as well, and undergoes the autolysis during calpain activation. It is suggested that this hydrophobic domain binds to phospholipids and is important for anchoring the subunit to cell membrane (Imajoh et al., 1986; Saido et al., 1994; Sorimachi et al., 2011a). Domain VI, the COOH terminal of small subunit, is similar to domain IV of large subunits and consists of five EF-hand Ca^{2+}-binding motifs. The fifth motif of both subunits interacts with each other and forms the heterodimers (Imajoh et al., 1987; Sorimachi et al., 2011a). Recently a novel small subunit of ubiquitous calpains, CAPNS2, was identified which has a sequence similarity to CAPNS1. The similarity is mostly restricted to C-terminal domain of two small subunits since two oligo-Gly clusters are missing from N-terminal of CAPNS2. Given that the CAPNS2 binds to the large subunits more weakly than CAPNS1; and that the disruption of CAPNS1 eliminates both ubiquitous calpains activities in the presence of CAPNS2, it is suggested that either the novel small subunit is not significant for calpain function or it acts functionally in a tissue specific manner (Schad et al., 2002).

Figure 1.1. Schematic diagram of human calpain, its 80 kDa large subunit and 30 kDa small subunit. Calpain-1/calpain-2 are heterodimers consist of a distinct large subunit and a common small subunit called catalytic and regulatory subunit respectively. The large subunit includes four domains (dI-dIV) comprising autolysis domain, catalytic domain, C2-like domain, and penta-EF-hand domain. The small subunit consists of two domains, Gly-rich domain (dV) and penta-EF-hand domain (dVI).

Large Subunit (80 kDa)



Small Subunit (30 kDa)



1.3. Calpain activity

The majority of the calpain molecules expressed in cell is thought to be inactive. Calpain is activated by elevation of intracellular calcium concentration either in response to activation of plasma membrane receptors and channels, for instance ligand-gated receptors/channels and Ca^{2+} channels, or by release of calcium from intracellular stores such as endoplasmic reticulum and mitochondria (Sareen et al., 2007). Studies to determine how calcium activates calpain have been hampered by two main difficulties: dissociation of small and large subunits of ubiquitous calpains and aggregation of large subunit in the presence of calcium (Khorchid and Ikura, 2002; Goll et al., 2003). Lately, examination of recombinant mini calpains has provided valuable information regarding calpain activation. Studies by Davis and colleagues revealed that calpain activation by Ca^{2+} is a two-step mechanism (Moldoveanu et al., 2002). In the first step, calcium binding to EF-hand motifs results in conformational changes in dIV and dVI domains that lead to withdrawal of the interaction between N-terminal α-helix of dI and the second EF-hand motif of dVI. This might also support structural changes in dIII by bringing the catalytic parts close together. In the second step, Ca^{2+} bind to two non-EF-hand Ca^{2+} binding site in dII resulting in realignment of active site cleft in domain II (Moldoveanu et al., 2002).

One of the main remaining questions regarding the activation of calpain by Ca^{2+} is that calcium concentrations required for calpain-1 and calpain-2 activities are much higher than intracellular Ca^{2+} amount. Calpain-1 and calpain-2 are activated in the range of 5-50 μM and 200-1000 μM respectively, while physiological intracellular Ca^{2+} level is 50-300 nM and it can increase to 1 μM at most (Khorchid and Ikura, 2002; Goll et al., 2003). A

number of proposals have been suggested some mechanisms which might assist the systems to lower the Ca^{2+} level required for calpain activity. These include:

- Dissociation of small subunit (Yoshizawa et al., 1995; Pal et al., 2001; Li et al., 2004)
- Post-translational modification of calpains such as phosphorylation and intermolecular autolysis (Glading et al., 2004; Li et al., 2004)
- Binding to phospholipid and plasma membrane (Kuboki et al., 1990; Saido et al., 1991; Tompa et al., 2001; Leloup et al., 2010)
- Participation of possible protein activators (Melloni et al., 1998; Melloni et al., 2000).

In addition, calpain-2 has been recently shown to be activated by Brain-derived neurotrophic factor (BDNF) and epidermal growth factor (EGF) in the absence of calcium (Zadran et al., 2010b).

All these mechanism have suggested reducing calcium requirement for calpain to some level, though they do not drop the ion level to actual concentration in living cell (Goll et al., 2003; Glading et al., 2004). The amounts of calcium required for calpain activity *in vivo* and mechanisms regulate this concentration need further investigation.

Calpastatin is another direct regulator of calpain activity in the cells. Calpastatin is the only known endogenous calpain-1 and calpain-2 inhibitor found in vertebrates (Goll et al., 2003). Calpain and calpastatin are both localized in cytoplasm and the inhibitor needs to bind to both calpain subunits for inhibitory activity. One calpastatin molecule can inhibit up to four molecules of calpain (Wendt et al., 2004). Studies on calpastatin transgenic models have suggested that the inhibitor causes no effect on calpain activity under normal conditions and that the inhibitory function occurs under pathological circumstances (Takano et al., 2005). The structure and function of calpastatin and

calpain/calpastatin interaction will be discussed in more details in the section “Calpain inhibitors”.

1.4. Calpain substrates

Although cleavage of some substrates by calpain leads to degradation of the proteins, the protease cleaves most of its substrates at a limited number of sites that produces a stable functionally active fragment of the target protein. Therefore, calpain is commonly called a “modulator” protease (Goll et al., 2003; Franco and Huttenlocher, 2005).

Analyses, predominantly *in vitro*, have suggested that calpain cleaves a large number of proteins proposed to mediate various physiological and pathological events particularly in the CNS. The substrates mainly include cytoskeletal proteins (e.g. spectrin, tubulin, tau), synaptical and transmembrane proteins (e.g. glutamate receptors, calcium channels, amyloid precursor proteins), signalling/activator proteins (kinases, phosphatases, and kinase activators e.g. PKC, CaM kinase II, p35), and transcription factors and gene expression regulators (e.g. p53, c-Jun, NFκB) (Chan and Mattson, 1999; Goll et al., 2003; Sedarous et al., 2003; Franco and Huttenlocher, 2005; Smith et al., 2006).

The cytoskeletal protein spectrin, and particularly the αII spectrin subunit, is a well-known endogenous calpain substrate. Calpain cleaves spectrin at specific sites resulting in two calpain-mediated spectrin breakdown products (SBDP) of ~150 kDa and 145 kDa (Siman et al., 1984; Wang, 2000). This proteolytic event has been reported in both physiological and pathological processes and is temporally and spatially related to calpain activation in various cellular processes. In the CNS in particular, SBDPs have

been related to synaptogenesis, synaptic reorganization, stimulation of glutamate receptors, and learning and memory in hippocampus (Czogalla and Sikorski, 2005). Moreover, in many pathological events such as Alzheimer's disease (Vanderklish and Bahr, 2000), Parkinson's disease (Crocker et al., 2003), and ischemic stroke (Seubert et al., 1989), calpain-mediated cleavage of spectrin has been used to estimate calpain activation (Czogalla and Sikorski, 2005). It is not clear yet whether calpain-cleavage of spectrin initiates or causes the pathologic events or if it is simply a result of calpain activation in these systems (Czogalla and Sikorski, 2005). Proteins involved in synaptic plasticity are among other major calpain substrates. Several studies have reported that different isoforms of ionotropic *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors undergo calpain-mediated truncation which is part of their post-translational modification events. The modifications are assumed to further enhance the functions of ion channels associated to these receptors subunits (Wu and Lynch, 2006). However, cleavage of metabotropic glutamate receptors such as mGluR1 by calpain is linked to excitotoxicity (Xu et al., 2007). In addition to the receptors, calpain is suggested to truncate postsynaptic density proteins such as PSD95 and kinases in synapses which in turn regulate synaptic structure and long term potentiation (LTP) (Wu and Lynch, 2006).

Many proteins, involved in various pathological events, have been considered to be calpain substrates examined by *in vitro* or *in vivo* models. Calpain modulation of cytoskeletal proteins [e.g. microtubule- associated proteins (MAP) and tau], cysteine-aspartic proteases (caspase) families, nuclear transcription factor p53, Bcl-2 family of cell death regulators, and kinases and their activators (e.g. PKA, PKC, p35/p39) are associated

with neuronal apoptosis and necrosis, reviewed in Vosler et al., 2008 (Vosler et al., 2008). One of these key proteins is p35, a cyclin dependent kinase (Cdk5) activator. Calpain-mediated cleavage of p35 to p25, a stable pathologic fragment, has been attributed to many neuronal injuries such as Parkinson's disease (Lew et al., 1994; Smith et al., 2006; Vosler et al., 2008). Calpain-mediated cleavage of p35 to p25 and its association with neuronal death is discussed in more details in the section of "Calpain and Cdk5/p35/p25 system".

1.5. Calpain inhibitors

The role of calpain in many cellular functions, and more significantly in human neurological disorders, has raised interest in finding proper calpain inhibitors.

Calpain inhibitors can be categorized as peptide/peptidomimetic and non-peptide inhibitors (Donkor, 2011). Calpastatin is an endogenous peptide-based calpain inhibitor and the only identified inhibitor specific for calpain-1 and calpain-2 (Goll et al., 2003). Calpastatin structure consists of five domains: an N-terminal domain called domain L with unknown function and four repeating inhibitory domains, dI-dIV, each with three conserved subdomains A, B, C. Each repeating subdomain is capable of binding and inhibiting a single calpain molecule in the presence of Ca^{2+}, however there is no evidence that calpastatin binds calcium (Goll et al., 2003; Wendt et al., 2004). A series of studies confirmed that subdomain B is inhibitory whereas subdomains A and C do not have inhibitory activities. However, they bind to domain IV and domain VI of calpain respectively and these interactions are necessary for calpastatin inhibitory function (Hanna et al., 2008). Subdomain B inhibits calpain by interacting with area near the active site

cleft in domain IIa and domain IIb (Hanna et al., 2008). While calpastatin is the only known calpain specific inhibitor, the limitation of using it in calpain studies is its poor cell permeability (Donkor, 2011). To circumvent this problem researchers have focused on generating functional truncated forms or a short peptide of calpastatin conjugated to the fragments which enhance cell penetration (Gil-Parrado et al., 2003; Donkor, 2011). A truncated form of calpastatin, CP1B, encoded by exon 1B of its gene is a very potent and selective inhibitor of calpain (Gil-Parrado et al., 2003). Association of this fragment with different cell-penetrating peptide-constructs selectively inhibits calpain activity in different cells in some events. For instance, poly-arginine-fused calpastatin peptide significantly inhibit both calpain-mediated cleavage of αII spectrin and neuronal death induced by glutamate in primary neuronal culture (Wu et al., 2003; Fiorino et al., 2007; Pfizer et al., 2008). Although, these approaches provide better tools to study calpain functions, they have been limited by some restrictions. Since blockage of calpain activity requires the interaction of the calpastatin subdomains to penta-EF-hand domains of large and small subunits, a proteolytic core of calpain generated by autolysis or a calpain monomer might be resistant to calpastatin. Besides, extra calpain molecules may escape the inhibition system or excessive activation of calpain may result in cleavage of calpastatin (Moldoveanu et al., 2004).

Aside from calpastatin, all other available calpain inhibitors suffer from some limitation such as lack of potency, permeability, and more critically, specificity for calpain. Factors considered to be the key obstacles in developing calpain inhibitors are wide cleavage specificity of calpain as well as having similar specificities with other related proteases that altogether cause the calpain inhibitors to suppress the related

cysteine proteases as well (Cuerrier et al., 2007). Significant efforts have gone into the development of potent calpain inhibitors with enhanced selectivity. In the peptidomimetic category, aldehyde- and α-ketoamide-based calpain blockers are among the most commonly used inhibitors. Approaches aimed at enhancing the specificity, potency, and permeability of the compounds, such as modification of non-specific residues adjacent to warhead of these peptide-like inhibitors, resulted in production of some improved calpain inhibitors such as MDL-28170, leupeptin, and E64 (Neffe and Abell, 2005; Donkor, 2011). The modified compounds exhibit good properties in inhibition of calpain, mainly tested in calpain-mediated injuries. However, they are still capable of blockage of some other proteases such as cathepsin, and this makes it complex to properly interpret the data as reflective of calpain functions. Beside the lack of specificity, available calpain inhibitors suffer from other restrictions such as poor *in vivo* stability, limited water solubility, and toxicity (Donkor, 2000, 2011).

1.6. Physiological functions of calpain in the CNS

Extensive studies have suggested that calpain has the capability to significantly influence cell physiology. Under normal conditions calpain is believed to get activated following controlled rise in intracellular calcium concentration and modulates cellular functions. The physiological roles of calpain in the brain have been investigated relative to either CNS development such as cell proliferation/differentiation (Santella et al., 1998; de Azevedo-Pereira et al., 2011; Santos et al., 2012), cell migration (Franco and Huttenlocher, 2005), apoptosis (Gil-Parrado et al., 2002; Li et al., 2009), and dendrites/axon development (Wilson et al., 2000; Sedarous et al., 2003; Mingorance-Le

Meur and O'Connor, 2009); or brain functions like signal transduction (Sato and Kawashima, 2001), synaptic structure (Zadran et al., 2010a), and synaptic plasticity (Wu and Lynch, 2006). For the purpose of this thesis, I will discuss cell proliferation, developmental apoptosis, dendrite development, and synaptic plasticity, which are most relevant to my research.

1.6.1. Cell proliferation

Reports on the role of calpain in cell proliferation have mostly relied on the involvement of calpain in cell cycle. The cell cycle consists of four distinct phases; (i) growth phase (G1): cells prepare for replication of chromosome; (ii) synthesis phase (S): DNA duplication happens; (iii) G2 phase: cell grows and sets up everything to enter to mitosis; (iv) finally, mitotic phase (M): cell is duplicated. The progression of the cell cycle is mainly governed by cyclin dependent kinases (Cdks) and their regulators, cyclins, during the different phases. For instance, Cdk4/cyclin D1 complex is required for G1/S progression and degradation of cyclins is one of the regulatory mechanisms for chromosome segregation and transition from mitosis (M) and growth phase (G1) to synthesis phase (S) (Arellano and Moreno, 1997; Goll et al., 2003). Several reports have shown that calpain mediates cyclin D1 degradation by utilizing calpain inhibitors. For example, calpain inhibitors reverse the loss of cyclin D1 in serum-deprived NIH 3T3 cells (Choi et al., 1997). Also, degradation of cyclin D1 was increased in the presence of pure calpain activated by $CaCl_2$ in different cell lines (Choi et al., 1997). Zhang and colleagues showed that administration of calpain inhibitor in WI-38 fibroblast entirely prevented the progression of cells into S-phase and inhibited cell proliferation at late G1 phase (Zhang et al., 1997). However, the level of cyclin D1 protein was unchanged in that case. Finally, a

recent finding suggested that inhibition of calpain by synthetic calpain inhibitor decreased proliferation of neural stem cells (Santos et al., 2012).

Concerns regarding the importance of calpain in proliferation have been raised by the finding that embryos deficient in both calpain-1 and calpain-2 activities survived until E10.5 and fibroblasts derived from these embryos proliferated normally (Arthur et al., 2000; Tan et al., 2006b). In addition, germline calpain-1 knock-out mice were viable, however germline disruption of calpain-2 expression in mouse caused lethality prior to implantation stage at E.2.5 (Dutt et al., 2006).

The involvement of calpain in cell growth has not been directly examined. In addition, the related results achieved by calpain knock-out animal models are controversial. Therefore, the role of calpain in cell proliferation and more specifically in neuronal proliferation is yet to be deciphered.

1.6.2. Apoptosis

The term apoptosis was first applied to non-necrosis cell loss in 1972, and is defined as a managed, programmed form of cell death (PCD) activated by intercellular death signals (Kerr et al., 1972; Raff, 1992). It is characterized by membrane blebbing, cell shrinkage, DNA fragmentation and chromatin condensation. Finally, the cell breaks into small membrane-bound fragments called apoptotic bodies, and is phagocytized by neighbouring cells or macrophages (Raff, 1992; Naruse and Keino, 1995).

Apoptosis can happen in both physiological and pathological circumstances. Physiological apoptosis is principally beneficial for the organisms and includes a series of highly regulated biochemical and signalling cascades leading to removal of excessive cells and elimination of unwanted and harmful cells. It predominantly takes place during

embryonic and postnatal development (Raff, 1992; Blomgren et al., 2007). During distinct periods of CNS development in vertebrates more than half of nerve cells die in brain shortly after they are formed. It is mostly to maintain the cellular homeostasis (Naruse and Keino, 1995). Developmental apoptosis is mediated by both extracellular effectors, such as neurotrophic growth factor, and intracellular proteolytic cascades including cysteine proteases (Blomgren et al., 2007). Caspases are by far the most recognized participants in apoptosis process (Danial and Korsmeyer, 2004). By now 14 members of mammalian caspases have been detected, 7 of them are involved in apoptosis including caspase-2, -3, -6, -7, -8, -9, and -10 (Riedl and Shi, 2004). Assessment of caspase expression in rat brain revealed that cytosolic caspase-3, -7, -8, and -10 are highly expressed during development and abruptly downregulated after birth, implicating them as important elements in brain growth (Shimohama et al., 2001). All caspases are synthesised as catalytically inactive proteins called pro-caspases and are activated by proteolytic cleavage during apoptosis (Riedl and Shi, 2004).

The involvement of calpain in programmed cell death has drawn attention by finding that calpain activation increased after inducing apoptosis in thymocytes and that inhibition of calpain blocked the related neuronal death (Squier et al., 1994). Afterward, it has been found that calpain mediates cleavage of important factors in developmental neuronal death such as caspases and Bcl-2 family members (Lopatniuk and Witkowski, 2011). Related to caspases, investigation revealed that calpain and caspases share similar substrate targets and that they are even each other's substrate (Lopatniuk and Witkowski, 2011). For instance, calpain was primarily demonstrated to be activated prior to caspases during radiation-induced apoptosis. The report suggested that calpain cleaves and activates

caspase-3 in the apoptosis processing (Waterhouse et al., 1998). Utilizing the B cell lymphoma cell line WEHI 231, Ruiz-Vela and colleagues reported that calpain engaged in B cell apoptosis during development by triggering the processing and activation of caspase-7 (Ruiz-Vela et al., 1999). Additionally, calpain has been shown to cleave procaspase-7 into two large subunits in test tube and human embryonic kidney 293T cells, leading to the amplification of their activities (Gafni et al., 2009). Both caspase-7 and caspase-3 play pivotal roles in apoptosis in different cells including neurons (Kuida et al., 1996; Lakhani et al., 2006).

Bcl-2 protein family is another key component in apoptosis suggested to be calpain substrates. The family consists of proteins divided into proapoptotic members such as Bax, Bak, Bid, and Bcl-Xs, or antiapoptotic members like Bcl-2 and Bcl-X_L (Burlacu, 2003). Bcl-2 family members are localized at intracellular membranes mainly in outer mitochondrial membrane, endoplasmic reticulum (ER) membrane, and nuclear envelope with their N-terminal facing the cytosol (Burlacu, 2003). However, most proapoptotic members are proposed to be initially localized in cytosol or cytoskeleton and incorporated into mitochondrial outer membrane upon a death signal (Hsu et al., 1997). N-terminal cleavage of proapoptotic proteins generates truncated forms that target outer membrane in mitochondria, interact with channels and open the membrane pore which results in release of mitochondrial proteins, mainly cytochrome *c*, and then activation of apoptotic signals (Gross et al., 1999; Burlacu, 2003).

The first evidence suggested a direct interaction between calpain and Bcl-2 proteins displayed that pure calpain-1 and calpain-2 were able to cleave N-terminus of Bax protein in a calcium dependent manner. The cleavage was inhibited by calpain

inhibitors, including E-64, in HL-60 cells after drug-induced apoptosis (Wood et al., 1998). The Bax fragment, mediated by calpain cleavage, is able to target mitochondria and elicit cytochrome *c* release and apoptosis (Goping et al., 1998; Gao and Dou, 2000; Tan et al., 2006a). Bid, another Bcl-2 family member, is also truncated by calpain and induces cytochrome *c* release and cell death in human melanoma cells after drug-induced apoptosis (Mandic et al., 2002). Calpain cleaves Bid at its N-terminus and calpain inhibitors block Bid truncation and apoptosis (Mandic et al., 2002). The role of calpain in apoptosis was further investigated in mouse embryonic fibroblasts (MEFs) generated from calpain germline knock-out. Calpain-deficient cells are resistant to some apoptotic agents such as camptothecin and ultraviolet light, although the cleavage of Bcl-2 family members have not been observed in these processes (Tan et al., 2006a).

Altogether, the preliminary findings suggest a role for calpain in programmed cell death and developmental apoptosis. However, the investigations are controversial, indirect, and mostly rely on calpain inhibitors which have non-specific natures. Hence, the importance of calpain in physiological apoptosis, particularly in the CNS, needs to be addressed precisely.

1.6.3. Dendrite and spine development

(I) Dendrite outgrowth/branching: Dendrite structure/arborisation is critical for processing of neuronal signalling and, accordingly, for neuronal functions (Cline, 2001; Jan and Jan, 2001). Understanding how neurons regulate dendrite development and maintain their morphology is critical for the characterization of neuronal plasticity, the most fascinating and important property of the CNS, as well as neuronal responses to injury and repair (Mingorance-Le Meur and O'Connor, 2009).

Dendrite/axon outgrowth initiates from soma and proceeds through five stages: (1) formation of lamellipodia; (2) generation of short neurites; (3) axonal outgrowth; (4) dendrite outgrowth; and (5) maturation of axon and dendrite and synaptogenesis (Dotti et al., 1988). Dendritic branching is part of a developmental process that shapes the territories of neuronal dendrites called dendritic field. It covers the area in where the dendrite communicates with axons in synapses, receives signals from axons and integrates them before sending them to other neurons.

Dendritic development is a very dynamic process and suggested to happen in parallel with synapse formation. In other words, the shaping of dendritic structure/branching is highly related to neuronal activity (Bailey and Kandel, 1993; McAllister, 2000). Several studies have been shown that alternations in level of neuronal afferent affect dendritic growth. For example, a primary finding revealed that visual deprivation from dark-rearing resulted in reduction in dendritic length and branching in visual cortex of cat (Coleman and Riesen, 1968). Activity-dependent dendrite development was also evidenced by an *in vivo* study in which blockage of NMDA or AMPA receptors decreased dendrite branching and length of the arbors in tadpole (Rajan and Cline, 1998).

Both intrinsic and extrinsic factors are engaged in activity-dependent dendritic outgrowth/arborisation. Among the extracellular signaling which regulate dendritic growth are neurotrophic and neuronal growth factors such as NGF and BDNF. A series of studies demonstrate the ability of these growth factors to control and maintain dendritic arbor (Bibel and Barde, 2000; McAllister, 2000). For instance, application of above

neurotrophins to a slice of ferret visual cortex enhanced length and complexity of the dendrite of cortical neurons (McAllister et al., 1995).

Cytoskeletal elements including actin and microtubules are central components of dendrite structures; hence the factors that regulate these proteins and their dynamics appear to be key parts of intrinsic signals such as CaMKII activity (McAllister, 2000; Jan and Jan, 2001, 2003, 2010). CaMKs are activated in the presence of Calcium/Calmodulin. CaMK/Calcium/Calmodulin regulates cytoskeletal function since calmodulin binds to several of these proteins like spectrin and MAP2 (Sobue et al., 1988; Sobue, 1993). CaMKII is required for transition from rapid dendritic arbor growth phase to slower growth rate and is necessary for stabilization of dendrite elaboration (Wu and Cline, 1998). Activation of *N*-methyl-D-aspartate receptors (NMDAR), a class of glutamate receptor, by glutamate mediates calcium entry to the neurons and results in activation of kinases such as CaMK (Cline, 2001).

Given that many of the factors involved in dendrite development are either calpain substrate such as CaMK, spectrin, and microtubules, (Shoeman RL, 1990) or calpain activators like calcium and BDNF (Zadran et al., 2010b), the cysteine protease is proposed to have a role in dendritic development. A report from Saito and colleagues showed that leupeptin, a calpain inhibitor, stimulates *de novo* neurite outgrowth in PC12h cells (Saito and Kawashima, 1989). Additionally, dendrite retraction and suppression of axon elongation by a calcium-induced repressor, A23187, are blocked by calpain inhibitors EST and MDL 28170 in cultured hippocampal neurons (Song et al., 1994). Examination of expression and activation of calpain and calpastatin in SH-SY5Y cells and human brain suggested that calpain contributes to neurite outgrowth (Grynspan et al., 1997). Further

studies analyzed the pathways by which glutamate mediates dendrite outgrowth in hippocampal pyramidal neurons by utilizing calmodulin and calpain inhibitors. Short time glutamate exposure leads to a transient increase in intracellular calcium concentration and then calmodulin-associated enhancement in dendrite outgrowth, while dendrite retraction happens after the longer glutamate exposure and calpain activation via sustained calcium level in the neurons. Dendrites regression by calpain is correlated to decrease in microtubules (MTs) level in dendrite (Wilson et al., 2000).

(II) Dendritic spines: Dendritic spines are small protrusion of the dendritic membrane. They are generally characterized by a bulbous tip, ~0.5-2 μm in diameter, attached to the dendrite by a narrow stalk, 0.04-1 μm long, called spine neck (Zhang and Benson, 2000; Calabrese et al., 2006). Each spine forms a single synapse with presynaptic axon. It makes the postsynaptic compartment that receives the majority of excitatory signalling from axons in most neurons within central nervous system (Calabrese et al., 2006). Ultrastructural studies of dendritic spines show an apparent zone just beneath the membrane that called postsynaptic density (PSD). This compact matrix consists of neurotransmitter receptors (e.g. glutamate receptors), channels (e.g. calcium channel), organelles [e.g. smooth endoplasmic reticulum (SER)], protein translational machinery, signalling molecules, and scaffolding proteins (e.g. PSD95) (Sheng, 2001; Nimchinsky et al., 2002; Calabrese et al., 2006).

Spine generation and plasticity occur during both development and adulthood (Garcia-Lopez et al., 2010). Depending on the neuronal cell type and life stages, spine density, represented by distribution of spines, varies from 1 to 10 spines per micrometer stretch of dendrite (Calabrese et al., 2006). Spine density escalates to maximum level

during late development when synaptic plasticity is at its peak and then decreases to a stable level maintained through adulthood (Zhang and Benson, 2000).

Spines thus serve one of critical neuronal function, synaptic activity; and alternation in spine density and structure are thought to be critical for synaptic plasticity and its fundamental responsibility, learning and memory (Chakraborti et al., 2012). Spine is rich in actin filament and actin interacts with both synaptic membrane and PSD in spine (Matus et al., 1982; Calabrese et al., 2006). Actin regulation is fundamental for maintenance of spine number, shape, motility, and stability. Thus, changes in actin structure affects spine properties and accordingly synaptic function (Baudry et al., 2011; Sheng and Kim, 2011; Rochefort and Konnerth, 2012; Baudry et al., 2013).

There has been intense interest in mechanisms underlying actin regulation and polymerization in dendritic spine (Nimchinsky et al., 2002; Calabrese et al., 2006; Baudry et al., 2011). Studies over the years suggested that calpain modulates several actin regulatory pathways. Spectrin, one of the major calpain substrates, is concentrated in PSD and has been associated to synapse modification long ago. αII spectrin is attached to plasma membrane in synapses and binds to major spine cytoskeletal proteins, actin and calmodulin (Lynch and Baudry, 1984, 1987). Cleavage of spectrin by calpain is believed to result in reorganization of these structural proteins and spine morphological changes (Bennett, 1990; Baudry et al., 2013). In hippocampal culture neurons calpain activation by BDNF and epidermal growth factor (EGF) in dendrites and dendritic spines was associated with actin polymerization. The effect was entirely blocked by calpain inhibitor (Zadran et al., 2010b). Rho and Rac proteins, family members of Rho family of GTPases, function as major regulators of actin dynamics and have proposed to impact spine

formation, shape, and numbers (Tashiro et al., 2000; Govek et al., 2005). Cortical and hippocampal pyramidal neurons transfected by constitutively active Rac or Rho demonstrated different spine reorganization. Active Rac enhances spine density whereas active Rho decreases spine density and length. Inhibition of activity of each protein prevent the related effects (Tashiro et al., 2000). Rho can be cleaved by calpain in both test tube and cell culture and the fragment has a dominant negative effect that prevents actin filament assembly and cell spreading. The finding connects calpain to actin polymerization through Rho-GTPase activation (Kulkarni et al., 2002). Finally, the link between calpain and spine formation has been demonstrated by a recent report in which overexpression of p25, a calpain-mediated product of p35, in mouse forebrain enhances the number of spine and synapses (Fischer et al., 2005).

The total examinations propose the engagement of calpain in neuronal structures regarding to axon/dendritic tree organization and spinogenesis/synaptogenesis. The importance of these analyses become more significant knowing that synapses in neurons can undergo the structural reorganization, such as changes in spine numbers and shape, related to stimuli and plasticity in both development and maturity. Calpain has been associated to long term potentiation by several findings. Therefore, the necessity of direct investigations to clarify the role of calpain in neuronal development at early stage and adulthood is largely required.

1.6.4. Synaptic plasticity

Plasticity is perhaps the most significant and fascinating property of the mammalian brain. It is defined as the ability of neuronal circuitry to be modified by their activity and it underlies the emotions, behaviours, and thoughts of the individual (David,

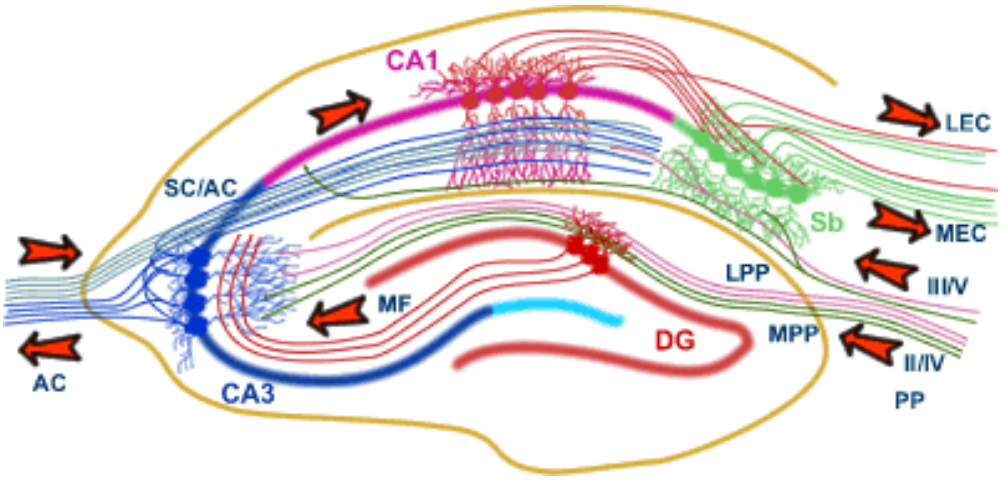
2002). Neuronal activity can alter neuronal wiring in at least two ways: (1) by inducing modification of neuronal structure such as changes in the overall number of synapses (2) by leading to strengthening or weakening of the synapses in response to related activities (David, 2002). Synaptic plasticity refers to the latter case, the ability of synaptic transmission in utilizing the information and transient experience and translating them into memories (David, 2002). Synaptic transmission can be improved or diminished in response to the nature of the activity and the modification can last for a short period or result in long lasting changes in synaptic connections (Nicoll et al., 1988; Lynch, 2004). One such activity-dependent synaptic enhancement is LTP, proposed to be one of the major cellular mechanisms for information storage that underlies learning and memory (Lynch, 2004).

The basic properties of LTP make it a fascinating model in the study of learning and memory. First, LTP can be induced rapidly and lasted long by repetition of stimulation: synaptic transmission is detected within seconds or minutes following the stimulation and the response can last for hours in *in vitro* and even days in *in vivo* experiments (Bliss and Lomo, 1973; Malenka and Nicoll, 1999). Second, LTP is input-specific: enhancement in synaptic strength of one set of synapses does not affect other synapses on the same cell. Third, LTP is cooperative and associative: weak stimulation of many pathways to a synapse cooperatively induces LTP; and weak stimulation of synapses that cannot trigger LTP by itself will provoke LTP when it is coupled with a strong input (Malenka and Nicoll, 1999; David, 2002). Finally, LTP can be induced by tetanic stimulation including theta burst and pairing protocol, a brief high frequency induction at the end of directly depolarization of the cell (Hernandez et al., 2005). LTP

has been detected at excitatory synapse in many regions of mammalian brains including hippocampus, cerebral cortex, amygdale and cerebellum (Malenka and Bear, 2004). Since, LTP shares many fundamental features with learning and memory there is substantial motivation to understand its underlying mechanisms (David, 2002; Lynch, 2004).

Most of the findings about LTP mechanisms have been drawn from studies on Cornu Ammonis (CA1) pyramidal neurons in mammalian hippocampus (Ho et al., 2011). The hippocampus has long been linked with formation of new memories and their long-term storage through different cell layers and sections classified by a series of connections. Simplistically, there are three uni-directional synaptic routes that connect the main pyramidal and granule cell layers together and with other brain structures: (1) the Perforant pathway (PP) is the main signal entry to the hippocampus. It is the axons from entorhinal cortex (EC) that project to granule cells of the dentate gyrus (DG); (2) the Mossy Fiber (MF) pathway comprises the axons deriving from dentate gyrus cells that reach the CA3 pyramidal neurons; (3) the Schaffer Collateral (SC) pathway is the axons from CA3 neurons that extend to the CA1 region. The major output from hippocampus comes from these neurons (CA1) to the entorhinal cortex **(Fig. 1.2)** (Neves et al., 2008; Ho et al., 2011).

Figure 1.2. Hippocampal cell layers and connection pathways. Input signals go to hippocampus through Perforant Pathway, and then pass the long axons, Mossy Fiber, to reach CA3. Pyramidal cells in CA3 transmit the signal to the region CA1 of hippocampus via Schaffer Collateral pathway. The CA1 axons send the signals from hippocampus to entorhinal cortex **(http://www.bristol.ac.uk/synaptic/pathways/).**


CA1
LEC
SC/AC
Sb
MEC
LPP
III/V
MF
DG
MPP
II/IV
AC
CA3
PP

1.6.4.1. Excitatory synaptic transmission

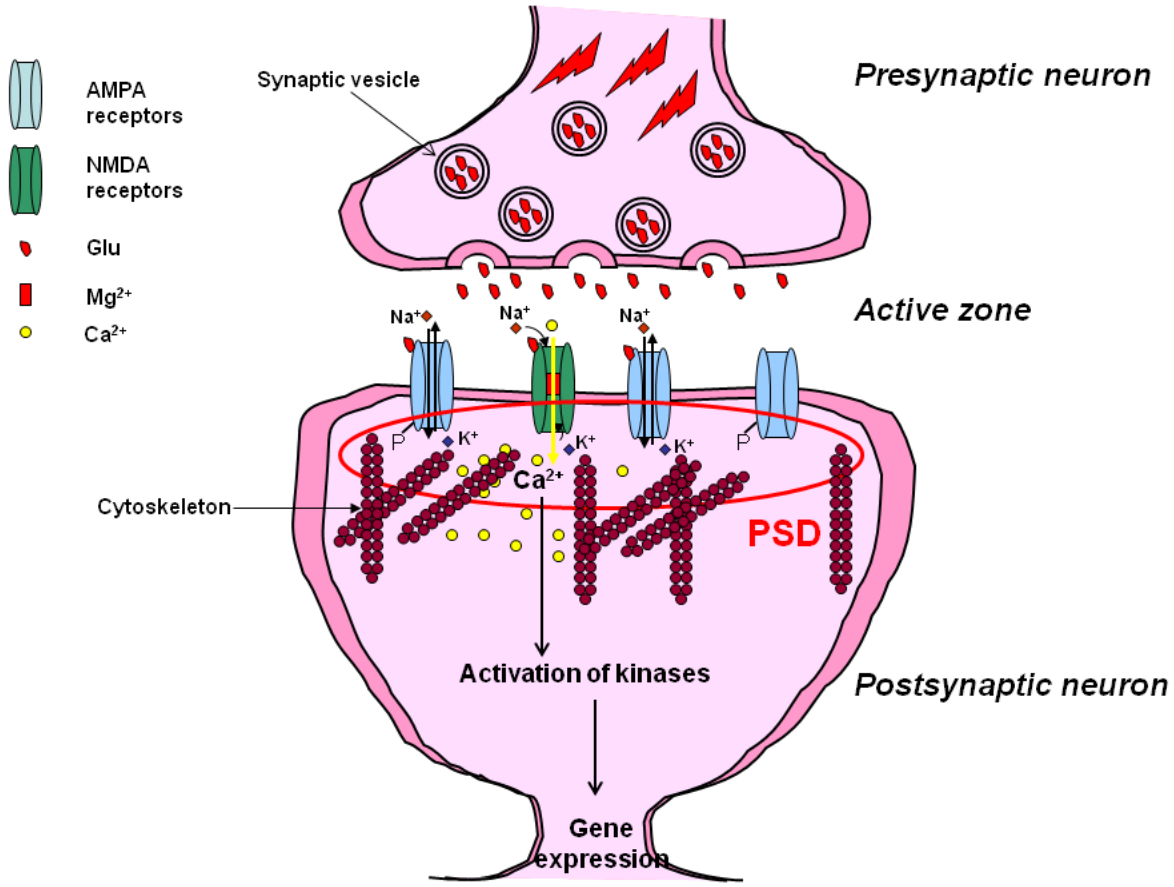
Excitatory synapses in mammalian CNS are usually located on dendritic spines and are composed of presynaptic neuron, postsynaptic neuron, and active zone or synaptic cleft. Each compartment harbours molecules and organelles involved in synaptic transmission and plasticity (Chua et al., 2010). The action potential reaches the axon terminal at the presynaptic neuron and triggers the activation of voltage-gated calcium channels. It causes the entry of calcium into presynaptic bouton where finally the high calcium concentration triggers exocytosis of vesicles and release of neurotransmitter into synapse active zone. Stimulation of receptors on postsynaptic neurons results in activation of ion channels, passage of cation and activation of postsynaptic density components including cell adhesion molecules, scaffolding proteins, cytoskeletal elements, and signalling molecules. Postsynaptic density is the leading spot for signal transduction and signal processing in synaptic plasticity (Siekevitz, 1985; Kennedy, 2000).

Glutamate is a key excitatory neurotransmitter in mammalian brain. It orchestrates brief depolarization of postsynaptic membrane, termed excitatory postsynaptic potential (EPSP), by activation of ligand-gated ion channels and flow of cation such as Na^+, K^+, and Ca^{2+} into postsynaptic cells. Repeated stimulation and combination of EPSPs result in possible action potential in postsynaptic cells (Sheng and Hoogenraad, 2007; Chua et al., 2010).

Glutamate activates various membrane receptors at postsynaptic neurons that are mainly divided into two large groups; metabotropic receptors (mGluR), members of G-protein-coupled receptors; and ionotropic receptors, ligand-gated ion channels. Metabotropic receptors, mGluR1-mGluR8, indirectly activate ion channels upon binding

to GTP- (G-proteins) followed by a series of biochemical cascades involving second messengers and enzymes such as cyclic adenosine monophosphate (cAMP) and protein kinases A (PKA) (Ozawa et al., 1998; Traynelis et al., 2010). Ionotropic receptors open ion channels directly upon glutamate interaction. Biological and electrophysiological properties of these receptors have been object of intense investigations due to their intriguing capability to provide a ready passage for vital cations, Na^+, K^+, and Ca^{2+}, in signalling process **(Fig. 1.3)** (Ozawa et al., 1998; Traynelis et al., 2010).

Figure 1.3. Overview of main components and general function of an excitatory synapse in the CNS. Synaptic vesicle in presynaptic neuron fuses with the membrane and release glutamate into active zone (Synaptic cleft). The neurotransmitters bind to ionotropic receptors located on the membrane of postsynaptic neuron. Activation of the receptors results in opening of ion channels and ions transportation through the postsynaptic membrane and finally depolarization of the postsynaptic neuron.


AMPA receptors
NMDA receptors
Glu
Mg2+
Ca2+
Synaptic vesicle
Presynaptic neuron
Active zone
Na+
Na+
Na+
K+
K+
K+
P
P
Ca2+
Cytoskeleton
PSD
Activation of kinases
Postsynaptic neuron
Gene expression

1.6.4.2. Ionotropic glutamate receptors

These glutamate receptors are classified into three major subgroups on the basis of their affinity preference for three different types of agonists: kainate, α-amino-3-hydroxy-5-methyl-4-isoxaxolepropionic acid (AMPA), and N-methyl-D-aspartate (NMDA).

Kainate receptors are present in the brain in much lesser extent than other ionotropic receptors and their functions remain poorly defined. They are permeable to Na^+, K^+, and, slightly, Ca^{2+} cations. These receptors are detected in both pre- and postsynaptic areas with suggested distinct properties. Activation of presynaptic kainate receptors are involved in decrease of neurotransmitter release by Schaffer collateral onto CA1 neurons, whereas the postsynaptic receptors play a part in eliciting EPSC at mossy fiber synapses (Huettner, 2003).

AMPA and NMDA receptors are widely expressed throughout the mammalian brain and play key roles in excitatory synaptic transmission and plasticity (Ozawa et al., 1998; Traynelis et al., 2010). Their structures are mostly heteromeric tetramers composed of different subunits. All subunits share the same structure containing an extracellular N-terminus domain comprising ligand-binding sites, a transmembrane domain playing an important part in the formation of the core of ion channels, and a C-terminal intracellular domain, a most divergent region. The C-terminal domain varies in sequence and length and undergoes modification such as post-transcriptional alternation, cleavage, and phosphorylation during the activity. This region is involved in receptor trafficking, anchoring and binding to signalling molecules (Derkach et al., 2007; Traynelis et al., 2010).

AMPA receptors (AMPAR) with rapid kinetics are the major transducers of fast excitatory transmission in mammalian brain. They conduct flow of cation including Na^+ and K^+ and play pivotal roles in synaptic current under basal conditions and in activity-dependent synaptic transmission during LTP. These receptors are made of four different subunits, GluA1-GluA4 (Gouaux, 2004; Hansen et al., 2007). The functional properties of the receptors are highly related to the diverse co-assemblies of the receptor subunits. The composition of receptor subunits varies depending on the developmental stage, brain region, and cell type. Most AMPARs in adult CA1 pyramidal neurons are heterodimers consisting of GluA1/GluA2 predominantly and GluA3/GluA2 in some cases. GluA2 subunit is impermeable to Ca^{2+} in adult brains due to replacement of a glutamine (Q) residue with arginine (R), termed Q/R site, in receptor's ion channel. Therefore, considering the predominant distribution of GluA2-containing receptors in adults, it is believed that most AMPAR in hippocampus are incapable of Ca^{2+} passage (Ozawa et al., 1998; Gouaux, 2004; Hansen et al., 2007; Traynelis et al., 2010). However, recent findings indicate that Ca^{2+} conduction of AMPARs might be altered as a result of transient incorporation of GluA2-lacking AMPARs in the synapse (Plant et al., 2006).

The number and type of AMPA receptors represented at synapses are very dynamic because of continuously trafficking of receptors to or from synapses by exocytosis, endocytosis, recycling, and reinsertion (Shepherd and Huganir, 2007). The receptors' incorporation appears to take place in different ways. There is a pool of AMPA receptors nearby postsynaptic density that are assumed to get incorporated to synapses by two possible methods: (1) the receptors are inserted at extrasynaptic sites and laterally diffuse into PSD, where they become anchored by cytoskeleton and proteins; (2) the

receptors move intracellularly to the spines by cytoskeleton-associated activity and directly inserted at synapses (Malinow and Malenka, 2002; Bredt and Nicoll, 2003; Shepherd and Huganir, 2007). Also, the receptors are suggested to be synthesised locally in dendrites compartment and directly added to the PSD through interaction with synaptic protein (Sans et al., 2001; Shepherd and Huganir, 2007).

NMDA receptors are essential mediators of brain plasticity, due to their unique features that differentiate them from other ionotropic receptors. NMDA receptors have unique requirement for occupancy of another ligand, glycine, a co-agonist with glutamate for the receptors activation. Glycine is absolutely required for the NMDA receptors channel to enter the open state (Johnson and Ascher, 1987). In addition to binding to both glutamate and glycine, the receptors require postsynaptic depolarization to promote channel opening. The postsynaptic depolarization is needed for removal of Mg^{2+}, the cation blocking the receptors channel in a voltage-dependent behaviour (Crunelli and Mayer, 1984; Traynelis et al., 2010). Opening of ion-conduction pore by expelling of Mg^{2+} allows a great influx of cation Na^{+}, K^{+}, and most importantly Ca^{2+} into postsynaptic cells. NMDA receptors ion channels are highly permeable to Ca^{2+}, a vital second messenger orchestrates many physiological and pathological functions in mammalian CNS (Burnashev, 1996; Traynelis et al., 2010).

Similar to AMPA receptors, the functional properties of the NMDA receptors are associated with specificity, arrangement, and co-assembly of their subunits. The NMDA receptors subunits are currently organized into three subfamilies, GluN1, GluN2A-D, and GluN3A-B (Traynelis et al., 2010; Paoletti et al., 2013). The heteromeric tetramer of the receptor contains of GluN1 with one of GluN2 subunits, although the mix of GluN2 and

GluN3 build the receptors in some case. GluN1subfamilies are widely found in the brain largely in hippocampus while GluN2 expression is spatiotemporally modulated (Cull-Candy et al., 2001; Paoletti et al., 2013). The dominant GluN2 subfamilies at mature synapses in adult hippocampus are GluN2A and GluN2B. The two subunits appear to be sensitive to Mg^{2+} blockage to a large degree and show high Ca^{2+} conductance compare to another two subfamilies (Cull-Candy et al., 2001; Traynelis et al., 2010). NMDA receptors also display slower activation/deactivation kinetics compared to AMPA receptors, a fact that contributes to brain plasticity by facilitation of EPSCs temporal summation (Cull-Candy et al., 2001; Traynelis et al., 2010; Paoletti et al., 2013).

For a long time NMDA receptors were considered to be tightly linked to PSD and strongly anchored to the synaptic membrane (Allison et al., 1998). However, recent studies have proposed the trafficking of the receptors to or from the synaptic site by exocytosis or lateral diffusion (Carroll and Zukin, 2002). Furthermore, the receptor trafficking and targeting appeared to contribute to synaptic plasticity in hippocampus (Carroll and Zukin, 2002; Tovar and Westbrook, 2002). Nevertheless, and in contrast to the case of AMPA receptors, NMDA receptors transportation and its role in synaptic plasticity need to be examined in more details.

1.6.4.3. Postsynaptic structural and molecular modifications in LTP

Increase in Ca^{2+} concentration triggers a series of proteins and mechanisms such as proteases, kinases, phosphatases, cytoskeletal reorganization, and postsynaptic membrane restructure. It ultimately results in initiation of protein synthesis reflecting synaptic plasticity in dendritic spine within early and late phase of LTP (David, 2002; Lynch, 2004; Luscher and Malenka, 2012). Significant evidence indicates that protein kinases play

critical roles in both induction and expression of LTP by modulation of AMPA and NMDA receptors; and in LTP maintenance by contribution to new protein synthesis (Lynch, 2004; Abraham and Williams, 2008). Some of the key kinases involved are Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), a required component of LTP, protein kinase C (PKC), cAMP-dependent protein kinase (PKA), and mitogen-activated protein kinase (MAPK). Phosphorylation of Ser831 by CaMKII and of Ser845 by PKA on GluA1 modulate the receptor single channel conductance and trafficking respectively (Lisman et al., 2002; Shepherd and Huganir, 2007; Paoletti et al., 2013).

These kinases by themselves cannot regulate postsynaptic changes in LTP processing if not for the PSD cytoskeleton and scaffolding proteins being part of the factors. In fact, these structural proteins are key regulators of many signalling pathways in postsynaptic membrane. They mediate changes in synaptic composition and shape and link receptors and channels to downstream signalling molecules such as kinases and phosphatases (Sheng and Kim, 2011). A large number of scaffolding proteins in PSD, including PSD95 and SAP97, are PDZ domain-containing family members. PDZ, a long binding domain, interacts with short amino acid motifs at the end of C-terminal of binding proteins (Kim and Sheng, 2004). SAP97 binds to GluA1 and promotes its targeting to the synapses during AMPA receptor activity (Kim and Sheng, 2004). PSD95 is a major component and probably the most famous member of the family. It interacts with a series of proteins in PSD such as receptors, ion channels, and cytoplasmic proteins and is proposed to be important for proteins clustering and localization at postsynaptic membrane. NMDA receptors interaction with PSD95 stabilizes channel presentation and inhibits PKC-mediated internalization of the receptors at the synapse surface (Carroll and

Zukin, 2002; Kim and Sheng, 2004). Furthermore, PSD95 interacts with a number of signalling molecules such as kinases and keeps them close to the synapse, regulates synaptic transmission, and controls the synapse size and, consequently, synapse strength (Kim and Sheng, 2004).

As mentioned before, synaptic activity and changes in synapse composition alter morphology, size, and density of synaptic spine that appears to be crucial for brain plasticity (Luscher et al., 2000). Reorganization of spine cytoskeleton within LTP expression is linked with PSD and spine enlargement and thus synapse strength (Rochefort and Konnerth, 2012). As previously mentioned, actin is the principal cytoskeleton protein that drives changes in spine shape and size. Evidence show that actin density increases in spines after LTP. Synaptic activity drives augmentation in actin polymerization and, consequently, filament stability and spine enlargement as a result (Krucker et al., 2000; Kramar et al., 2006; Gordon-Weeks and Fournier, 2013). In addition, these results have led to some models proposing that synaptic strength is related to splitting of existing spines or production of new ones (Luscher et al., 2000; Hering and Sheng, 2001; Rochefort and Konnerth, 2012). Indeed, several reports reflect that changes in spine shape and number can predict translation of short-term changes into long-lasting alternations in structure, connection, and functions of synapses in brain plasticity (Yuste and Bonhoeffer, 2001). Perhaps the main early reports that link learning and memory to spine plasticity came from examination of visual cortex (Hering and Sheng, 2001). Mice that experienced complete light deprivation since birth revealed significant decrease in spine density in pyramidal cells in cortex (Valverde, 1967). Then, activity-related changes in the development and maintenance of dendritic spine has been also investigated in

hippocampus following induction of LTP by several methods (Luscher et al., 2000). In a noteworthy study, Engert and colleagues limited LTP induction to a small area on dendrite of postsynaptic neurons and demonstrated the growth of new spines corresponding to long-lasting LTP induction in CA1 region of rat hippocampus (Engert and Bonhoeffer, 1999).

Taken as a whole, structure and signalling changes in postsynaptic neurons after LTP stimulation may be able to interpret the relationship among physiological modification, synaptic plasticity, and learning and memory in the CNS.

1.6.4.4. Calpain and excitatory synapse and LTP

Since 1980s, studies of the activity-dependent biochemical changes in synapses have generated considerable interest in calpain (Lynch and Baudry, 1984). Calpain is localized in soma, dendrites, and spine in pyramidal neurons in hippocampus. The protease is suggested to be activated following an increase in intracellular calcium concentration in neurons as a result of NMDA receptors-dependent synaptic activity (Lynch and Baudry, 1984). Calpain has been associated with excitatory synaptic function and LTP through many lines of investigation. Several evidences, principally based on pharmacological inhibition of calpain, have examined the relevance of the protease to modulation of glutamate receptors structure and function mainly AMPA and NMDA receptors (Doshi and Lynch, 2009). *In vitro* and *in situ* examination of synaptic membrane extract and brain sections treated with either calcium or calpain suggest that the C-terminal of both AMPA and NMDA receptor subunits, to higher degree GluA1 and GluN2, are cleaved by calpain and the production of fragments are blocked by calpain inhibitors (Bi et al., 1994; Bi et al., 1996; Bi et al., 1997; Bi et al., 1998a; Bi et al., 1998b).

Further studies proposed that calpain-dependent cleavage of GluA1 leads to internalization of the generated fragment and reduction in AMPA receptor current under excitatory and ischemic conditions (Yuen et al., 2007). Activation of calpain by stimulation of NMDA receptor in transfected cell line such as HEK293t results in truncation of GluN2A and GluN2B. The cleaved fragment of GluN2A is unstable on the surface and the functional properties of the receptor including glutamate antagonist binding (MK801) or calcium uptake are diminished after calpain-mediated cleavage (Guttmann et al., 2001; Guttmann et al., 2002). Experiments from different neuronal cultures perhaps demonstrate more precise facts than those from *in vitro* assessments. In hippocampal cultures calpain is activated after NMDA receptor overstimulation and it drives cleavage of NMDA receptor subunits particularly GluN2B. The generated fragment is active and remains on the surface and its production can be blocked by calpain inhibitor (Simpkins et al., 2003). Afterwards, some data demonstrated that overstimulation of NMDA receptor leads to sustained suppression of NMDA receptor current and degradation of GluN2A and GluN2B. The subunits degradation is blocked by calpain inhibitor (Wu et al., 2005). The overall findings therefore propose that calpain may engage in synaptic transmission by regulation of glutamatergic receptors structure and thus their functions. However, due to lack of appropriate approaches, particularly *in vivo*, calpain-mediated cleavage of receptor subunits and the subsequent effects on synaptic plasticity remain still unclear.

Calpain is also linked to brain plasticity by cleavage of various other proteins such as PSD95, PKC, and CaMKII which are believed to be crucial modulator of synaptic structure and activity (Zadran et al., 2010a). The significant involvement of PSD95 in

spine structure and synaptic receptor anchoring was highlighted in previous section. PSD95 has been proposed to be a calpain substrate and the truncation is suggested to contribute to turnover of PSD95 protein and thus play a part in anchoring of membrane receptors (Lu et al., 2000). Analysis of expression of PSD95 in postnatal brain and increase in products of calpain-mediated degradation of PSD95 in neonatal hippocampus promoted the hypothesis that calpain may interrupt the interaction of PSD95 with some other cytoskeleton proteins and thus plays a pivotal role in synapse formation during hippocampus development (Lu et al., 2000).

To further test the hypothesis regarding the role of calpain in synaptic plasticity a selection of calpain inhibitors were applied to both hippocampal slices and animals *in vivo*. The finding proposed that the inhibitors have no effect on basal synaptic transmission, but significantly blocks LTP in the Schaffer collateral-CA1 pathway (Staubli et al., 1988; Oliver et al., 1989; del Cerro et al., 1990; Denny et al., 1990). Rats genetically deficient in calpastatin, a condition expected to promote calpain activation, demonstrated enhanced LTP produced by high frequency stimulations in hippocampus (Muller et al., 1995). Vanderklish and colleagues treated cultured hippocampal slices with antisense oligonucleotide and reduced the activity of Calpain-1 to 50% by translational suppression. Similar to the result from calpain inhibitors, suppression of calpain-1 activity significantly blocked generation of LTP in the absence of any insufficiency in basal synaptic response (Vanderklish et al., 1996). Generation of a viable calpain-1 knock-out mice (Azam et al., 2001) provided an experimental tool for the analysis of the involvement of calpain in LTP and learning and memory. Surprisingly, mice lacking

calpain-1 activity did not display any detectable changes in LTP and learning and memory tested by related behavioural paradigms such as fear conditioning (Grammer et al., 2005).

In relation with the above finding, Baudry and his colleagues proposed that calpain-2 might play a more prominent role in neuronal function, in particular synaptic plasticity. By utilizing a Rabies-Virus-Glycoprotein (RVG) targeting peptide, calpain-2 was selectively down-regulated in mouse brain. This calpain-2 downregulation led to LTP elimination and impairments in learning and memory (Zadran et al., 2013).

Finally, calpain substrate, p35, as well as the associated cyclin-dependent kinase 5 (Cdk5), have been described as potential modulators of LTP (Angelo et al., 2006). Cdk5 is a serine-threonine kinase with multiple functions (Angelo et al., 2006). Calpain is suggested to cleave p35 to p25, a brain specific regulatory subunit that activates Cdk5 (Lew et al., 1994; Tsai et al., 1994; Lee et al., 2000). The mechanism will be discussed later in more details in neurodegenerative disorders. Fisher and colleagues employed the tet-off system to induce p25 in mouse forebrain and study the role of the regulator in synaptic plasticity (Cruz et al., 2003). The authors reported that transient expression of p25 in mouse forebrain enhances hippocampal LTP and improves performance in associative and spatial learning and memory tasks. The authors proposed that the increase in the number of spine/dendrite induced by overexpression of p25, may be the underlying mechanism for LTP facilitation (Fischer et al., 2005). However, other studies revealed that inducible conditional knock-down of Cdk5 in mouse brain causes improved LTP and development in associative and spatial learning and memory performance (Hawasli et al., 2007). Examination of GluN2B expression and relative NMDA receptor current lead to the proposal that the facilitation of synaptic plasticity in transgenic mice is associated with

downregulation of calpain-mediated degradation of GluN2B (Hawasli et al., 2007). This finding opened a controversial discussion regarding the role of calpain activity in regulation of synaptic plasticity, at least in term of calpain downstream in LTP.

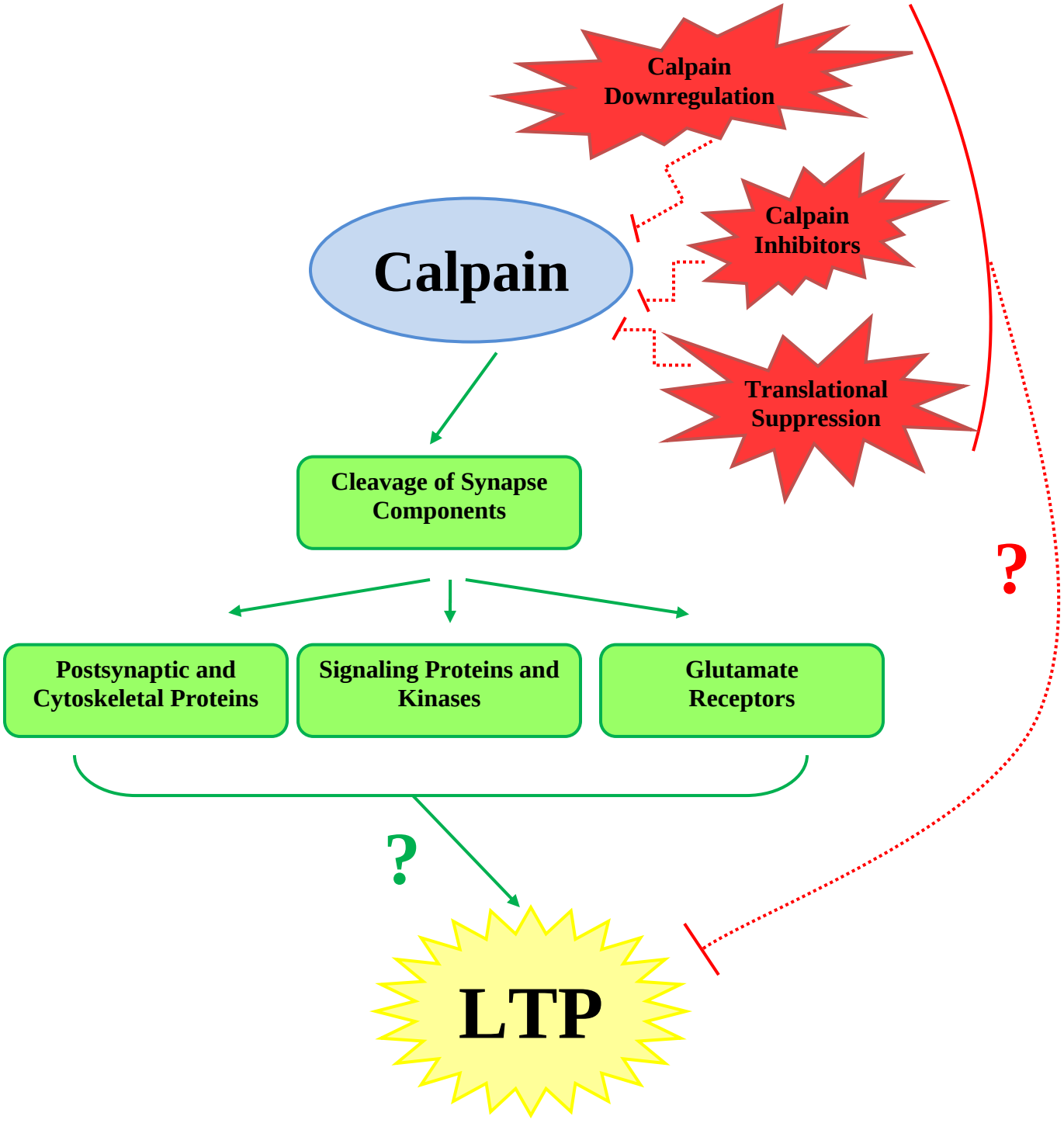
As we highlighted here, several observations have led to the idea that activation of calpain may modulate the functional properties of its synaptic substrates and may translate Ca^{2+} signalling into well-known long-lasting changes in synapse, LTP **(Fig. 1.4)**. However, there are some major caveats in these studies that limit their interpretation. A large body of evidence related to calpain and LTP rely on experiments by calpain inhibitors. As discussed before, a problem frequently encountered when using the inhibitors is their low specificity for calpain, making it difficult to determine if the findings reflect suppression of the protease itself or of related enzymes. This might explain the contradictory in findings related to calpain activity in LTP.

The approach from Baudry and colleagues to knock-down calpain-2 in mouse brain supports the hypothesis proposing the beneficial effect of calpain on LTP (Zadran et al., 2013). However, the conclusions are permeated with the concern about the possible compensatory role of calpain-1 in the system, e.g. in activation of distinct downstream pathways.

Synaptic plasticity is a key element of learning and memory. Therefore, understanding the precise mechanism underlying brain plasticity is crucial for discovering the proper therapeutic strategy for learning and memory disorders. Calpain has long been considered as a potential target of such therapies, based on its hypothesized central role in regulating synaptic activity. However, the overall findings leave many questions still

unanswered, particularly those regarding the direct involvement of calpain in LTP, due to a lack of proper experimental tools.

Figure 1.4. General overview of proposed roles of calpain in LTP. The importance of calpain in LTP has been highlighted by several findings in which inhibition or downregulation of calpain hinders LTP. Activation of calpain in synapse proposes to exert multiple functions to regulate LTP. Significant effects are modulation of glutamate receptors, postsynaptic and cytoskeletal proteins, and kinases.


Calpain Downregulation
Calpain Inhibitors
Calpain
Translational Suppression
Cleavage of Synapse Components
?
Postsynaptic and Cytoskeletal Proteins
Signaling Proteins and Kinases
Glutamate Receptors
?
LTP

1.7. Pathological functions of calpain in the CNS

Calpain has been associated with a variety of neuronal normal functions under physiological condition and its presence has been shown to be vital for mammalian viability (Arthur et al., 2000; Goll et al., 2003). However, sustained increase in Ca^{2+} intracellular concentration under pathological circumstances results in overactivation of calpain that has been linked to neuronal death in range of disorders such as Huntington's disease (HD), Alzheimer's disease (AD), Multiple Sclerosis (MS), Parkinson's disease (PD), and ischemic stroke (Goll et al., 2003; Ray and Banik, 2003; Liu et al., 2008).

For the purpose of this study, I review the summation of major findings regarding to Parkinson's disease and ischemic stroke relevant to our research.

1.7.1. Parkinson's disease (PD)

Parkinson's disease is the second common neurodegenerative disorder, after Alzheimer's disease, estimated to impact seven to 10 million people worldwide. The disease was first described in 1817 by Dr. James Parkinson and is characterized by progressive loss of movement control. This can lead to the symptoms including resting tremor, bradykinesia or akinesia, rigidity, postural instability, and even dementia. The high rate of the PD patients as well as the long-term implication of PD on the quality of the individuals' lives impose a large social and financial burden on the families and societies. As stated by the Parkinson's Society of Canada almost 100,000 Canadians live with PD with approximately 5,500 new cases diagnosed each year. According to the report drawn by Health Canada in 1998 the economic burden of PD disease in Canada is 558.1 million per year. The cost is expected to be rise significantly due to increase in population

aging and, consequently, the rate of disorder incidence (Sources: Parkinson's Society of Canada www.parkinson.ca, Parkinson's disease Foundation www.pdf.org).

Despite advances toward PD symptomatic medication, so far researchers and physicians have found no way to stop or prevent the disease. Hence, a large body of work has focused on the pathology and pathogeny of this progressive debilitating disorder (Sources: Parkinson's Society of Canada www.parkinson.ca, Parkinson's disease Foundation www.pdf.org).

1.7.1.1. Pathology and etiology of PD

PD causes progressive neurodegeneration in the central nervous system that particularly affects, but is not restricted to, neuromelanin-pigmented neurons called dopaminergic (DA) neurons in Substantia Nigra Pars compacta (SNc) in the midbrain. The consequent result is loss of dopaminergic innervations to the caudate and putamen in basal ganglia (the nigro-striatal tract) (Blandini et al., 2000). The neurotransmitter dopamine plays a central role in important brain functions such as movement control, cognition, and emotion (Arias-Carrion et al., 2010). Dopamine is synthesised from tyrosine; tyrosine is converted to L-dopa and then dopamine by involvement of enzymes tyrosine hydroxylase (TH) and aromatic amino acid decarboxylase (AADC) respectively (Elsworth and Roth, 1997). TH is used as a marker of dopaminergic neuron in substantia nigra in histological studies (Weihe et al., 2006).

Dopaminergic neurons in SNc project their axons to striatum in basal ganglia where they exert their controlling effect on the voluntary movement followed by a series of inhibitory and excitatory signalling. The final outcome of dopamine signals is less obstruction on thalamus and subsequent activation of frontal cortex to start a movement.

Dopamine depletion in PD shifts basal ganglia effects toward excessive inhibition on cerebral cortex that results in difficulty in the generation of movement (Obeso et al., 2008b; Obeso et al., 2008a).

Dopaminergic neuronal loss is characterized by a significant decline in pigmented neurons in SNc of post-mortem brain (Braak et al., 2003). The other typical pathological hallmark of Parkinson's disease is the presence of Lewy bodies (LBs), abnormal fibrillar cytoplasmic inclusions, located in cell body, axon, and dendrites of surviving DA neurons. The primary constituent of LBs is the protein alpha synuclein (α-synuclein), plus a wide range of other molecules such as neurofilament and ubiquitin (Braak et al., 2003; Braak et al., 2004). The link between the presence of Lewy body and dysfunction of neurons has yet to emerge (Braak et al., 2003; Braak et al., 2004; Hartmann, 2004)

Currently, the potential causes of neuronal loss in PD are considered genetics, environmental factors or combination of both. Almost 85-95% of PD cases are sporadic with unknown origin, but several genes have been identified as, when mutated, lead to familial forms (5-15%) (Dauer and Przedborski, 2003; Warner and Schapira, 2003). The familial cases comprise young population as early as 20 years old; hence in most cases called young-onset or early-onset (Lucking et al., 2000). Polymeropoulos and colleagues identified first a mutation in α-synuclein gene (called also PARK1/PARK4), linked to autosomal dominant familial PD in 1990s (Polymeropoulos et al., 1996; Polymeropoulos et al., 1997). Since this breakthrough, many other genes have been linked to familial PD; to name the most well-known among them are parkin (PARK2), PINK1 (PARK6), DJ-1 (PARK7), and LRRK2 (PARK8) as the leading cause of genetically inherited PD (Kitada et al., 1998; Funayama et al., 2002; Bonifati et al., 2003; Valente et al., 2004). The

discovery of genes related to PD is very exciting since the mutations may not be very common but they can lead to better understanding of the facts and mechanisms underlying both familial and sporadic PD (Klein and Westenberger, 2012).

Idiopathic or sporadic PD (late-onset) embraces a majority of PD cases in which brain becomes progressively damaged over a number of years. The clinical symptoms appear when 50%-70% of dopaminergic neurons in SNc have been lost at or after the age of 50 (Davie, 2008). Like other age-related diseases, the causative origins and molecular pathways that govern neuronal death in these PD cases have been the focus of intense investigations (Mattson and Magnus, 2006).

The causes of sporadic PD remain essentially unknown. Currently, PD is mainly linked to environmental factors in addition to the role of gene vulnerability in development of the illness (Warner and Schapira, 2003). Main environmental factors that raise the risk of PD include toxins and rural living associated with prolonged exposure to herbicide and insecticide (Kieburtz and Wunderle, 2013). Toxin-based PD animal model utilizing toxin 6-hydroxydopamine (6-OHDA), herbicide paraquat, insecticide rotenone, and drug-related toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) have been employed in primates and rodents to mimic PD pathology and symptoms and to dissect the molecular events occurring during neuronal damage (Duty and Jenner, 2011).

1.7.1.2. MPTP: Parkinson-related toxin

Perhaps the most important observation supporting the direct contribution of environmental agents to PD is accidental exposure to MPTP, an inadvertent by-product of manufacturing the narcotic drug, 1-Methyl-4-phenyl-4-propionoxypiperidine (MPPP). In 1970s Davis *et al*. reported severe features of PD in a young student abusing MPPP. Loss

of dopamine neurons and presence of LBs were detected in the SNc of the brain during autopsy (Davis et al., 1979). Afterwards, application of MPPP, contaminated with MPTP, was linked to development of Parkinsonian-like symptoms within 7-14 days in a young patient group (Langston et al., 1983). Since then, MPTP animal models of Parkinson's disease have been used to study clinical, biochemical and pathological features of the illness. MPTP produces irreversible and striking Parkinsonian syndromes in primate and rodents identical to that seen in PD patients including movement abnormality, nigrostriatal damage, LBs occurrence, and response to the medical therapy, levodopa (L-dopa, the precursor of dopamine) (Tieu, 2011; Porras et al., 2012). MPTP is not toxic by itself; the neurotoxic metabolite is 1-methyl-4-phenylpyridinium (MPP+), an oxidized form of MPTP by action of the enzyme monoamine oxidase B (MAO-B) (Przedborski and Vila, 2003; Tieu, 2011). MPP+ is taken up by dopamine neurons via the dopamine transporter. MPP+ neurotoxicity depends on its selective concentration in mitochondria. It results in complex-I, mitochondrial electron chain enzyme, inhibition which are interfering with mitochondrial respiration, energy production, and intracellular Ca^{2+} concentration (Przedborski and Vila, 2003; Tieu, 2011). Indeed, all known toxins which destroy dopamine neurons are complex I inhibitor, propose consequence signalling of mitochondria dysfunction as one of major culprit in dopaminergic neurodegeneration in PD (Przedborski and Vila, 2003; Tieu, 2011).

1.7.1.3. Pathogenesis of PD

The main pathogenesis of PD still needs to be precisely defined. Currently vulnerability of SNc dopamine neurons in aging and gene mutations, for at least the most part, is associated with perturbation of mitochondria to maintain energy levels and to

restrain reactive oxygen species (ROS) production. This is leading to impaired restoring intracellular Ca^{2+} concentration, particularly in the face of environmental risk factors or genetics (Mounsey and Teismann, 2010; Hirsch et al., 2013).

Although calcium is essential for a great variety of cellular processes in the CNS including activity-dependent signaling, dysregulation of basal cytosolic Ca^{2+} is highly associated with cellular death in a diverse number of neurodegenerative and aging disorders (Simons, 1988; Gleichmann and Mattson, 2011). Regulation of Ca^{2+} homeostasis is a critical process in neurons in the CNS. Due to sustained extracellular level of Ca^{2+} in brain, internal concentration of ions is maintained via diverse machineries located in plasma membrane and cytosol. Ca^{2+} streaming into neurons via receptors or channels must either be extruded out against gradient across the plasma membrane or collected by key cytoplasmic organelles including mitochondria and ER. A common feature of both systems is their great dependency on cellular energy (Gleichmann and Mattson, 2011; Grienberger and Konnerth, 2012). However, regarding to nigral neurons, the burden on cells to extrude back the entering Ca^{2+} is much larger due to steady influx of the ions into the cells. One specific feature of SNc dopamine neurons is their autonomous activity uncommonly maintained by calcium channels instead of Na^{+} passages seen in most other neurons (Surmeier, 2007; Chan et al., 2009) . This characteristic is suggested to impose extra metabolite stress on neurons regarding to cytosolic Ca^{2+} maintenance (Perier and Vila, 2012; Hirsch et al., 2013).

Several lines of evidence support alternation in cytosolic Ca^{2+} levels and its impact on neurodegeneration in PD-related models or PD individuals. Administration of neurotoxin MPP+ to mesencephalic neurons selectively enhanced intracellular Ca^{2+}

amount in the presence or absence of extracellular Ca^{2+}. Interestingly, the amount of cation was left unchanged in cortical neurons by the same treatment (Chen et al., 1995). Also, cells transformed by mitochondria obtained from PD patients, exhibited impaired Ca^{2+} buffering after MPP+ application. This supports the possible ultimate effect of complex I inhibition on dysregulation of Ca^{2+} homeostasis (Sheehan et al., 1997). Additional support came from long term rotenone-treatment of SH-SY5Y cells when partial decreased in complex I activity created oxidative damage and changes in Ca^{2+} dynamics. The proposed ultimate outcome is vulnerability of the cells to Ca^{2+} stress followed by neuronal death (Sherer et al., 2001).

Significant evidence linking Ca^{2+} toxicity to loss of dopamine cells have been also reported from examination of function and dynamics of calcium channels in nigral neurons. Interesting findings suggested an increase in the number of these channels in SNc dopamine neurons by aging (Chan et al., 2007). By utilizing PD-related toxin, Chan and colleagues proposed that cation channels on mitochondrial membrane switch to more presence of calcium channels by aging followed by increase in calcium entries and risk of ion toxicity. Suppression of channels activity protects the SNc in PD-animal models *in vitro* and *in vivo*. The relevance of the investigation lead to the proposition of calcium channel blockers as candidates for neuroprotection in PD (Chan et al., 2007; Surmeier, 2007; Chan et al., 2009; Olanow et al., 2009).

In general, regardless of original mechanisms responsible for SNc neuronal loss in PD, marked increase in free intracellular Ca^{2+} level and alternation in Ca^{2+}- mediated signalling are possible initial steps in the path to neurodegeneration, triggering either necrotic or apoptotic pathways.

1.7.1.4. Cell death pathways in PD

Necrosis and apoptosis (PCD) are two main death pathways identified in neurons. In contrast to apoptosis, defined previously, necrosis is rapid cell death characterized by membrane disruption, massive ionic inflows across the plasma membrane, ATP depletion, cell swelling, metabolic collapse, and finally plasma membrane rupture and subsequent loss of intracellular contents (Olanow and Tatton, 1999).

Necrosis has been initially regarded as the major pathogenesis of PD. Conclusions, in part, are based on studies in which acute administration, high concentration or rapid delivery, of PD-related toxins induces fast and massive neuronal loss (Olanow and Tatton, 1999; Kostrzewa, 2000). However, growing lines of evidence propose that neuronal death happening in PD is through an apoptotic process, at least to some degree. The key observations supporting the role of apoptosis in SNc neuronal loss are mostly drawn from studies on identification of apoptotic morphology and alternation in the molecules related to apoptosis (Lev et al., 2003; Tatton et al., 2003; Vila and Przedborski, 2003). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, which detects DNA fragmentation resulting from apoptosis, suggests apoptotic death in *in vitro* and *in vivo* PD models, yet controversially in post-mortem PD brains (Lev et al., 2003). Electron microscopy examinations revealed ultrastructural changes in SNc dopamine neurons related to apoptosis in brain autopsies from PD patients. Changes in the level of Bcl-2 family proteins and upregulation of proteolytic enzymes, mainly caspases, as key mediators of apoptosis, are demonstrated in PD experimental models as well as from PD cases. Overexpression of antiapoptotic molecule, bax, or suppression of proapoptotic

factor, bcl-2, has protective effects *in vivo* (Lev et al., 2003; Tatton et al., 2003; Vila and Przedborski, 2003).

1.7.1.5. Calpain in PD

Calpain is considered a major player in degradation of key cellular enzymes and proteins that mediate necrosis and apoptosis in neuronal injuries. Both apoptosis and intracellular Ca^{2+} upregulation observed in PD animal models and patients point directly to the importance of calcium-dependent proteases, specifically calpain, in pathology of PD (Vosler et al., 2008; Esteves et al., 2010). Presumably, the first evidence comes from the demonstration of upregulation in calpain-2 levels in the mesencephalon, particularly SN, of PD cases but not in the age-matched controls (Mouatt-Prigent et al., 1996). Moreover, enhancement in expression and activity of calpain has been reported in SNc, hippocampus and particularly spinal cord of mice following MPTP intoxication (Ray et al., 2000; Chera et al., 2002). However, changes in protein level or proteolysis activity of calpain may not necessarily reflect the contribution of the protease to neuronal death. Hence, several research efforts, mainly utilizing calpain inhibitors in toxin-based animal models, have been established to determine the significance of calpain activation in PD-related pathology and symptoms. Attractive supporting data regarding calpain-mediated neuronal loss in PD mouse models have been previously reported from our lab (Crocker et al., 2003). In MPTP mouse model, administration of pharmacological calpain inhibitor or adenoviral vector expressing calpastatin not only significantly blocked dopaminergic neurodegeneration, but ameliorated the deficiency in locomotor activity. In agreement with the prior work by others, calpain activity was elevated by MPTP and in PD cases (Crocker et al., 2003).

The overall findings provide important evidence to link calpain, as a major Ca^{2+}-dependent enzyme with diverse number of substrates, to dopamine neuronal loss in PD. However, most of these studies relied on approaches with potential deficiencies to provide direct evidence regarding the functional importance of calpain in PD. Hence, clear understanding of calpain role in PD pathology has yet to be established.

1.7.2. Stroke in the CNS

Stroke is defined as rapid loss of brain function as a result of transient or permanent disruption in blood flow in some part of the brain. The interruption occurs when blood vessel supplies of oxygen and nutrients, glucose in particular, to the neurons is either blocked (ischemic stroke) or ruptured (haemorrhagic stroke). The result of this deprivation is the activation of several pathological cascades of intercellular consequences within the affected tissue that will ultimately lead to cellular death and disturbance of brain function (Sources: www.heartandstroke.com) (www.canadianstrokenetwork.ca).

Stroke is a leading cause of death and serious disability in the world. In Canada, 50,000 strokes happen every year, about one every ten minutes. Sixty percent of individuals who experience acute stroke suffer lifetime disability and need help; consequently exerting huge financial, mental and social impacts on the survivors, their families and societies. Accordingly, the cost of stroke-related damages is an enormous burden on health care Canada, about $3.6 billion annually for injuries treatments such as hospital care, rehabilitation, and researches. The cost is expected to raise because of boosting in aging population (Donnan et al., 2008) (Sources: www.heartandstroke.com) (www.canadianstrokenetwork.ca).

1.7.2.1. Ischemic stroke and associated experimental models

Ischemic stroke accounts for approximate 80% of the incidents. When the blood flow decreases to less than 25% of its normal values and the condition persists for a few minutes the inner area around the blocked vessel, called core area, undergoes irreversible sever injury and instant cell death. While penumbra region, the outer tissue surrounding the core area, can be viable for several hours and slowly progresses to activation of pathological pathways and neuronal death. Thus, the peripheral tissues are the target of therapeutic intervention through enhancement of blood flow or prevention of pathological cascades (Deb et al., 2010).

Reperfusion of blood flow, if it happens in an appropriate time frame, may save the vulnerable neurons in penumbra region. Hence, one of the current targets of pharmaco-therapies is the quick removal of blood clot to prevent further damage to the brain. However, despite numerous efforts, the treatments utilizing to date are narrowed to a very limited numbers of drug which are suffering noticeable drawbacks including side effects and short time window (Markus et al., 2004; Deb et al., 2010). Cells in the penumbra area can be rescued by impeding signalling pathways leading to cell death. Neuronal cells in penumbra region are considered to be non-functional but still metabolically active (Belayev et al., 1997; Ginsberg, 2003). Thus, preventing or minimizing molecular, biochemical, and genetics mechanisms of neuronal damage may open an opportunity to save the vitality and functionality of these vulnerable cells (Ginsberg, 2003; Markus et al., 2004; Donnan et al., 2008; Deb et al., 2010).

Various cellular and molecular studies of ischemia have been developed by means of several *in vitro* and *in vivo* stroke models including oxygen and glucose deprivation

(OGD), hypoxia, and global and focal ischemia animal models. In global ischemia like 4-vessel occlusion (4-VO) the blood deprivation to induce pathological mechanisms is produced in the whole brain. In focal ischemia such as middle cerebral artery occlusion (MCAO), on the other hand, the blood starvation is locally restricted to a specific region in the brain including cortex and striatum (Graham et al., 2004).

Despite considerable improvement in ischemia studies, the lack of essential knowledge of key molecular mechanisms underlying neuronal death hampers the discovery of effective neuroprotective strategies in current post-stroke therapies.

1.7.2.2. Pathophysiology of ischemic stroke

Different types of cell death happen in ischemic brain depending on multiple factors such as duration and severity of the insult and type and vulnerability of the cells. Still, all of the pathological events originate from the same point: lack of primary sources of energy, mainly oxygen and glucose. Neurons require a great quantity of these substances for oxidative phosphorylation to meet their high need for energy (Dirnagl et al., 1999). Accordingly, to keep pace with this metabolic demand they rely on constant and sufficient blood supply. In cerebral ischemia, due to oxygen and glucose deprivation, mitochondrial malfunction and subsequent failure in energy generation initiate complex series of molecular, biochemical, and physiological mechanisms that impair neuronal function. It is because of a collapse of cellular integrity provoked by ionic imbalance, necrosis, excitotoxicity, calcium overload, oxidative stress, inflammation, and apoptosis (Lipton, 1999; Won et al., 2002). Here, we briefly define profound pathological events that happen in cells affected by ischemic injury.

(I) Necrosis/Oncosis : ATP depletion diminishes function of energy-dependent ion pumps with subsequent disruption in maintenance of ionic gradients across neuronal membranes. Hence, loss of intracellular K^+ in exchange of Na^+, Cl^-, and Ca^{2+} causes massive accumulation of ions followed by water entry to maintain osmotic equilibrium. The consequences are swelling of cytoplasmic organelles, damage to plasma membrane, leakage of cellular components, and ultimate cell lysis (Deb et al., 2010). Necrosis/oncosis is not systematically as complex as other neuronal death mechanisms such as apoptosis. It is usually distinguished by disruption of cellular membrane, swelling of cytoplasm and mitochondria, and disintegration of cytoplasmic organelles (Vanlangenakker et al., 2008; Deb et al., 2010).

(II) Apoptosis: Neuronal apoptosis has been an intense subject of investigations on cerebral ischemia due to its potential time frame for stroke therapy. Many key morphological, structural, and biochemical features of programmed cell death have been identified in *in vitro* and *in vivo* stroke models and even in human stroke (Love, 2003). Mitochondrial malfunction in cerebral stroke causes the release of cytochrome *c* and activation of procaspase-9 resulting in formation of the apoptosome and activation of caspase-9. Finally, stimulation of the caspase cascade initiated by caspase-9 leads to ultimate activation of a well-known apoptotic caspase, caspase-3 and cell death. Both release of cytochrome *c* and activation of caspases have been demonstrated in stroke animal models. Activation of caspase 3 has been detected in post-mortem samples of human brain after ischemic stroke (Broughton et al., 2009). In addition, it is proposed that cleavage and activation of cytosolic proapoptotic proteins from the Bcl-2 family triggers their translocation to mitochondria membrane with subsequent opening of

mitochondrial membrane pores and release of apoptotic components such as cytochrome *c*. Similar to caspases family, cleavage and activation of Bcl-2 family proapoptotic proteins have been identified following experimental stroke models. Inhibition of proapoptotic proteins or overexpression of antiapoptotic members of the Bcl-2 family has shown to be neuroprotective in *in vitro* and *in vivo* models (Lipton, 1999; Love, 2003; Broughton et al., 2009).

(III) Excitotoxicity: Excitotoxicity is regarded as the central mediator of neuronal death in ischemic stroke, revealed in both experimental and clinical stroke (Choi and Rothman, 1990; Waxman and Lynch, 2005). Following excessive Na^+ influx and K^+ efflux via cell membrane as a result of energy-dependent ion pumps failure, neuronal membranes are depolarized with subsequent activation of voltage-gated calcium channels and Ca^{2+} entry into the cells. Increase in intercellular Ca^{2+} leads to release of excitotoxic amino acids particularly glutamate which activates glutamate receptors including ionotropic and metabotropic receptors. Accumulation of extracellular glutamate is amplified by impairment in energy-dependent reuptake of amino acid by neurons and astrocytes (Deb et al., 2010). Although, glutamate is necessary for normal neuronal functions, prolonged excessive exposure to the neurotransmitter triggers massive stimulation of associated receptors, enhancement in Ca^{2+} influx, hyper-activation of various enzymes, and ultimate neuronal damage; all together described as excitotoxicity (Wang and Qin, 2010). It is believed that all family members of glutamate receptors participate in excitotoxicity, however, NMDA receptors, as ligand-gated cation channels permeable to Ca^{2+}, are considered to have the major contribution to the event. AMPA receptors usually do not allow the passage of Ca^{2+} due to their GluA2 impermeability, yet the concentration of the

subunit is proposed to be reduced after ischemia allowing receptors to pass Ca^{2+} (Waxman and Lynch, 2005; Wang and Qin, 2010). Disruption in cytosolic Ca^{2+} homeostasis, due to excessive ion influx or release from internal storage, is suggested to play a critical regulatory role in excitotoxic event by overactivation of several enzymes including proteases, endonucleases, and phospholipases. Uncontrolled activation of the enzymes is capable of producing several pathological changes such as cytoskeletal breakdown, DNA damage, mitochondrial dysfunction, production of free radicals, and cell membrane disruption leading to both necrotic and apoptotic neuronal death (Choi and Rothman, 1990; Castillo et al., 1997; Waxman and Lynch, 2005; Deb et al., 2010).

1.7.2.3. Calpain in ischemic stroke

High cytosolic Ca^{2+} overload in ischemic tissue leads the direction of molecular studies to the role of proteases and in particular cytosine protease calpain in pathology of ischemia (Bevers and Neumar, 2008). Calpain has been suggested to play an important role in necrosis/oncosis, one of the key features of ischemic injury. Calpain is proposed to contribute to necrosis/oncosis by two mechanisms: (1) weaken the plasma membrane integrity by cleavage of spectrin and its related cytoskeleton such as actin, (2) changes in cell osmotic balance by accelerating Ca^{2+} entry into the cell or release of Ca^{2+} from intracellular stores, reviewed in Liu et al., 2004 (Liu et al., 2004). Calpain-mediated proteolysis of spectrin was demonstrated in gerbil hippocampus after transient ischemia and gerbil hippocampal slice subjected to hypoxia. The break-down products of spectrin was detected at initial phase prior to reoxygenation. The result indicated that the cleavage of spectrin is a key response to ischemia that precedes the consequent damage of reoxygenation (Lee et al., 1991). Calpains is believed to interrupt the ion homeostasis in

the cells by modulation of Ca^{2+}-regulating proteins during the ischemic damage. For instance, cleavage of Ca^{2+} channels and plasma membrane Ca^{2+}-ATPase may disrupt the channels function results in increase in influx of extracellular Ca^{2+}. In addition, calpain perhaps enhances the release of calcium from intracellular storages such as ER and mitochondria by interfering with their Ca^{2+}-regulating proteins such as inositol (1,4,5)-trisphosphate receptor (IP3) receptor and sodium-calcium exchanger (NCX) protein respectively (Liu et al., 2004)

The initial evidence associating calpain activity with post-ischemic neurodegeneration has been collected mainly from two approaches: (1) investigation of spatial and temporal patterns of calpain activity in ischemic regions (Bevers and Neumar, 2008) and (2) administration of calpain inhibitors before or after ischemic injury (Bevers and Neumar, 2008). Several examinations have shown increase in calpain-mediated SBDPs in different regions of rodent forebrain including cortex, striatum, and hippocampus after focal and global ischemia treatments (Saido et al., 1993; Roberts-Lewis et al., 1994; Bevers and Neumar, 2008). For instance calpain-mediated cleavage of fodrin (brain spectrin) was demonstrated to occur as early as 15 mins following cerebral ischemic treatment in gerbil forebrain and hippocampus in particular. NMDA receptor antagonist MK-801 precluded calpain activation, suggesting participation of calpain in excitotoxicity in this ischemia model (Saido et al., 1993; Roberts-Lewis et al., 1994; Neumar et al., 2001; Bevers and Neumar, 2008). The suggested involvement of calpain in ischemic neurodegeneration was strengthen by various studies demonstrating that calpain inhibitors, applied before or after ischemic treatment, suppressed neuronal loss. Inhibition of calpain activity protected neurons against both cytotoxic hypoxia and global transient ischemia in

rat forebrain. A time course examination revealed that a pharmacological calpain inhibitor exerted its survival effect up to 6 hours after the focal ischemia insult (Rami and Krieglstein, 1993; Markgraf et al., 1998; Bevers and Neumar, 2008). Additional support for involvement of calpain overactivation in excitotoxicity came from the test in which reduction in calpain-1 translation utilizing antisense oligonucleotide in hippocampal slice culture resulted in neuroprotection following NMDA treatment (Bednarski et al., 1995).

Further examinations of calpain activation in ischemia focused on the wide range of calpain putative substrates; those functional alternations have been demonstrated in ischemic pathology. As previously mentioned, following ischemia multiple cellular components undergo functional disruption such as membrane receptors, plasma membrane integrity, signalling proteins, mitochondria, and nucleus. Calpain is hypothesized to cleave some key elements in these parts, e.g. AMPA and NMDA receptors, PKC and p35, during the stress process (Bevers and Neumar, 2008; Vosler et al., 2008).

Despite all the efforts up to date, the precise role of calpain in ischemic pathology remains poorly defined due to correlative evidence and the use of calpain inhibitors. The direct role of calpain in ischemic neuronal injury, particularly *in vivo*, and the key substrates by which calpain mediates neuronal death are still open questions.

1.7.3. Calpain and Cdk5/p35/p25 system

Cyclin-dependent kinase 5 (Cdk5) is a serine/threonine kinase protein, a member of Cdk family, abundantly expressed and activated in rodent brain (Tsai et al., 1993). In contrast to other Cdks, Cdk5 activity is not detected in proliferating cells and in control of cell cycle, while the kinase has shown a vital role in CNS development. Generation of knock-out mice targeting Cdk5 gene revealed prenatal lethality with significant deficiency

in brain development including cortical, hippocampal, and cerebellar structures (Ohshima et al., 1996; Gilmore et al., 1998; Ohshima et al., 1999). Furthermore, the postmitotic activity of the kinase has been linked to several cellular processes such as synaptic structure and plasticity, dopaminergic signalling, cytoskeletal dynamics, and microtubule stability (Dhavan and Tsai, 2001; Su and Tsai, 2011). Conversely, growing evidence indicates that inappropriate activation of the kinase is damaging to neurons. The most basic and significant finding on this regard is the abnormal phosphorylation of microtubule-associated protein tau by Cdk5, connected to pathology of many neurodegenerative disorders such as Alzheimer's disease (Pei et al., 1998). Hence, overall evidence has revealed that Cdk5 has critical and still opposing functions in the CNS; dual roles that may depend upon the character of the kinase activators.

Cdk5 activity appears to predominantly rely on two neurospecific regulatory proteins, p35 and p39, which restrict the kinase activity to the CNS (Tsai et al., 1994; Ko et al., 2001). p35 is suggested to bind to Cdk5 and activates the kinase principally under physiological condition that are essential for normal brain development and function (Ko et al., 2001). For example, examination of viable p35 null mice displayed a similar structural deficiency in the CNS as the Cdk5 knock-out (Tsai et al., 1994; Chae et al., 1997). Transfection of rat cortical neurons with vectors expressing antisense p35 inactivated cdk5 and inhibited neuronal outgrowth (Nikolic et al., 1996). Mice lacking p35 protein exhibit impaired depotentiation of LTP and deficiency in spatial learning and memory (Ohshima et al., 2005).

The main mechanism by which Cdk5 mediates death signalling is likely governed by calpain-mediated turnover of p35. p25, a truncated form of p35, along with Cdk5, has

been associated with phosphorylated tau *in vitro* and *in vivo*, including in the brain of AD patients (Patrick et al., 1999). In contrast to p35, p25 is a more stable protein without myristoylation signal at N-terminal, causing prolonged activation and mislocalization of Cdk5 with subsequent pathological functions (Lew et al., 1994). Conversion of p35 to p25 is induced by different neurotoxicity such as calcium overloading and administration of hydrogen peroxide and glutamate (Kusakawa et al., 2000; Lee et al., 2000). Interestingly, use of calpain inhibitor suppressed cleavage of p35 to p25 *in vitro* and *in vivo*, a fact that implicated calpain in the Cdk5/p35/p25 pathological pathway (Kusakawa et al., 2000; Lee et al., 2000).

The importance of p35/p25 as downstream target of calpain in neurodegeneration has been reported in neurodegenerative disorders such as PD, ALS, and ischemic stroke (Nguyen et al., 2001; Wang et al., 2003; Smith et al., 2006). In the studies from our lab, administration of MPTP in PD mouse model enhanced Cdk5 activity with an increase in the ratio of p25/p35 in substantia nigra. Calpain inhibitors significantly reduced the level of p25 in this mouse model (Smith et al., 2003; Smith et al., 2006). Moreover, in DNA damage insult in neuronal culture, calpain-mediated cleavage of p35 to p25 has been implicated as a potential mechanism for translocalization of p25/Cdk5 in nucleus with subsequent neuronal loss (O'Hare et al., 2005).

Although, p25 is considered a prime candidate for calpain-mediated neurodegeneration, there are some key questions needed to be addressed regarding to exclusive cleavage of p35 by the protease and the requirement of the proteolytic product in cell death.

1.8. Statement of research problem, rational and objectives

Calpain-1/calpain-2 have been suggested to cleave an array of protein substrates in the CNS and hence the consequence of protease activity in brain function and neurodegeneration has long been investigated. However, the exact function of calpain in the CNS and the role of calpain-mediated modification of most substrates are largely unknown because of correlative, indirect, and controversial findings. The major problem regarding calpain study is the lack of appropriate system to regulate calpain activity in the CNS and particularly *in vivo*. Utilizing calpain inhibitors raise some problematic issues mainly related to impermeability and non-specific effects such as inhibition of other proteases. Generation of knock-out calpain-1/calpain-2 has failed to provide an animal model to precisely examine calpain tasks in the CNS. Hence, these together leave several important questions unanswered:

1) Is calpain activity necessary for the CNS development?

2) Does calpain enhance or abolish LTP, a main feature of synaptic plasticity? And what is the major target of calpain in this system?

3) Does calpain directly mediates neuronal death in different neurodegenerative paradigms? And what is the mechanism upon which calpain exert its death signaling?

Accordingly, the research presented in this thesis was designed with the aim of exploring the role of calpain related to the CNS development, function and neurodegeneration. By utilizing a novel calpain-1/calpain-2 deficient animal **we hypothesize that calpain is essential for synaptic plasticity and learning and memory under the basal condition. Yet, under the stress conditions calpain mediates neuronal**

death in PD and ischemic stroke that may provide a useful pharmacological target for treating neurodegenerative disease.

Objectives

1) Determine whether calpain activity is necessary for the CNS development and in particular for the structures related to synaptic plasticity. This objective is presented in chapter 2.

2) Assess whether calpain activity is critical for LTP, a key feature of synaptic plasticity, and learning and memory. This objective presented in Chapter 2.

3) Determine whether calpain deficiency protects neurons against mitochondrial toxicity and excitotoxic stress related to PD and ischemic stroke *in vitro* models? This objective presented in Chapter 3.

4) Determine the downstream target of calpain in neuronal death in the PD and ischemic stroke injuries? This objective presented in Chapter 3.

Chapter 2

Conditional disruption of calpain in the CNS alters dendrite morphology and impairs LTP and learning and memory

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Statement of author contribution

The experiments presented in this manuscript were predominantly completed by Mandana Amini with the assistance of Dr. Chun-lei Ma (former post-doc in laboratory of Dr. Richard Bergeron) and contribution of Dr. Rasoul Farazifard (former post-doc in laboratory of Dr. David S. Park), Dr. Guoqi Zhu (Post-doc in Dr. Michel Baudry's lab), Fadi Hage (summer student), Dr. Jacqueline Vanderluit (former post-doc in the laboratory of Dr. Ruth S. Slack) and Dr. Joanna Susie Zoltewicz (Senior Scientist in Banyan Biomarkers, Inc). Original *Nestin-Cre* mice and *capns1*$^{flox/flox}$ mice were generated in the laboratories of Dr. Ruth S. Slack and Dr. Peter A. Greer respectively. All the backcrossing, interbreeding, and genotyping of *Nestin-Cre* and *capns1*$^{flox/flox}$ mice and progeny were performed by Mandana Amini. All experiments involving conditional knock-out verification and characterization including southern blot, western blot, and immunohistochemistry; western blot analysis of synaptic proteins, and behavioural tests were done by Mandana Amini. Confirmation of calpains activity utilizing spectrin substrate was performed by Dr. Joanna Susie Zoltewicz. Proliferation study (BrdU-staining) was carried out by Mandana Amini with assistance of Fadi Hage. Golgi staining was done by Dr. Rasoul Farazifard and Mandana Amini and data analysing was performed by Neurodigitech, LLC (San Diego, CA), Dr. Rasoul Farazifard, and Mandana Amini. The electrophysiology experiments were carried out by Dr. Chun-lei Ma, Dr. Rasoul Farazifard, and Dr. Guoqi Zhu. Dr. Diane Lagace supported with behavioural facilities and related data analysing. Dr. Richard Bergeron and Dr. Jean-Claude Beique contributed to valuable tools and inputs into synaptic studies. The figures and text for the manuscript were predominantly prepared by Mandana Amini. Figures and text related to

electrophysiology tests and Golgi staining were prepared with assistance of Dr. Chun-lei Ma and Dr. Rasoul Farazifard. The manuscript was written by Mandana Amini with the assistance and guidance from Dr. David S. Park.

Conditional disruption of calpain in the CNS alters dendrite morphology and impairs LTP and learning and memory.

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Abstract

Ubiquitous classical (typical) calpains, calpain-1 and calpain-2, are Ca^{2+}-dependent cysteine proteases which are abundantly expressed in the CNS and have been associated with numerous physiological and pathological cellular events. They have been related to CNS development/function including cell proliferation, apoptosis, signal transduction, and synaptic plasticity under normal condition; and neuronal death under stress in many neurodegenerative disorders. However, exploring the exact role(s) of calpains in the CNS has been hampered by the lack of appropriate deletion paradigms in the brain. In this study, we describe a unique model of conditional deletion of both calpain-1 and calpain-2 in mouse brain that more definitively assesses the role of these ubiquitous proteases in brain development and function. Surprisingly, we show that these calpains are not critical for gross brain development including cell proliferation and developmental programmed cell. However, our study reveals that calpain-1/calpain-2 loss leads to reduced dendritic branching complexity, spine density deficits, and reduction in expression of glutamate receptor subunits. These alterations are associated with major deterioration in hippocampal long term potentiation (LTP) and spatial memory. Our results define for the first time the direct role of calpain in CNS development and function related to synaptic plasticity; and calpain-related mechanism underlying LTP.

Introduction

Calpain is an intracellular calcium-dependent cysteine protease thought to regulate a diverse set of biological processes (Goll et al., 2003). There are at least 15 human calpain genes broadly divided into classical (typical) and non-conventional isoforms (Sorimachi et al., 2011b). The best characterized calpains are the ubiquitously expressed classical calpain-1/calpain-2 isoforms. These are heterodimers consisting of a distinct catalytic large subunit encoded by *capn1* or *capn2* genes, respectively, and a common regulatory small subunit encoded by *capns1* (previously known as *capn4*). They are also traditionally known as μ- and m-calpain, respectively, referring to micro- and milli-molar Ca^{2+} concentrations required for their activation *in vitro*. Although a large number of cellular processes have been ascribed to calpain-1/calpain-2 activities, the exact roles of these calpains, particularly in the nervous system, have never been completely clear due to lack of appropriate selective inhibition paradigms.

Calpain-1/calpain-2 are widely expressed in the CNS and have been proposed to have both normal and pathological roles (Goll et al., 2003; Ray and Banik, 2003). Functions potentially pertinent to nervous system development/function include cell death (Gil-Parrado et al., 2002; Li et al., 2009), cell proliferation (Goll et al., 2003; Konig et al., 2003), cell signalling (Sato and Kawashima, 2001), synaptic restructuring (Zadran et al., 2010a), and synaptic plasticity (Lynch and Baudry, 1984; Denny et al., 1990; Vanderklish et al., 1996). Particularly relevant to the latter has been the notion that calpain may contribute to synaptic plasticity. Pharmacological evidence suggested that calpain initiates anatomic changes in synapses, specifically in dendritic spines, by proteolysis of cytoskeletal proteins such as spectrin (Lynch and Baudry, 1987). In addition, there are a

number of other molecules postulated to be calpain substrates in synapses. These include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl D-aspartate (NMDA) receptors subunits and their anchoring proteins (Lu et al., 2001; Doshi and Lynch, 2009), post synaptic density PSD95 (Lu et al., 2000), and enzymes like PKC and CamKIIα (Goll et al., 2003). Modifications of these substrates have been proposed to account for the role of calpains in synaptic transmission. Indeed, a variety of pharmacological calpain inhibitors can block modification of these substrates and affect LTP (Denny et al., 1990). Initially, it was suggested that calpain-1 was the isoform most likely involved in LTP. However, recent work with germline calpain-1 deficient animals showed no differences in LTP (Grammer et al., 2005).

Germline disruption of CAPNS1, common small subunit, deletes calpain-1/calpain-2 activities, yet it is embryonic lethal (Arthur et al., 2000). This combined with the controversial nature of pharmacological inhibitors, all of which possess non-specific effects, has led to questions about the direct role of calpain in CNS development and function importantly synaptic plasticity.

To overcome this problem, we generated a CNS-specific calpain-1/calpain-2 deficient mouse utilizing a floxed *capns1* allele combined with the *Nestin-Cre* transgenic driver. Using this paradigm we have explored the role of calpain-1/calpain-2 in brain development and function. Given previous reports describing the essential roles of calpain in CNS development, such as cell proliferation (Konig et al., 2003; Honda et al., 2004) and developmental death (Sedarous et al., 2003; Vosler et al., 2008), we were surprised to find that calpain-deficient animals survived to adulthood and that brain morphology appeared grossly normal. However, further phenotypic characterization of these mice

revealed critical functions for calpain in dendrite morphology, synaptic plasticity, and spatial memory.

Together, our data provide significant evidence to support the direct role of calpain in synaptic plasticity and learning and memory connected to synaptic structure. Moreover, our findings indicate that although calpain activity is essential for some developmental events it is not critical for general CNS growth.

Materials and methods

Generation of CNS-specific CAPNS1 knock-out mice and genotyping. Experimental studies were approved by the University of Ottawa Animal Care Committee and conformed to the guidelines set forth by the Canadian Council on Animal Care and Canadian Institutes of Health Research, and by the IACUC from Western University of Health Sciences. Both male and female mice were used in all experiments. Conditionally targeted (floxed) *capns1* and transgenic *Nestin-Cre* were generated as previously described (Berube et al., 2005; Tan et al., 2006c). *capns1*$^{flox/flox}$ and *Nestin-Cre* mice were backcrossed for eight generations onto an FVB/N background, then interbred to obtain hemizygous *Nestin-Cre* transgenic mice that were homozygous for the loxP targeted (floxed) *capns1* gene *(Nestin-Cre; capns1*$^{flox/flox}$*).* These mice were referred to as *CAPNS1-Nestin-cKO* mice. Controls were the littermates carrying a single floxed allele of *capns1 (capns1* $^{+/flox}$). PCR was used to determine *Cre* sequences in tail biopsy DNA samples of *Nestin-Cre*, as described before (Berube et al., 2005). The *capns1* floxed and wild type alleles were detected using the primer set: forward - 5'-GTGGTAGCCGCTGAAACTCC-3'; reverse - 5'-TGTTCCCGCTCTCATCTGC-3'. The products were 550 bp for the floxed allele and 515 bp for the wild type allele. Nonradioactive DIG labelling Southern blot hybridization analysis of brain DNA was used to confirm the excision of *loxP*-flanked (floxed) sequences, indicated by a 5.1-kbp PstI fragment from the wild type *capns1* locus, a 3.2-kbp PstI fragment from the pre-Cre floxed allele, and a 4.3-kbp fragment from Cre recombinase-excised (post-Cre floxed) allele, as previously described (Tan et al., 2006c).

Immunoblotting and casein zymogram. Total brain fractions of the *CAPNS1-Nestin-cKO* mice and control littermates were resolved by SDS-PAGE and PVDF immunoblots were prepared and probed using a rabbit polyclonal antisera raised against rat calpain-2 that recognizes both the small subunit (28 kDa/CAPNS1) and calpain-2 large subunit (80 kDa/CAPN2), as previously described (Tan et al., 2006c). Casein zymogram of the same brain lysates was used to detect calpain-1 and calpain-2 activities as previously described (Tan et al., 2006c). In brief, total brain lysis was resolved on 8% nondenaturing polyacrylamide gels containing casein (1.5 mg/ ml). Calpain was activated by incubating the gels overnight with 5 mM $CaCl_2$ and its activity was assessed by visualization of the casein-cleared regions of the gel after staining with Coomassie Brilliant Blue. AlphaII-spectrin break down protein (αII-spectrin BDP) and active calpain-1 analysis was described previously (Martinez et al., 2010). Briefly, brain lysates from *CAPNS1-Nestin-cKO* or control mice were made using 1% TritonX-100. 100µg of total protein was incubated with the following additives: 1µg purified calpain-2 + 10mM $CaCl_2$, or 10mM $CaCl_2$, or 10mM EGTA, and incubated at room temperature for 30 minutes to allow digestion to occur. 50µg of each reaction was resolved on a 4-20% gel Tris-glycine gel, blotted to PVDF and immunoblotted with antibodies against calpain cleaved αII-spectrin or active calpain-1. Immunoblot analysis of hippocampus protein fractions for GluN1, GluN2A, GluN2B, PSD95, GluA1, and GluA2/3 was previously described (Imamura et al., 2008). In brief, hippocampal regions from the brain of 4-6-week-old mice were dissected and homogenized using ice cold tobacco etch virus protease (TEVP) lysis buffer. After a series of centrifugations, the membrane protein fractions were collected and protein concentrations were determined using Bio-Rad protein assay. Primary antibodies:

anti-calpain-2 (a kind gift of Dr. Peter A. Greer, 1:1000) (Tan et al., 2006c); anti-αII-spectrin (α-Fodrin) (mouse monoclonal, Enzo, 1:3000); anti-activated calpain-1 (rabbit polyclonal generated in laboratory of Banyan Biomarkers Inc. FL, USA, 1:300; antibody was raised against the peptide LGRHENA corresponding to the new N-terminus produced by calpain-1 autolysis); anti-NMDAR1 (GluN1) (Rabbit polyclonal, Cell Signalling, 1:1000); anti-NMDAR2A (GluN2A) and anti-NMDAR2B (GluN2B) (Rabbit polyclonal and mouse monoclonal respectively, LifeSpan BioScience, Inc, 1:750); anti-GluR1 (GluA1) (Rabbit monoclonal, Upstate Cell Signalling Solutions, 1:5000); anti GluR2/3 (GluA2/3) (Rabbit polyclonal, Chemicon, 1:1000); anti-PSD95 (Rabbit polyclonal, Cell Signalling, 1:1000).

Tissue preparation, immunohistochemistry, and phenotypic analysis. Tissue preparation was previously described (Rashidian et al., 2005; Vanderluit et al., 2007). Briefly, coronal serial sections (14 μm) were obtained from whole fixed cryoprotected brains of 4-6-week-old mice, and embryos at embryonic days (E) 13.5 and 17.5. Analyses of CA1 pyramidal cell density was performed using cresyl violet-stained sections from 4-6-week-old mice. CA1 neurons of hippocampus were counted using ImageJ Program in two representative sections per animal in a field of 70 mm^2. For short-term bromodeoxyuridine (BrdU) incorporation experiments, pregnant dams at 13.5 days received a single intraperitoneal injection of BrdU (100 μg/g of body weight) and the embryos were collected 2 h after injection. To detect BrdU labelled nuclei, sections were denatured in 2 N HCl at 37°C for 30 min and incubated in rat anti-BrdU (1:500; Accurate Chemicals & Scientific, WESTBURY, NY), as previously done (Vanderluit et al., 2007). $BrdU^+$ neurons were counted on 70 mm^2 of VZ and SVZ field of 4 representative sections

per embryos using ImageJ program. TUNEL assay was performed using the Roche TdT kit. Briefly, embryonic brains at E17.5 were fixed, and 14 µm sections were washed with 0.5% TritonX-100 in PBS incubated with TdT and dUTP at 37ºC, and then blocked with 10% BSA. TUNEL+ cells were detected by staining with Alexa Streptavidin 594 and counted throughout whole sections of different regions of brains, 12 sections per brain.

Golgi staining. Golgi staining was completed using FD Rapid GolgiStain™ Kit (FD NeuroTechnologies, Inc. Catonsville, MD). Briefly, 4-6-week-old naïve mice were intracardially perfused with 0.9% cold saline. Forebrains within the region ~0.50 to ~ -3.5 mm (relative to the bregma position) were separated and stained according to the manufacturer's instructions. Tissues were sectioned at 150 µm and mounted on gelatin-coated slides. Morphology of apical and basal dendrites of hippocampal CA1 pyramidal neurons were blindly analysed by Neurodigitech, LLC, San Diego, CA. Commercially stereology-based software (Microbrightfield, VT), installed on a Dell PC workstation that controlled a Zeiss Axioplan 2 image microscope with Optronics MicroFire CCD camera (1600 x 1200 digital camera, motorized X, Y, and Z-focus for high-resolution image acquisition and digital quantitation) was used. For cell selection, the sampling process for candidate pyramidal cells was conducted as follows: (1) previewing the entire anterior-to-posterior axis of CA1 region, under low-and high-mag Zeiss objectives (10x and 20x), (2) comparing and locating those cells with the least truncations of distal dendrites as possible under higher-mag Zeiss objectives (40x and 63x), (3) after verifications, capturing low-magnification images for archiving purpose, and then (4) using a Zeiss 100x objective with immersion oil (N.A=1.25) to perform 3D dendritic reconstruction. For spine sampling, only spines orthogonal to the dendritic shaft were readily resolved and included

in this analysis, whereas spines protruding above or beneath the dendritic shaft were not sampled. Spines were counted throughout the entire dendritic trees. For Scholl analysis, a series of concentric circles (30 μm interval) radiating from the soma was set up. All data represented the average for neurons.

Electrophysiology. *Whole-cell recordings*. Coronal brain slices containing the hippocampus were obtained as described (Imamura et al., 2008). In brief, 4-6-week-old mice were anaesthetized with isofluorane in agreement with the guidelines of the Canadian Council of Animal Care. The brain was removed and placed in an oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF) at 4°C, containing (in mM) 126 NaCl, 2.5 KCl, 1 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$ and 10 glucose. The osmolarity of the ACSF was adjusted to 300 mOsmol and the pH to 7.2. A vibrating microtome (VT 1000S; Leica, Bannockburn, IL, USA) was used to obtain coronal sections (300 μm) containing the hippocampus. Acute brain slices were stored for at least 1 h in an oxygenated chamber at room temperature before recording. Voltage-clamp recordings of CA1 pyramidal cells were obtained with a Multiclamp 700A amplifier (Axon Instruments, Foster City, CA, USA) under visual control using differential interference contrast and infrared video microscopy (IR-DIC) (DMLFSA, Leica, Germany). Excitatory postsynaptic currents (EPSCs) were evoked by electrical stimulation of the Schaffer collaterals with a bipolar microelectrode positioned in stratum radiatum (str. rad.) with 100 μs current pulses (0.1-1 mA, 0.3-0.01 Hz), which were adjusted to evoke a current amplitude in the range of 60-120 pA at Vm = -65 mV. Patch-clamp recordings were obtained in pyramidal cells (PCs) of CA1 region in hippocampus in normal ACSF. For recordings of excitatory postsynaptic currents mediated by glutamate receptors (both AMPA receptor and NMDA

receptor EPSCs), brain slices were bathed in ACSF containing picrotoxin (50 μM) and strychnine (1 μM); For recordings of NMDA receptor-mediated excitatory postsynaptic currents (NMDA receptor-EPSCs) brain slices were bathed in low Mg^{2+} ACSF containing picrotoxin (50 μM), strychnine (1 μM) and 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[*f*]quinoxaline (NBQX) (5 μM); for recordings of miniature excitatory postsynaptic currents (mEPSCs) brain slices were perfused in low Mg^{2+} ACSF containing TTX (1μM), picrotoxin (50 μM), strychnine (1 μM), CGP52432 (10 μM); for LTP brain slices were bathed in ACSF containing picrotoxin (50 μM) and strychnine (1 μM). The pH of the perfusion solution was adjusted to 7.2 and osmolarity to 300 mOsm. Recording electrodes were filled with an intracellular solution (in mM): 130 Cs^{+}-methanesulphonate, 10 HEPES, 10 KCl, 2 $MgCl_2$, 0.2 EGTA, 2 ATP-Mg and 0.2 GTP-tris (hydroxymethyl) aminomethane and lidocaine N-ethyl bromide (QX-314, 5mM). The pH of the intracellular solutions was adjusted to 7.2 and the osmolarity was 280-290 mOsm. The pipettes had a resistance of 3-7 MΩ. For eliciting LTP a pairing protocol composed of three brief high-frequency trains (50 pulses at 100 Hz) repeated at 4 s intervals given at the end of a long depolarization (3 min at 0 mV) was used. This protocol has been successfully used to induce an increase of synaptic responses lasting for more than 40 min (Chen et al., 1999; Martina et al., 2004). Data were collected by Pclamp 9 software (Molecular Devices, Sunnyvale, CA, USA). Analyses were performed using Igor software (WaveMetrics, Lake Oswego, OR, USA).

Field potential recording. Three-4-month-old male mice were anesthetized with halothane and decapitated. Brains were quickly removed and transferred to oxygenated, ice-cold, high-magnesium cutting medium (in mM: 124 NaCl, 26 $NaHCO_3$, 10 glucose, 3 KCl, 1.25

KH_2PO_4, 5 $MgSO_4$, and 3.4 $CaCl_2$). Hippocampal transversal slices (400 μm-thick) were prepared using a McIlwain-type tissue chopper and transferred to an interface recording chamber and exposed to a warm, humidified atmosphere of 95%O_2/5%CO_2 and continuously perfused with oxygenated and preheated (33 ± 0.5 °C) ACSF (in mM: 110 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.5 $MgSO_4$, 1.24 KH_2PO_4, 10 glucose, 27.4 $NaHCO_3$) at 1.4 ml/min. After 1.5 h incubation at 33 ± 0.5 °C in the recording chamber, a single glass pipette filled with 2 M NaCl was used to record field EPSPs (fEPSPs) elicited by stimulation of the Schaffer collateral pathway with twisted nichrome wires (single bare wire diameter, 50 μm) placed in CA1 stratum radiatum (str. rad.). Responses were recorded through a differential amplifier (DAM 50, World Precision Instruments, USA) with a 10 kHz high-pass and 0.1 Hz low-pass filter. Before each experiment, the input/output (I/O) relation was examined by varying the intensity of the stimulation. Data were collected and digitized by Clampex, and the amplitude of fEPSP was analyzed.

All data are expressed as means ± SEM. and statistical significance of differences between means was calculated with appropriate Student's *t-test*.

Behavioural analysis. All behavioural tests were completed in the Behaviour Core Facility at the University of Ottawa using standardized protocols. Animals were habituated to the testing room ~1 h prior to testing. One cohort of mice (3-4-month-old) was used for beam break, rotarod, elevated plus maze and open field within at least 2 days between tests. Due to aging deficits in vision that can occur in the FVB/N strain, a separate cohort of younger mice (4-6-week-old) was examined for the water maze paradigm.

Beam Break: Locomotor activity over a 24 h period was analysed by using the computer-assisted beam break, MicroMax system (Accuscan, Columbus, OH) as previously reported (Smith et al., 2006).

Rotarod: The rotating rod test was performed on an accelerating rotarod (IITC Life Science; length of the test 2 min; accelerating speed 1-45 rpm). The time until the animal falls off the rotating rod was measured in four tests performed on two consecutive days.

Elevated Plus Maze: The arms were ~12 cm wide and ~50 cm long and the maze was raised ~1 meter off the floor. The times spent in the open arms, closed arms, and center of the elevated plus-maze apparatus were recorded for 10 min using the Ethovision software.

Open Field: Mice were placed in the corner of a wooden box measuring 45 cm × 45 cm × 45 cm and allowed to freely explore for 10 min. The main outcome measures included the time spent in the center, distance traveled, and velocity were detected by a video camera using the Ethovision software.

Morris Water Maze: The water maze pool was filled with opaque water and heated to remain at 21°C. A white platform was submerged 1 cm below the water's surface in the center of the target quadrant. Mice were randomly placed on one of starting points in one of the four quadrants and given 60 s to find the hidden platform. Mice that did not find the platform at the end of the 60 s period were led to the platform and were allowed to stay on it for 20 s. Each mouse had 4 trials per day for 11 consecutive days. An animal was scored as successfully finding the platform if it succeeded in 2/4 trials per day. In the visual tests, mice were placed on one of four quadrants and allowed to find the visible platform in 60 s. The swimming path of the mice was recorded by a video camera and analyzed by the Ethovision 7 XT.

Data and statistical analysis. Statistical analysis was carried out using by SPSS Statistics 18 and included use of either; (1) two tailed Student's *t*-test for comparison of two groups, (2) one-way ANOVA for comparison of four groups on one outcome measure, (3) two-way ANOVA for more than one group on more than one outcome measure, (4) repeated ANOVA (for repeated measure on same animal), and (5) chi-square (χ^2) to examine the distribution of animals achieving preset criteria. All posthoc tests were completed using Bonferroni correction. In analyses that included both male and female animals, sex differences were also tested. However, as all comparisons between sexes did not reveal any differences, only grouped data for controls and mutants are shown. Significance was marked by * when $P<0.05$.

Results

Elimination of calpain-1 and calpain-2 activities in mouse brain

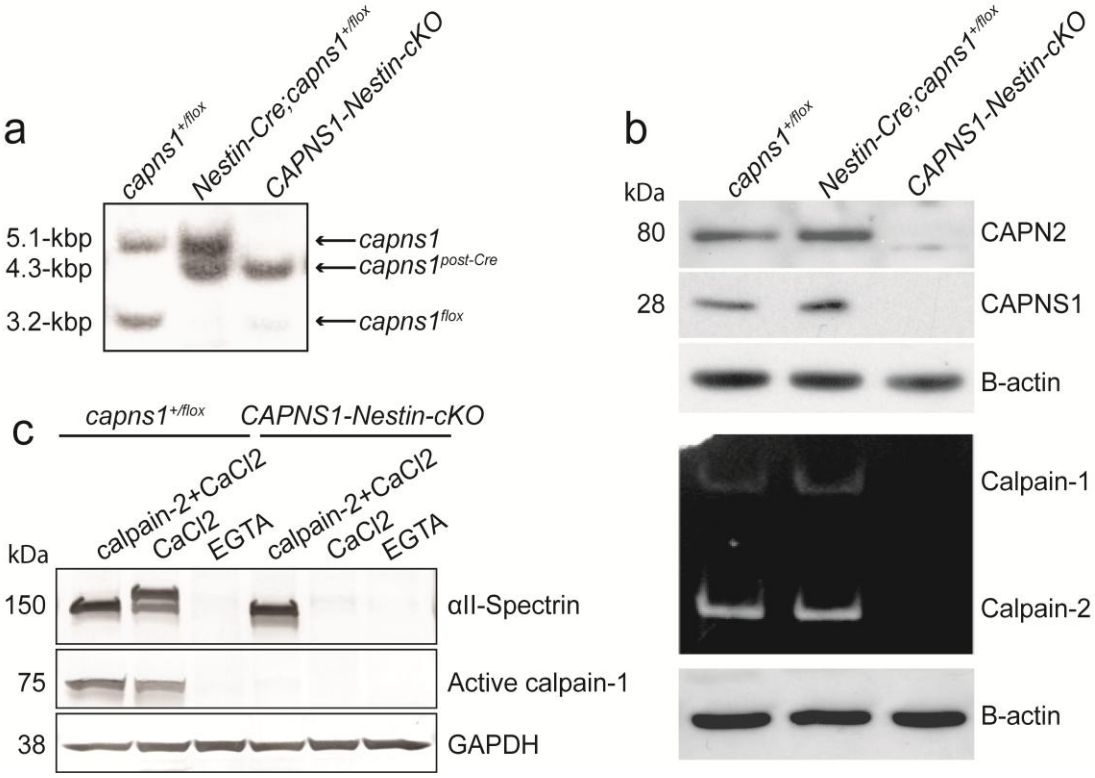
We used a Cre/loxP-mediated recombination strategy to conditionally target the *capns1* locus in the mouse brain. Exons 9, 10, and 11 of *capns1* were flanked by *loxP* sites as described previously (Tan et al., 2006c). To obtain CNS-specific disruption of calpain-1/calpain-2 activities, *capns1*$^{flox/flox}$ mice were crossed with *Nestin-Cre* transgenic mice expressing Cre recombinase under the control of the *nestin* promoter, to create *Nestin-Cre*; *capns1*$^{flox/flox}$ *(CAPNS1-Nestin-cKO*) mice. Nestin is an intermediate filament expressed specifically in the CNS precursor cells (Berube et al., 2005). The *Nestin-Cre* transgenic construct contains the Nestin neural-specific enhancer, indicating the specific expression of Cre recombinase in neurons. Surprisingly, the *CAPNS1-Nestin-cKO* were viable, fertile, and were born at approximate Mendelian ratios (n=49/225; 21.7%)

Southern blot analysis of genomic DNA revealed that complete excision of *capns1* occurred only in the brains of mice that were transgenic for *Nestin-Cre* and homozygous for the *loxP* targeted (flox) *capns1* gene. These *CAPNS1-Nestin-cKO* mice brains demonstrated near-complete excision of floxed *capns1* alleles, as determined by the generation of a 4.3-kbp size post-Cre allele fragment **(Fig. 2.1a)**. To assess calpain expression in the brains of these mice, we first performed immunoblot analysis using an antibody raised against calpain-2, which recognizes both the 28 kDa *capns1* and 80 kDa *capn2* gene products. As expected, the brains of *CAPNS1-Nestin-cKO* mice displayed complete loss of the 28 kDa subunit. Importantly, and as previously reported, loss of CAPNS1 also led to the loss of 80 kDa CAPN2 protein presumably due to the destabilizing effects of CAPNS1 loss (Arthur et al., 2000; Chung et al., 2004) **(Fig. 2.1b)**.

The effect of *capns1* targeting in brain on calpain protease activity was assessed using two methods. First, casein zymogram analyses of brain lysates showed no detectable calpain-1 or calpain-2 activity in the brains of *CAPNS1-Nestin-cKO* mice **(Fig. 2.1b)**, whereas robust activity for both calpain isoforms was observed in controls. Second, endogenous calpains were activated *in vitro* in whole brain lysates by adding $CaCl_2$, which yielded the well-characterized calpain-specific spectrin breakdown products (Zhang et al., 2009), SBDP145 and 150, in control but not in *CAPNS1-Nestin-cKO* brain extracts (Martinez et al., 2010). Disruption of calpain activity was further supported using an antibody specific for the active form of calpain-1, which did not detect the active 80 kDa catalytic subunit in *CAPNS1-Nestin-cKO* brain lysates **(Fig. 2.1c)**. Together, these results provided compelling evidence of calpain-deficiency in the brains of *CAPNS1-Nestin-cKO* mice.

Figure 2.1. Disruption of calpain-1 and calpain-2 expression and activities in the *CAPNS1-Nestin-cKO* mouse brain. (a) Southern blot analysis of brain DNA samples from *CAPNS1-Nestin-cKO* (*Nestin-Cre; capns1* $^{flox/flox}$) and control littermates (*Nestin-Cre; capns1* $^{+/flox}$ or *capns1* $^{+/flox}$). The probe distinguishes 5.1, 4.3 and 3.2-kbp PstI fragments corresponding to untargeted (*capns1*) or the floxed allele after (*capns1*$^{post\text{-}Cre}$) or before (*capns1*^{flox}) Cre-mediated excision, respectively. **(b)** Top, Representative immunoblot of calpain-2 (CAPN2) (80 kDa) and CAPNS1 (28 kDa) proteins extracted from brains of *CAPNS1-Nestin-cKO* and control littermates probed with calpain-2 antibody; Bottom, Casein zymogram of the brain total fraction to analyse calpain-1 and calpain-2 activities (n=5 per genotype). **(c)** Representative immunoblot of brain total protein extracts probed with indicated antibodies for αII-spectrin and active calpain-1 (n=3 per genotype). $CaCl_2$ triggered cleavage of spectrin to its calpain-specific 145 and 150 kDa breakdown products in control but not *CAPNS1-Nestin-cKO* brain extracts, while addition of rat calpain-2 enzyme cleaved spectrin to 150 kDa in brain lysates of both genotypes.

Figure 2.1

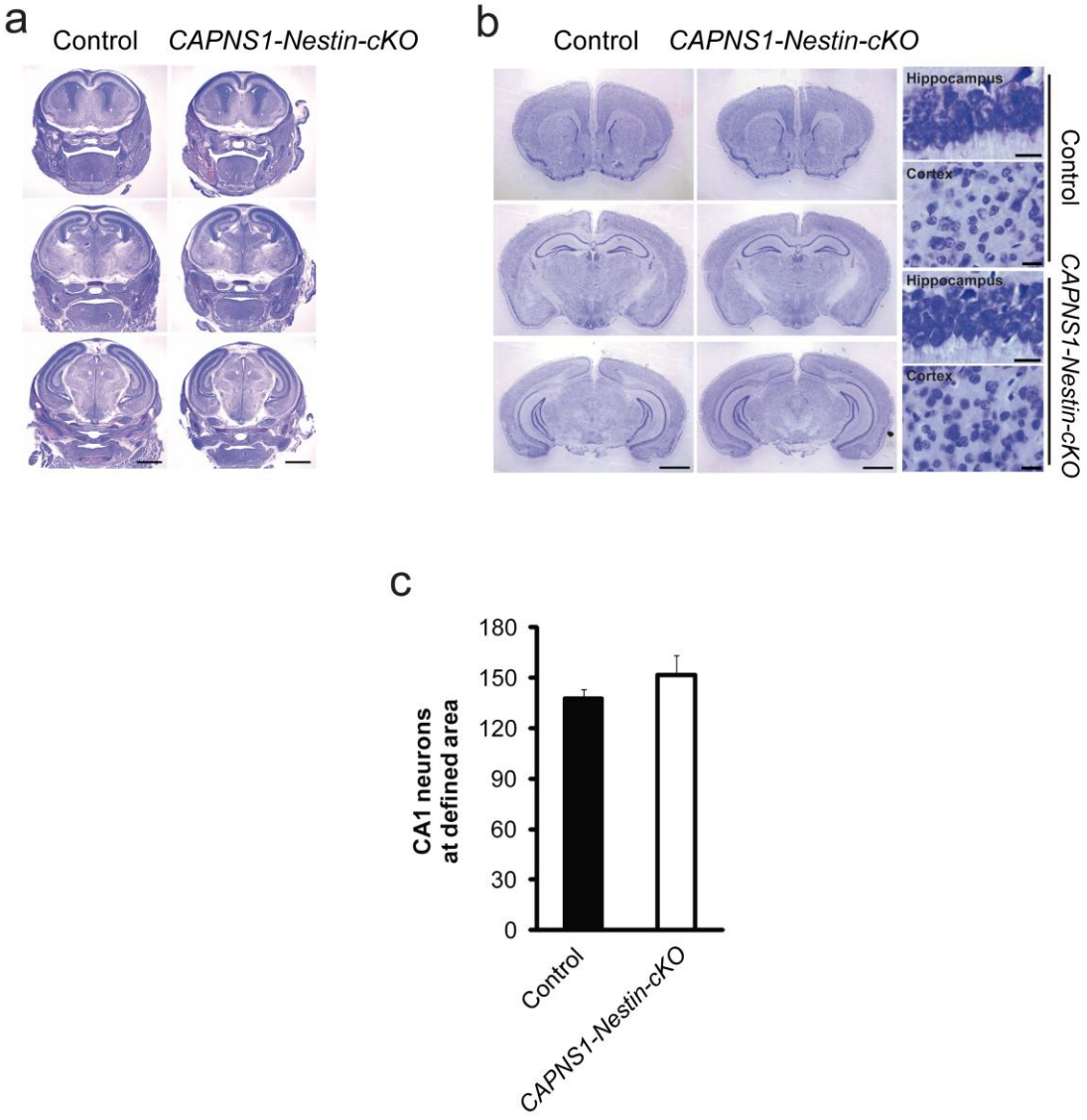

a
capns1+/flox
Nestin-Cre;capns1+/flox
CAPNS1-Nestin-cKO
5.1-kbp
4.3-kbp
3.2-kbp
capns1
capns1post-Cre
capns1flox
b
kDa
80
28
CAPN2
CAPNS1
B-actin
Calpain-1
Calpain-2
B-actin
c
capns1+/flox
CAPNS1-Nestin-cKO
calpain-2+CaCl2
CaCl2
EGTA
kDa
150
75
38
αII-Spectrin
Active calpain-1
GAPDH

Calpain-deficient mice do not show detectable gross morphological abnormalities

The importance of calpain in early murine development has been previously established by the lethality observed in both *capns1* and *capn2* germline knock-out mice (Arthur et al., 2000; Dutt et al., 2006). The viability of adult *CAPNS1-Nestin-cKO* animals with brain-specific calpain-deficiency allowed us to explore the role of calpain in CNS development. Serial coronal sections of brains from mutant and control littermates at developmental and adult stages were stained with cresyl violet. Unexpectedly, we did not detect any gross morphological or structural abnormalities in the brain regions and neurons of the mutants either at E13.5 (data not shown), E17.5 or 4-6-week-old adult mice **(Fig. 2.2a-b)**. Quantification of hippocampal CA1 neurons in the mutants demonstrated the same number of pyramidal neurons as in controls **(Fig. 2.2c)**.

Figure 2.2. Histological assessment of *CAPNS1-Nestin-cKO* mice compared to control littermates. (a-b) Representative cresyl violet staining of coronal sections through different regions of brains from **(a)** E17.5 and **(b)** 4-6-week-old adult *CAPNS1-Nestin-cKO* mice and controls, respectively, including different regions (scale bar represents 1 mm), and neurons in hippocampus and cortex (scale bar represents 20 μm). **(c)** Number of CA1 neurons in defined fields at 4-6-week-old age (n=3 per genotype).

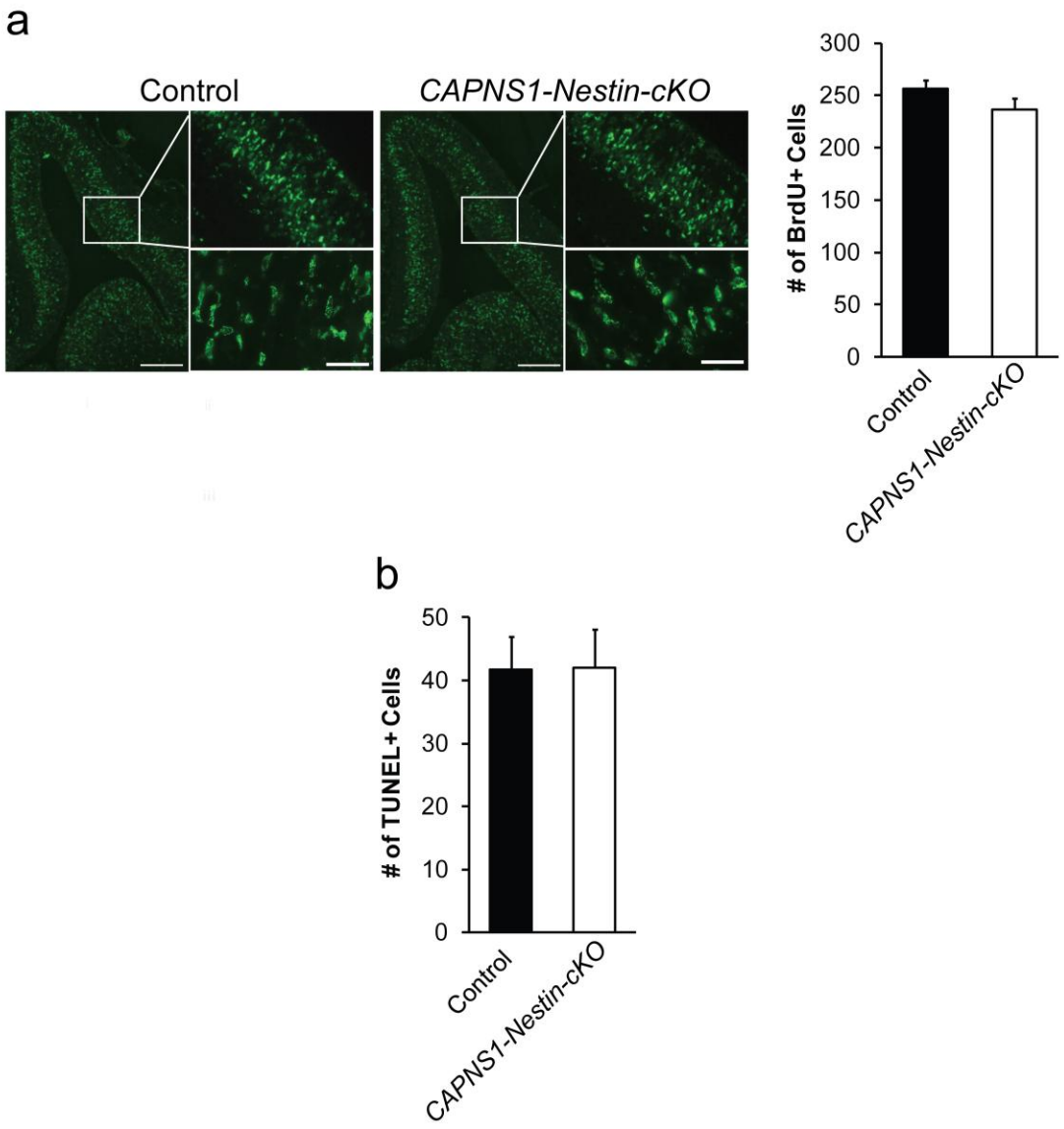
Figure 2.2


a
Control
CAPNS1-Nestin-cKO
b
Control
CAPNS1-Nestin-cKO
Hippocampus
Cortex
Hippocampus
Cortex
Control
CAPNS1-Nestin-cKO
c
CA1 neurons at defined area
180
150
120
90
60
30
0
Control
CAPNS1-Nestin-cKO

We also examined whether neuronal precursor proliferation was altered in mutant mice. Embryos at E13.5 were subjected to short-term BrdU labeling and BrdU-positive nuclei were evaluated in the ventricular zone (VZ) and subventricular zone (SVZ) of the brain. No differences in the number of proliferating cells were detected between mutant and control mice (**Fig. 2.3a).** Similarly, developmental programmed cell death, i.e., apoptosis, was detected by TUNEL staining at E17.5. Equal numbers of scattered TUNEL-positive cells were observed in both genotypes **(Fig. 2.3b)**. These results indicated that calpain-1/calpain-2 isoforms do not play essential roles in regulating developmental cell proliferation/apoptosis or the gross organization or structure of the developing and adult brains.

Figure 2.3. Histological assessment of *CAPNS1-Nestin-cKO* mice compared to control littermates. (a) Representative photomicrographs of BrdU-stained sections of embryonic brains from *CAPNS1-Nestin-cKO* and control littermates. Scale bars represent 175 µm and 20 µm in left and right panels respectively. Number of BrdU-positive cells were counted in defined fields of sections, (n=4 per genotype). **(b)** Number of TUNEL-positive cells throughout whole brain sections (n=3 per genotype).

Figure 2.3

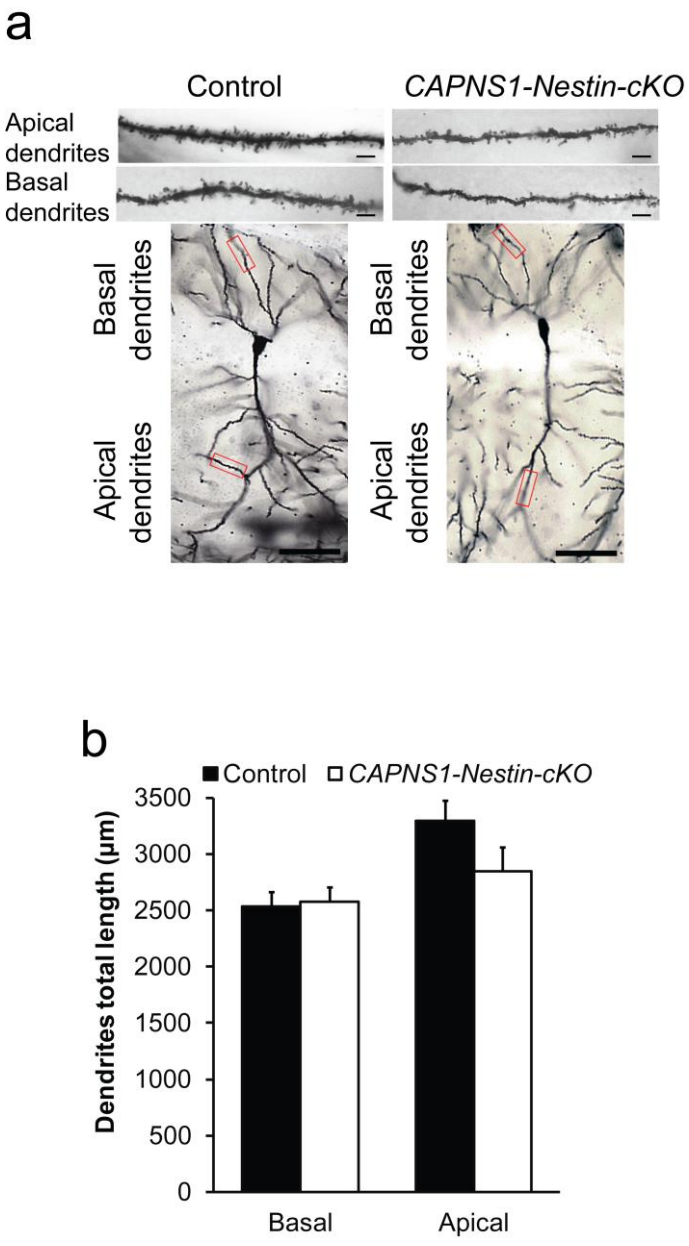

a
Control
CAPNS1-Nestin-cKO
# of BrdU+ Cells
300
250
200
150
100
50
0
Control
CAPNS1-Nestin-cKO
b
# of TUNEL+ Cells
50
40
30
20
10
0
Control
CAPNS1-Nestin-cKO

Dendrite morphology alterations in calpain-deficient CA1 pyramidal neurons

Since calpain has been suggested to modify cytoskeletal proteins (Wilson et al., 2000), we sought to investigate whether dendritic structures of CA1 hippocampal neurons were altered in *CAPNS1-Nestin-cKO*. We thus performed Golgi staining of dorsal hippocampus in 4-6-week-old controls and conditionally calpain-deleted brains. Representative photomicrographs of CA1 pyramidal neurons and segments of apical and basal dendrites in control littermates and *CAPNS1-Nestin-cKO* mice are shown in **Fig. 2.4a**. No significant changes were observed in dendritic lengths with calpain-deficiency **(Fig. 2.4b).**

Figure 2.4. Morphology of hippocampal CA1 neuron dendrites in *CAPNS1-Nestin-cKO* mice. (a) Representative segments of apical and basal dendrites of CA1 neurons in *CAPNS1-Nestin-cKO* and control littermates. Scale bar represents 5 µm. Bottom, representative photomicrograph of CA1 pyramidal neurons. Scale bar represents 50 µm. **(b)** Representative graphs of basal and apical dendrites total length. (n=27 neurons for control littermates and n=20 neurons for *CAPNS1-Nestin-cKOs*).

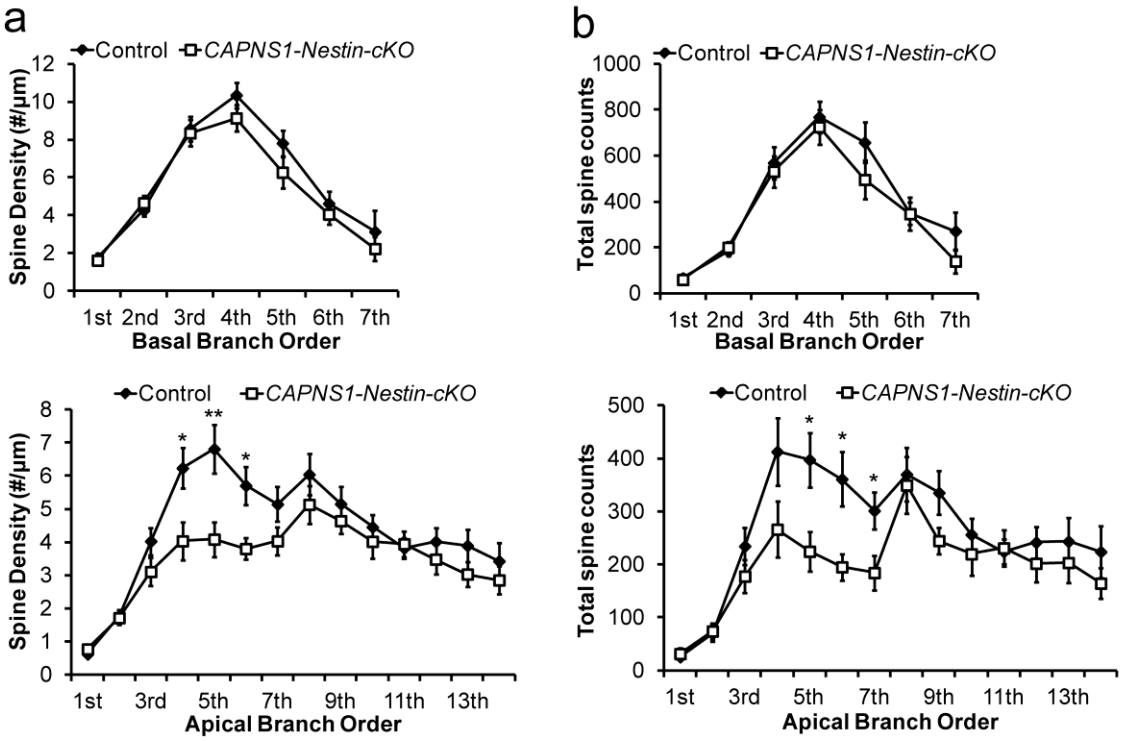
Figure 2.4


a
Control
CAPNS1-Nestin-cKO
Apical dendrites
Basal dendrites
Basal dendrites
Apical dendrites
Basal dendrites
Apical dendrites
b
Control
CAPNS1-Nestin-cKO
Dendrites total length (μm)
3500
3000
2500
2000
1500
1000
500
0
Basal
Apical

However, we observed a significant reduction in the density of dendritic spines along the apical branches of CA1 pyramidal neurons of the mutants. This decrease in spine density was also accompanied by a significant decline in total spine counts in apical dendrites in calpain-deficient mice **(Fig. 2.5a-b)**.

Figure 2.5. Altered morphology of hippocampal CA1 neuron dendrites in *CAPNS1-Nestin-cKO* mice. (a) Representative graphs of spine density of basal and apical dendrites per branch orders. Spine density is expressed as the mean spine number per 1 µm dendritic segment. **(b)** Representative graphs of total spine count of basal and apical dendrites per branch orders. (n=27 neurons for control littermates and n=20 neurons for *CAPNS1-Nestin-cKOs*). Data are represented as means ± SEM, *Repeated ANOVA*, $*P < 0.05$, $**P < 0.01$

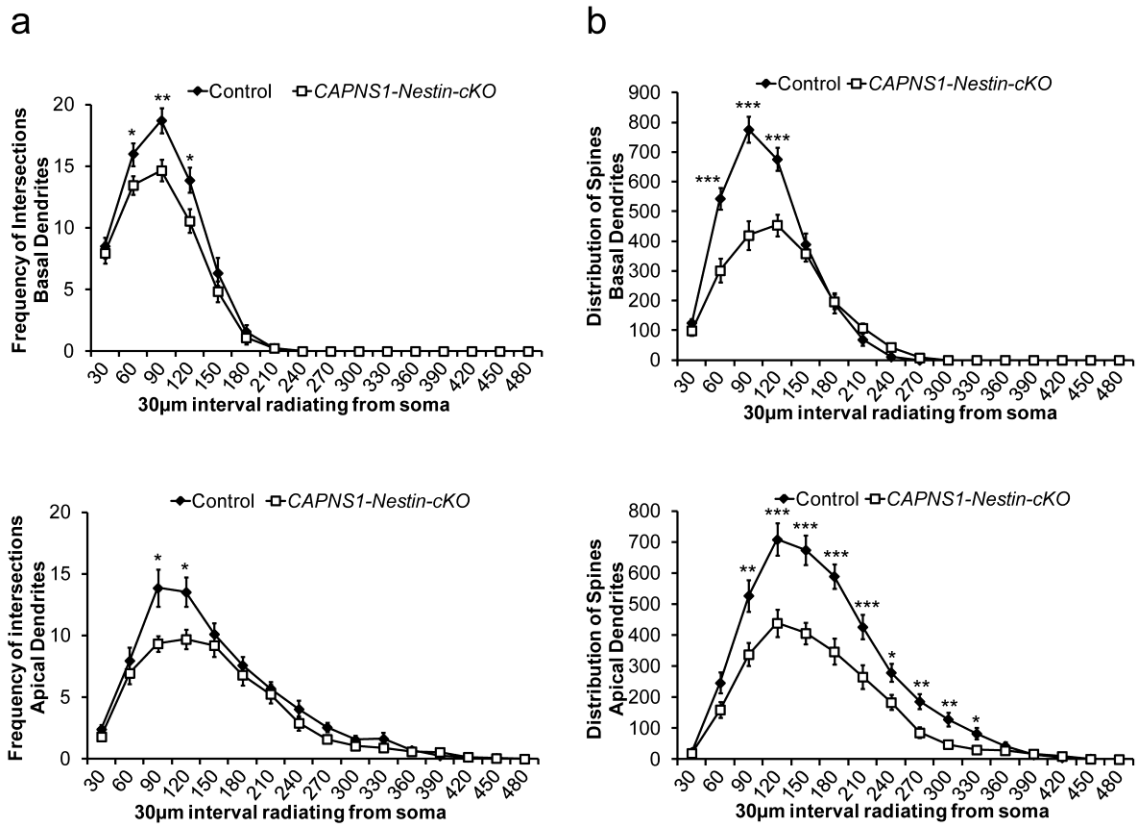
Figure 2.5


a
Control
CAPNS1-Nestin-cKO
Spine Density (#/µm)
0
2
4
6
8
10
12
1st 2nd 3rd 4th 5th 6th 7th
Basal Branch Order
b
Control
CAPNS1-Nestin-cKO
Total spine counts
0
200
400
600
800
1000
1st 2nd 3rd 4th 5th 6th 7th
Basal Branch Order
Control
CAPNS1-Nestin-cKO
Spine Density (#/µm)
0
1
2
3
4
5
6
7
8
*
**
*
1st 3rd 5th 7th 9th 11th 13th
Apical Branch Order
Control
CAPNS1-Nestin-cKO
Total spine counts
0
100
200
300
400
500
*
*
*
1st 3rd 5th 7th 9th 11th 13th
Apical Branch Order

Moreover, apical and basal dendritic complexity and spine distribution were reduced in CA1 pyramidal neurons of *CAPNS1-Nestin-cKO,* as revealed by Scholl analysis **(Fig. 2.6a-b)**. These results suggest a critical role of calpain in regulation/organization of dendritic trees in hippocampal CA1 neurons.

Figure 2.6. Altered morphology of hippocampal CA1 neuron dendrites in *CAPNS1-Nestin-cKO* mice. (a) Scholl analysis of intersection pattern of basal and apical dendrites in CA1 neurons. **(b)** Distribution of spines in basal and apical dendrites at 30-μm interval radiating distances from the soma in CA1 neurons. (n=27 neurons for control littermates and n=20 neurons for *CAPNS1-Nestin-cKOs*). Data are represented as means ± SEM, *Repeated ANOVA*, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

Figure 2.6

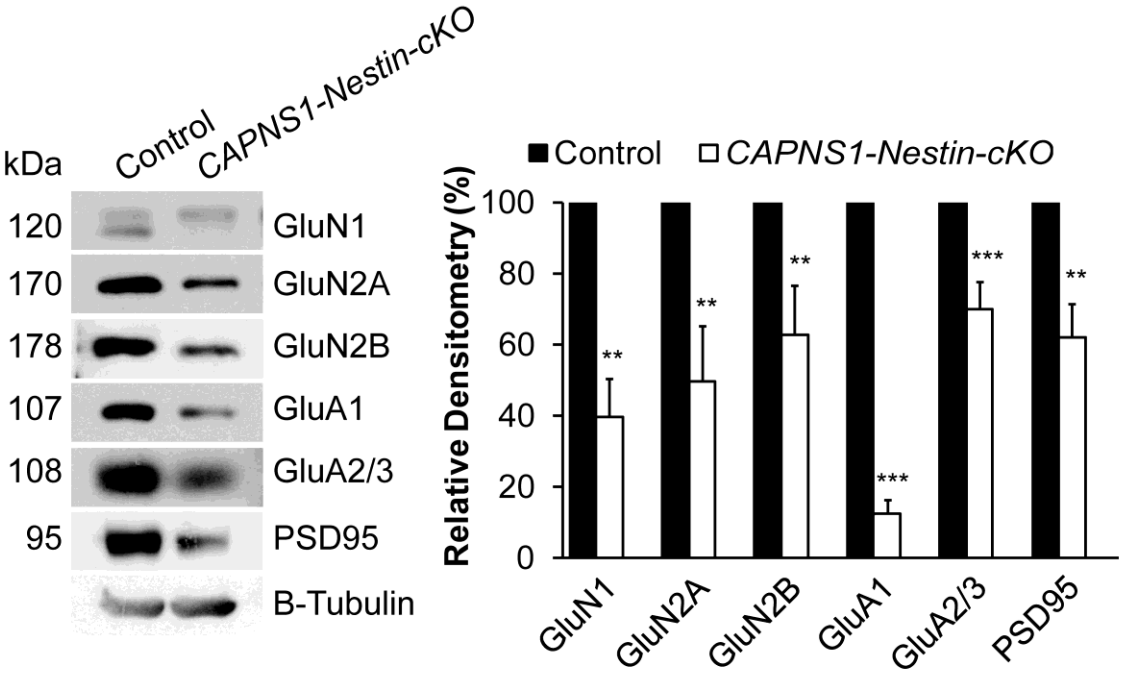

a
b
Control
CAPNS1-Nestin-cKO
Frequency of Intersections Basal Dendrites
Distribution of Spines Basal Dendrites
Frequency of intersections Apical Dendrites
Distribution of Spines Apical Dendrites
30µm interval radiating from soma

Effect of calpain-deficiency on glutamatergic synaptic transmission and LTP

The overall alterations observed in dendritic complexities and spine density in *CAPNS1-Nestin-cKO* suggested that calpain may play a role in modulating glutamatergic synaptic transmission. To begin addressing this possibility, we measured the expression of a number of AMPA and NMDA receptor subunits in isolated membrane fractions from hippocampi of 4-6-week-old *CAPNS1-Nestin-cKO* and controls. Intriguingly, we observed robust reductions in the levels of the NMDA receptor subunits GluN1, GluN2A and GluN2B as well as of the AMPA receptor subunits GluA1 and GluA2/3. Finally, the expression of PSD95 was also reduced in mutant mice, as compared to control littermates **(Fig. 2.7)**.

Figure 2.7. Hippocampal membrane protein distribution. Representative immunoblots of membrane protein fractions of hippocampi from control and *CAPNS1-Nestin-cKO* probed with the indicated antibodies and densitometric quantification of changes in grey values expressed as means ± SEM, Student's *t-test,* $^{**}P < 0.01$, $^{***}P < 0.001$ (per genotype: n=4 for GluN1 and GluA1; n=5 for GluN2A, GluN2B, and PSD95; n=3 for GluA2/3).

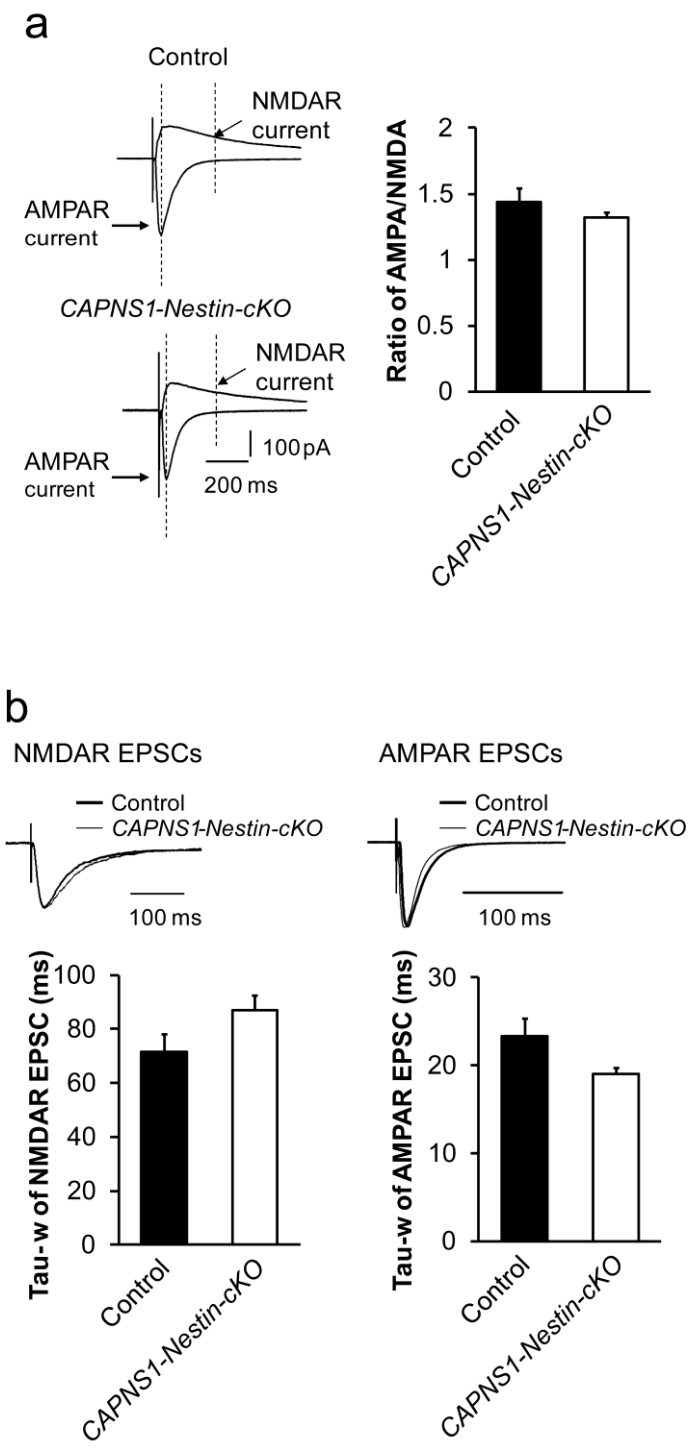
Figure 2.7


kDa
Control
CAPNS1-Nestin-cKO
120
GluN1
170
GluN2A
178
GluN2B
107
GluA1
108
GluA2/3
95
PSD95
B-Tubulin
Control
CAPNS1-Nestin-cKO
Relative Densitometry (%)
100
80
60
40
20
0
**
**
**
***
***
**
GluN1
GluN2A
GluN2B
GluA1
GluA2/3
PSD95

These results prompted us to examine in greater detail metrics of glutamatergic synaptic transmission by whole-cell recordings of CA1 pyramidal cells (PCs) in acute slices from *CAPNS1-Nestin-cKO* and control mice. We first determined the ratio of AMPA to NMDA receptor components of evoked excitatory postsynaptic currents (eEPSCs) in both types of mice. When holding the cell at +40 mV, both AMPA and NMDA receptors are activated by synaptically released glutamate, and their respective contribution to the eEPSCs can be approximated because AMPA receptor- and NMDA receptor-mediated synaptic responses are kinetically distinguishable. Under these conditions, the AMPA/NMDA ratio of eEPSCs was indistinguishable between *CAPNS1-Nestin-cKO* and control mice (**Fig. 2.8a**). Moreover, the decay kinetics of both isolated AMPA receptor- or NMDA receptor-mediated (in low Mg^{2+}) EPSCs were likewise not altered in mutant mice (**Fig. 2.8b**), suggesting that no obvious changes in surface glutamate receptor subunit composition occurred in these mice.

Figure 2.8. Glutamatergic synaptic transmission in hippocampal CA1 neurons of *CAPNS1-Nestin-cKO* and controls. (a) Representing traces of AMPAR and NMDAR EPSCS at V-hold of -65 and +40 mV, respectively (*CAPNS1-Nestin-cKO* 1.32 ± 0.04, n=9 neurons from 3 mice; and controls 1.4 ± 0.1, n=8 neurons from 4 mice). At -65 mV the peak current is mainly contributed by AMPA receptors, while at +40 mV the peak current comes from both AMPA and NMDA receptors. **(b)** Representative traces and weighted decay time constants of NMDAR and AMPAR EPSCs in *CAPNS1-Nestin-cKO* (NMDAR 87.07 ± 5.46 ms, n=11 neurons from 4 mice and AMPAR 19.05 ± 0.64 ms, n=9 neurons from 4 mice) and controls (NMDAR 71.55 ± 6.42 ms, n=6 neurons from 4 mice and AMPAR 23.36 ± 2.01 ms, n=9 neurons from 4 mice).

Figure 2.8

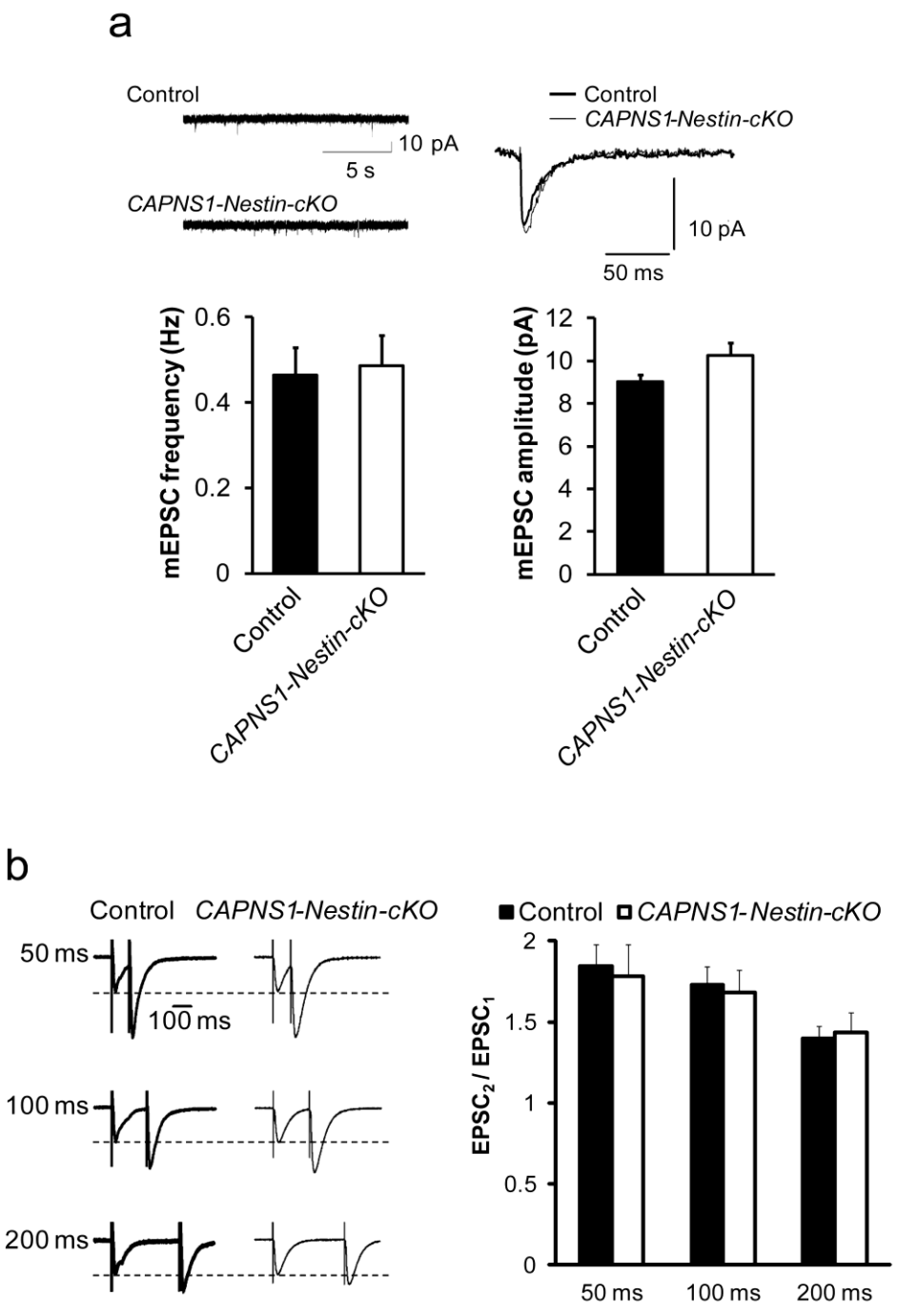

a
Control
NMDAR current
AMPAR current
CAPNS1-Nestin-cKO
NMDAR current
AMPAR current
100 pA
200 ms
Ratio of AMPA/NMDA
2
1.5
1
0.5
0
Control
CAPNS1-Nestin-cKO
b
NMDAR EPSCs
AMPAR EPSCs
Control
CAPNS1-Nestin-cKO
100 ms
100 ms
Tau-w of NMDAR EPSC (ms)
100
80
60
40
20
0
Control
CAPNS1-Nestin-cKO
Tau-w of AMPAR EPSC (ms)
30
20
10
0
Control
CAPNS1-Nestin-cKO

We next recorded spontaneous miniature EPSCs (mEPSCs) in CA1 pyramidal neurons and observed no significant changes in the amplitudes or the frequency of mEPSCs in calpain-deficient mice, as compared to control animals (**Fig. 2.9a**). These results are in agreement with those of the AMPA/NMDA receptor ratio and suggest that deletion of calpains did not alter the expression of AMPA receptors at individual synapses, or the probability of release of glutamate. The latter possibility was further examined by paired pulse ratio analysis, a measure of presynaptic neurotransmitter release probability. As expected for CA1 synapses, paired pulse facilitation was observed at intervals of 50, 100 and 200 ms and this ratio was not altered in *CAPNS1-Nestin-cKO* mice **(Fig. 2.9b)**.

Altogether, these observations argue that while calpain-deficiency was accompanied by reduced levels of a variety of synaptic proteins; this was not associated with measurable deficits in basal excitatory glutamatergic neurotransmission.

Figure 2.9. Glutamatergic synaptic transmission in hippocampal CA1 neurons of *CAPNS1-Nestin-cKO* and controls. (a) Representative traces and averages of spontaneous AMPAR-mediated mEPSCs recorded from neurons of *CAPNS1-Nestin-cKO* (amplitude, 10.27 ± 0.59 pA; frequency, 0.48 ± 0.07 Hz, n=8 neurons from 3 mice) and control mice (amplitude, 9.01 ± 0.34 pA; frequency, 0.46 ± 0.07 Hz, n=8 neurons from 4 mice). **(b)** Representative traces and average of paired pulse ratios of EPSCs (AMPAR+NMDAR) from neurons of *CAPNS1-Nestin-cKO* mice (50 ms, 1.78 ± 0.2; 100 ms, 1.68 ± 0.14; 200 ms, 1.44 ± 0.12, n=9 neurons from 3 mice) and controls (50 ms, 1.85 ± 0.13; 100 ms, 1.73 ± 0.11; 200 ms, 1.39 ± 0.07, n=12 neurons from 3 mice).

Figure 2.9


a
Control
10 pA
5 s
CAPNS1-Nestin-cKO
Control
CAPNS1-Nestin-cKO
10 pA
50 ms
mEPSC frequency (Hz)
0.6
0.4
0.2
0
Control
CAPNS1-Nestin-cKO
mEPSC amplitude (pA)
12
10
8
6
4
2
0
Control
CAPNS1-Nestin-cKO
b
Control
CAPNS1-Nestin-cKO
50 ms
100 ms
100 ms
200 ms
Control
CAPNS1-Nestin-cKO
EPSC2 / EPSC1
2
1.5
1
0.5
0
50 ms
100 ms
200 ms

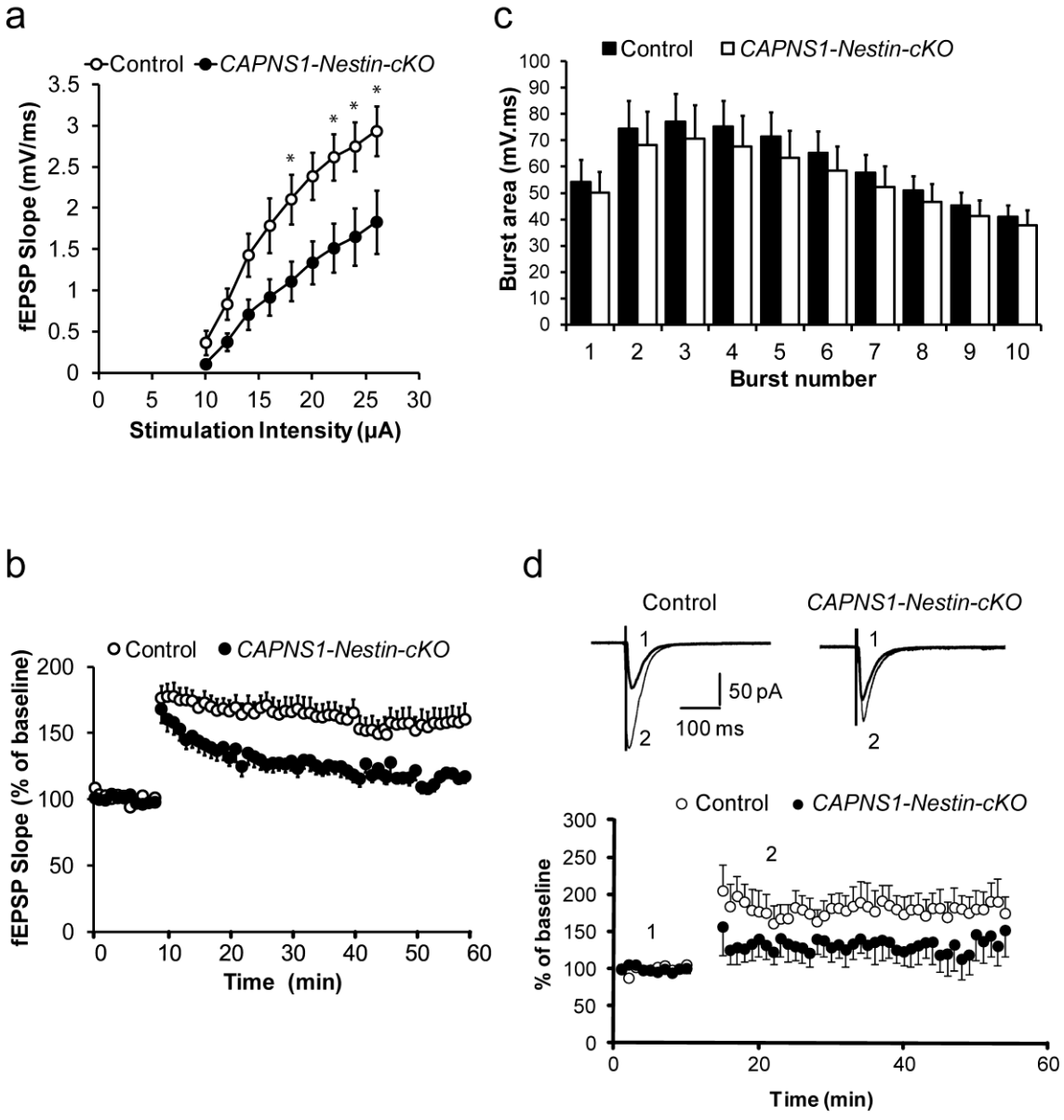
We further analyzed properties of synaptic transmission in the CA1 field of hippocampal slices from control and *CAPNS1-Nestin-cKO* mice by field recording. No alteration was observed in paired-pulse facilitation in the absence of calpain (data not shown). However, the input/output curve (stimulation intensity to evoked response) was significantly shifted downwards in the mutant as compared to controls **(Fig. 2.10a)**. The decrease in field excitatory postsynaptic potentials (fEPSPs) was about 30-40%, broadly matching the decrease in the density of synapses observed in the mutant CA1 pyramidal neurons. We also determined the effects of theta burst stimulation (TBS) on fEPSPs **(Fig. 2.10b)**. Interestingly, TBS induced LTP was significantly reduced in hippocampal slices from *CAPNS1-Nestin-cKO* mice (field EPSP slope, compared with baseline, at 50 min after LTP induction: control 161 ± 10%; mutant 123 ± 6%; * $p < 0.001$). Analysis of the burst responses during TBS revealed that, while the absolute levels of each burst responses were lower in the mutant than in the control, the overall increase observed during successive bursts exhibits a similar pattern in control and mutant mice **(Fig. 2.10c)**.

The magnitude of LTP was also studied with whole-cell recording using a pairing protocol consisting of 3 brief high frequency tetani (50 pulses at 100 Hz, 4 s intervals) given at the end of a 3 min long depolarization step at 0 mV. This protocol induced a 90.9% ± 11.9 (control, $n = 6$; $p < 0.005$) increase in the amplitude of EPSC lasting for more than 40 min. This LTP was NMDA receptor-dependent since it was prevented by application of DL-2-amino-5-phosphonovaleric acid (AP-5, 50 μM; -9.50% ± 8.77 above baseline; $n = 4$; $p > 0.05$, data not shown). Interestingly, the same pairing protocol administered to slices from *CAPNS1-Nestin-cKO*s induced only minimal LTP (control 181 ± 24%; mutant 132 ± 29% at 30-40 min after LTP induction) **(Fig. 2.10d)**.

Together, these data indicate that calpain plays critical role in LTP, although the deficiency of the protease activity does not affect the basal excitatory glutamatergic neurotransmission.

Figure 2.10. Features of synaptic transmission and plasticity in calpain-deficient mice assessed with field and patch clamp recording. (**a)** Input/output curves. Amplitudes of the field EPSPs were determined for various intensities of stimulation. The curve is significantly shifted downwards in the mutant mice. The results are means ± SEM, Student's *t-test*, $*P < 0.05$ (n=5-6 slices from 5 mice per genotype) **(b)** TBS-induced LTP. Theta burst stimulation (TBS) was delivered after 10 min of baseline recording and slopes of the fEPSPs monitored for an additional 50 min. The values are normalized to the baseline and are means ± SEM, Student's *t-test*, $***P < 0.001$ (n=5-6 slices from 5 mice per genotype) **(c)** Areas of the burst responses during theta burst stimulation (10 bursts of 4 pulses delivered at 100 Hz, with an interburst interval of 200 ms). Results are means ± SEM with no statistical differences between the two groups of mice. (5-6 slices from 5 mice per genotype) **(d)** Patch clamp recording of LTP. Representative traces of EPSC amplitudes 10 min before (1) and 50 min after (2) HFS pairing paradigm. Bottom, time course of relative changes of EPSCs (for control n=6 neurons from 4 mice, for *CAPNS1-Nestin-cKO* n=5 neurons form 4 mice). Data are means ± SEM, Student's *t-test*, p value is from the data comparison of 30-40 min EPSCs average of mutants to those of control, $***P < 0.001$.

Figure 2.10

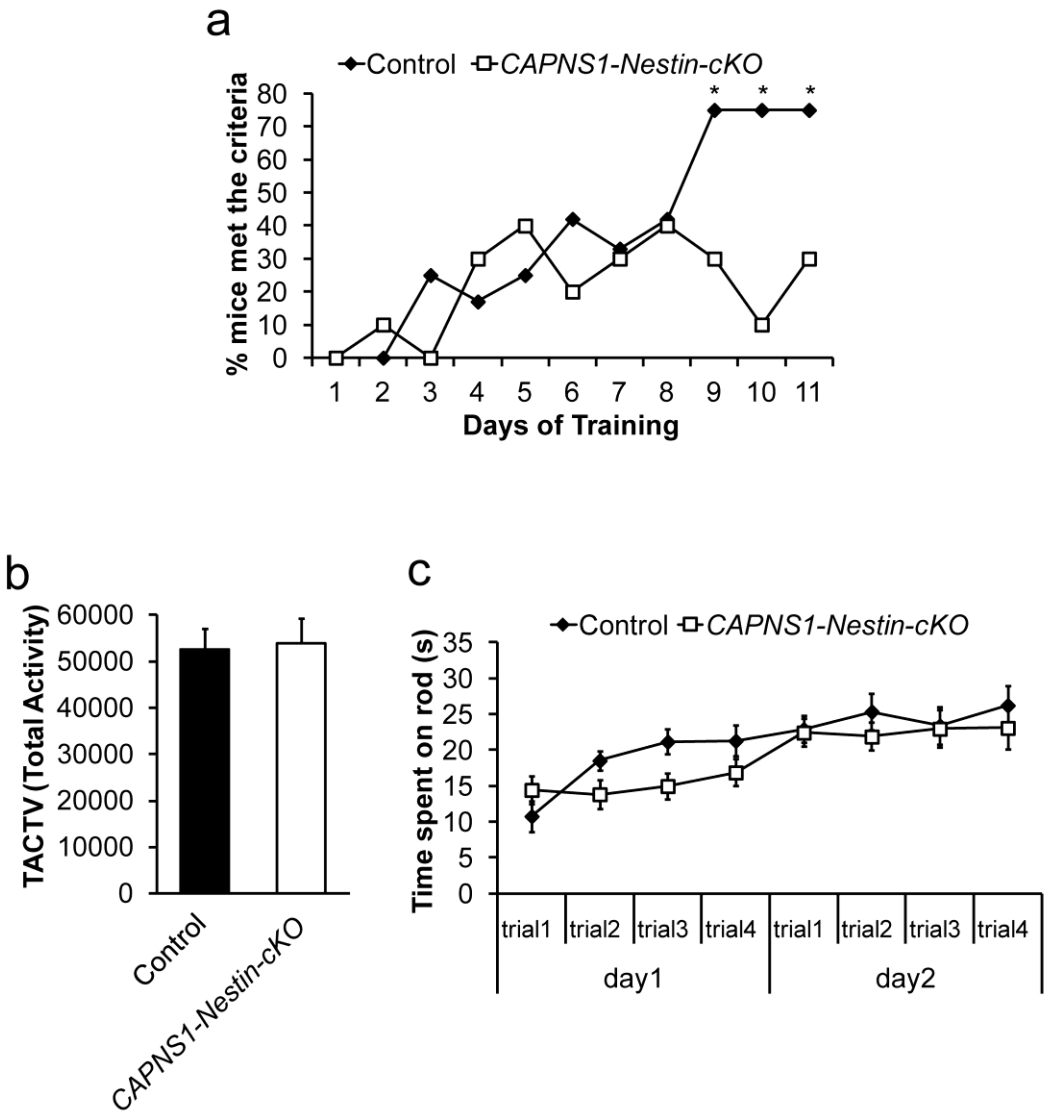

a
Control
CAPNS1-Nestin-cKO
fEPSP Slope (mV/ms)
Stimulation Intensity (μA)
c
Control
CAPNS1-Nestin-cKO
Burst area (mV.ms)
Burst number
b
Control
CAPNS1-Nestin-cKO
fEPSP Slope (% of baseline)
Time (min)
d
Control
CAPNS1-Nestin-cKO
50 pA
100 ms
1
2
Control
CAPNS1-Nestin-cKO
% of baseline
Time (min)

Impairments in spatial memory in calpain-deficient mice

We next determined whether the robust reduction in hippocampal LTP observed in *CAPNS1-Nestin-cKO* would be accompanied with deficits in hippocampal-dependent spatial memory. We thus tested *CAPNS1-Nestin-cKO* in the Morris water maze (MWM) task. Significantly less *CAPNS1-Nestin-cKO* mice were able to reach the hidden platform on the last three days of training when compared to littermate control mice suggesting that *CAPNS1-Nestin-cKO* have impaired spatial memory (**Fig. 2.11a)**. This behavioural deficit was not related to deficiencies in vision since there was no significant difference in the average time to find the visual platform (data not shown). In addition, examination of general motor activity by beam break and rotarod tests did not reveal any differences in the mutants (**Fig. 2.11b-c)**.

Figure 2.11. Spatial learning is deficient in *CAPNS1-Nestin-cKO* mice while motor activity appears normal. (a) Percentage of *CAPNS1-Nestin-cKO* (males n=6, females n=4) and control littermate (males n=5, females n=7) mice successfully reaching the criteria of finding the platform in the Morris Water Maze, as described in the methods. **(b)** Locomotor activity assessed in a novel cage test **(c)** motor coordination examined by rotarod test. Data are represented as means ± SEM, chi-square (χ_2) and *Repeated ANOVA*, *$P < 0.05$, (controls: males n=10, females n=11; *CAPNS1-Nestin-cKO*: males n=11, females n=10).

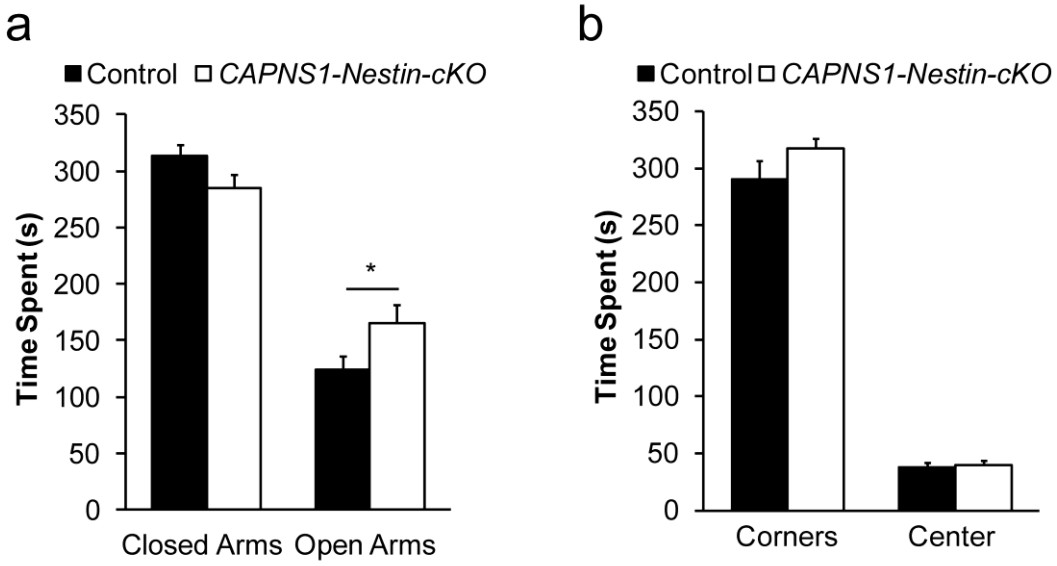
Figure 2.11


a
Control
CAPNS1-Nestin-cKO
% mice met the criteria
Days of Training
b
TACTV (Total Activity)
Control
CAPNS1-Nestin-cKO
c
Control
CAPNS1-Nestin-cKO
Time spent on rod (s)
trial1
trial2
trial3
trial4
day1
day2

Finally, while the calpain-deficient mice showed less anxiety behaviour in elevated plus maze test **(Fig. 2.12a)**, the differences in the open field anxiety test were not observed **(Fig. 2.12b)**. Collectively, these observations suggest that calpain is necessary for hippocampal LTP and learning and memory, and this may be related to a role in modulating the structure of neuronal dendrites and synapses.

Figure 2.12. Anxiety behaviour appears normal in calpain-deficient mice. Anxiety observation carried out with elevated plus maze **(a)** and open field tests **(b)**. Data are represented as means ± SEM, Student's *t-test* and *Repeated ANOVA*, $*P < 0.05$, (controls: males n=10, females n=11; *CAPNS1-Nestin-cKO*: males n=11, females n=10).

Figure 2.12


a
Control
CAPNS1-Nestin-cKO
Time Spent (s)
350
300
250
200
150
100
50
0
*
Closed Arms
Open Arms
b
Control
CAPNS1-Nestin-cKO
Time Spent (s)
350
300
250
200
150
100
50
0
Corners
Center

Discussion

Although four decades have elapsed since the discovery of the ubiquitous calpains, the biological roles of calpain remain elusive. Animal models to identify the function of these proteases in the CNS have been limited due to early embryonic lethality or lack of phenotype in germline knock-out mice. CAPN2 knock-out mice (lacking calpain-2) died prior to the implantation stage of development and germline deletion of CAPNS1 (disrupting both calpain-1 and calpain-2) caused embryonic lethality due to cardiovascular defects (Arthur et al., 2000; Dutt et al., 2006). Although CAPN1 knock-out mice (lacking calpain-1) survived to adulthood, they did not display brain phenotypes such as alterations in synaptic plasticity (Grammer et al., 2005). In the present studies, we describe a unique conditional deletion model of both calpain-1 and calpain-2 to assess the roles of these proteases in brain development and function.

Calpain in Development

Studies utilizing pharmacological calpain inhibitors have supported a role for calpain in several biological processes critical for CNS development. Our own experience (Sedarous et al., 2003) as well as others (Luo and O'Leary, 2005; Touma et al., 2007) has shown that pharmacological calpain inhibitors can cause dramatic and rapid axon retraction *in vitro*, implicating calpain in the biochemistry of axon maturation and maintenance (Qin et al., 2010). In addition, calpain has been implicated in developmental neuronal death, a process critical for sculpting the mature brain (Gil-Parrado et al., 2002; Li et al., 2009). However, as previously mentioned, pharmacological calpain inhibitors suffer from considerable drawbacks, importantly non-specificity.

Given these observations, it was surprising that mice with brain calpain-deficiency are viable and born with normal general morphology and unaltered developmental apoptosis and proliferation in the brain. Considering that nestin expression begins at E.7.5 (Berube et al., 2005), a time when the earliest processes of CNS development occurs (Theiler, 1989), our findings strongly suggest that although calpain may play crucial roles in development of other organ systems, it appears dispensable for general CNS establishment.

While gross brain development and architecture was normal, calpain-deficiency resulted in a decline in spine populations and deterioration of branch ramifications/bifurcations in basal and apical dendrites in hippocampal CA1 pyramidal neurons. A caveat to these observations is that we cannot rule out the possibility that loss of staining rather than loss of spine structures is occurring. However, these staining differences would have to occur specifically in spines since soma and dendritic structures are clearly present. Our results are also supported by previous work suggesting that calpain mediates changes in cytoskeletal structure and organization (Wilson et al., 2000; Fischer et al., 2005) by cleaving potential substrates including spectrin and MAP2 (Fischer et al., 1991; Zadran et al., 2010a).

Calpain in synaptic plasticity

The changes in neuronal structural complexity led us to examine whether this was associated with altered synaptic transmission. Indeed, calpain has also been suggested to cleave a number of substrates relevant to excitatory transmission (Doshi and Lynch, 2009). Our results show that brain-specific calpain-deficiency leads to reduced levels of

some glutamate receptor components and PSDs. These findings suggest that calpain does not directly cleave these substrates, but may actually regulate their levels of expression. This is possibly through effects of calpain on signaling pathways regulating gene expression. Calpain is implicated in the cleavage of several transcription factors such as c-Jun and c-Fos (Goll et al., 2003), MEF2 (Smith et al., 2006), and NFκB (Sedarous et al., 2003). These transcriptional factors are proposed to regulate expression of NMDA receptor subunits (Bai and Hoffman, 2009). Intriguingly, the ultrastructural and synaptic protein deficits observed in the *CAPNS1-Nestin-cKOs* did not translate into obvious basal defects at individual glutamatergic CA1 synapses, since the amplitude of mEPSCs were unaltered. This may reflect a general robust compensatory ability of synapses to maintain faithful transmission despite deletion of key synaptic components [e.g., GluA1 (Zamanillo et al., 1999), TARP γ-8 (Rouach et al., 2005), GluA2 (Toyoda et al., 2009)]. The overall decrease in input/output responses observed in CA1 region *CAPNS1-Nestin-cKOs* may reflect the overall decrease in spine density observed.

Calpain-deleted mice displayed a significant reduction in LTP supporting a central role for calpain in synapse plasticity. This is also consistent with our results showing reduced memory as evaluated in the Morris water maze test. The observed reduction in LTP with calpain loss is consistent with a number of previous observations. First, pharmacological as well as antisense-based inhibitors of calpains blocked LTP (del Cerro et al., 1990; Denny et al., 1990; Vanderklish et al., 1996). Furthermore, calpains are localized in spines and can be activated by threshold levels of inducing stimulation (Baudry and Lynch, 2001; Zadran et al., 2010b). However, and in contrast to these findings, calpain-1 knock-out mice did not display impaired LTP (Grammer et al., 2005).

Whether this discrepancy is due to differences in the LTP paradigm utilized is unclear at present. Here, we provide compelling genetic evidence that calpain is required for LTP. Considering the absence of an LTP phenotype in calpain-1 knock-out mice (Grammer et al., 2005), our observations suggest that either calpain-2-deficiency is responsible for this phenotype in *CAPNS1-Nestin-cKO* mice, or that there is functional redundancy between calpain-1/calpain-2 isoforms with respect to this function. The former interpretation is also consistent with recent reports indicating that calpain-2 can be activated by Ca^{2+}-independent systems (Zadran et al., 2010b) giving a possible explanation for calpain-2 activation with physiological intracellular calcium concentration; and that calpain-2 down-regulation using a novel rabies-virus glycoprotein-chimeric peptide to deliver calpain-2 siRNA to the brain resulted in impairments in LTP and learning and memory (Zadran et al., 2012). Finally, it is important to emphasize that we cannot presently distinguish between subtle developmental defects, which may affect LTP/behavior in these calpain-deficient mice versus more specific effects of calpain-deficiency on signaling processes in the adult brain.

In summary, our analysis of a conditional model of calpain-deficiency in the CNS has provided a clearer understating of calpain biology in the brain regarding CNS development and synaptic plasticity. We believe that this conditional model will continue to generate critical information on calpain function in the brain.

Chapter 3

Classical calpain promotes neuronal death by cleavage of p35, a cdk5 co-activator, to p25 in Parkinson's and ischemic related injuries

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David S. Park

Statement of author contribution

The experiments presented in this manuscript were predominantly completed by Mandana Amini. Original *Nestin-Cre* mice, *capns1*$^{flox/flox}$ mice, and *TH-Cre* mice were generated in the laboratories of Dr. Ruth S. Slack, Dr. Peter A. Greer, and Dr. Joseph M. Savitt respectively. All the backcrossing, interbreeding, and genotyping of the transgenic mice and progeny were performed by Mandana Amini. All experiments involving cell culturing, activity assays, survival experiments, and western blot were done by Mandana Amini. AAV construct was made by Yi Zhang and Mandana Amini. Midbrain neuronal survival was done by Mandana Amini with the assistance of Yi Zhang. The figures and text for the manuscript were prepared by Mandana Amini. The manuscript was written with the assistance and guidance from Dr. David S. Park.

Classical calpain promotes neuronal death by cleavage of p35, a cdk5 co-activator, to p25 in Parkinson's and ischemic related injuries.

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Key words: Calpain; neuronal death; Parkinson's disease; Ischemic stroke; Cdk5; p35; p25;

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Abstract

While classical ubiquitous calpains, calpain-1 and calpain-2, are necessary for neuronal functions, their deregulation has been associated with many neurodegenerative diseases under stress conditions. Elevation of calpain activity after neuronal injuries and related cell protection by calpain inhibitors support involvement of calpain in neuronal death process. However, the precise role of calpain and particularly their key targets in neuronal loss still remain controversial, mostly because of the characteristic limits in the approaches. By utilizing brain-specific knock-out of calpain we showed that calpain-1/calpain-2 deficient neurons are significantly resistant to injury induced by excitotoxic stress or mitochondrial toxicity mimicking *in vitro* models of ischemia and Parkinson's disease. Examination of downstream target revealed that conversion of the Cdk5 activator, p35, to pathogenic p25 form, occurred only in the presence of calpain and that it played a major role in calpain-mediated neuronal death. Our data directly demonstrated a key role of calpain and its downstream target, p35/p25, in neuronal loss in different neurodegeneration paradigms.

Introduction

Understanding the mechanisms of neuronal loss in neurodegenerative diseases such as ischemic stroke and Parkinson's disease (PD), has been the major subject of current research due to the great physical, financial, and emotional burden on society. Though the pathology of these neurodegenerative conditions is not well understood, supporting evidence link proteases and above all, calpain, to neuronal loss process (Lendeckel and Hooper, 2005).

Calpains are a highly conserved family of calcium-dependent cysteine proteases which divided into classical (typical) and non-conventional isoforms. The classical ones include two well identified calpains, calpain-1 and calpain-2, which are ubiquitously expressed, dominantly in the central nervous system (CNS) (Goll et al., 2003; Sorimachi et al., 2011b). They both are heterodimers consist of a unique catalytic large subunit encoded by *capn1* or *capn2* genes, respectively, and a common regulatory small subunit encoded by *capns1* (previously known as *capn4*) (Sorimachi et al., 2011b). Calpain-1/calpain-2 are proposed to act in two modes; while they regulate some of major neuronal functions such as synaptic plasticity in physiological condition (Denny et al., 1990; Vanderklish et al., 1996; Liu et al., 2008; Zadran et al., 2012; Amini et al., 2013), they are suggested to undergo hyperactivation under the stress situation and trigger neuronal death. A body of evidence indicates that calpain-1/calpain-2 regulate neuronal death in *in vitro* and *in vivo* rodent models of diverse neurodegenerative/injury conditions such as Huntington's disease (HD), Alzheimer's disease (AD), ischemic stroke and Parkinson's disease (PD) (Mouatt-Prigent et al., 1996; Crocker et al., 2003; Smith et al., 2006; Bevers and Neumar, 2008; Vosler et al., 2008). Several findings, including from our lab, suggest

that calpain activity increases after neuronal injury and that calpain inhibitors block neuronal loss after death insults including those linked to Parkinson's disease and stroke (Bednarski et al., 1995; Crocker et al., 2003; Sedarous et al., 2003; Czogalla and Sikorski, 2005; Donkor, 2011). In fact, further studies from our lab revealed that there is an increase in calpain-mediated cleavage of p35, a co-activator of cyclin-dependent kinase 5 (Cdk5), to a more pathogenic form, p25, in dopaminergic neurons after treatment with PD-related toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Smith et al., 2003; Smith et al., 2006). While all these findings provide a better understanding of calpain pathology and its downstream targets, particularly in dopaminergic neurons death, the exact pathological function of calpain in neurodegeneration is yet to be deciphered. This is mostly because of the limits in the characters of the approaches (Czogalla and Sikorski, 2005; Donkor, 2011). Indeed, the major related findings are based on utilizing calpain inhibitors which raise some considerable caveats such as impermeability and non-specificity.

Germline disruption of *capns1*, which encodes the common small subunit of calpain-1 and calpain-2, has been previously shown to disrupt the activities of both these isoforms (Arthur et al., 2000). However, CAPNS1-deficient embryos die at midgestation (E11.5) probably due to defects in cardiovascular development, which precludes study of calpain in the nervous system using this particular animal model. Calpain-2 deficient mice are embryonic lethal at very early stage; and although calpain-1 knock-out mice are viable, they do not demonstrate any CNS functional impairment such as synaptic plasticity (Grammer et al., 2005; Dutt et al., 2006). Study of the classical calpain isoforms is further

complicated by the possibility that the two calpain isoforms may compensate for each other.

To overcome this problem, we previously generated a CNS-specific calpain-1/calpain-2 deficient mouse utilizing a floxed *capns1* allele combined with the *Nestin-Cre* transgenic driver. Utilizing this paradigm, we explored the role of calpain-1/calpain-2 in brain development and function under normal condition. In the present study, we provide more direct evidence of significance of calpain in neuronal loss in excitotoxic injury and mitochondrial toxicity related to stroke and PD *in vitro* models. In addition, we demonstrated p35/p25 as an essential calpain-mediated system in neurodegeneration which has important implications for therapeutic targeting of calpain in injury-induced or age-related degenerative diseases.

Material and methods

Generation of CNS-specific CAPNS1 knock-out mouse and genotyping. All animal experimental studies were approved by the University of Ottawa Animal Care Committee and conformed to the guidelines set forth by the Canadian Council on Animal Care and Canadian Institutes of Health Research. Conditionally targeted (floxed) *capns1,* transgenic *Nestin*-Cre and *TH-Cre* mice were generated as previously described (Berube et al., 2005; Savitt et al., 2005; Tan et al., 2006c). Breeding strategy was as described before (Amini et al., 2013). Briefly, *capns1*$^{flox/flox}$ and *Nestin-Cre* mice were backcrossed interbred to obtain hemizygous *Nestin-Cre* transgenic mice that were homozygous for the *loxP* targeted (floxed) *capns1* gene (*Nestin-Cre; capns1*$^{flox/flox}$). These mice were referred to as *CAPNS1-Nestin-cKO* mice. Controls were the littermates carrying a single floxed allele of *capns1* (*capns1* $^{+/flox}$). Similarly, the *capns1*$^{flox/flox}$ and *TH-Cre* (Savitt et al., 2005) mice were backcrossed for ten generations onto C57BL/6 strain and then interbred to obtain TH-specific calpain-deficient mice *(CAPNS1-TH-cKO).* All experiments were performed with *CAPNS1-Nestin-cKO* (FVB/N) mice, except the midbrain cultures, which were from *CAPNS1-TH-cKO* (C57BL/6J) mice. PCR was used to determine *Cre* or *capns1* sequences in tail biopsy DNA samples of *Nestin-Cre* or *TH-Cre* mice, as described before (Berube et al., 2005; Savitt et al., 2005; Amini et al., 2013). The *capns1* floxed and wild-type alleles were detected using the primer set as previously described (Amini et al., 2013). Nonradioactive DIG labelling Southern blot hybridization analysis of DNA extracted from brain of embryo or pup was used to confirm the excision of *loxP*-flanked (floxed) sequences, indicated by a 5.1-kbp PstI fragment from the wild type *capns1* locus, a 3.2-

kbp PstI fragment from the pre-Cre floxed allele, and a 4.3-kbp fragment from Cre recombinase-excised (post-Cre floxed) allele, as previously described (Tan et al., 2006c).

Neuronal culture. Cortical neuron cultures were prepared as previously described from 14-15 days *CAPNS1-Nestin-cKO* or *capns1* $^{+/flox}$ mouse embryos (Kim et al., 2005). Equal numbers of neurons were plated into proper dishes coated with Poly-D-Lysine (Sigma) in serum-free medium (500 ml Neuorbasal media supplemented with 0.5 ml N2 10X, 1 ml B27 50X, 125 µl l-glutamine 200 mM, 250 Penicillin-Streptomycin 1X). For midbrain cultures, mesencephalic neurons were collected from day 13–14 embryos as described previously (Kim et al., 2005) and plated the same as cortical neurons. Cerebellar granule neurons (CGN) were harvested from 7-8 days pups as reported before (O'Hare et al., 2000). Neurons were maintained in complete medium [Eagle's minimum essential medium (Sigma) containing 2 mm glutamine, 25 mm glucose, 0.02 mg/ml gentamycin (Sigma), 10% dialyzed FBS supplemented with K^+ to a final concentration of 25 mm]. An equal number of neurons were plated in proper dishes coated with Poly-D-Lysine (Sigma). The antimitotic Arabinofuranosyl Cytidine (Ara-C) (10 µm final concentration; Sigma), which kills dividing cells, was added 18–24 h after plating to reduce the amount of glia cells in the culture.

Casein zymogram and Immunoblotting. Casein zymogram of cell lysates was used to detect calpain-1 and calpain-2 activities as previously described (Tan et al., 2006c). Whole-cell protein was resolved on 8% nondenaturing polyacrylamide gels containing casein (1.5 mg/ ml). Calpain was activated by incubating the gels overnight with 5 mM $CaCl_2$ and its activity was assessed by visualization of the casein-cleared regions of the gel after staining with Coomassie Brilliant Blue. For analysis of p35/p25 expression, whole-

cell protein lysates were collected from cortical neurons treated with MPP+ or from CGNs transiently treated with glutamate for the indicated times (O'Hare et al., 2005). The lysates were separated by 12% SDS-PAGE and immunoblots were probed with anti p35/25 antibody as described before (O'Hare et al., 2005). Primary antibodies: anti-p35 (p35 C-19, sc-820, Santa Cruz Biotechnology, 1:1000).

Assessment of *in vitro* neuronal survival. Cortical neurons were treated with 1-methyl-4-phenylpyridinium (MPP+) at a final concentration of 20 µM after two days *in vitro* (DIV) for 24 h and 48 h. For midbrain cultures MPP+ treatment started on 7 DIV at 20 µM final concentration for desired time periods. Cells were stained with anti-tyrosine hydroxylase (TH) antibody (ImmunoStar, Hudson, WI; 1:2,500) as primary antibody and Hoechst as the control. Fluorescent stained TH+ neurons were counted and evaluated for nuclear, dendrite, and axon morphology. Seven-day CGNs were transiently treated with glutamate to the 50 µM for 70 min in the presence or absence of 10 µM MK801, an NMDA receptor blocker. Cells were washed off with conditional medium and re-incubated for 1.5 h. For hypoxia-related excitotoxic death paradigm, CGNs were incubated in hypoxia chamber [a humidified environmental chamber (Coy Laboratory Products, Ann Arbor, MI) set at 37°C, 1% O2, and 5% CO2] for 4-5 h in the presence or absence of MK801 and then reoxygenated for 1 h (Rashidian et al., 2005). For all survival assays, except TH+ neurons, numbers of viable neurons were evaluated by lysis of cultures followed by counting intact nuclei as described (Kim et al., 2005). The percentage of surviving neurons is expressed relative to untreated control wells. For rescuing survival phenotype, CGNs were infected with Adeno-associated virus (AAV) expressing p25 or with AAV-GFP (multiplicity of infection, 25) at the time of plating. Glutamate treatments were completed on 8 DIV as

described. Neurons viability was evaluated by lysis of cell membrane followed by counting of intact nuclei as described (Kim et al., 2005).

Adeno-Associated Viral (AAV) Construct. cDNA sequences of human p25 were subcloned into the BamHI–EcoRI sites of the AM/CBA-pl-WPRE-bGH plasmid, a recombinant AAV (rAAV1) vector. The virus was then generated and purified as described (Rashidian et al., 2005). EGFP was excised from Clontech pEGFP-N2 vector (cat# 6081-1) using BamHI and a blunted NotI. This fragment was subsequently subcloned into the same AAV vector, AM CBA-pl-WPRE-bGH, into the BamHI and EcoRV sites to produce AAV-CBA-EGFP-WPRE-bGH.

Results

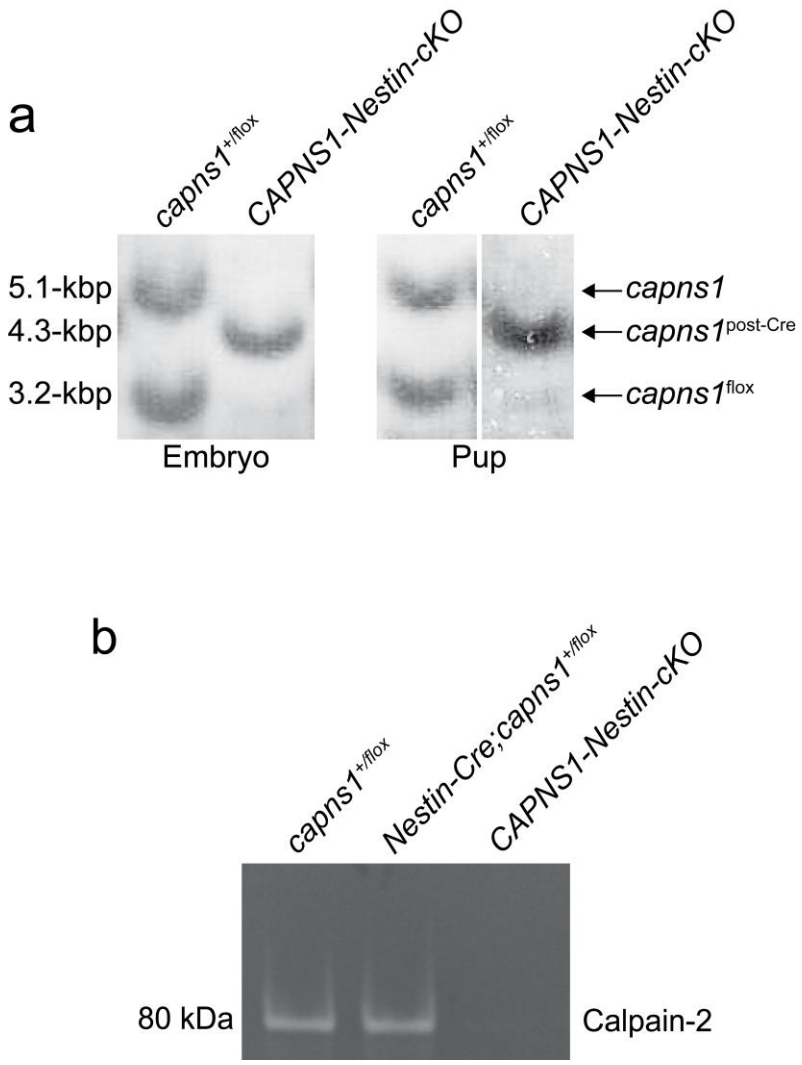
Elimination of calpain-1/calpain-2 activities in mouse neurons in the CNS

We employed a Cre/loxP-mediated recombination strategy to conditionally target calpain-1/calpain-2 small subunit, *capns1* locus, in the mouse brain. We previously reported that directed excision of *capns1* occurred only in the mutants, *CAPNS1-Nestin-cKO*, compare to other transgenic littermates (Amini et al., 2013). Besides, the brain of the adult mutants showed neither CAPNS1 expression nor calpain-1/calpain-2 activities (Amini et al., 2013).

Here, southern blot analysis of brain genomic DNA extracted from embryo or pups revealed the similar result as adult mutants, where complete excision of *capns1* appeared only in the brain of mice that were transgenic for *Nestin-Cre* and homozygous for the *loxp* targeted (flox) *capns1* gene *(CAPNS1-Nestin-cKO)* **(Fig. 3.1a)**. The effect of *capns1* targeting on calpain protease activity was assessed by casein zymogram analysis of individual neuronal cultures from the mutants and control littermates. Casein zymogram analyses of whole cell lysates from cortical neurons (data not shown) and CGNs showed no detectable calpain-2 activity in the neurons of *CAPNS1-Nestin-cKO* mice **(Fig. 3.1b**), while robust activity of calpain-2 isoform was observed in neurons from controls. We could not detect calpain-1 activity in the neurons either from the mutants or the controls. Protein instability might be a possible explanation for this result.

Figure 3.1. Disruption of calpain small subunit, capns1 locus, and elimination of calpain-1/calpain-2 activities in the *CAPNS1-Nestin-cKO* mouse brain. (a) Southern blot analysis of brain DNA samples from embryo and pup from *CAPNS1-Nestin-cKO* (*Nestin-Cre; capns1* $^{flox/flox}$) and control littermate (*capns1* $^{+/flox}$). The probe distinguishes 5.1, 4.3, and 3.2 kbp PstI fragments corresponding to untargeted (*capns1*) or the floxed allele after (*capns1*$^{Post\text{-}Cre}$) or before (*capns1*^{flox}) Cre-mediated excision, respectively. **(b)** Casein zymogram of the cell lysates to analyze calpain-1 and calpain-2 activities (n=3 per genotype).

Figure 3.1


a
capns1+/flox
CAPNS1-Nestin-cKO
capns1+/flox
CAPNS1-Nestin-cKO
5.1-kbp
4.3-kbp
3.2-kbp
capns1
capns1post-Cre
capns1flox
Embryo
Pup
b
capns1+/flox
Nestin-Cre;capns1+/flox
CAPNS1-Nestin-cKO
80 kDa
Calpain-2

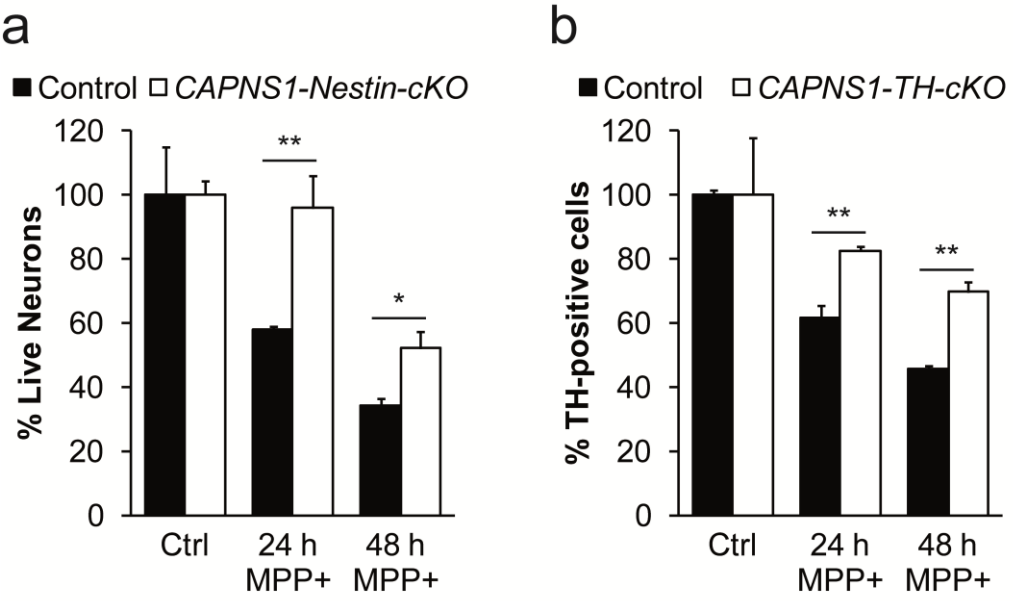
Calpain regulates neuronal cell death in Parkinson's disease *in vitro* model

Classical calpains have previously been proposed to participate in neuronal death in several pathological conditions (Vosler et al., 2008). However, the direct role of calpain and its precise target in neurodegeneration need to be definitively demonstrated. Accordingly, we employed this genetic model system to test the role of calpain in neuronal loss in PD *in vitro* model. 1-methyl-4-phenylpyridinium (MPP+), a metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), is a mitochondrial complex 1 toxin which promotes selective death of both dopaminergic neurons *in vivo* and several neuronal types *in vitro,* including cortical and midbrain cultures (Crocker et al., 2003; Smith et al., 2006; Qu et al., 2007; Huang et al., 2010). To test whether calpain plays a role in neuronal death induced by this toxin, we first examined whether embryonic cortical cultures, derived individually from control or calpain-deficient animals, were differentially sensitive to MPP+. Cortical neurons from *CAPNS1-Nestin-cKO* embryos were significantly less sensitive to the toxin compare to the ones from controls [(95.80± 10.23% vs. 58± 0.72% at 24 hrs; 52.1± 5.32% vs. 34.07± 2.17% at 48 hrs)] **(Fig. 3.2a)**. Because TH-positive neurons are grossly affected in PD, we also examined whether calpain may play a role in dopaminergic neuronal death as well. To do this, we crossed the floxed *capns1* mice with a *TH-Cre* driver (Savitt et al., 2005) to specifically eliminate calpain-1/calpain-2 activities in TH-positive neurons. Neuronal cell cultures individually obtained from embryonic midbrains of *CAPNS1-TH-cKO* and control animals were treated with MPP+. Cell survival was assessed by evaluating the number of TH-positive neurons. Consistent with cortical cultures generated from *Nestin-Cre* mice, neuronal survival was

significantly higher in calpain-deficient TH+ neurons compared to controls [(82.59± 1.44% vs. 61.45± 3.83% at 24 h; 69.7± 3.06% vs. 45.49± 0.96% at 48 h)] **(Fig. 3.2b)**

Figure 3.2. Calpain mediates excitotoxic neuronal death in PD-related *in vitro* models. (a) MPP+ treatment of cortical neurons at 20 μM final concentration for 24 h and 48 h revealed that calpain-deficient neurons were resistant to toxin-induced death [(95.80± 10.23% vs. 58± 0.72% at 24 h; 52.1± 5.32% vs. 34.07± 2.17% at 48 h)] (n=4 per genotype). **(b)** MPP+ treatment of midbrain neurons at 20 μM final concentration for 24 h and 48 h revealed the similar results as cortical neurons [(82.59± 1.44% vs. 61.45± 3.83% at 24 h; 69.7± 3.06% vs. 45.49± 0.96% at 48 h)] (n=4 per genotype). Data are represented as mean ± SEM, Student's *t* test, **P<0.05, **P< 0.01*.

Figure 3.2

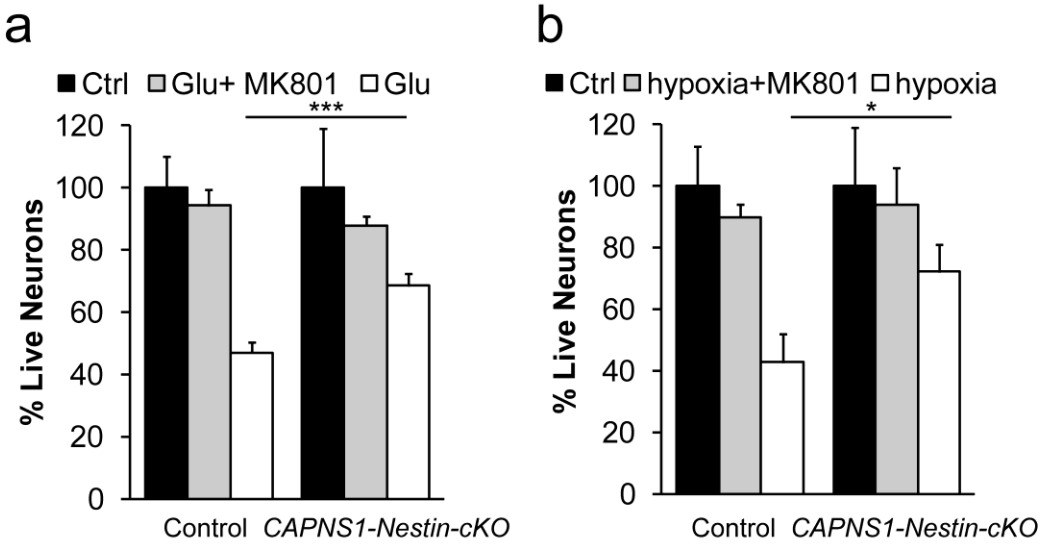

a
Control
CAPNS1-Nestin-cKO
% Live Neurons
120
100
80
60
40
20
0
**
*
Ctrl
24 h MPP+
48 h MPP+
b
Control
CAPNS1-TH-cKO
% TH-positive cells
120
100
80
60
40
20
0
**
**
Ctrl
24 h MPP+
48 h MPP+

Calpain is mediator of neuronal death in ischemic *in vitro* models

To further evaluate the importance of calpain in neuronal injury, we also tested models of ischemic cell death utilizing *in vitro* glutamate- or hypoxia-induced excitotoxicity in cerebellar granule neurons (CGNs). Indeed, very early finding suggested that *in vitro* translational suppression of calpain-1 reduced excitotoxic damage related to NMDA receptor activation (Bednarski et al., 1995). Here, CGNs derived from postnatal cerebellum of *CAPNS1-Nestin-cKO* and controls were transiently subjected to glutamate in the presence or absence of MK801, a NMDA receptor blocker, and neuronal survival was assessed by nuclear integrity. Calpain-deficient neurons were significantly less sensitive to glutamate than control littermates (68.49± 3.87% vs. 46.76± 3.6%) **(Fig. 3.3a)**. Similarly, CGNs obtained from the mutants were significantly protected against hypoxia-induced cell death in a considerable extent (72.06± 9.1% vs. 42.75± 8.81%) **(Fig. 3.3b)**. Taken together, these findings as well as results from PD-related toxin in cortical cultures suggest a direct role of calpain in multiple mechanisms of neuronal death.

Figure 3.3. Calpain mediates excitotoxic neuronal death in ischemic-related *in vitro* models. (a) Transient glutamate treatment of CGNs at 50 µM final concentration for 70 mins following by 1.5 h reoxygenation in the presence and absence of 10 µM MK801 (68.49± 3.87% vs. 46.76± 3.6%) (n=6 per genotype), **(b)** Hypoxia (1% oxygen) treatment of CGNs for 4-5 h followed by 1-2 h reoxygenation in the presence and absence of 10 µM MK801 (72.06± 9.1% vs. 42.75± 8.81%) (n=4 per genotype). Data are represented as mean ± SEM, Student's *t* test *$P<0.05$, ***$P < 0.001$.

Figure 3.3

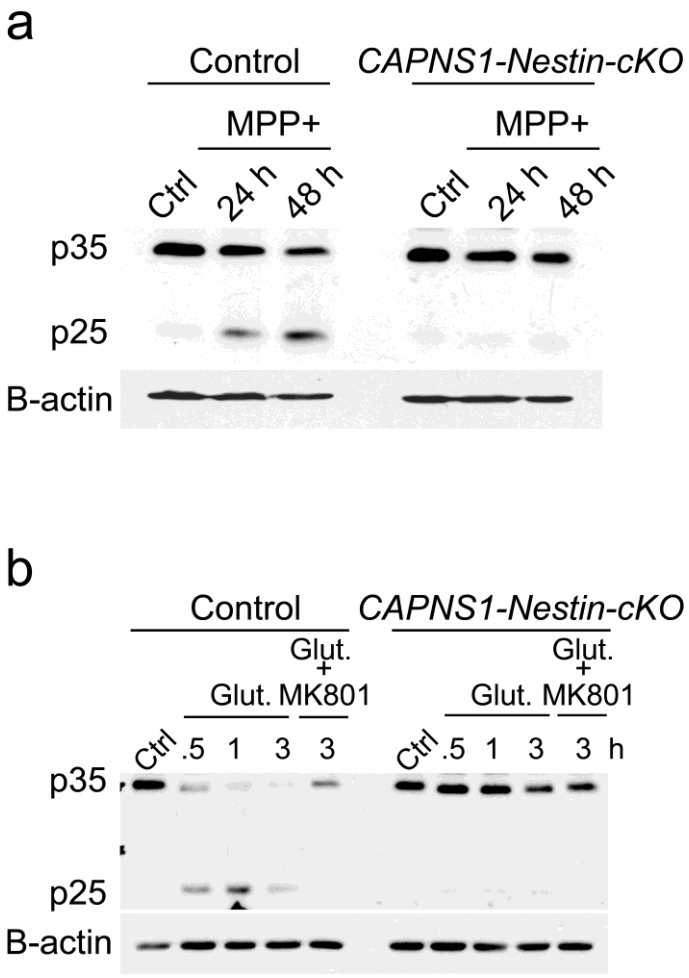

a
Ctrl
Glu+ MK801
Glu
***
% Live Neurons
120
100
80
60
40
20
0
Control
CAPNS1-Nestin-cKO
b
Ctrl
hypoxia+MK801
hypoxia
*
% Live Neurons
120
100
80
60
40
20
0
Control
CAPNS1-Nestin-cKO

Calpain-mediated cleavage of p35, the Cdk5 regulatory subunit, plays a critical role in neuronal damage.

The generation of calpain-deficient neurons afforded the opportunity to ascertain the importance of selected substrates of calpain in neuronal death. Previous studies, including from our lab, suggested that calpain-mediated cleavage of p35, a cyclin dependent kinase 5 (Cdk5) regulatory subunit, to p25 elicits inappropriate activation of Cdk5, which drives neuronal cell death (Lee et al., 2000; Smith et al., 2006). However, it is not completely clear whether p35 cleavage to p25 is mediated exclusively by calpain, or how critical this cleavage is to the neuronal death process (O'Hare et al., 2005). To explore this question, cortical cultures from *CAPNS1-Nestin-cKO* or control animals were exposed to MPP+ for 24 h or 48 h. Expression of p35 and p25 was assessed by immunoblot analysis of cell lysates using a C-terminal polyclonal antibody that recognizes both the full-length p35 and the p25 cleaved product. We observed a dramatic increase in p25 expression associated with a reduction in p35 protein levels after MPP+ in controls **(Fig. 3.4a)**. The same result has obtained with glutamate treatment of CGNS in controls **(Fig. 3.4b)**. Importantly, we could not detect p25 formation or loss of p35 in calpain-deficient neurons following MPP+ or glutamate treatment **(3.4a-b)**. These observations demonstrate an essential role of calpain in cleavage of p35 to p25 in injured neuron.

Figure 3.4. p35 is a target of calpain, and the p25 formation increases after neuronal injuries. Representative immunoblots showing the increase in p35 cleavage to p25 after **(a)** MPP+ and **(b)** glutamate treatment in cortical neurons and CGNs, respectively, from control mice but not *CAPNS1-Nestin-cKO* (n=5 per genotype).

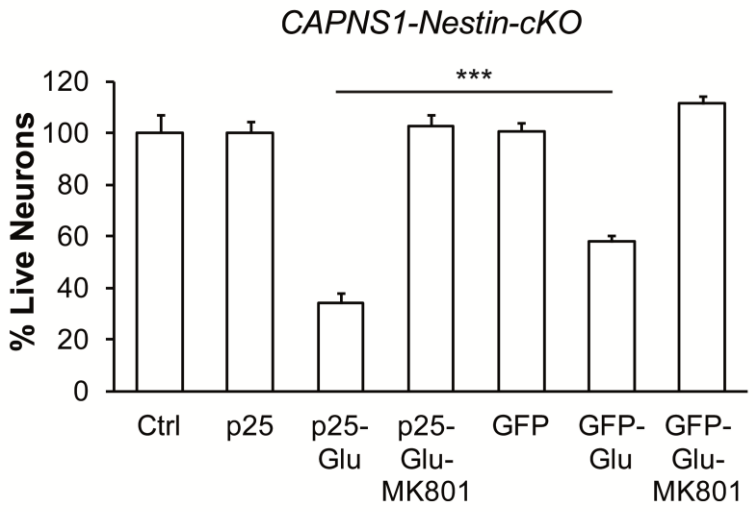
Figure 3.4


a
Control
CAPNS1-Nestin-cKO
MPP+
MPP+
Ctrl
24 h
48 h
Ctrl
24 h
48 h
p35
p25
B-actin
b
Control
CAPNS1-Nestin-cKO
Glut. + Glut. MK801
Glut. + Glut. MK801
Ctrl
.5
1
3
3
Ctrl
.5
1
3
3 h
p35
p25
B-actin

To functionally test the involvement of calpain-induced p25 formation in cell death, adeno-associated virus was used to rescue p25 expression and to determine whether the protection associated with calpain-deficiency could be reversed. As predicted, CGN cultures from *CAPNS1-Nestin-cKO* infected with a control GFP-expressing adeno-associated virus displayed less neuronal death than wild type cells upon transiently glutamate challenge. In contrast, infection with p25 expressing adeno-associated virus restored glutamate-induced cell death in calpain-deficient neurons to the same levels seen in control cells (34± 3.8% vs 58± 2.6%) **(Fig. 3.5)**. Interestingly, consistent with what has shown before (Fischer et al., 2005) short-term overexpression of p25 did not initiate neuronal death by itself. Taken together, these results provide compelling evidence for the critical role of calpain-mediated conversion of p35 to p25 in two different models of neuronal cell death.

Figure 3.5. p25, the cleavage product of p35 mediates excitotoxic neuronal death. Graphical representation of sensitization of CGNs to glutamate and the re-sensitization of calpain-deficient neurons by AAV-directed p25 over-expression (n=3 per genotype). Data are represented as mean number of intact nuclei ± SEM, Student's *t* test, ***$P < 0.001$.

Figure 3.5


CAPNS1-Nestin-cKO
***
% Live Neurons
120
100
80
60
40
20
0
Ctrl
p25
p25-Glu
p25-Glu-MK801
GFP
GFP-Glu
GFP-Glu-MK801

Discussion

Although a significant amount of studies have linked classical calpain, calpain-1 and calpain-2, to neuronal death, the central pathological functions of the protease need to be definitively demonstrated. Clarification of exact role of calpain-1/calpain-2 in neuronal death has been complicated mostly because of lack of a proper system to regulate calpain activity, in particular *in vivo*. Transgenic animal models to explore the function of calpain in neuronal injuries have been restricted caused by early embryonic lethality or lack of phenotype presumably due to compensatory mechanisms (Arthur et al., 2000; Grammer et al., 2005; Dutt et al., 2006; Takano et al., 2011). To overcome this issue we recently introduced a unique conditional deletion model of calpain-1/calpain-2 activity. Our previous investigations demonstrated that CNS-specific calpain-1/calpain-2 knock-out mice are viable and do not show general morphology defects (Amini et al., 2013). However, calpain-1/calpain-2 deficiency significantly impacts dendrite structures, LTP, and learning and memory in CA1 neurons of hippocampus (Amini et al., 2013). In current study we employed this conditional knock-out to clarify the precise role of calpain in neuronal death, focusing on PD and ischemic stroke *in vitro* models.

Calpain activation has been associated with a number of death paradigms, both *in vitro* and *in vivo* including those linked to stroke and PD (Crocker et al., 2003; Ray and Banik, 2003; Bevers and Neumar, 2008). Relevant to the former, both *in vitro* and *in vivo* experiments have shown that proteolysis of calpain-specific substrates is elevated in the hippocampal and neocortical areas in focal and global ischemia (Hong et al., 1994; Bartus et al., 1995; Bednarski et al., 1995). Also several studies have provided evidence that pre- or post-treatment with calpain inhibitors has neuroprotective action in *in vitro* and *in vivo*

ischemic models (Vosler et al., 2008; Peng et al., 2011). In terms of PD, we have previously shown in our lab that calpain-1 and calpain-2 are activated in both *in vitro* and *in vivo* models of neuronal loss induced by the dopaminergic toxin MPTP/MPP+. Calpain inhibitors also protected dopaminergic neurons from death in these paradigms (Crocker et al., 2003; Qu et al., 2007; Huang et al., 2010). The intrinsic problems with calpain inhibitors are impermeability or their effect on nonspecific targets (Donkor, 2011). Additionally, data from our lab reported that neurons treated with calpain inhibitors demonstrated fewer neuritic processes (Sedarous et al., 2003). This cannot be related to blockage of calpain activity since neuronal cultures from our CNS-specific calpain deficient animals look normal. Together, all these drawbacks make interpretation of experiments from calpain inhibitors difficult. Our present genetic work provides further support for the key role of calpain in neuronal injury. We found that calpain-deficiency promotes survival under multiple contexts of hypoxia, excitotoxicity and mitochondrial dysfunction. MPP+, the active metabolite of MPTP, has been shown to impair mitochondrial function and lead to dopaminergic neurotoxicity mimic key features of PD (Crocker et al., 2003). We showed that calpain deficient neurons are significantly resistant to this neurotoxin. To be more precise, we were able to target calpain-1/calpain-2 activity specifically in TH+ neurons, the target cells in PD, utilizing *TH-CAPNS1-cKO*. Our data revealed that calpain-deficiency prevents TH+ neurodegeneration in midbrain culture under the similar injury paradigm.

In term of ischemia, we employed hypoxia or glutamate treatment mimicking ischemic stroke *in vitro* models. High dose glutamate or long-time oxygen deficiency trigger the stream of overactivation of glutamate receptor, Ca^{2+} intracellular influx, and

proteases activity, including calpain (Ankarcrona et al., 1995; Bickler and Donohoe, 2002). Our results indicate that calpain deficiency is significantly protective against excitotoxicity insult, one of the major death factors in ischemic stroke.

Calpain involvement in death induced by injury contrasts with developmental programmed death examined in the embryo in our previous study (Amini et al., 2013). What is the mechanism by which calpain promotes injury-mediated death? We and others have previously proposed that calpain cleaves the Cdk5 activator p35 to a more stable active p25 form (Lee et al., 2000; O'Hare et al., 2005; Smith et al., 2006). In the contexts of stroke and PD, we have shown that this is associated with increased Cdk5 activation (Smith et al., 2006). Our calpain-deficiency model allowed us to more concretely examine whether calpain is indeed critical to p25 formation and whether this formation is central to the death process. In multiple paradigms of neuronal death, we show that calpain-deficient neurons have significantly impaired p25 formation when compared to controls. The observed data are consistent with the previous ones in our lab displaying the increase in p35/p25 turnover and Cdk5 activity after excitotoxicity insults (Smith et al., 2003; O'Hare et al., 2005; Smith et al., 2006). We also have shown before that Cdk5/p35/p25 regulates both life and death in neurons after DNA damage injury depending on the cellular localization of the kinase and related regulators (O'Hare et al., 2005). Therefore, our knock-out model will assist us with better understanding of prosurvival/pro-death functions of p35/p25 and cdk5 in the future.

Conversely, the protection afforded by calpain-deficiency can be reversed by ectopic expression of p25. This supports a central role for calpain-mediated p25 formation in the paradigms of neuronal death presently examined. Given that p25 is also reported to

be involved in synaptic plasticity (Fischer et al., 2005), it is intriguing to speculate that cleavage of p35 to p25 might be one of the central events that connects calpain to some of its physiological and pathophysiological functions. Finally, it is important to note that whether or not genetic calpain-deficiency plays a role in adult degeneration/injury has not been presently studied.

In summary, our analysis of a conditional model of calpain-deficiency in the CNS has provided a clearer understating of calpain biology in the brain regarding specific neuronal death paradigms. We believe that this conditional model will continue to generate critical information on calpain function in neurodegeneration relevant to diverse numbers of disease.

General Discussion

Summary

In the research presented in this thesis, we have focused on the consequences of the absence of conventional calpain activity, calpain-1/calpain-2, in rodent brain regarding development, synaptic functionality, and induced neuronal loss. As reviewed in the Chapter 1, rapid grow in the number of identified calpain substrates as well as blockage of some of pathological and physiological signalling by the protease inhibitors in the brain have suggested an outstanding range of possible functions for this calcium-dependent enzyme in the CNS. However, the lack of a proper animal model and the non-specific effects of the pharmacological inhibitors confounded the nature of calpain's roles in these processes. The main goal of this work was to address some central questions directly associating calpain activity to brain structure and functions including partial morphology, synaptic activity, and neurodegeneration. For this purpose, we assessed the objectives by means of our novel mouse model in which the proteolytic activity of the calpain-1/calpain-2 isoforms was properly eliminated in the brain.

In this section I briefly present and dissect the fundamental findings of my research with possible future lines of work.

4.1. Calpain mediates specific CNS morphology including dendrite development rather than general structure

In chapter 2 we first presented a novel mouse model with deletion in CAPNS1 expression and consequent deficiency in calpain-1/calpain-2 activities in the CNS. Unlike previous calpain germline knock-outs (Arthur et al., 2000; Dutt et al., 2006), our conditional deletion did not cause embryonic lethality, becoming the first calpain knock-

out mouse model available for investigations of the role of the protease in the CNS. General brain morphology of these mice looked normal and some of the main features of embryogenesis, including apoptosis and proliferation, were unaltered in the absence of calpain. This is interesting because calpain has been proposed to mediate pathological apoptosis in some injuries. Also calpains and caspases, proteases classically linked to both developmental and injury-related apoptosis, have been hypothesized to regulate each other's activities in cell death (Broker et al., 2005; Lopatniuk and Witkowski, 2011). Thus, our data strongly supports the difference between physiological and pathological signaling in CNS apoptosis. Although, we did not directly examine differentiation of the cells in the mutant brain, our gross morphogenesis study and particularly observations of the cells in neuronal culture revealed inconspicuous abnormality. Overall, due to early embryonic deletion of calpain-1/calpain-2 activities in the mutant CNS, the viability of our postnatal mutant and normal gross morphology of the brain argue against the significance of calpain in general CNS development. Our western blot analysis of other calpain isoforms such as calpain-3 and calpain-5 ruled out the possible compensatory role of these members of the protease family (data not shown).

Previous findings including those utilizing calpain inhibitors as well as developmental and postnatal changes in calpain-1 /calpain-2 protein levels and activities in the brain, suggested a role for calpain in CNS development (Arthur et al., 2000; Dutt et al., 2006; Li et al., 2009). Then, does calpain preferably mediates more specific structures in embryogenesis/adult CNS development? Our results showed that calpain deficient neurons in hippocampal CA1 region suffered significant impairment in dendritic

branching and spine density in basal and apical dendrites. This is the first direct evidence linking calpain to dendrite development.

Changes in dendrite and spine morphology are believed to happen both during development and adulthood. Hence, better understanding of the event could lead us to better knowledge of synaptic activity and strategies for aiding neuronal repair after injury. Our *in vivo* animal model excitingly provides a better opportunity for further detailed analysis of the signalling underlying dendrite and spine growth. For example, future research could be done to directly examine the influence of calpain activity on actin polymerization and its consequence in dendrite and spine growth. Dendrite growth critically relies on changes in microfilaments and microtubules such as the polymers of actin and tubulin respectively, and all intrinsic and extrinsic pathways involved in the event have been shown to affect polymerization and depolymerisation of these molecules. As discussed in the introductory section of this research, calpain catalyzes the truncation of cytoskeleton components such as spectrin and has been associated with reconstruction of microfilament and microtubules in dendrites and synapse (Lynch and Baudry, 1984; Wilson et al., 2000; Baudry et al., 2013). Recently, evidence has been reported showing an important function of calpain downstream target Cdk5/p35/p25 in dendrite and spine development/structural changes. Overexpression of p25 increased the number of spines in hippocampal CA1 neurons (Fischer et al., 2005). Moreover, p35 has been suggested to directly bind to filamentous actin (F-actin) and stabilize the protein structure (He et al., 2011). Several studies including utilizing Cdk5 dominant-negative mutants revealed supportive roles for Cdk5 in neurite outgrowth through regulation of chemorepellent and chemoattractant or phosphorylation of cytoskeletal components (Cheung and Ip, 2007; Fu

et al., 2007). This would be an important issue for future research to test whether calpain mediates dendrite development through targeting microfilaments and microtubules by its downstream Cdk5/p35/p25 system.

4.2. Calpain regulates LTP in the absence of alternation in glutamatergic synaptic transmission

The above-mentioned finding led us to the next question of whether calpain-mediated deficiency in dendrite and spine structure contributes to alternation in excitatory synaptic transmission. In fact, prior studies have also suggested a role for calpain in glutamatergic synaptic transmission by cleavage of a number of related synaptic components (Doshi and Lynch, 2009). In the chapter 2, our initial investigation revealed that CNS-specific calpain deficiency resulted in decreased amounts of synaptic proteins such as NMDA and AMPA receptors subunits and PSDs, rather than cleavage of the substrates. There are at least three possible explanations for this observation. The first and most likely interpretation is that calpain is actually required for the expression of these proteins through engaging in signalling pathways regulating gene expression. Indeed, calpain has been implicated in modulating several transcription factors such as c-Jun and c-Fos, NFκB, and MEF2. Calpain has been suggested to involve in cleavage of c-Jun and c-Fos and thus, in gene expression related to these transcription factors (Hirai et al., 1991; Pariat et al., 2000). Moreover, phosphorylation of MEF2D by calpain resulted in inactivation of the transcription factor while calpain-mediated degradation of IκB, an inhibitor of NFκB, activated the NFκB transcription factor (Han et al., 1999; Shumway et al., 1999; Smith et al., 2006). Interestingly, all of these transcription factors have been

demonstrated to have a positive influence in regulating the transcription rate of NMDA receptor subunits with binding either to a site in the promoter or to an activator of the promoters. Additionally, MEF2 expression was also synchronizing with the subunits protein levels in developing CNS; and NFκB family members are known to be contributors to neuronal plasticity (Bai and Kusiak, 1993; Leifer et al., 1993; Morgan and Curran, 1995; Krainc et al., 1998; Qiang and Ticku, 2005; Bai and Hoffman, 2009). The second possible explanation is that cleavage of the synaptic proteins is smaller than what could be detected by our techniques. Accordingly, although the adult mice experienced synaptic plasticity and LTP during development and postnatal living, the truncation might be only distinguished after LTP induction in the mouse brain. The third and last possibility is that the reduced level of the synaptic components in calpain mutant brain may also reflect other alternative mechanisms such as lack of inhibitory role of calpain in the proteins degradation.

Enigmatically, the individual glutamatergic transmission in CA1 synapses, examined by presynaptic glutamate release, simultaneous synapse activity and NMDAR and AMPA receptor functions and distributions, was not evidently altered in the absence of key proteins and neuronal ultrastructure. This may indicate the compensatory ability of synapses to maintain faithful transmission by counterbalancing the defects of critical components. To name some noteworthy examples, mutant mice lacking GluA1 or GluA2 subunits revealed unchanged synaptic transmission current (Zamanillo et al., 1999; Toyoda et al., 2009). Beside compensatory mechanisms, another explanation could be that our observed reduction in synaptic proteins in hippocampus reflected largely the level of protein of extrasynaptic components and accordingly, does not impact synapse basal

activity. However, the level of some of the proteins was so dramatically weakened that cast doubt on this possibility.

Finally, our calpain-deficient mice revealed a significant impairment in LTP evaluated by both whole cell and field recording. The finding indicates an essential role for calpain in synaptic plasticity supported by insufficiency in spatial learning and memory. The importance of the observation can be discussed from several points of view. First, our observation supports the lines of evidence that are in favour of calpain as a necessary molecule in LTP. Pharmacological and antisense-based inhibitors as well as studies on calpain's target, Cdk5/p35/p25, suggested a positive regulatory role for calpain in long term potentiation (del Cerro et al., 1990; Denny et al., 1990; Vanderklish et al., 1996; Fischer et al., 2005; Guan et al., 2011). On the contrary, mice lacking calpain-1 did not show any CNS dysfunction relevant to learning and memory and LTP. Furthermore, an inducible conditional knock-out of Cdk5 displayed enhancement in LTP (Grammer et al., 2005; Hawasli et al., 2007). Considering all these inconsistent results, here we provided compelling evidence that calpain is required for LTP. Our finding is also supported by the observation that downregulation of calpain-2, using a novel rabies-virus glycoprotein-chimeric peptide to deliver calpain-2 siRNA to the brain, resulted in impairments in LTP and learning and memory (Zadran et al., 2013). In fact, calpain-2 has been shown to be activated by neurotrophic factors independently of calcium (Zadran et al., 2010b). Accordingly, the question has been raised of whether calpain-2 is the essential isoform mediating LTP. Our results cannot discriminate the significance of isoforms in this paradigm. Additionally, some previous reports have suggested that calpain-1/calpain-2 have functional redundancy in cells (Takano et al., 2011). Hence, the issue awaits

further studies. The second significant point in our observation is that regulation of spine population maybe at least one of the mechanisms upon which calpain acts in synaptic plasticity. Our results are consistent with the observation that overexpression of p25 leads to enhancement in long term potentiation accompanying by increase in spine density (Fischer et al., 2005). Several studies have demonstrated that learning and memory or LTP induction alter the number and morphology of spines in dendrites. For instance, a very early study revealed that light-deprivation in different rodents including mouse and rat caused significant decrease in the number of dendritic spines in visual cortex (Valverde, 1967; Fifkova, 1968; Parnavelas et al., 1973; Chakraborti et al., 2012). Hence, the question has been raised of whether or not the above proposal will be correct the other way around. In other words, does increase in the spine populations enhance LTP? Here, we evidently presented that changes in dendrite and spine structure affects LTP and related learning and memory. The conclusion is also supported by the examination of input/output curve which showed declined in the input from Schaffer collateral to the CA1 neurons via apical dendrites in the mutant. The data did reflect impairment in postsynaptic depolarization. Interestingly, the 30%-40% level of reduction in fEPSP coincided with the decrease in the spine density observed in calpain-deficient mice. At this point we cannot discern if the disturbance in the synaptic plasticity of our mutant is due to developmental defects or is a consequence of calpain deficiency on signalling processes. Moreover, our research does not rule out the possibility of other mechanisms by which calpain may regulate synaptic plasticity. Calpain has been suggested to contribute to synaptic activity by cleavage of many substrates including intracellular mediators (e.g. CaMKII, PKC, SCOP). Calpain-mediated cleavage of these proteins suggested its regulation over some

signalling processes such as phosphorylation of receptors or gene expression in synaptic activity (Wu and Lynch, 2006; Liu et al., 2008). Indeed, a recent report from Wang and colleagues demonstrated that calpain-1 potentiates synaptic plasticity at early phase by degradation of an inhibitory protein, suprachiasmatic nucleus circadian oscillatory protein (SCOP), while calpain-2 restricted LTP by activation of SCOP resynthesis at later stages (Wang et al., 2014). The finding defined an additional role of calpain in LTP as well as supporting the requirement for the functions of both isoforms in this neuronal activity.

Finally, we reported that the impairment in spatial learning and memory of mutant mice is not due to anxiety or lack of motor activity.

4.3. Calpain modulates neuronal death in the CNS under stress conditions

Exploring the role of calpain in pathological events was the main aim in chapter 3 of this research. Our data demonstrated that calpain deficiency promotes neuronal survival against several stress conditions including mitochondrial dysfunction, excitotoxicity and hypoxia. Calpain has been long studied as a mediator of multiple neuronal death paradigms both *in vivo* and *in vitro* relevant to a variety of neurodegenerative disorders. However, for reasons outlined in the previous chapters of this thesis, there was little direct evidence particularly *in vivo* to define the specific role of calpain in these aspects. Our calpain-deficient model allowed us to investigate the direct effect of calpain on cell susceptibility subjected to diverse death-inducing circumstances in the CNS. Utilizing multiple neuronal death *in vitro* models relevant to PD and ischemic stroke we found that calpain contributes to both apoptotic and excitotoxic neurodegeneration. With regards to PD, we have previously shown in our lab that calpain-1/calpain-2 are activated in neuronal

loss induced by dopaminergic toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Calpain inhibitors protected dopaminergic neurons from death in this paradigm (Crocker et al., 2003). Our results further support calpain participation in PD-related neuronal injury. More interestingly, we demonstrated that TH-specific calpain deficient neurons are resistant to the above neuronal insult as well. Considering that there are less general developmental effects of calpain deficiency on our TH-specific calpain knock-outs, our findings implied the specific involvement of the protease in dopaminergic neuronal death. The above findings also bring up at least another important point. Since neuronal death occurring in MPP+-treated cell cultures is apoptotic as previously illustrated in our lab (Huang et al., 2010), our results indicated that calpain-mediated apoptosis in the CNS is highly regulated since it switches on upon the stress condition but not under developmental programmed cell death. Hence, further study could reveal the significant mechanisms modulate apoptosis in neurodegenerative disorders.

In terms of ischemic stroke, previous reports showing neuronal survival by pre-treatment or post-treatment of calpain inhibitors as well as detection of increased calpain activation following ischemic injury suggested the contribution of calpain in this neuronal loss paradigm (Vosler et al., 2008). In our model, downregulation of calpain in neurons was significantly protective against both glutamate- and hypoxia-mediated neuronal death. The data provides a concrete link between calpain activity and excitotoxicity particularly hypoxia as a major factor of ischemic-mediated death. The additional importance of the finding is that excitotoxicity not only occurs in ischemia but is a process that has been linked to pathogenesis of a diverse number of neurodegenerative disorders (Dong et al.,

2009). Therefore, our results provide insight into better understanding of one of the principal mechanisms involved in injury-mediated death.

How does calpain mediate neuronal death? Although our findings support a pivotal role for calpain activity in neuronal injury, the protease cannot be a target for neurodegeneration therapy because of its essential role in normal CNS function such as plasticity. Therefore, a critical downstream target of calpain in injury paradigms is required to be defined. Our genetic model provides us with a novel possibility to examine the above issue. We previously discussed the regulatory role of calpain in Cdk5/p35/p25 activation and consequence of this activation on neuronal death in PD and ischemia. In fact, cleavage of p35/p25 has been associated with neuronal death in a number of neurodegenerative disorders such as Amyotrophic Lateral Sclerosis and Huntington's disease (Su and Tsai, 2011). In our research we investigated the requirement of calpain in formation of p25 and the significance of the proteolytic result in neurodegeneration. We demonstrated a substantial impairment in the production of p25 in calpain-deficient neurons following multiple death-inducing models; revealing that the protease is essential for the atypical expression of p25. We were also enabled to confirm the findings by a novel approach in which we expressed p25 in mutant neurons and demonstrated the subsequent reverse in protection provided by calpain deficiency following injuries. Overall, the above data supports calpain-mediated ectopic formation of p25 as a central component of neuronal loss in the death paradigms presented here. Given that p25 has been linked to some of brain physiological functions including plasticity (Angelo et al., 2003; Fischer et al., 2005), it is likely that Cdk5/p35/p25 is the key downstream target of

calpain in regulating both physiological and pathological functions in brain. Further studies are necessitated for better understanding of this hypothesis.

Conclusions and future views

In summary, in this project we examined consequences of calpain activity in both life and death under basal and stress conditions. Our conditional calpain animal model revealed that although calpain is required for normal neuronal functions such as plasticity, the protease has also potential to turn to a death signal following excitotoxic and apoptotic injuries. The goal of various investigations on neuropsychiatric, aging, and neurodegeneration are to find a proper cure or therapeutic strategy missing for years. The conditional animal model introduced in this research is believed to generate more critical *in vivo* and *in vitro* information on calpain brain functions and injuries that can be translated into therapeutic approaches.

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Appendix II: Reprint published articles

Conditional disruption of calpain in the CNS alters dendrite morphology, impairs LTP, and promotes neuronal survival following injury. (2013) The Journal of Neuroscience, 33(13):5773-84

Cellular/Molecular

Conditional Disruption of Calpain in the CNS Alters Dendrite Morphology, Impairs LTP, and Promotes Neuronal Survival following Injury

Mandana Amini,[1] Chun-lei Ma,[2] Rasoul Farazifard,[1] Guoqi Zhu,[3] Yi Zhang,[1] Jacqueline Vanderluit,[4] Joanna Susie Zoltewicz,[5] Fadi Hage,[1] Joseph M. Savitt,[6] Diane C. Lagace,[1] Ruth S. Slack,[1] Jean-Claude Beique,[1] Michel Baudry,[3] Peter A. Greer,[7] Richard Bergeron,[1,2] and David S. Park[1,8]

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Ubiquitous classical (typical) calpains, calpain-1 and calpain-2, are Ca^{+2}-dependent cysteine proteases, which have been associated with numerous physiological and pathological cellular functions. However, a clear understanding of the role of calpains in the CNS has been hampered by the lack of appropriate deletion paradigms in the brain. In this study, we describe a unique model of conditional deletion of both calpain-1 and calpain-2 activities in mouse brain, which more definitively assesses the role of these ubiquitous proteases in brain development/function and pathology. Surprisingly, we show that these calpains are not critical for gross CNS development. However, calpain-1/calpain-2 loss leads to reduced dendritic branching complexity and spine density deficits associated with major deterioration in hippocampal long-term potentiation and spatial memory. Moreover, calpain-1/calpain-2-deficient neurons were significantly resistant to injury induced by excitotoxic stress or mitochondrial toxicity. Examination of downstream target showed that the conversion of the Cdk5 activator, p35, to pathogenic p25 form, occurred only in the presence of calpain and that it played a major role in calpain-mediated neuronal death. These findings unequivocally establish two central roles of calpain-1/calpain-2 in CNS function in plasticity and neuronal death.

Introduction

Calpains are intracellular calcium-dependent cysteine proteases thought to regulate a diverse set of biological processes (Goll et al., 2003). There are at least 15 human calpain genes broadly divided into classical (typical) and nonconventional isoforms (Sorimachi et al., 2011). The best characterized calpains are the ubiquitously expressed classical calpain-1 and calpain-2 isoforms. These are heterodimers consisting of a distinct catalytic large subunit encoded by *capn1* or *capn2* genes, respectively, and a common regulatory small subunit encoded by *capns1* (previously known as *capn4*). Calpains are widely expressed in the brain (Goll et al., 2003). Functions potentially pertinent to CNS development/function include cell death (Gil-Parrado et al., 2002; Li et al., 2009), cell proliferation (Konig et al., 2003), cell signaling (Sato and Kawashima, 2001), synaptic restructuring (Zadran et al., 2010a), and synaptic plasticity (Lynch and Baudry, 1984; Denny et al., 1990; Vanderklish et al., 1996). A body of evidence also suggests that calpain regulates neuronal death in *in vitro* and *in vivo* rodent models of diverse neurodegenerative/injury conditions, such as Huntington's disease, Alzheimer's disease, ischemic stroke, and Parkinson's disease (PD) (Mouatt-Prigent et al., 1996; Crocker et al., 2003; Smith et al., 2006; Bevers and Neumar, 2008; Vosler et al., 2008). However, questions remain regarding the exact roles of calpain-1/calpain-2, particularly in the nervous system. Study of the classical calpain isoforms is further complicated by the possibility that the two calpain isoforms may compensate for each other. Germline disruption of *capns1*, which encodes the common small subunit of calpain-1 and calpain-2,

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The authors declare no competing financial interests.

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has been previously shown to disrupt the activities of both these isoforms (Arthur et al., 2000). However, CAPNS1-deficient embryos die at midgestation (E11.5) because of defects in cardiovascular development, which precludes study of the nervous system using this particular animal model.

To overcome this problem, we generated a CNS-specific calpain-1/calpain-2-deficient mouse using a floxed *capns1* allele combined with the *Nestin-Cre* transgenic driver. Using this paradigm, we have explored the role of calpain-1/calpain-2 in nervous system development and function. We were surprised to find that calpain-deficient animals survived to adulthood and brain development appeared grossly normal, given previous reports describing the essential roles of calpain in nervous system development, such as cell proliferation (Konig et al., 2003; Honda et al., 2004) and death (Sedarous et al., 2003; Vosler et al., 2008). However, phenotypic characterization of these mice revealed critical functions for calpain in synaptic plasticity and neuronal injury, which has important implications for therapeutic targeting of calpain in injury-induced or age-related degenerative diseases.

Materials and Methods

Generation of CNS-specific CAPNS1 knock-out mice and genotyping

All animal experimental studies were approved by the University of Ottawa Animal Care Committee and conformed to the guidelines set forth by the Canadian Council on Animal Care and Canadian Institutes of Health Research, and by the Institutional Animal Care and Use Committee from Western University of Health Sciences. Both male and female mice were used in all experiments. Conditionally targeted (floxed) *capns1* and transgenic *Nestin-Cre* and *TH-Cre* mice were generated as previously described (Berube et al., 2005; Savitt et al., 2005; Tan et al., 2006). *capns1*$^{flox/flox}$ and *Nestin-Cre* mice were backcrossed for eight generations onto an FVB/N background and then interbred to obtain hemizygous *Nestin-Cre* transgenic mice that were homozygous for the loxP targeted (floxed) *capns1* gene (*Nestin-Cre*; *capns1*$^{flox/flox}$). These mice were referred to as *CAPNS1-Nestin-cKO* mice. Controls were the littermates carrying a single floxed allele of *capns1* (*capns1*$^{+/flox}$). Similarly, the *capns1*$^{flox/flox}$ and *TH-Cre mice* were backcrossed for 10 generations onto C57BL/6 strain and then interbred to obtain tyrosine hydroxylase (TH)-specific calpain-deficient mice (*CAPNS1-TH-cKO*). All experiments were performed with *CAPNS1-Nestin-cKO* (FVB/N) mice, except the midbrain cultures, which were from *CAPNS1-TH-cKO* (C57BL/6J) mice. PCR was used to determine *Cre* sequences in tail biopsy DNA samples of *Nestin-Cre* and *TH-Cre* mice, as described previously (Berube et al., 2005; Savitt et al., 2005). The *capns1* floxed and wild-type alleles were detected using the primer set: forward, 5′-GTGGTAGCCGCTGAAACTCC-3′; reverse, 5′-TGTTCCCGCTCTCATCTGC-3′. The products were 550 bp for the floxed allele and 515 bp for the wild-type allele. Nonradioactive DIG labeling Southern blot hybridization analysis of brain DNA was used to confirm the excision of *lox*P-flanked (floxed) sequences, indicated by a 5.1 kbp PstI fragment from the wild-type *capns1* locus, a 3.2 kbp PstI fragment from the pre-Cre floxed allele, and a 4.3 kbp fragment from Cre recombinase-excised (post-Cre floxed) allele, as previously described (Tan et al., 2006).

Immunoblotting and casein zymogram

Total brain fractions of the *CAPNS1-Nestin-cKO* mice and control littermates were resolved by SDS-PAGE and PVDF immunoblots were prepared and probed using a rabbit polyclonal antisera raised against rat calpain-2 that recognizes both the small subunit (28 kDa/CAPNS1) and calpain-2 large subunit (80 kDa/CAPN2), as previously described (Tan et al., 2006). Casein zymogram of brain lysates was used to detect calpain-1 and calpain-2 activities as previously described (Tan et al., 2006). αII-Spectrin breakdown protein and active calpain-1 analysis was described previously (Martinez et al., 2010). Briefly, brain lysates from *CAPNS1-Nestin-cKO* or control mice were made using 1% Triton X-100. A total of 100 μg of total protein was incubated with the following additives: 1 μg purified calpain-2 + 10 mM $CaCl_2$, or 10 mM $CaCl_2$, or 10 mM EGTA, and incubated at room temperature for 30 min to allow digestion to occur. A total of 50 μg of each reaction was resolved on a 4–20% gel Tris-glycine gel, blotted to PVDF, and immunoblotted with antibodies against calpain cleaved αII-spectrin or active calpain-1. For analysis of p35/p25 expression, whole-cell protein lysates were collected from cortical neurons treated with 1-methyl-4-phenylpyridinium (MPP^+) or from cerebellar granule neurons (CGNs) treated with glutamate for the indicated times. The lysates were separated by 12% SDS-PAGE, and immunoblots were probed with anti-p35/25 antibody as described previously (O'Hare et al., 2005). Immunoblot analysis of hippocampal protein fractions for GluN1, GluN2A, GluN2B, PSD95, GluA1, and GluA2/3 was previously described (Imamura et al., 2008). Briefly, hippocampi from the brain of 4- to 6-week-old mice were dissected and homogenized using ice-cold tobacco etch virus protease lysis buffer. After a series of centrifugations, the membrane protein fractions were collected and protein concentrations were determined using the Bio-Rad protein assay. Primary antibodies included the following: anti-calpain-2 (1:1000) (Tan et al., 2006); anti-αII-spectrin (α Fodrin, mouse monoclonal, Enzo; 1:3000); anti-activated calpain-1 (rabbit polyclonal generated in the laboratory of Banyan Biomarkers; 1:300; antibody was raised against the peptide LGRHENA corresponding to the new N terminus produced by calpain-1 autolysis); anti-NMDAR1 (GluN1) (rabbit polyclonal, Cell Signaling Technology; 1:1000); anti-NMDAR2A (GluN2A) and anti-NMDAR2B (GluN2B) (rabbit polyclonal and mouse monoclonal, respectively, LifeSpan BioScience; 1:750); anti-GluR1 (GluA1) (rabbit monoclonal, Millipore Cell Signaling Technology Solutions; 1:5000); anti GluR2/3 (GluA2/3) (rabbit polyclonal, Millipore Bioscience Research Reagents; 1:1000); anti-PSD95 (rabbit polyclonal, Cell Signaling Technology;1:1000); and anti-p35 (p35 C-19, sc-820, Santa Cruz Biotechnology; 1:1000).

Tissue preparation, immunohistochemistry, and phenotypic analysis

Tissue preparation was previously described (Rashidian et al., 2005; Vanderluit et al., 2007). Briefly, coronal serial sections (14 μm) were obtained from whole fixed cryoprotected brains of 4- to 6-week-old mice, and embryos at embryonic days (E) 13.5 and 17.5. Analyses of CA1 pyramidal cell density were performed using cresyl violet-stained sections from 4- to 6-week-old mice. CA1 neurons of hippocampus were counted using ImageJ Program in two representative sections per animal in a field of 70 mm^2. For short-term BrdU incorporation experiments, pregnant dams at 13.5 d received a single intraperitoneal injection of BrdU (100 μg/g of body weight) and the embryos were collected 2 h after injection. To detect BrdU-labeled nuclei, sections were denatured in 2 N HCl at 37°C for 30 min and incubated in rat anti-BrdU (1:500; Accurate Chemicals & Scientific), as previously done (Vanderluit et al., 2007). $BrdU^+$ neurons were counted on 70 mm^2 of ventricular zone and subventricular zone field of 4 representative sections per embryos using ImageJ program. TUNEL assay was performed using the Roche Diagnostics TdT kit. Briefly embryonic brains at E17.5 were fixed, and 14 μm sections were washed with 0.5% Triton X-100 in PBS incubated with TdT and dUTP at 37°C, and then blocked with 10% BSA. $TUNEL^+$ cells were detected by staining with Alexa streptavidin-594 and counted throughout whole sections of different regions of brains, 12 sections per brain.

Golgi staining

Golgi staining was completed using FD Rapid GolgiStain Kit (FD NeuroTechnologies). Briefly 4- to 6-week-old naive mice were intracardially perfused with 0.9% cold saline. Forebrains within the region ~0.50 to ~−3.5 mm (relative to the bregma position) were separated and stained according to the manufacturer's instructions. Tissues were sectioned at 150 μm and mounted on gelatin-coated slides. Morphology of apical and basal dendrites of hippocampal CA1 pyramidal neurons were blindly analyzed by Neurodigitech. Commercially stereology-based software (Microbrightfield), installed on a Dell PC workstation that controlled a Zeiss Axioplan 2 image microscope with Optronics MicroFire CCD camera (1600 × 1200 digital camera, motorized *x*, *y*, and *z*-focus for high-

resolution image acquisition and digital quantitation) was used. For cell selection, the sampling process for candidate pyramidal cells was conducted as follows: (1) previewing the entire anterior-to-posterior axis of CA1 region, under low and high magnification Zeiss objectives (10× and 20×); (2) comparing and locating those cells with the least truncations of distal dendrites as possible under higher magnification Zeiss objectives (40× and 63×); (3) after verifications, capturing low-magnification images for archiving purpose; and then (4) using a Zeiss 100× objective with immersion oil (NA = 1.25) to perform 3D dendritic reconstruction. For spine sampling, only spines orthogonal to the dendritic shaft were readily resolved and included in this analysis, whereas spines protruding above or beneath the dendritic shaft were not sampled. Spines were counted throughout the entire dendritic trees. For Scholl analysis, a series of concentric circles (30 μm interval) radiating from the soma was set up. All data represented the average for neurons.

Electrophysiology

Whole-cell recordings. Coronal brain slices containing the hippocampus were obtained as described previously (Imamura et al., 2008). In brief, 4- to 6-week-old mice were anesthetized with isofluorane in agreement with the guidelines of the Canadian Council of Animal Care. The brain was removed and placed in an oxygenated (95% O_2, 5% CO_2) artificial CSF (ACSF) at 4°C, containing (in mM) 126 NaCl, 2.5 KCl, 1 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, and 10 glucose. The osmolarity of the ACSF was adjusted to 300 mOsmol and the pH to 7.2. A vibrating microtome (VT 1000S; Leica) was used to obtain coronal sections (300 μm) containing the hippocampus. Acute brain slices were stored for at least 1 h in an oxygenated chamber at room temperature before recording. Voltage-clamp recordings of CA1 pyramidal cells were obtained with a Multiclamp 700A amplifier (Molecular Devices) under visual control using differential interference contrast and infrared video microscopy (Leica). EPSCs were evoked by electrical stimulation of the Schaffer collaterals with a bipolar microelectrode positioned in stratum radiatum with 100 μs current pulses (0.1–1 mA, 0.3–0.01 Hz), which were adjusted to evoke a current amplitude in the range of 60–120 pA at $V_m = -65$ mV. Patch-clamp recordings were obtained in pyramidal cells of CA1 region in hippocampus in normal ACSF. For recordings of EPSCs mediated by glutamate receptors (both AMPA receptor and NMDA receptor EPSCs), brain slices were bathed in ACSF containing picrotoxin (50 μM) and strychnine (1 μM). For recordings of NMDA receptor-mediated EPSCs, brain slices were bathed in low Mg^{2+} ACSF containing picrotoxin (50 μM), strychnine (1 μM), and 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo-[*f*]quinoxaline (5 μM); for recordings of miniature EPSCs (mEPSCs), brain slices were perfused in low Mg^{2+} ACSF containing TTX (1 μM), picrotoxin (50 μM), strychnine (1 μM), CGP52432 (10 μM); for LTP, brain slices were bathed in ACSF containing picrotoxin (50 μM) and strychnine (1 μM). The pH of the perfusion solution was adjusted to 7.2 and osmolarity to 300 mOsm. Recording electrodes were filled with an intracellular solution (in mM): 130 Cs^+-methanesulphonate, 10 HEPES, 10 KCl, 2 $MgCl_2$, 0.2 EGTA, 2 ATP-Mg, and 0.2 GTP-Tris (hydroxymethyl) aminomethane and lidocaine *N*-ethyl bromide (QX-314, 5 mM). The pH of the intracellular solutions was adjusted to 7.2, and the osmolarity was 280–290 mOsm. The pipettes had a resistance of 3–7 MΩ. For eliciting LTP a pairing protocol composed of three brief high-frequency trains (50 pulses at 100 Hz) repeated at 4 s intervals given at the end of a long depolarization (3 min at 0 mV) was used. This protocol has been successfully used before to induce an increase of synaptic responses lasting for >40 min (Chen et al., 1999; Martina et al., 2004). Data were collected by Pclamp 9 software (Molecular Devices). Analyses were performed using Igor software (WaveMetrics).

Field potential recording. Three- to 4-month-old male mice were anesthetized with halothane and decapitated. Brains were quickly removed and transferred to oxygenated, ice-cold, high-magnesium cutting medium (in mM: 124 NaCl, 26 $NaHCO_3$, 10 glucose, 3 KCl, 1.25 KH_2PO_4, 5 $MgSO_4$, and 3.4 $CaCl_2$). Hippocampal transversal slices (400 μm thick) were prepared using a McIlwain-type tissue chopper and transferred to an interface recording chamber and exposed to a warm, humidified atmosphere of 95%O_2/5%CO_2 and continuously perfused with oxygenated and preheated (33 ± 0.5°C) ACSF (in mM: 110 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.5 $MgSO_4$, 1.24 KH_2PO_4, 10 glucose, 27.4 $NaHCO_3$) at 1.4 ml/min. After 1.5 h incubation at 33 ± 0.5°C in the recording chamber, a single glass pipette filled with 2 M NaCl was used to record field EPSPs (fEPSPs) elicited by stimulation of the Schaffer collateral pathway with twisted nichrome wires (single bare wire diameter, 50 μm) placed in CA1 stratum radiatum. Responses were recorded through a differential amplifier (DAM 50, World Precision Instruments) with a 10 kHz high-pass and 0.1 Hz low-pass filter. Before each experiment, the input/output relation was examined by varying the intensity of the stimulation. Data were collected and digitized by Clampex, and the amplitude of fEPSP was analyzed.

All data are expressed as mean ± SEM, and statistical significance of differences between means was calculated with appropriate Student's *t* test.

Behavioral analysis

All behavioral tests were completed in the Behavior Core Facility at the University of Ottawa using standardized protocols. Animals were habituated to the testing room ~1 h before testing. One cohort of mice (3- to 4-month-old) was used for beam break, rotarod, elevated plus maze, and open field within at least 2 d between tests. Because of aging deficits in vision that can occur in the FVB/N strain, a separate cohort of younger mice (4- to 6-week-old) was examined for the water maze paradigm.

Beam Break. Locomotor activity over a 24 h period was analyzed by using the computer-assisted beam break, MicroMax system (Accuscan) as previously reported (Smith et al., 2006).

Rotarod. The rotating rod test was performed on an accelerating rotarod (IITC Life Science; length of the test 2 min; accelerating speed 1–45 rpm). The time until the animal falls off the rotating rod was measured in four tests performed on two consecutive days.

Elevated Plus Maze. The arms were ~12 cm wide and ~50 cm long and the maze was raised ~1 m off the floor. The times spent in the open arms, closed arms, and center of the elevated plus-maze apparatus were recorded for 10 min using the Ethovision software.

Open field. Mice were placed in the corner of a wooden box measuring 45 cm × 45 cm × 45 cm and allowed to freely explore for 10 min. The main outcome measures included the time spent in the center, distance traveled and velocity were detected by a video camera using the Ethovision software.

Morris Water Maze. The water maze pool was filled with opaque water and heated to remain at 21°C. A white platform was submerged 1 cm below the water's surface in the center of the target quadrant. Mice were randomly placed on one of starting points in one of the four quadrants and given 60 s to find the hidden platform. Mice that did not find the platform at the end of the 60 s period were led to the platform and were allowed to stay on it for 20 s. Each mouse had 4 trials per day for 11 consecutive days. An animal was scored as successfully finding the platform if it succeeded in two of four trials per day. In the visual tests, mice were placed on one of four quadrants and allowed to find the visible platform in 60 s. The swimming path of the mice was recorded by a video camera and analyzed by the Ethovision 7 XT.

Assessment of in vitro neuronal survival

Primary cortical neuron cultures were prepared from 14 to 15 d embryos individually as previously described (Kim et al., 2005). Cultured neurons were treated with MPP^+ at a final concentration of 20 μM after 2 DIV. Numbers of viable neurons were evaluated by lysis of cultures followed by counting intact nuclei as described previously (Kim et al., 2005). For midbrain cultures, mesencephalic neurons were collected from day 13 to day 14 embryos as described previously (Kim et al., 2005). MPP^+ treatment started on 7 DIV at 20 μM final concentration for desired time periods. Cells were stained with anti-TH antibody (ImmunoStar; 1:2500) as primary antibody and Hoechst as the control. Fluorescent-stained TH^+ neurons were counted and evaluated for nuclear, dendrite, and axon morphology. CGNs were harvested from 7 to 8 d pups, and glutamate- or hypoxia-induced excitotoxicity was performed as described previously (Rashidian et al., 2005). Cell survival was evaluated as indicated previously (Kim et al., 2005). For rescuing survival phenotype, both cortical neurons and CGNs were infected with adeno-associated virus (AAV) expressing p25 or with AAV-GFP (multiplicity of infection,

25) at the time of plating. MPP^+ was added to cortical neurons on 6 DIV at a final concentration of 20 μM. Glutamate treatments were completed on 8 DIV. Neuron viability was evaluated by counting the number of intact nuclei as described previously (Kim et al., 2005).

AAV construct

cDNA sequences of human p25 were subcloned into the BamHI–EcoRI sites of the AM/CBA-pl-WPRE-bGH plasmid, a recombinant AAV (rAAV1) vector. The virus was then generated and purified as described previously (Rashidian et al., 2005). EGFP was excised from Clontech pEGFP-N2 vector (catalog #6081-1) using BamHI and a blunted NotI. This fragment was subsequently subcloned into the same AAV vector, AM CBA-pl-WPRE-bGH, into the BamHI and EcoRV sites to produce AAV-CBA-EGFP-WPRE-bGH.

Data and statistical analysis

Statistical analysis was performed using SPSS Statistics 18 and included use of one of the following: (1) two-tailed Student's *t* test for comparison of two groups, (2) one-way ANOVA for comparison of four groups on one outcome measure, (3) two-way ANOVA for more than one group on more than one outcome measure, (4) repeated ANOVA (for repeated measure on same animal), and (5) χ^2 to examine the distribution of animals achieving preset criteria. All *post hoc* tests were completed using Bonferroni correction. In analyses that included both male and female animals, sex differences were also tested. However, as all comparisons between sexes did not reveal any differences, only grouped data for controls and mutants are shown. Significance is indicated when $p < 0.05$ (*).

Results

Elimination of calpain-1 and calpain-2 activities in mouse brain

We used a Cre/loxP-mediated recombination strategy to conditionally target the *capns1* locus in the mouse brain. Exons 9, 10, and 11 of *capns1* were flanked by *loxP* sites as described previously (Tan et al., 2006). To obtain CNS-specific disruption of calpain-1/calpain-2 activity, $capns1^{flox/flox}$ mice were crossed with *Nestin-Cre* transgenic mice expressing Cre recombinase under the control of the *nestin* promoter, to create *Nestin-Cre*; $capns1^{flox/flox}$ (*CAPNS1-Nestin-cKO*) mice. Nestin is an intermediate filament expressed specifically in CNS precursor cells (Berube et al., 2005). Surprisingly, the *CAPNS1-Nestin-cKO* were viable, fertile, and born at approximate Mendelian ratios ($n = 49$ of 225; 21.7%).

Southern blot analysis of genomic DNA revealed that complete excision of *capns1* occurred only in the brains of mice that were transgenic for Nestin-Cre and homozygous for the *loxP* targeted (flox) *capns1* gene. These *CAPNS1-Nestin-cKO* mice brains demonstrated near-complete excision of floxed *capns1* alleles, as determined by the generation of a 4.3 kbp size post-Cre allele fragment (Fig. 1*a*). To assess calpain expression in the brains of these mice, we first performed immunoblot analysis using an antibody raised against calpain-2, which recognizes both the 28 kDa *capns1* and 80 kDa *capn2* gene products. As expected, the brains of *CAPNS1-Nestin-cKO* mice displayed complete loss of the 28 kDa subunit. Importantly, and as previously reported, loss of CAPNS1 also led to the loss of 80 kDa CAPN2 protein presumably because of the destabilizing effects of CAPNS1 loss (Arthur et al., 2000; Chung et al., 2004) (Fig. 1*b*). The effect of *capns1* targeting in brain on calpain protease activity was assessed using two methods. First, casein zymogram analyses of brain lysates showed no detectable calpain-1 or calpain-2 activity in the brains of *CAPNS1-Nestin-cKO* mice (Fig. 1*b*), whereas robust activity for both calpain isoforms was observed in controls. Second, endogenous calpains were activated *in vitro* in whole brain lysates by adding $CaCl_2$, which yielded the well-characterized calpain-specific spectrin breakdown products (Zhang et al., 2009), SBDP145 and 150, in control but not in *CAPNS1-Nestin-cKO* brain extracts (Martinez et al., 2010). Disruption of calpain activity was further supported using an antibody specific for the active form of calpain-1, which did not detect the active 80 kDa catalytic subunit in *CAPNS1-Nestin-cKO* brain lysates (Fig. 1*c*). Together, these results provided compelling evidence of calpain deficiency in the brains of *CAPNS1-Nestin-cKO* mice.

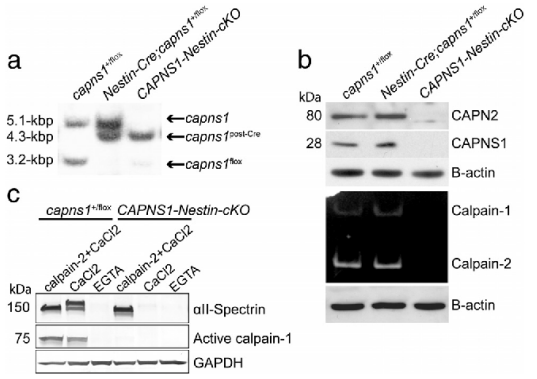

Figure 1. Disruption of calpain-1 and calpain-2 expression and activities in the *CAPNS1-Nestin-cKO* mouse brain. ***a***, Southern blot analysis of brain DNA samples from *CAPNS1-Nestin-cKO* (*Nestin-Cre*; $capns1^{flox/flox}$) and control littermates (*Nestin-Cre*; $capns1^{+/flox}$ or $capns1^{+/flox}$). The probe distinguishes 5.1, 4.3, and 3.2 kbp PstI fragments corresponding to untargeted (*capns1*) or the floxed allele after ($capns1^{post\text{-}Cre}$) or before ($capns1^{flox}$) Cre-mediated excision, respectively. ***b***, Top, Representative immunoblot of calpain-2 (CAPN2) (80 kDa) and CAPNS1 (28 kDa) proteins extracted from brains of *CAPNS1-Nestin-cKO* and control littermates probed with calpain-2 antibody. Bottom, Casein zymogram of the brain total fraction to analyze calpain-1 and calpain-2 activities ($n = 5$ per genotype). ***c***, Representative immunoblot of brain total protein extracts probed with indicated antibodies for αII-spectrin and active calpain-1 ($n = 3$ per genotype). $CaCl_2$ triggered deavage of spectrin to its calpain-specific 145 and 150 kDa breakdown products in control but not *CAPNS1-Nestin-cKO* brain extracts, whereas addition of rat calpain-2 enzyme deaved spectrin to 150 kDa in brain lysates of both genotypes.

Calpain-deficient mice do not show detectable gross morphological abnormalities

The importance of calpain in early murine development was underscored by the lethality observed in both *capns1* and *capn2* germline knock-out mice (Arthur et al., 2000; Dutt et al., 2006). The viability of adult *CAPNS1-Nestin-cKO* animals with brain-specific calpain deficiency allowed us to explore the role of calpain in CNS development. Serial coronal sections of brains from mutant and control littermates at developmental stages and adults were stained with cresyl violet. Unexpectedly, we did not detect any gross morphological or structural abnormalities in the brain regions and neurons of the mutants either at E13.5 (data not shown), E17.5, or 4- to 6-week-old adult mice (Fig. 2*a*,*b*). Quantification of hippocampal CA1 neurons in the mutants demonstrated the same number of pyramidal neurons as in controls (Fig. 2*c*). We also examined whether neuronal precursor proliferation was altered in mutant mice. Embryos at E13.5 were subjected to short-term BrdU labeling and BrdU-positive nuclei were evaluated in the ventricular zone and subventricular zone of the brain. No differences in the number of proliferating cells were detected between mutant and control mice (Fig. 2*d*). Similarly, developmental programmed cell death (i.e., apoptosis) was detected by TUNEL staining at E17.5. Equal numbers of scattered TUNEL-positive cells were observed in both genotypes (Fig. 2*e*).

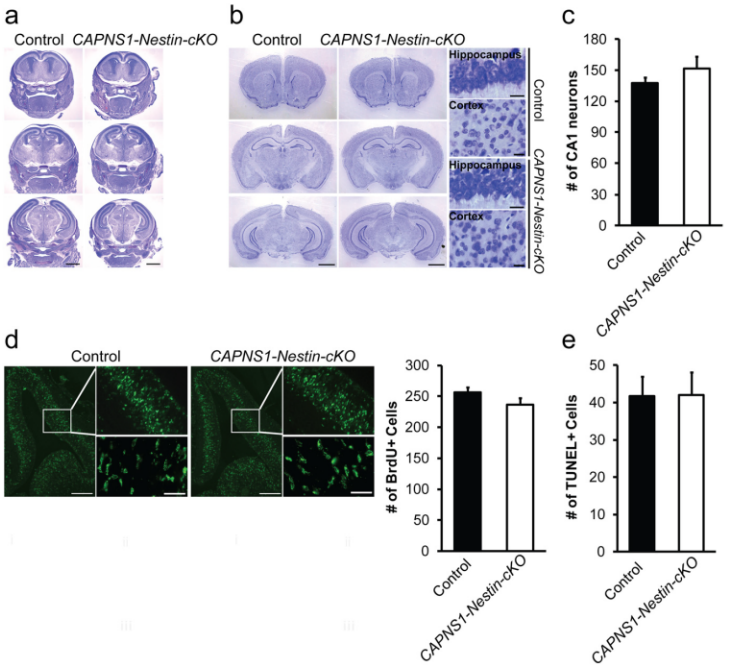

Figure 2. Histological assessment of *CAPNS1-Nestin-cKO* mice compared with control littermates. ***a***, ***b***, Representative cresyl violet staining of coronal sections through different regions of brains from (***a***) E17.5 and (***b***) 4- to 6-week-old adult *CAPNS1-Nestin-cKO* mice and controls, respectively, including different regions (scale bar, 1 mm) and neurons in hippocampus and cortex (scale bar, 20 μm). ***c***, Number of CA1 neurons in defined fields at 4 to 6 weeks of age ($n = 3$ per genotype). ***d***, Representative photomicrographs of BrdU-stained sections of embryonic brains from *CAPNS1-Nestin-cKO* and control littermates. Scale bar: left, 175 μm; right, 20 μm. Number of BrdU-positive cells were counted in defined fields of sections ($n = 4$ per genotype). ***e***, Number of TUNEL-positive cells throughout whole brain sections ($n = 3$ per genotype).

These results indicate that calpain-1/calpain-2 isoforms do not play essential roles in regulating developmental cell proliferation/apoptosis or the gross organization or structure of the developing and adult brain.

Dendrite morphology alterations in calpain-deficient CA1 pyramidal neurons

Because calpain has been suggested to modify cytoskeletal proteins (Wilson et al., 2000), we sought to investigate whether dendritic structures of CA1 hippocampal neurons were altered in *CAPNS1-Nestin-cKO*. We thus performed Golgi staining of dorsal hippocampus in 4- to 6-week-old controls and conditionally calpain-deleted brains. Representative photomicrographs of CA1 pyramidal neurons and segments of apical and basal dendrites in control littermates and *CAPNS1-Nestin-cKO* mice are shown in Figure 3*a*. We observed a significant reduction in the density of dendritic spines along the apical branches of CA1 pyramidal neurons of the mutants. This decrease in spine density was also accompanied by a significant decline in total spine counts in apical dendrites in calpain-deficient mice (Fig. 3*b*,*c*). Moreover, apical and basal dendritic complexity and spine distribution were reduced in CA1 pyramidal neurons of *CAPNS1-Nestin-cKO*, as revealed by Scholl analysis (Fig. 3*d*,*e*). Finally, no significant changes were observed in dendritic lengths with calpain deficiency (Fig. 3*f*). These results suggest a critical role of calpain in regulation/organization of dendritic trees in hippocampal CA1 neurons.

Effect of calpain deficiency on glutamatergic synaptic transmission and LTP

The overall alterations observed in dendritic complexities and spine density in *CAPNS1-Nestin-cKO* suggested that calpain may play a role in modulating glutamatergic synaptic transmission. To begin addressing this possibility, we measured the expression of a number of AMPA and NMDA receptor subunits in isolated membrane fractions from hippocampi of 4- to 6-week-old *CAPNS1-Nestin-cKO* and controls. Intriguingly, we observed robust reductions in the levels of the NMDA receptor subunits GluN1, GluN2A, and GluN2B as well as of the AMPA receptor subunits GluA1 and GluA2/3. Finally, the expression of PSD95 was also reduced in mutant mice compared with control littermates (Fig. 4*a*).

These results prompted us to examine in greater detail metrics of glutamatergic synaptic transmission by whole-cell recordings of CA1 pyramidal cells in acute slices from *CAPNS1-Nestin-cKO* mice and controls. We first determined the ratio of AMPA to NMDA receptor components of evoked EPSCs in both types of mice. When holding the cell at +40 mV, both AMPA and NMDA receptors are activated by synaptically released glutamate, and their respective contribution to the evoked EPSCs can be approximated because AMPA receptor- and NMDA receptor-mediated synaptic responses are kinetically distinguishable. Under these conditions, the AMPA/NMDA ratio of evoked EPSCs was indistinguishable between *CAPNS1-Nestin-cKO* and control mice (Fig. 4*b*). Moreover, the decay kinetics of both isolated AMPA receptor- or NMDA receptor-mediated (in low Mg^{2+}) EPSCs were likewise not altered in mutant mice (Fig. 4*c*), suggesting that no obvious changes in surface glutamate receptor subunit composition occurred in these mice. We next recorded spontaneous mEPSCs in CA1 pyramidal neurons and observed no significant changes in the amplitudes or the frequency of mEPSCs in calpain-deficient mice, compared with control animals (Fig. 4*d*). These results are in agreement with those of the AMPA/NMDA receptor ratio and suggest that deletion of calpains did not alter the expression of AMPA receptors at individual synapses, or the probability of release of glutamate. The latter possibility was further examined by paired pulse ratio analysis, a measure of presynaptic neurotransmitter release probability. As expected for CA1 synapses, paired pulse facilitation was observed at intervals of 50, 100, and 200 ms, and this ratio was not altered in *CAPNS1-Nestin-cKO* mice (Fig. 4*e*). Together, these observations argue that, although calpain deficiency was accompanied with reduced levels of a variety of synaptic proteins, this was not associated with measurable deficits in basal excitatory glutamatergic synaptic neurotransmission.

We further analyzed properties of synaptic transmission in the CA1 field of hippocampal slices from control and *CAPNS1-Nestin-cKO* mice by field recording. No alteration was observed in paired-pulse facilitation in the absence of calpain (data not

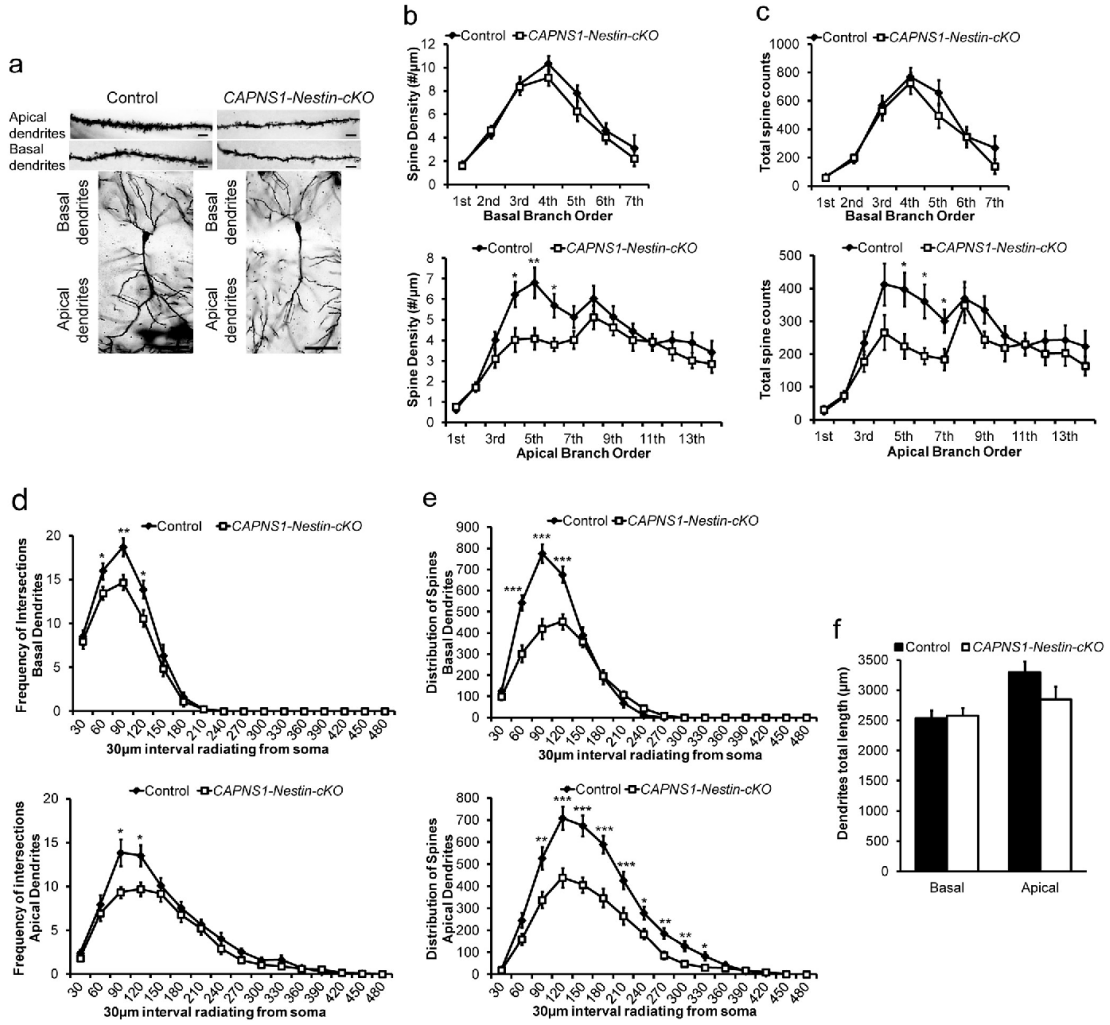

Figure 3. Altered morphology of hippocampal CA1 neuron dendrites in *CAPNS1-Nestin-cKO* mice. ***a***, Representative segments of apical and basal dendrites of CA1 neurons in *CAPNS1-Nestin-cKO* and control littermates. Scale bar: 5 μm. Bottom, Representative photomicrograph of CA1 pyramidal neurons. Scale bar: 50 μm. ***b***, Representative graphs of spine density of basal and apical dendrites per branch orders. Spine density is expressed as the mean spine number per 1 μm dendritic segment. ***c***, Representative graphs of total spine count of basal and apical dendrites per branch orders. ***d***, Scholl analysis of intersection pattern of basal and apical dendrites in CA1 neurons. ***e***, Distribution of spines in basal and apical dendrites at 30 μm interval radiating distances from the soma in CA1 neurons. ***f***, Representative graphs of basal and apical dendrites total length ($n = 27$ neurons for control littermates and $n = 20$ neurons for *CAPNS1-Nestin-cKOs*). Data are mean ± SEM. *$p <$ 0.05 (repeated ANOVA). **$p < 0.01$ (repeated ANOVA). ***$p < 0.001$ (repeated ANOVA).

shown). However, the input/output curve (stimulation intensity to evoked response) was significantly shifted downward in the mutant compared with controls (Fig. 5*a*). The decrease in fEPSPs was ~30–40%, broadly matching the decrease in the density of synapses observed in the mutant CA1 pyramidal neurons. We also determined the effects of θ burst stimulation (TBS) on fEPSPs (Fig. 5*b*). Interestingly, TBS-induced LTP was significantly reduced in hippocampal slices from *CAPNS1-Nestin-cKO* mice (fEPSP slope, compared with baseline, at 50 min after LTP induction: control 161 ± 10%; mutant 123 ± 6%; $p < 0.001$). Analysis of the burst responses during TBS revealed that, although the absolute levels of each burst responses were lower in the mutant than in the control, the overall increase observed during successive bursts exhibits a similar pattern in control and mutant mice (Fig. 5*c*).

The magnitude of LTP was also studied with whole-cell recording using a pairing protocol consisting of 3 brief high-frequency tetani (50 pulses at 100 Hz, 4 s intervals) given at the end of a 3-min-long depolarization step at 0 mV. This protocol induced a 90.9 ± 11.9% (control, $n = 6$; $p < 0.005$) increase in the amplitude of EPSC lasting for >40 min. This LTP was NMDA receptor-dependent because it was prevented by application of DL-2-amino-5-phosphonovaleric acid (AP-5, 50 μM; 9.50 ± 8.77% above baseline; $n = 4$; $p > 0.05$, data not shown). Interestingly, the same pairing protocol administered in slices from *CAPNS1-Nestin-cKOs* induced

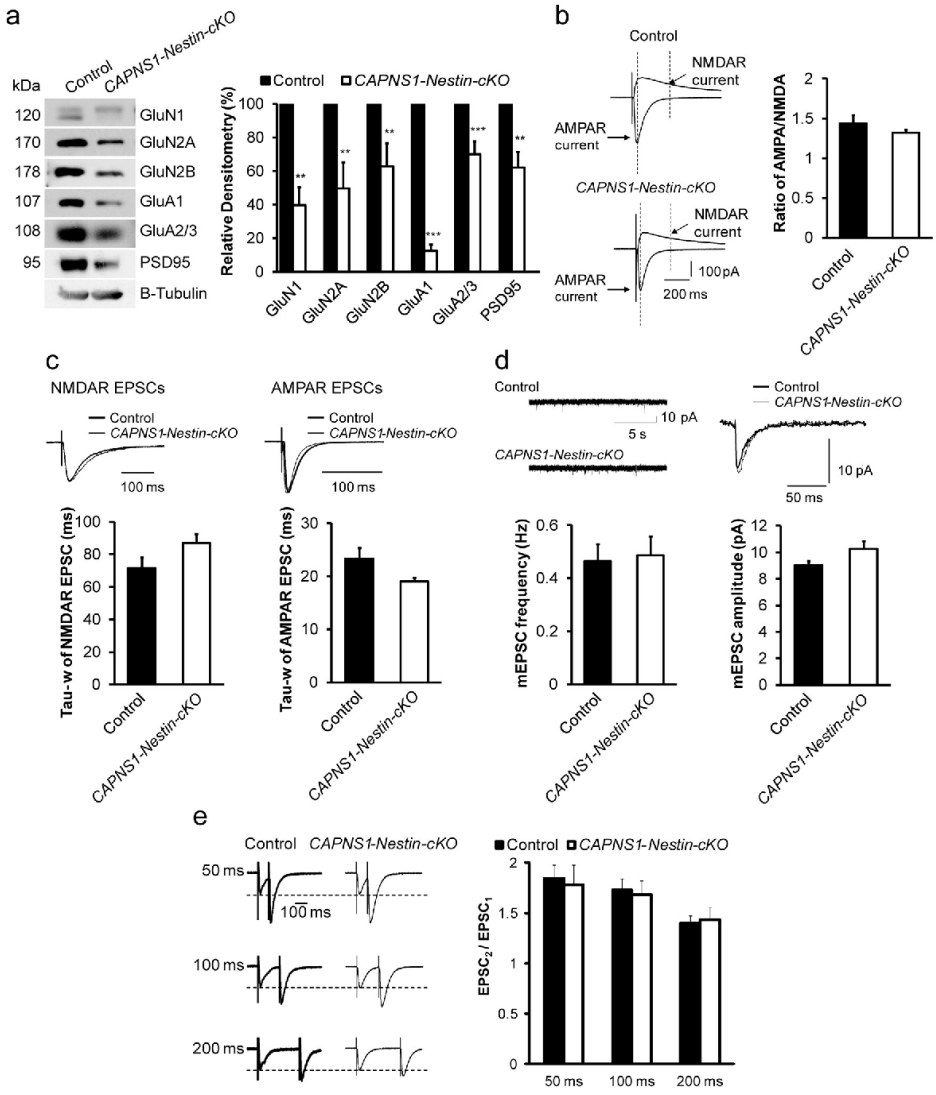

Figure 4. Hippocampal membrane protein distribution and glutamatergic synaptic transmission in hippocampal CA1 neurons of *CAPNS1-Nestin-cKO* and controls. ***a***, Representative immunoblots of membrane protein fractions of hippocampi from control and *CAPNS1-Nestin-cKO* probed with the indicated antibodies and densitometric quantification of changes in gray values expressed as mean ± SEM. $^{**}p < 0.01$ (Student's *t* test). $^{***}p < 0.001$ (Student's *t* test). Per genotype: $n = 4$ for GluN1 and GluA1; $n = 5$ for GluN2A, GluN2B, and PSD95; $n = 3$ for GluA2/3. ***b***, Representing traces of AMPAR and NMDAR EPSCs at ¥-hold of −65 and +40 m¥, respectively (*CAPNS1-Nestin-cKO* 1.32 ± 0.04, $n = 9$ neurons from 3 mice; and controls 1.4 ± 0.1, $n = 8$ neurons from 4 mice). At −65 m¥, the peak current is mainly contributed by AMPA receptors, whereas at +40 m¥ the peak current comes from both AMPA and NMDA receptors. ***c***, Representative traces and weighted decay time constants of NMDAR and AMPAR EPSCs in *CAPNS1-Nestin-cKO* (NMDAR 87.07 ± 5.46 ms, $n = 11$ neurons from 4 mice; and AMPAR 19.05 ± 0.64 ms, $n = 9$ neurons from 4 mice) and controls (NMDAR 71.55 ± 6.42 ms, $n = 6$ neurons from 4 mice and AMPAR 23.36 ± 2.01 ms, $n = 9$ neurons from 4 mice). ***d***, Representative traces and averages of spontaneous AMPAR-mediated mEPSCs recorded from neurons of *CAPNS1-Nestin-cKO* (amplitude, 10.27 ± 0.59 pA; frequency, 0.48 ± 0.07 Hz, $n = 8$ neurons from 3 mice) and control mice (amplitude, 9.01 ± 0.34 pA; frequency, 0.46 ± 0.07 Hz, $n = 8$ neurons from 4 mice). ***e***, Representative traces and average of paired pulse ratios of EPSCs (AMPA + NMDAR) from neurons of *CAPNS1-Nestin-cKO* mice (50 ms, 1.78 ± 0.2; 100 ms, 1.68 ± 0.14; 200 ms, 1.44 ± 0.12, $n = 9$ neurons from 3 mice) and controls (50 ms, 1.85 ± 0.13; 100 ms, 1.73 ± 0.11; 200 ms, 1.39 ± 0.07, $n = 12$ neurons from 3 mice).

only minimal LTP (control, 181 ± 24%; mutant, 132 ± 29% at 30–40 min after LTP induction; Fig. 5*d*).

Impairments in spatial memory in calpain-deficient mice

We next determined whether the robust reduction in hippocampal LTP observed in *CAPNS1-Nestin-cKO* would be accompanied with deficits in hippocampal-dependent spatial memory. We thus tested *CAPNS1-Nestin-cKO* in the Morris Water Maze task. Significantly less *CAPNS1-Nestin-cKO* mice were able to reach the hidden platform on the last 3 d of training compared with littermate control mice, suggesting that *CAPNS1-Nestin-cKO* have impaired spatial memory (Fig. 6*a*). This behavioral

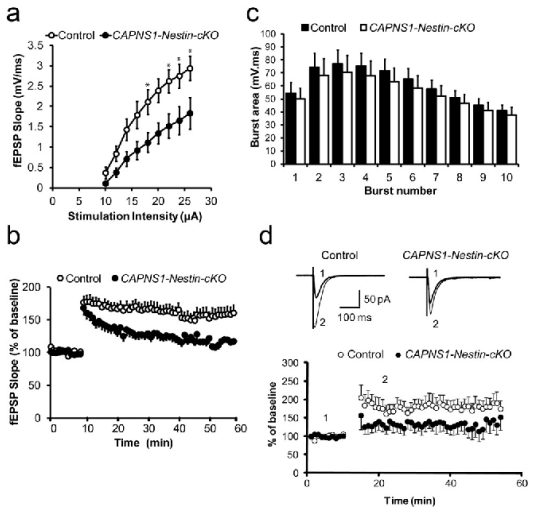

Figure 5. Features of synaptic transmission and plasticity in calpain-deficient mice assessed with field and patch-clamp recording. ***a***, Input/output curves. Amplitudes of the fEPSPs were determined for various intensities of stimulation. The curve is significantly shifted downward in the mutant mice. The results are mean ± SEM; $n = 5$ or 6 slices from 5 mice per genotype. $^{*}p < 0.05$ (Student's *t* test). ***b***, TBS-induced LTP. TBS was delivered after 10 min of baseline recording and slopes of the fEPSPs monitored for an additional 50 min. The values are normalized to the baseline and are mean ± SEM; $n = 5$ or 6 slices from 5 mice per genotype. $^{***}p < 0.001$ (Student's *t* test). ***c***, Areas of the burst responses during TBS (10 bursts of 4 pulses delivered at 100 Hz, with an interburst interval of 200 ms). Results are mean ± SEM with no statistical differences between the two groups of mice; n = 5 or 6 slices from 5 mice per genotype. ***d***, Patch-clamp recording of LTP. Representative traces of EPSC amplitudes 10 min before (1) and 50 min after (2) HFS pairing paradigm. Bottom, Time course of relative changes of EPSCs (for control $n = 6$ neurons from 4 mice, for *CAPNS1-Nestin-cKO* $n = 5$ neurons form 4 mice). Data are mean ± SEM. *p* value is from the data comparison of 30–40 min EPSC average of mutants with those of control. $^{***}p < 0.001$ (Student's *t* test).

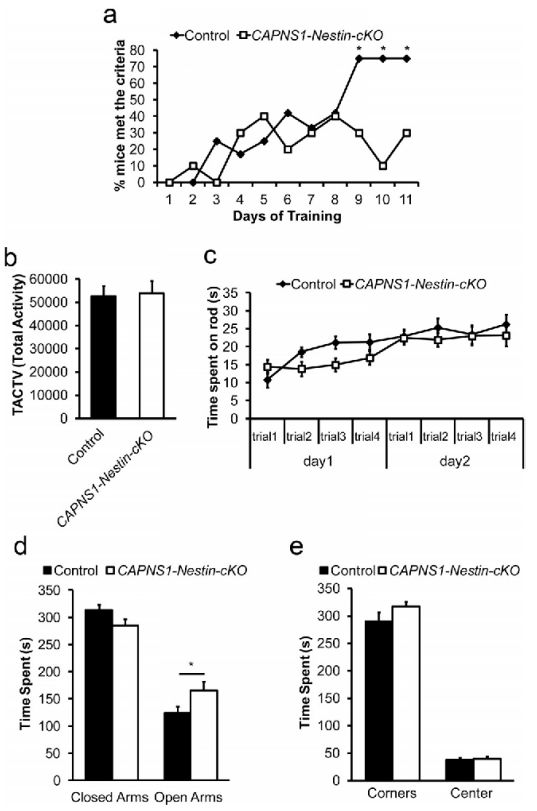

Figure 6. Spatial learning is deficient in *CAPNS1-Nestin-cKO* mice, whereas motor activity and anxiety behavior appear normal. ***a***, Percentage of *CAPNS1-Nestin-cKO* (males, $n = 6$; females, $n = 4$) and control littermate (males, $n = 5$; females, $n = 7$) mice successfully reaching the criteria of finding the platform in the Morris Water Maze, as described in Materials and Methods. ***b***, Locomotor activity assessed in a novel cage test. ***c***, Motor coordination examined by rotarod test. ***d***, ***e***, Anxiety observation performed with elevated plus maze (***d***) and open field test (***e***). Data are mean ± SEM. $^{*}p < 0.05$ (Student's *t* test and repeated ANOVA). Controls: males, $n = 10$; females, $n = 11$; *CAPNS1-Nestin-cKO*: males, $n = 11$; females, $n = 10$.

deficit was not related to deficiencies in vision because there was no significant difference in the average time to find the visual platform (data now shown). In addition, examination of general motor activity by beam break and rotarod tests did not reveal any differences in the mutants (Fig. 6*b*,*c*). Finally, although the calpain-deficient mice showed less anxiety behavior in elevated plus maze test (Fig. 6*d*), the differences in the open field anxiety test were not observed (Fig. 6*e*). Collectively, these observations suggest that calpain is necessary for hippocampal LTP and learning and memory, and this may be related to a role in modulating the structure of neuronal dendrites and synapses.

Calpain mediates neuronal cell death

In contrast to their physiological roles in the CNS, classical calpains have previously been proposed to participate in neuronal cell death in several pathological conditions (Vosler et al., 2008). However, the direct role of calpain and its precise target in neurodegeneration need to be definitively demonstrated. Accordingly, we used this genetic model system to test the role of calpain in neuronal loss paradigms, including PD-related toxin exposure and ischemic stroke *in vitro*. MPP^+, a metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, is a mitochondrial complex 1 toxin, which promotes selective death of both dopaminergic neurons *in vivo* and several neuronal types *in vitro*, including cortical and midbrain cultures (Crocker et al., 2003; Smith et al., 2006; Qu et al., 2007; Huang et al., 2010). To test whether calpain plays a role in neuronal death induced by this toxin, we first examined whether embryonic cortical cultures, derived from control or calpain-deficient animals, were differentially sensitive to MPP^+. Neurons from *CAPNS1-Nestin-cKO* embryos were significantly less sensitive to the toxin (Fig. 7*a*). Because TH^+ neurons are grossly affected in PD, we also examined whether calpain may play a role in dopaminergic neuronal cell death as well. To do this, we crossed the floxed *capns1* mice with a *TH-Cre* driver (Savitt et al., 2005) to specifically eliminate calpain-1/calpain-2 activity in TH^+ neurons. Neuronal cell cultures obtained from embryonic midbrains of *CAPNS1-TH-cKO* and control animals were treated with MPP^+. Cell survival was assessed by evaluating the number of TH^+ neurons. Consistent with the result in cortical cultures generated from *Nestin-Cre* mice, neuronal survival after MPP^+ treatment was significantly higher in calpain-deficient TH^+ neurons compared with controls (Fig. 7*b*).

To further evaluate the importance of calpain in neuronal injury, we also next tested models of ischemic cell death using *in vitro* glutamate- or hypoxia-induced excitotoxicity in cerebellar granule neurons (CGNs). Indeed, very early finding suggested

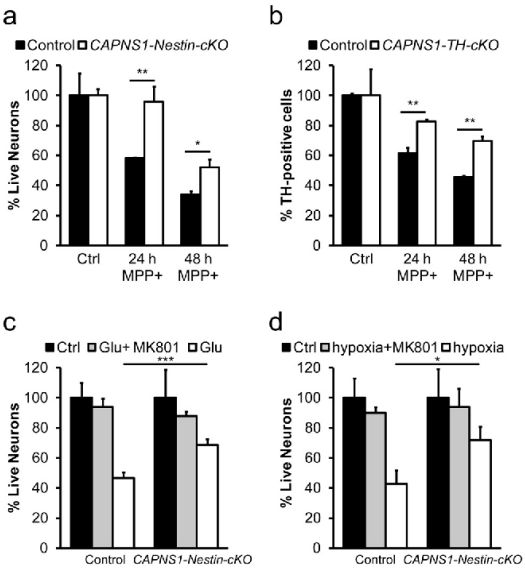

Figure 7. Calpain mediates excitotoxic neuronal death in PD- and ischemic-related *in vitro* models. ***a***, ***b***, MPP^+ treatment of (***a***) cortical neurons and (***b***) midbrain neurons, respectively, at 20 μM final concentration for 24 and 48 h revealed that calpain-deficient neurons were resistant to toxin-induced death (survival rate, 96 ± 10% vs 58 ± 1% at 24 h; 52 ± 5% vs 34 ± 2% at 48 h and 83 ± 1% vs 61 ± 4% at 24 h; 70 ± 3% vs 45 ± 1% at 48 h, respectively; $n = 4$ per genotype). ***c***, Transient glutamate treatment of CGNs at 50 μM final concentration for 70 min followed by 1.5 h reoxygenation in the presence and absence of 10 μM MK801 (survival rate, 68 ± 4% vs 47 ± 4%; $n = 6$ per genotype). ***d***, Hypoxia (1% oxygen) treatment of CGNs for 4–5 h followed by 1–2 h reoxygenation in the presence and absence of 10 μM MK801 (survival rate, 72 ± 9% vs 43 ± 9%; $n = 4$ per genotype). Data are mean ± SEM. $^*p < 0.05$ (Student's *t* test). $^{**}p < 0.01$ (Student's *t* test). $^{***}p < 0.001$ (Student's *t* test).

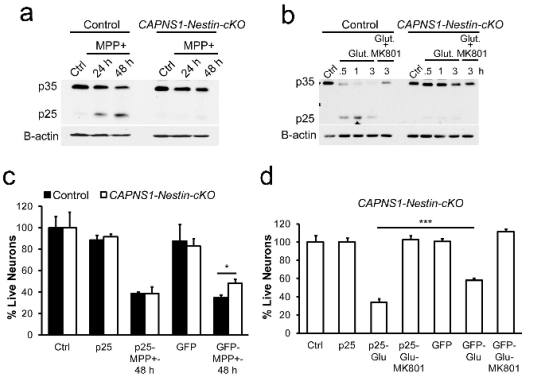

Figure 8. p35 is a target of calpain, and the p25 cleavage product mediates excitotoxic neuronal death. ***a***, ***b***, Representative immunoblots showing the increase in p35 cleavage to p25 after (***a***) MPP^+ and (***b***) glutamate treatment in cortical neurons and CGNs, respectively, from control mice but not *CAPNS1-Nestin-cKO* ($n = 5$ per genotype). ***c***, ***d***, Graphical representation of sensitization of (***c***) cortical neurons and (***d***) CGNs to MPP^+ and glutamate, respectively, and the resensitization of calpain-deficient neurons by AAV-directed p25 overexpression ($n = 3$ per genotype). Data are mean ± SEM of the number of intact nuclei. ***c***, $^*p < 0.05$, controls versus mutant neurons expressing GFP and treated with MPP^+ (Student's *t* test). ***d***, $^{***}p < 0.001$, neurons from mutants expressing either p25 or GFP and treated with glutamate (Student's *t* test).

that *in vitro* translational suppression of calpain-1 reduced excitotoxic damage resulting from NMDA receptor activation (Bednarski et al., 1995). Here, CGNs derived from postnatal cerebellum of *CAPNS1-Nestin-cKO* and controls were transiently subjected to glutamate in the presence or absence of MK801, an NMDA receptor blocker, and neuronal survival was assessed by nuclear integrity. Calpain-deficient neurons were less sensitive to glutamate than control littermates (Fig. 7*c*). Similarly, CGNs obtained from the mutants were significantly protected against hypoxia-induced cell death (Fig. 7*d*). Together, these results suggest a direct role of calpain in multiple mechanisms of neuronal cell death.

Calpain-mediated cleavage of p35, the Cdk5 regulatory subunit, plays a critical role in neuronal damage

The generation of calpain-deficient neurons afforded the opportunity to ascertain the importance of selected substrates of calpain in neuronal death. Previous studies, including from our laboratory, suggested that calpain-mediated cleavage of p35, a cyclin-dependent kinase 5 (Cdk5) regulatory subunit, to p25 elicits inappropriate activation of Cdk5, which drives neuronal cell death (Lee et al., 2000; Smith et al., 2006). However, it is not completely clear whether p35 cleavage to p25 is mediated exclusively by calpain, or how critical this cleavage is to the neuronal cell death process (O'Hare et al., 2005). To explore this question, cortical cultures from *CAPNS1-Nestin-cKO* and control animals were exposed to MPP^+ for 24 or 48 h. Expression of p35 and p25 was assessed by immunoblot analysis of cell lysates using a C-terminal polyclonal antibody that recognizes both the full-length p35 and the p25 cleaved product. We observed a dramatic increase in p25 expression associated with a reduction in p35 protein levels after MPP^+ or glutamate treatment in controls (Fig. 8*a*,*b*). Importantly, we could not detect p25 formation or loss of p35 in calpain-deficient neurons after MPP^+ or glutamate treatment (Fig. 8*a*,*b*). These observations demonstrate an essential role of calpain in cleavage of p35 to p25 in injured neurons. To functionally test the involvement of calpain-induced p25 formation in cell death, AAV was used to rescue p25 expression and to determine whether the protection associated with calpain deficiency could be reversed. As predicted, cortical cultures from *CAPNS1-Nestin-cKO* infected with a control GFP-expressing AAV displayed less neuronal death than wild-type cells upon MPP^+ challenge. In contrast, infection with p25 expressing AAV restored MPP^+-induced cell death in calpain-deficient cells to the same levels seen in control cells (Fig. 8*c*). Glutamate-induced cell death was also restored in calpain-deficient neurons by rescuing p25 expression (Fig. 8*d*). Interestingly, consistent with what has been shown previously (Fischer et al., 2005), short-term overexpression of p25 did not initiate neuronal death by itself. Together, these results provide compelling evidence for the critical role of calpain-mediated conversion of p35 to p25 in two different models of neuronal cell death.

Discussion

Although it has been four decades since the discovery of the ubiquitous calpains, its biological roles remain elusive. Animal models to identify the function of these proteases in the CNS have been limited because of early embryonic lethality or lack of phenotype in germline knock-out mice. CAPN2 knock-out mice (lacking calpain-2) died before the implantation stage of development and germline deletion of CAPNS1 (disrupting both calpain-1 and calpain-2) caused embryonic lethality because of cardiovascular defects (Arthur et al., 2000; Dutt et al., 2006).

Although CAPN1 knock-out mice (lacking calpain-1) survived to adulthood, they did not display brain phenotypes, such as alterations in synaptic plasticity (Grammer et al., 2005). In the present studies, we describe a unique conditional deletion model of both calpain-1 and calpain-2 to assess the roles of these proteases in brain development/function and pathology.

Studies using pharmacological calpain inhibitors have supported a role for calpain in several biological processes critical for CNS development. Our own experience (Sedarous et al., 2003) as well as that of others (Luo and O'Leary, 2005; Touma et al., 2007) has shown that pharmacological calpain inhibitors can cause dramatic and rapid axon retraction *in vitro*, implicating calpain in the biochemistry of axon maturation and maintenance (Qin et al., 2010). In addition, calpain has been implicated in developmental neuronal death, a process critical for sculpting the mature brain (Gil-Parrado et al., 2002; Li et al., 2009). Given these observations, it was surprising that mice with CNS calpain deficiency are viable and born with normal general morphology and unaltered developmental apoptosis and proliferation in the brain. Considering that nestin expression begins at E.7.5 (Berube et al., 2005), a time when the earliest processes of CNS development occurs (Theiler, 1989), our findings strongly suggest that, although calpain may play crucial roles in development of other organ systems, it appears dispensable for basic CNS establishment.

Although gross brain development and architecture was normal, calpain deficiency resulted in a decline in spine density and deterioration of branch ramifications/bifurcations in basal and apical dendrites in hippocampal CA1 pyramidal neurons. A caveat to these observations is that we cannot rule out the possibility that loss of staining rather than loss of spine structures is occurring. However, these staining differences would have to occur specifically in spines because soma and dendritic structures are clearly present. Our results are also supported by previous work suggesting that calpain mediates changes in cytoskeletal structure and organization (Wilson et al., 2000; Fischer et al., 2005) by cleaving potential substrates, including spectrin and MAP2 (Fischer et al., 1991; Zadran et al., 2010a).

These changes in neuronal structural complexity led us to examine whether this was associated with altered synaptic transmission. Indeed, calpain has also been suggested to cleave a number of substrates relevant to excitatory transmission (Doshi and Lynch, 2009). Our results show that brain-specific calpain deficiency leads to reduced levels of some glutamate receptor components and PSDs. These findings suggest that calpain does not directly cleave these substrates but may actually regulate their levels of expression. This is possibly through effects of calpain on signaling pathways regulating gene expression. Calpain is implicated in the cleavage of several transcription factors, such as c-Jun and c-Fos (Goll et al., 2003), MEF2 (Smith et al., 2006), and NFκB (Sedarous et al., 2003). These transcriptional factors are proposed to regulate expression of NMDA receptor subunits (Bai and Hoffman, 2009). Intriguingly, the ultrastructural and synaptic protein deficits observed in the *CAPNS1-Nestin-cKOs* did not translate into obvious defects at individual glutamatergic CA1 synapses, as the amplitude of mEPSCs was unaltered. This may reflect the general robust compensatory ability of synapses to maintain faithful transmission despite deletion of key synaptic components, for instance, GluA1 (Zamanillo et al., 1999), TARP γ-8 (Rouach et al., 2005), GluA2 (Toyoda et al., 2009), and others. The overall decrease in input/output responses observed in CA1 region of *CAPNS1-Nestin-cKOs* may reflect the overall decrease in spine density observed.

Calpain-deleted mice displayed a significant reduction in LTP, supporting a central role for calpain in synapse plasticity. This is also consistent with our results showing reduced memory as evaluated in the Morris Water Maze Test. The observed reduction in LTP with calpain loss is consistent with a number of previous observations. First, pharmacological as well as antisense-based inhibitors of calpains blocked LTP (del Cerro et al., 1990; Denny et al., 1990; Vanderklish et al., 1996). Furthermore, calpains are localized in spines and can be activated by threshold levels of inducing stimulation (Baudry and Lynch, 2001; Zadran et al., 2010b). However, and in contrast to these findings, calpain-1 knock-out mice did not display impaired LTP (Grammer et al., 2005). Whether this discrepancy is the result of differences in the LTP paradigm used is unclear at present. Here, we provide compelling genetic evidence that calpain is required for LTP. Considering the absence of an LTP phenotype in calpain-1 knock-out mice (Grammer et al., 2005), our observations suggest that either calpain-2 deficiency is responsible for this phenotype in *CAPNS1-Nestin-cKO* mice or that there is functional redundancy between calpain-1/calpain-2 isoforms with respect to this function. The former interpretation is also consistent with recent reports indicating that calpain-2 can be activated by Ca^{+2}-independent systems (Zadran et al., 2010b), giving a possible explanation for calpain-2 activation with physiological intracellular calcium concentration; and that calpain-2 downregulation using a novel rabies-virus glycoprotein-chimeric peptide to deliver calpain-2 siRNA to the brain resulted in impairments in LTP and learning and memory (Zadran et al., 2012). Finally, it is important to emphasize that we cannot presently distinguish between subtle developmental defects, which may affect LTP/behavior in these calpain-deficient mice versus more specific effects of calpain deficiency on signaling processes in the adult brain.

In addition to defining a role of calpain in brain function under normal conditions, our results interestingly indicate that it can participate in neuropathological processes under stress situations. Calpain activation has been associated with a number of death paradigms, both *in vitro* and *in vivo*, including those linked to stroke and PD (Crocker et al., 2003; Ray and Banik, 2003; Bevers and Neumar, 2008). Relevant to the former, both *in vitro* and *in vivo* experiments have shown that proteolysis of calpain-specific substrates is elevated in the hippocampal and neocortical areas in focal and global ischemia (Hong et al., 1994; Bartus et al., 1995; Bednarski et al., 1995). In addition, several studies have provided evidence that pretreatment or post-treatment with calpain inhibitors has neuroprotective action in *in vitro* and *in vivo* ischemic models (Vosler et al., 2008; Peng et al., 2011). In terms of PD, we have shown that calpain-1 and calpain-2 are activated in both *in vitro* and *in vivo* models of neuronal loss induced by the dopaminergic toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine/MPP^+ (Crocker et al., 2003; Qu et al., 2007; Huang et al., 2010). Calpain inhibitors also protected dopaminergic neurons from death in these paradigms. Our present genetic work provides further support for the critical nature of calpains in neuronal injury. We find that calpain deficiency promotes survival under multiple contexts of hypoxia, excitotoxicity, and mitochondrial dysfunction. In addition, calpain involvement in death induced by injury contrasts with developmental death examined in the embryo. What is the mechanism by which calpain promotes injury-mediated death? We and others have previously proposed that calpain cleaves the Cdk5 activator p35 to a more stable active p25 form (Lee et al., 2000; O'Hare et al., 2005; Smith et al., 2006). In the contexts of stroke and PD, we have shown that

this is associated with increased Cdk5 activation (Smith et al., 2006). Our calpain deficiency model allowed us to more concretely examine whether calpain was indeed critical to p25 formation and whether this formation is central to the death process. In multiple paradigms of neuronal death, we show that calpain-deficient neurons have significantly impaired p25 formation compared with controls. Conversely, the protection afforded by calpain deficiency can be reversed by ectopic expression of p25. This supports a central role for calpain-mediated p25 formation in the paradigms of neuronal death presently examined. Given that p25 is also reported to be involved in synaptic plasticity (Fischer et al., 2005), it is intriguing to speculate that cleavage of p35 to p25 might be one of the central events that connects calpain to some of its physiological and pathophysiological functions. Finally, it is important to note that whether or not genetic calpain deficiency plays a role in adult degeneration/injury has not been presently studied.

In conclusion, our analysis of a conditional model of calpain deficiency in the CNS has provided a clearer understanding of calpain biology in the brain regarding CNS development, synaptic plasticity, and specific neuronal death paradigms. We think that this conditional model will continue to generate critical information on calpain function in the brain and in disease.

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Perturbation of Transcription Factor *Nur77* Expression Mediated by Myocyte Enhancer Factor 2D (MEF2D) Regulates Dopaminergic Neuron Loss in Response to 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)*

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Background: *Nur77* expression is regulated by MEF2D, which is found to be associated with the calpain-CDK5-MEF2D neuronal death pathway.
Results: *Nur77* deficiency results in hypersensitivity to neuronal toxicity with *Nur77* expression rescuing this loss.
Conclusion: *Nur77* reduces toxic neuronal insult regulated by the calpain-CDK5-MEF2 pathway.
Significance: Previously reported calpain-CDK5-MEF2 signaling is now further elucidated with regulation of *Nur77* in dopaminergic neuronal loss.

We have earlier reported the critical nature of calpain-CDK5-MEF2 signaling in governing dopaminergic neuronal loss *in vivo*. CDK5 mediates phosphorylation of the neuronal survival factor myocyte enhancer factor 2 (MEF2) leading to its inactivation and loss. However, the downstream factors that mediate MEF2-regulated survival are unknown. Presently, we define *Nur77* as one such critical downstream survival effector. Following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment *in vivo*, *Nur77* expression in the nigrostriatal region is dramatically reduced. This loss is attenuated by expression of MEF2. Importantly, MEF2 constitutively binds to the *Nur77* promoter in neurons under basal conditions. This binding is lost following 1-methyl-4-phenylpyridinium treatment. *Nur77* deficiency results in significant sensitization to dopaminergic loss following 1-methyl-4-phenylpyridinium/MPTP treatment, *in vitro* and *in vivo*. Furthermore, *Nur77*-deficient MPTP-treated mice displayed significantly reduced levels of dopamine and 3,4-Dihydroxyphenylacetic acid in the striatum as well as elevated post synaptic FosB activity, indicative of increased nigrostriatal damage when compared with WT MPTP-treated controls. Importantly, this sensitization in *Nur77*-deficient mice was rescued with ectopic *Nur77* expression in the nigrostriatal system. These results indicate that the inactivation of *Nur77*, induced by loss of MEF2 activity, plays a critical role in nigrostriatal degeneration *in vivo*.

Parkinson disease is characterized by progressive clinical hallmarks, including tremor, bradykinesia, rigidity, and postural instability (1) and loss of dopamine (DA)[3] neurons in the substantia nigra pars compacta (SNc). Current therapies are mainly directed toward replacing dopamine levels in the brain and, as such, provide only symptomatic relief (2). Attenuation of the underlying degeneration in Parkinson disease is a potentially more effective therapeutic strategy, and the mechanism(s) controlling this loss is not clear.

CDK5, a member of the cyclin-dependent kinase family (CDKs) (3) is primarily involved in regulation of neuronal function (4, 5). CDK5 is involved in regulation of normal physiological function with evidence, suggesting that this protein also mediates death signaling (6–9). Importantly, we have previously provided evidence for the importance of CDK5 in MPTP-induced dopaminergic death (6). For example, CDK inhibitors, dominant negative CDK5 expression, and the deficiency of the CDK5 regulatory activating partner p35 (10) block dopaminergic loss *in vivo*. MPTP induces calpain-dependent cleavage of p35 to form p25, resulting in increased CDK5 activity in the SNc (7).

We identified a number of potential CDK5 substrates that mediate dopaminergic cell loss. The first substrate identified

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[3] The abbreviations used are: DA, dopamine; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase; DAT, DA transporter; MPP^+, 1-methyl-4-phenylpyridinium; ANOVA, analysis of variance; CDK, cyclin-dependent kinase; MEF2, myocyte enhancer factor 2.

was myocyte enhancer factor 2 (MEF2), a transcription factor with a critical role in muscle development (11), neuronal differentiation (12), and neuronal survival (13–15). In this regard, we observed calpain-mediated cleavage of p35 and consequent activation of CDK5 increased phosphorylation and inactivation of MEF2D in an MPTP model of dopamine degeneration *in vivo* (7). In addition, expression of constitutively active MEF2D protects dopamine neurons from MPTP-induced insult. However, the critical question of how MEF2 regulates survival is not completely clear.

Recent evidence suggests *Nur77* as a potential MEF2 transcriptional target gene (16–18). The *Nur77* promoter contains putative binding sites for MEF2 (19). *Nur77* was originally identified as an inducer of apoptosis in thymocyte selection (16, 20, 21). However, it is now known to show remarkable functional versatility. *Nur77* has also been found to act as a survival factor in several apoptotic paradigms outside the CNS (22, 23). Interestingly, evidence suggests *Nur77* expression regulates dopaminergic cell biochemistry and dopamine metabolism (24).

A potential link between MEF2D and *Nur77* in dopaminergic cell loss and the critical need to understand how MEF2 may regulate dopaminergic survival, and we examined whether *Nur77* might be the mechanistic link driving a calpain-CDK5-MEF2D-mediated pathway of death. We provide evidence that *Nur77* is both regulated by CDK5-MEF2D and plays a critical role in the survival response to dopaminergic cell death.

EXPERIMENTAL PROCEDURES

Animals—Male *Nur77* KO mice (generously provided by Dr. Jeff Milbrandt, University of Washington, St. Louis, MO) (21, 25) on a C57BK/6J background were further backcrossed for six generations with C57BK/6J strain mice (The Jackson Laboratory). Animals were maintained on a 12-h light/dark cycle with lights on between 06:00 and 18:00 and were permitted an *ad libitum* diet of Ralston Purina mouse chow. The room temperature was kept at 21 °C. All experimental procedures meet the guidelines set out by the Canadian Council on animal care and were approved by the University of Ottawa Committee for Animal Care.

MPTP Administration—Intraperitoneal injections of MPTP (Sigma; 25 mg/kg, measured free base; MPTP-HCl) were administered to mice (8–10 weeks old) once a day for five consecutive days (26). Control animals received an equivalent volume of saline (0.9%). Mice were sacrificed 14 days following initial MPTP/saline treatment or at indicated time points.

Immunohistochemistry—Following overnight post-fixation in 4% paraformaldehyde, brains were cryoprotected in 10% sucrose with free-floating sections obtained as described previously (26, 27). Immunolabeling was performed as described previously using anti-tyrosine hydroxylase (TH) (ImmunoStar, 1:10,000), rat anti-DA transporter (DAT) (Millipore; 1:2000), or rabbit anti-ΔfosB (Santa Cruz; 1:1000). Primary antibodies were visualized using diaminobenzidine.

Assessment of Neuronal Survival—Dopaminergic neurons in the SNc of treated mice were assessed for survival using the dopaminergic cell marker TH. A total of six to eight animals per group were analyzed. Estimates of total TH^+ stained neurons in the SNc were analyzed by unbiased stereological estimates, applying optical fractionation (28) using Stereo Investigator (version 6; MicroBrightField, Williston, VT), as described previously (26, 29). In brief, 40-nm brain sections were examined within the rostral and caudal limits of the SNc (bregma, −2.54 to −3.88 mm) (65). For each brain, six coronal sections were examined. Following immunohistochemistry, mounting, defatting, and coverslipping, the mean section thickness, as measured with a *z* axis microdissector, was 18 μm. Sections were analyzed using a 100× lens.

Quantification of Striatal Immunohistochemistry—Striatal dopaminergic (TH and DAT) fiber density and FosB-positive nuclei were quantified using NIH ImageJ densitometry analysis (26). Each tissue quantified was initialed to its own non-stained background.

Adenovirus Delivery—Adenoviral constructs expressing MEF2D-S444A (7, 30) and *Nur77* (pcDNA generously provided by Dr. Jeff Milbrandt) (31) were constructed using the pAdEasy system as described previously (8, 32, 33). Adenoviruses were delivered unilaterally to the right striatum using coordinates as described previously (7), 7 days prior to initiation of MPTP/saline treatment. A GFP-containing construct was used as a control for all adenoviral experiments. A single unilateral injection of virus was given to each animal (2 μl, 1×10^7 particles per μl), delivered to the right striatum (0.5 mm rostral, 2.2 mm right of the bregma, and 3.4 mm below the skull surface). Each adenovirus injection was given at a constant rate of 0.5 μl/min using a syringe pump system (Harvard Apparatus). Brains were extracted at indicated times following the first MPTP injection.

Brain Microdissection—Following decapitation, brains were sectioned into serial 2.0-mm coronal slices using a plastic dissecting block. Employing a 2-mm diameter biopsy needle, the SNc and striatum were obtained by punch biopsy. Brain tissue samples were taken according to the Franklin and Paxinos mouse brain atlas (26).

Amine Analyses—HPLC analysis was used to evaluate the levels of DA and its metabolite DOPAC in brain microdissections, 14 days following initial MPTP treatment. Levels were determined by HPLC as described previously (26).

Real-time PCR—RNA was extracted from mouse SNc microdissections using TRIzol reagent (Invitrogen). The primer sequences were as follows: *Nur77*, 5′-TGATGTTCCCGCCTTTGC-3′ (forward) and 5′-CAATGCGATTCTGCAGCTCTT-3′ (reverse); GAPDH, 5′-CTGCACCACCAACTGCTTAG-3′ (forward) and 5′-GGGCCATCCACAGTCTTCT-3′ (reverse). *Nur77* and GAPDH primers used for gene expression and PCR conditions have been described previously (34), with the following cycling parameters: 55 °C for 10 min, 95 °C for 5 min, and 40 cycles of 95 °C for 30 s and 60 °C for 60 s.

Mesencephalic Embryonic Survival Cell Culture—Mesencephalic neurons were collected from day 13.5 embryos, adapted as described previously (35). 7 days *in vitro* cultures were treated with 20 μM 1-methyl-4-phenylpyridinium (MPP^+; Sigma) for 24 and 36 h. Cultures were fixed and stained for TH (1:2500) and Hoescht. Survival was assessed by quantifying live TH^+ cells.

RNA Interference—To disrupt *Nur77* expression, 3 days following plating, siRNA, against *Nur77* (Santa Cruz Biotechnology, sc-36109), as well as control siRNA (Santa Cruz Biotech-

nology, sc-37007) was transfected in cortical neuron cultures 1 day prior to MPP^+ (20 μM) treatment (36). At select time points, cells were lysed and assessed for survival (36).

In Situ Hybridization—*In situ* hybridization was performed as described previously (24).

Western Blot Analysis—Western blot was performed as described previously (37), using antibodies against *Nur77* (1:1000; BD Pharmingen) and β-actin (1:5000; Sigma) as a loading control.

Chromatin Immunoprecipitation—ChIP was performed as described previously with embryonic day 15 mouse cortical neurons, following a 0-, 24-, 36-, and 48-h MPP^+ time course (37). The primers used for PCR amplification of the mouse *Nur77* promoter region were 5′-CTGCGGGCACGGATTTACAACACC-3′ and 5′-GGCGAGCCCGACCCACATCTT-5′.

Behavioral Analysis—Behavioral analysis was carried out by utilizing the computer-assisted beam break, MicroMax system (Accuscan, Columbia, OH). Animals were monitored for a 24 h at 8 weeks of age as described (38).

Statistical Analysis—Histochemical and monoamine data analysis was carried out using one-way ANOVA, followed by a Newman-Keuls post hoc test.

RESULTS

Nur77 Expression Following MPTP and Relationship with MEF2—Our previous evidence indicated that loss of MEF2 activity, mediated by CDK5, was critical for dopaminergic loss induced by MPTP *in vivo* (7). This evidence suggested that loss of MEF2 mediated expression of downstream transcriptional targets may lead to neuronal loss. Several reports suggested *Nur77* as a candidate for MEF2 regulation (17, 39, 40). Therefore, we initially ascertained whether loss of *Nur77* expression could be detected in normal WT animals with MPTP treatment. We first assessed the levels of *Nur77* transcripts from nigral extracts from an MPTP time course by quantitative real-time PCR. Previous reports have shown *Nur77*/TH^+ colocalization in the SNc following haloperidol treatment, whereas quantitative analysis by *in situ* hybridization did not display detectable levels under basal conditions (24, 41). However, employing quantitative real-time PCR, we observed a dramatic loss of *Nur77* transcript expression in comparison with basal levels at 6 h to 7 days post initial MPTP treatment, ranging from a 44 to 80% reduction in *Nur77* mRNA (Fig. 1*A*). These results were corroborated via *in situ* hybridization analysis in the dorsolateral and ventrolateral striatum (Fig. 1, *B–D*). We next assessed whether MEF2D binding on the endogenous *Nur77* promoter may be affected by conditions that lead to neuronal loss using ChIP analysis. Because of the need for a more homogenous population of neurons, we utilized the MPP^+ treatment paradigm in cultured cortical neurons. We and others (8, 9, 42, 43) have shown that MPP^+ can induce death in non-dopaminergic neurons, such as ceberebellar granule and cortical neurons, by non-dopamine transporter related mechanisms. Importantly, we observed that there was detectable MEF2D binding at the *Nur77* promoter basally in neurons (Fig. 1*F*) and that this binding diminished following MPP^+ exposure (Fig. 1*G*).

We next examined whether the reduction in *Nur77* might relate to loss of MEF2 as previously reported to occur following MPTP treatment. In this regard, Smith *et al.* (7) showed that expression of non-phosphorylatable MEF2D attenuate DA cell death. Accordingly, we determined whether expression of this construct might also attenuate the loss of *Nur77* expression in the SNc *in vivo*. *Nur77* levels were examined in WT mice virally expressing MEF2D-S444A or a GFP control as reported previously (7). Adenoviral MEF2D-S444A significantly attenuated the decrease in *Nur77* at 1 day post-MPTP (Fig. 1*E*).

Nur77 siRNA Elevates Neuronal Cell Death in Vitro—The above results indicate that *Nur77* expression decreases in a MEF2D-dependent manner following MPTP treatment *in vivo*. However, whether the loss of *Nur77* plays any functional role in DA loss is unknown. Accordingly, we examined this question and reasoned that if our logic was true, loss of *Nur77* should reduce neuronal survival either basally or in the presence of stress.

We first evaluated whether *Nur77*-deficient mesencephalic TH^+ neurons might display sensitivity to MPP^+ treatment in culture. Midbrain neurons from WT and *Nur77*-deficient mice were cultured and treated with MPP^+. Survival of TH^+ neurons was then evaluated. *Nur77*-deficient TH^+ neurons showed a significant hypersensitivity at 24 h exposure to MPP^+ (13% survival with *Nur77* deficiency *versus* 39% survival in WT; Fig. 2*A*). As an interesting side note, we also examined how other neuron types might respond to loss of *Nur77*. In this case, we examined cortical neurons treated with siRNA for *Nur77*. Importantly, treatment of cultured neurons to control siRNA to *Nur77* siRNA induced significant toxicity even in the absence of any stress (91% survival with control siRNA *versus* 49% survival with *Nur77* siRNA) (Fig. 2*B*). MPP^+ treatment also diminished survival. However, the survival ratio remained relatively stable between the control and *Nur77* siRNA-treated cultures at the various time points. This suggested that acute loss in *Nur77* expression in neurons produces a basal detrimental effect that can be further exaggerated by toxin exposure. *Nur77* siRNA down-regulation was confirmed by Western blot analysis (Fig. 2*B*). These results indicated that *Nur77* can play a role in neuronal loss and provided the rational to proceed further to examine the role of *Nur77 in vivo*.

Nur77-deficient Mice Exhibit Attenuated MPTP-induced Degeneration of Dopaminergic Cell Bodies in the SNc—We next assessed dopaminergic neuron survival following saline or MPTP treatment of WT and *Nur77* KO mice on a C57BK/6J background following a subchronic MPTP dosing regime (Fig. 3). These animals were analyzed by stereological assessment for surviving TH^+ neurons over the entire SNc region. Interestingly, unlike *in vitro*, with acute down-regulation of *Nur77* in cortical neurons, no statistical difference in the number of TH^+ neurons in the SNc could be detected between saline-treated WT and *Nur77* KO mice (Fig. 3, *A–C*). Similarly, no statistical difference in basal striatal TH or DAT fiber density was detected by immunohistochemistry in saline-treated animals (Fig. 3, *D–G*). DAT is critical for the reuptake of DA and MPP^+, the active MPTP metabolite in dopaminergic neurons (44). Finally, no differences in levels of striatal DA or its metabolite DOPAC were detected in these mutant mice under basal con-

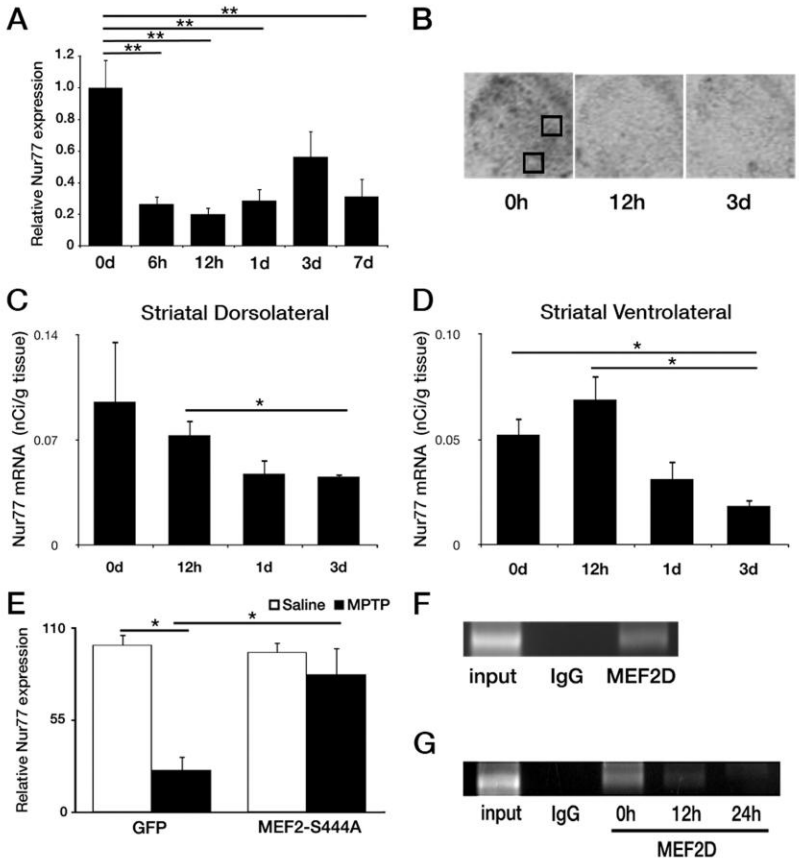

FIGURE 1. ***Nur77* expression and regulation following MPTP and MPP^+ treatment.** *A*, expression of *Nur77* mRNA in an MPTP time course, at 0, 6, 12, and 24 h and 3 and 7 days (*d*) post-initial MPTP treatment. *B*, representative photomicrographs illustrating *Nur77 in situ* hybridization of striatal tissue, following 0 and 12 h and 3 days MPTP. *Black boxes* designate dorsolateral (*upper*) and ventrolateral (*lower*) striatal quantified regions. *C* and *D*, quantification of striatal *Nur77 in situ* hybridization following no treatment, 12 h, and 1 and 3 days post-initial MPTP treatment. *C*, striatal dorsolateral. *D*, striatal ventrolateral. *E*, attenuation of *Nur77* loss as determined by quantitative real-time PCR (*qPCR*) following adenoviral (*AV*) MEF2-S444A expression and 1 day of MPTP treatment *in vivo*. *F*, ChIP assay for MEF2 binding to *Nur77* promoter in untreated cortical cultures. *G*, ChIP assay for MEF2 binding to *Nur77* promoter of cultures untreated (0 h) or treated with MPP^+ for 12 and 24 h. *Error bars* represent mean ± S.E. ANOVA, *, $p < 0.05$; **, $p < 0.01$; n = three to four animals per group.

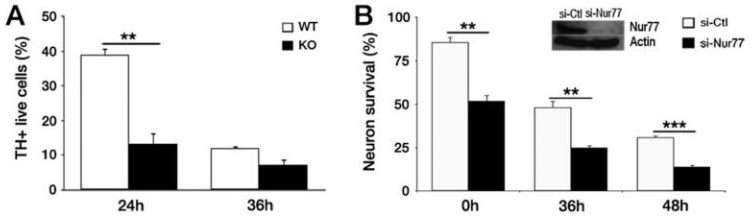

FIGURE 2. ***In vitro* neuronal cultures display sensitization to toxic insult with loss in *Nur77* expression.** *A*, mesencephalic WT and *Nur77*-deficient neuronal cultures treated with MPP^+ for 24 and 36 h. *B*, cortical neuronal cultures treated with *Nur77* and control siRNA (*si-Ctl*) and MPTP for 36 and 48 h. *Error bars* represent mean ± S.E. ANOVA, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n = three embryos per group.

ditions (Fig. 4, *C* and *D*). Furthermore, in our study, *Nur77*-deficient mice displayed no significant differences in 24 h of home cage activity (Fig. 3*H*). Similar groups of animals were also treated and assessed for potential differences in DA cell survival following MPTP. Following MPTP administration, there was a significant reduction (36.7%; $p < 0.001$) in TH^+ SNc neurons in WT mice (Fig. 3*B*). Interestingly, and consistent with results observed in culture, *Nur77*-deficient mice displayed a significantly greater loss of TH^+ cells than WT mice treated with MPTP (68.6%; $p < 0.001$). This observation of increased dopaminergic neuron loss was corroborated morphologically using cresyl violet staining, assessing neurons at the level of the medial terminal nucleus (bregma, −3.16 mm) within the SNc (Fig. 3*C*). Analysis was consistent with that of TH survival, showing no difference

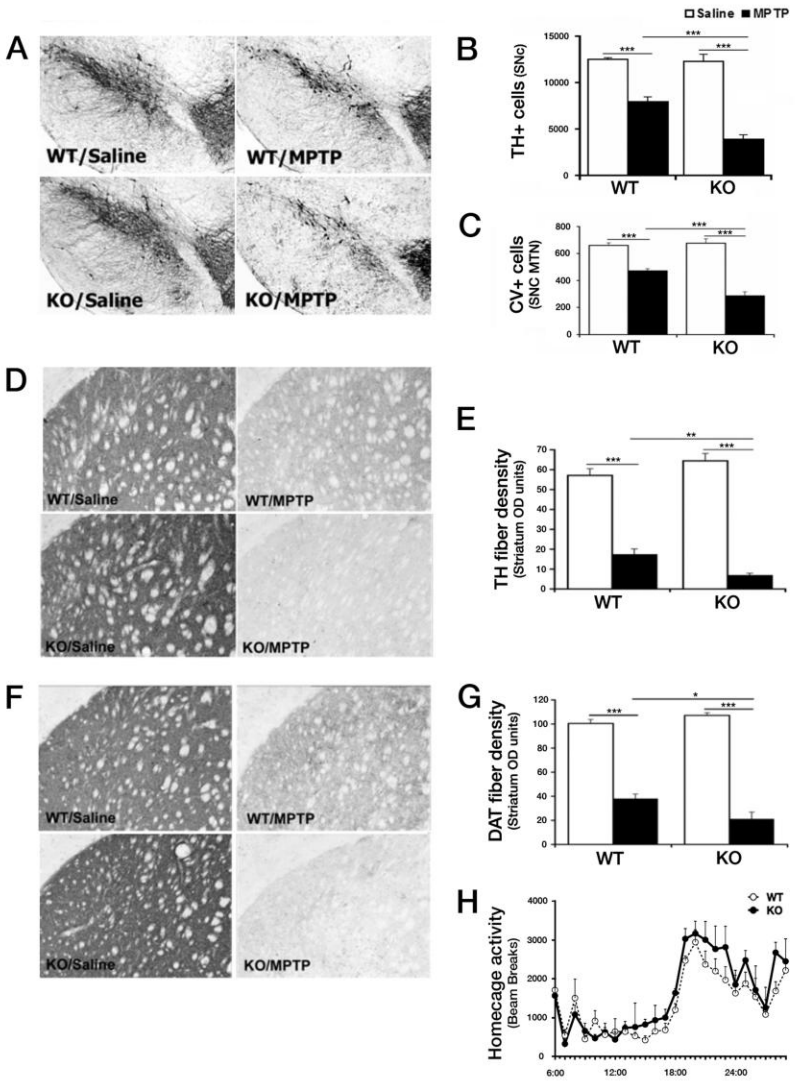

FIGURE 3. ***Nur77*-deficient mice display increased dopamine cell bodies and striatal terminals of MPTP-induced degeneration of SNc dopaminergic neurons.** *A*, representative photomicrographs illustrating TH immunoreactivity in the ventral midbrain SNc following indicated treatments. *B*, quantification of TH^+ neurons using stereological analysis, as described under "Experimental Procedures." *C*, quantification of cresyl violet-stained (*CV*) cells of the SNc (medial terminal nucleus (*MTN*) level). *D*, representative photomicrographs of striatal TH-immunoreactive sections from treated animal groups in *A*. *E*, quantification of striatal TH fiber optical density. *F*, representative photomicrographs of striatal DAT-immunoreactive sections from treated animal groups in *A*. *G*, quantification of striatal DAT fiber optical density. *H*, analysis of 24-h home cage locomotor activity for WT and *Nur77*-deficient mice. *Error bars* represent mean ± S.E. ANOVA, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; $n = 6–8$ animals per group. *CV*, cresyl-violet; *OD*, optical density.

between saline-treated groups, a significant loss of TH^+ neurons in WT MPTP-treated mice (28.7%; $p < 0.001$), and a significant increased loss in DA neurons in *Nur77*-deficient MPTP-treated animals (56.7%; $p < 0.001$).

MPTP derives its toxic effects once it crosses the blood-brain barrier and is converted to MPP^+, the active dopaminergic neural toxin, by monoamine oxidase B (45). The hypersensitivity observed in *Nur77*-deficient animals may be attributed to altered MPTP metabolism and therefore significant increased MPP^+. To examine this possibility, striatal tissue was evaluated from mice 90 min after a single injection of MPTP, and levels of MPP^+ in the striatum were determined using HPLC. It was observed that MPP^+ levels did not significantly differ between WT and *Nur77*-deficient animals (66.0 ± 5.4 and 74.5 ± 3.2, respectively, $p = 0.225$), suggesting that the increased sensitivity afforded by *Nur77* deficiency was not due to impaired MPTP metabolism.

Nur77-deficient Mice Exhibit Increased Degeneration of DA Terminals, Loss of Amines, and Markers of Deregulated Basal Ganglia Following MPTP Treatment—Our results above indicate that MPTP-induced loss of dopaminergic cell bodies in the

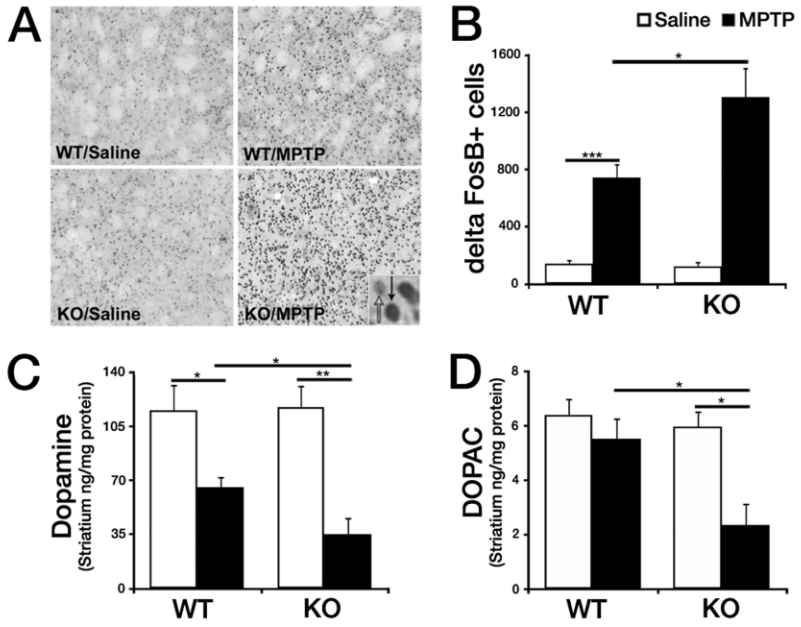

FIGURE 4. ***Nur77* KO mice treated with MPTP display increased striatal FosB, a marker for postsynaptic changes in the denervated striatum, as well as further reduced DA and DOPAC in comparison with WT MPTP-treated mice.** *A*, representative photomicrographs showing FosB staining in the striatum of mice treated as indicated (*inset*, *black arrow*, positive FosB; *white arrow*, negative FosB. *B*, quantification of $FosB^+$ cells/nuclei striatal photomicrographs. *C* and *D*, levels of DA (*C*) and its metabolite DOPAC (*D*) in the striatum were analyzed using HPLC on 14-day-old tissue after saline/MPTP injection. *Error bars* represent mean ± S.E. ANOVA, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; $n = 5–8$ animals per group.

SNc is increased in *Nur77*-deficient mice. To determine whether the striatal projections of the SNc neurons also exhibit hypersensitivity in *Nur77*-deficient mice, striatal dopaminergic terminal fiber density was assessed in response to MPTP. This analysis is important because this is the region where dopaminergic neurons express their activity through DA release. Densitometry analysis of TH striatal fiber staining showed a significant reduction in WT MPTP-treated animals compared with saline controls (69.9% *versus* 89.6%; $p < 0.001$) (Fig. 3, *D* and *E*) Examination of DAT levels also confirm these findings. Densitometric analysis of striatal DAT stained fibers showed a significant reduction (62%; $p < 0.001$) in WT MPTP-treated animals compared with saline controls (Fig. 3). *Nur77* KO animals exhibited a hypersensitivity to MPTP-induced reduction in striatal DAT density compared with WT-treated mice (80.2%; $p < 0.001$). These results indicate that striatal terminal fibers were further sensitized by *Nur77* deficiency, similar to that exhibited by dopaminergic cell bodies.

Treatment with MPTP previously has been demonstrated to induce expression of the transcription factor ΔFosB postsynaptically within the striatum (6). ΔFosB is suggested to mediate the supersensitivity of striatal DA receptors after denervation (46). Consistent with these reports, WT MPTP-treated mice displayed a significant increase in the number of $\Delta FosB^+$ cells (422.1%; $p < 0.001$) (Fig. 4, *A* and *B*) compared with saline controls. Importantly, MPTP-treated *Nur77*-deficient mice showed consistent hypersensitivity (929.3%; $p < 0.001$), a significant increase in ΔFosB staining over that observed in WT mice ($p < 0.05$).

We next examined how DA levels are affected in *Nur77*-deficient mice after MPTP treatment. Previous analyses report diminished DA and its metabolite DOPAC in the striatum 14 days following MPTP treatment (7, 26). The effects of *Nur77* deficiency parallel the observed dopaminergic cell survival results. In the striatum, MPTP significantly reduced the levels of DA in WT mice (43.1%; $p < 0.05$) with a greater diminishment in the KO animals (70.0%; $p < 0.001$) (Fig. 4*C*). Striatal DOPAC was significantly reduced in *Nur77* KO mice treated with MPTP (Fig. 4*D*). However, this significant loss was greater in the *Nur77* KO MPTP-treated mice than WT (60.5% *versus* 13.6%; $p < 0.05$).

Ectopic Expression of Nur77 Rescues Nur77-deficient Hypersensitivity to MPTP—The results described above demonstrate that germ line loss of *Nur77* does not lead to degeneration of DA basally but increases these neurons sensitivity to MPTP-induced degeneration *in vivo*. Because of the germ line nature of *Nur77* loss in the model system examined, we included some controls for potential confounding compensatory factors, which might account for the sensitization observed with chronic *Nur77* loss. To this end, we explored whether we could rescue the sensitivity observed with *Nur77* with ectopic WT *Nur77* expression. We expressed *Nur77* and control GFP in the SNc using an adenoviral system, in both *Nur77*-deficient and WT mice. Seven days following intracranial viral injection, the animals followed the same MPTP paradigm as previously employed. GFP expression in WT and *Nur77*-deficient mice produced similar results to those earlier exhibited in the *in vivo* survival experiments described above (Fig. 5, *A–C*). WT,

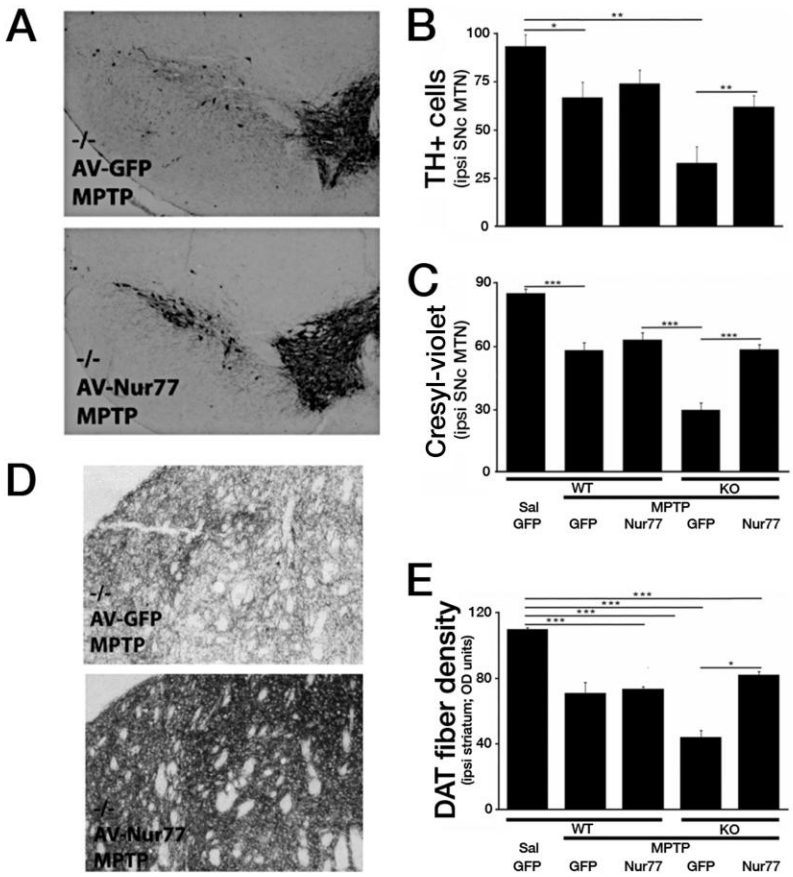

FIGURE 5. ***Nur77*-deficient hypersensitivity to MPTP can be attenuated with ectopic expression of *Nur77* in the nigrostriatal system.** *A*, representative photomicrographs illustrating TH immunoreactivity in the ventral midbrain SNc following indicated treatments. *B*, quantification of TH^+ neurons at the medial terminal nucleus (*MTN*) level of the SNc. *C*, quantification of cresyl violet-stained cells at the medial terminal nucleus level of the SNc. *D*, representative photomicrographs of striatal DAT immunoreactive. *E*, quantification of striatal DAT fiber optical density. *Error bars* represent mean ± S.E. ANOVA, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; $n = 5–8$ animals per group. *OD*, optical density; *AV*, adenoviral; *Sal*, saline.

MPTP-treated, GFP-expressing mice showed a 28.4% reduction in TH^+ neurons in comparison with the experimental control WT, saline-treated, GFP-expressing mice ($p < 0.05$). *Nur77*-deficient, GFP-expressing, MPTP-treated mice showed a similar heightened hypersensitivity, as observed in the initial *in vivo* experiments, with a 65.0% reduction in TH^+ neurons in comparison with control mice. Ectopic *Nur77* expression in *Nur77*-deficient animals dramatically and almost completely reversed the sensitivity observed in comparison with WT animals. These results were corroborated using the morphological cresyl violet analysis (Fig. 5*C*). Interestingly, *Nur77* expression in WT animals produced a slight, but non-significant increase in DA neuron numbers.

Examination of striatal DAT density produced similar results (Fig. 5, *D* and *E*). Ectopic expression of *Nur77* in *Nur77*-deficient animals almost completely reversed the sensitization induced by germ line loss of *Nur77*. Interestingly, *Nur77* expression again did not significantly attenuate fiber loss in the WT mouse. This suggests that levels of *Nur77* expression was insufficient to promote dramatically increased protection in WT cells but was sufficient to prevent the sensitization observed with full *Nur77* deficiency.

DISCUSSION

Previous work has suggested the importance of a calpain-CDK5-MEF2 signaling cascade in the adult *in vivo* MPTP model of dopaminergic loss (6, 7, 15, 30, 47). How CDK5-mediated repression of MEF2 activity leads to DA loss is unclear. Our findings are of significance as they delineate a novel player, *Nur77*, in DA loss and link this activity to the calpain-regulated pathway of death. We found that 1) *Nur77* is rapidly lost following MPTP treatment and that this loss is attenuated by expression of the transcription factor MEF2D; 2) *Nur77* loss results in hypersensitization of the nigrostriatal system to exogenous toxic stress and degeneration; and 3) this hypersensitization can be rescued by re-expression of exogenous *Nur77*.

Our observations of MEF2-mediated *Nur77* regulation is consistent with previous reports suggesting a relationship

between the two transcription factors in other systems. For example, MEF2 has been shown to regulate *Nur77* expression in thymocyte differentiation (48). In this context, MEF2 regulation of *Nur77* is mediated by molecular transcription associating proteins, including HDAC7 (40, 49) and Cabin1 (17, 39). In neurons, depolarization induced up-regulation of MEF2 has also been shown to increase *Nur77* levels (50). In addition, CREB-mediated induction of *Nur77* is also regulated by MEF2 in PC12 cells (51). Importantly, MEF2 sites exist in the *Nur77* promoter (19) with several groups reporting MEF2 binding to the *Nur77* promoter (17, 39). Importantly, we presently observed that MEF2 does constitutively occupy these sites endogenously, at least in cultured neurons. Therefore, an important caveat is that this analysis was not performed with SNc neurons. Taken together, we have proposed a model by which neuronal MEF2, known to act as a survival signal, mediates a constitutive level of NUR77 expression. This basal level of NUR77 activity is rapidly lost when calpain-mediated CDK5 activation leads to phosphorylation and inactivation of MEF2.

Several observations support a role for *Nur77* loss in facilitating DA loss. We showed that *Nur77*-deficient animals are hypersensitive to MPTP-induced degeneration. Importantly, we provide support, although not definitive proof, that this sensitivity relates to deficiency of *Nur77* in neurons themselves. First, TH^+ *Nur77*-deficient neurons cultured from neuronal midbrain cultures, under conditions where other contaminating cell types are limited, are more sensitive to MPP^+ than their WT counterparts. Second, acute suppression of *Nur77* by siRNA in cortical neurons leads to their demise even in the absence of stress. It is important to note that this NUR77 basal neuronal loss does not appear to occur with germ line *Nur77* loss, suggesting some compensatory events with germ line deficiency. Indeed, there are other Nur family members, including Nor1 and Nurr1 (52, 53). Nevertheless, *Nur77*-deficient neurons are sensitive to exogenous stress, and this sensitivity can be rescued by ectopic *Nur77* expression.

These results are consistent with other reports indicating a role for *Nur77* in survival, as seen in TNFα (23) and ceramide-induced cell death (22), and *Nur77*-NFκB inhibition of apoptosis (54). However, the role of *Nur77* appears context dependent. In other cases such as T cell selection (19, 55) and cancer cell death induction (56, 57), *Nur77* appears to induce death. Interestingly, NUR77 can localize to the mitochondria where it can interact and inhibit Bcl-2, inducing apoptosis (58, 59). Adding to the complexity is that *Nur77* can have functional activity outside of death regulation. In neurons, for example, *Nur77* has been associated with synaptic remodeling, response to L-DOPA, and behavioral modifications (41, 60, 61). Taken together, these observations belie the complexity of *Nur77* function. At least in DA neurons, however, in response to MPTP, we provide evidence for a unique MEF2-dependent function in DA survival.

What are potential targets of *Nur77* that might promote survival? In high metabolic cells such as muscle, *Nur77* expression appears to regulate genes involved in glycolysis, glycogenolysis, and the glycerophosphate shuttle (62). Overexpression and shRNA inhibition of *Nur77* in muscle cells, respectively, increased and decreased expression of glucose transporter 4 (GLUT4), muscle phosphofructokinase (Phkka1), and glycogen phosphorylase (Pygm). Furthermore, NUR77 was found to bind to the promoter regions of these metabolic controlling genes. Therefore, one attractive hypothesis is that interruption in expression of these metabolic controlling genes under conditions of elevated energy requirements, such as in neurons, may be detrimental. In this regard, maintenance of proper bioenergetics in neurons is critical for their survival, and loss of this capacity may sensitize to exogenous stresses, posing additional metabolic stress onto the cell, as observed with MPTP treatment.

Although our present results suggest *Nur77* as one critical effector downstream of MEF2, other MEF2-regulated pathways may also play a role in regulating dopaminergic loss following MPTP treatment. For example, She *et al.* (15) recently discovered MEF2 localized to the mitochondria, where it is bound to its consensus site on mitochondrial DNA containing and regulating the ND6 gene. ND6, a component of the mitochondrial complex I, controlling oxidative phosphorylation, is key to maintaining energy requiring homeostasis. MPTP-induced MEF2 degradation disrupted the expression of NG6 along with increased hydrogen peroxide concentration, reduced ATP production, and increased DA cell death. MEF2 has also been shown to be degraded in other toxic insult models, including 6-hydroxydopamine (63). Further inhibition of MEF2 contributing to neuronal toxicity is also presented by GSK3β phosphorylation (64). Finally, MEF2D itself acts alongside other regulatory targets of CDK5 to mediate DA loss following MPTP treatment. These additional CDK5 targets include both cytoplasmic and nuclear factors, Prx2 and APE1, respectively (8, 9). Prx2 is critical for handling oxidative stress in this paradigm, although APE1 acts to facilitate DNA repair. With all three targets, CDK5 mediated phosphorylation of these substrates leads to down-regulation of their respective activities, promoting death. In the case of MEF2, however, we have identified an additional critical downstream effector of this transcription factor.

In conclusion, we have shown that mice deficient in *Nur77* display a hypersensitization to MPTP-induced dopaminergic cell death and that levels of *Nur77* are regulated by MEF2. Our data suggest a new candidate in the calpain-CDK5-MEF2 pathway involving the demise of the nigrostriatal dopaminergic system. We propose that targeting and specifically inhibiting the calpain-CDK5-MEF2-NUR77 pathway may be effective in protecting dopamine neurons in Parkinson disease.

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