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Calcium-mediated regulation of autophagy and programmed cell death in adult hippocampal neural stem cells:

A story of calpains, ryanodine receptors, and presenilins

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A thesis submitted to the faculty of DGIST in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Brain and Cognitive Sciences. The study was conducted in accordance with Code of Research Ethics[1)].

05. 23. 2016

Approved by

Professor Seong-Woon Yu (Advisor) _____ (Signature)

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1) Declaration of Ethical Conduct in Research: I, as a graduate student of DGIST, hereby declare that I have not committed any acts that may damage the credibility of my research. These include, but are not limited to: falsification, thesis written by someone else, distortion of research findings or plagiarism. I affirm that my thesis contains honest conclusions based on my own careful research under the guidance of my thesis advisor.

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for the degree of Doctor of Philosophy

05. 23. 2016

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ABSTRACT

Importance of proper cell death regulation has received great recognitions in biomedical field as the greater understanding of cell death can provide better solutions to treatment of various human diseases, most notably neurodegenerative diseases. However, the underlying mechanisms of programmed cell death (PCD) are largely unknown, especially in neural stem cells (NSCs). Utilizing our well-established insulin withdrawal model of autophagic cell death (ACD) in adult hippocampal neural stem (HCN) cells, we explored the functional relevance of autophagic death of NSCs to pathogenesis of Alzheimer's disease (AD). Aberrant neuronal Ca^{2+} levels in brains of AD patients have given a rise to Ca^{2+} hypothesis of AD which states that the amyloidogenic pathway leads to ultimate cognitive impairment in affected individuals through dysregulation of neuronal Ca^{2+} signaling. The aberrant Ca^{2+} homeostasis consequently mediates the abnormal activation of calpains, the Ca^{2+}-dependent cysteine proteases which also play an essential role in diverse cellular events including cell development, differentiation and proliferation, and cell death.

We discovered that a switch of cell death mode in HCN cells occurs along with changes in calpain activities which is regulated by the proteasome-dependent degradation of calpain proteins. The dynamic change in calpain activity through the proteasome-mediated modulation of calpain level and intracellular Ca^{2+} rise can be the critical contributor to the demise of NSCs. The switching mechanism of the PCD mode by calpain is a novel discovery in the field of NSCs, and thereby may provide an insight into the complex mechanisms interconnecting autophagy and apoptosis and their roles in regulation of NSC death especially in neurodegeneration. Furthermore, by examining ryanodine receptors (RyRs) and IP_3 receptors (IP_3Rs) – the main Ca^{2+} release channels located in the endoplasmic reticulum (ER) membranes known to direct various cellular events such as autophagy and apoptosis – we investigated the intracellular Ca^{2+}-mediated regulation of survival and death of HCN cells utilizing the insulin withdrawal model of ACD. While treatment with the RyR agonist caffeine significantly promoted the autophagic death of insulin-deficient HCN cells, treatment with its inhibitor dantrolene prevented the induction of autophagy following insulin withdrawal. Furthermore, CRISPR/Cas9-mediated knockout of the RyR3 gene abolished autophagic cell death of HCN cells. Our finding delineates a distinct, RyR3-mediated ER Ca^{2+} regulation of autophagy and PCD in NSCs which may represent pathophysiological brain states. In addition, familial AD-related gene presenilin-2 is another key regulator of intracellular Ca^{2+} homeostasis that is located in the ER. Stemming from previous reports showed reduced the brain insulin levels along with the markedly elevated autophagy in brains of AD patients, we hypothesized that presenilin-2 plays an active, functional role in progression of AD pathology. Our results demonstrate that presenilin-2 is a direct upstream regulator of autophagy and ACD in HCN cells. Silencing and ectopic expression of presenilin-2 reduce and increase the level of autophagy, respectively, providing a hint for possible association of autophagy in AD pathogenesis. Our findings in novel functions of presenilin-2 in neural stem cell biology will open up a highly promising revenue for further NSC research.

Keywords: Neural stem cells, Autophagy, Programmed cell death, Calpain, Ryanodine receptors, Preseniln-2

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Table 1. A list of primers used for quantitative real-time qPCR analysis.

Target	**Gene**	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
β-Actin	ACTB	AGCCATGTACGTAGCCATCC	CTCTCAGCTGTGGTGGTGAA
Calpain 1	CAPN1	GCTCTCCTGGAGAAAGCCTATG	GAGTGACAACTCCTATGAGTG
Calpain 2	CAPN2	CATGCGTACTCCGTCACTGGA	GATTAGGTACTGAGGGTTCATC
RyR1	RYR1	CTTAACCAGCTCAGACACCTTC	GATTCGACCCAGGTATGGTTC
RyR2	RYR2	GGACTTGACATCCTCTGACAC	GGTTGGAAGTAGTTGAGGACAC
RyR3	RYR3	TGGGACAAGTTCGTGAAG	GCCCATGGCAATATCCAA
IP_3R1	ITPR1	TGTATGCAGAGGGATCTA	TCATCCAACGTCACTCTC
IP_3R2	ITPR2	AGGGCTCGGTCAATGGCT	CTTCGTTCCCTGCAGCATCC
IP_3R3	ITPR3	CCAGCTTTCTTCACATCG	CAGCCGCTTGTTCACCGTTAAG

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List of Abbreviations

ACD	Autophagic cell death
AD	Alzheimer's disease
APP	Amyloid precursor protein
Atg	Autophagy-related genes
BafA1	Bafilomycin A1
BrdU	5-Bromo-2-deoxyuridine
CEPIA1er	Ca^{2+}-measuring organelle-entrapped protein indicator 1 in the ER
DAPI	4′,6-Diamidino-2-phenylindole
ER	Endoplasmic reticulum
GFAP	Glial fibrillary acidic protein
GSK-3β	Glycogen synthase kinase-3β
HCN cells	Hippocampal neural stem cells
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IP_3R3	Inositol 1,4,5-triphosphate
LC3	Microtubule-associated protein light chain 3
LMP	Lysosomal membrane permeabilization
mTOR	Mammalian target of rapamycin
NSC	Neural stem cell
PBS	Phosphate-buffered saline
PCD	Programmed cell death

PE	Phosphatidylethanolamine
PS	Phosphatidylserine
PS1	Presenilin-1
PS2	Presenilin-2
RyR	Ryanodine receptor
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPases
SGZ	Subgranular zone
SVZ	Subventricular zone
TM	Transmembrane domain
UPS	Ubiquitin-proteasome system
Vcp	Valosin-containing protein

Acknowledgements

First and foremost, I would like to thank my supervisor, Dr. Seong-Woon Yu, for granting me with this invaluable learning opportunity and welcoming me into his laboratory. These past few years have been, unquestionably, of the upmost importance in my life. Through his guidance, patience and support, I have developed tremendously as a researcher, and I have contributed scientific knowledge that I am very proud of. His sincere mentorship has also allowed me to excel academically and instilled a great appreciation for scientific discovery. Additionally, his passion, determination, and accomplishments continuously inspire me in the pursuit of personal ambitions. As a result, this experience at Dr. Yu's laboratory has helped to shape a future that I could not be more excited for. Thank you.

I would also like to extend a sincere thank you to my thesis advisory committee, Dr. Seong Who Kim, Dr. Cheil Moon, Dr. Eun-Kyoung Kim, and Dr. Sung Bae Lee for their valuable insight and advice throughout my doctoral degree. And of course, my sincere appreciation goes to all current and past members of the Yu lab – with special thanks to every single one of them. Not only have they been outstanding co-workers I would opt to work with in a heartbeat, they have been the brothers and sisters. These last five years have been nothing but a blast and I will always cherish the memories. Lastly, but certainly not leastly, I thank my family for their unconditional love and support for me.

CHAPTER 1: General Introduction

1.1 Adult Neurogenesis and Neural Stem Cells

The discovery of adult neurogenesis – generation of new neurons – neural stem cells (NSCs) in neurogenic niches in the adult brain – has set a breakthrough in the contemporary understanding of adult brain plasticity (**Figure 1**). The excitement and anticipation of NSC research has arisen due to the prospect of promoting the generation and survival of the endogenous neurons in adult brain as a novel therapeutic target for the treatment of brain disorders. However, the ardent interest in NSCs and neurogenesis in adult mammalian brain from the scientific and medical community is seemingly an abrupt occurrence, considering that the concept of adult neurogenesis had been largely discredited and abandoned nearly for a century.

1.1.1 Adult Neurogenesis: The Discovery

Santiago Ramón y Cajal, who is perceived as the father of modern neuroscience, disavowed the existence of a phenomenon now known as adult neurogenesis in his famous *Degeneration and Regeneration of the Nervous System* article published in 1913 in which he commented "*Once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centers, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated* (Clarke, E, 1992)." However, evidence of adult neurogenesis were gradually discovered through a series of fundamental findings throughout the decades after Cajal's dismissal of generation of newborn cells in the brain.

It was not until 1960's that Cajal's statement was challenged by Joseph Altman and colleagues who reported the generation of new neurons in the adult rat brain by utilizing radiolabeled thymidine, a DNA nucleotide labeled with radioactivity, which enabled identification of dividing cells (Altman, 1962; Altman and Das, 1965a; Altman and Das, 1965b). Two decades later, Fernando Nottebohm and colleagues revealed the existence of adult neurogenesis through a series of systematic experiments using zebra finch as a model animal (Nottebohm, 1985; Nottebohm, 1989). Despite the lucid evidence of adult neurogenesis and integration of newborn neurons into neuronal circuits, these findings were considered irrelevant to mammalian brains.

In 1990's, by utilizing the newly developed synthetic thymidine analogue 5-bromo-3'-deoxyuridine (BrdU), which incorporates into the DNA of proliferating cells, to trace the generation of newborn neurons in conjunction with visualization of neuronal markers MAP-2 and NeuN through immunohistological (IHC) methods, Fred Gage and

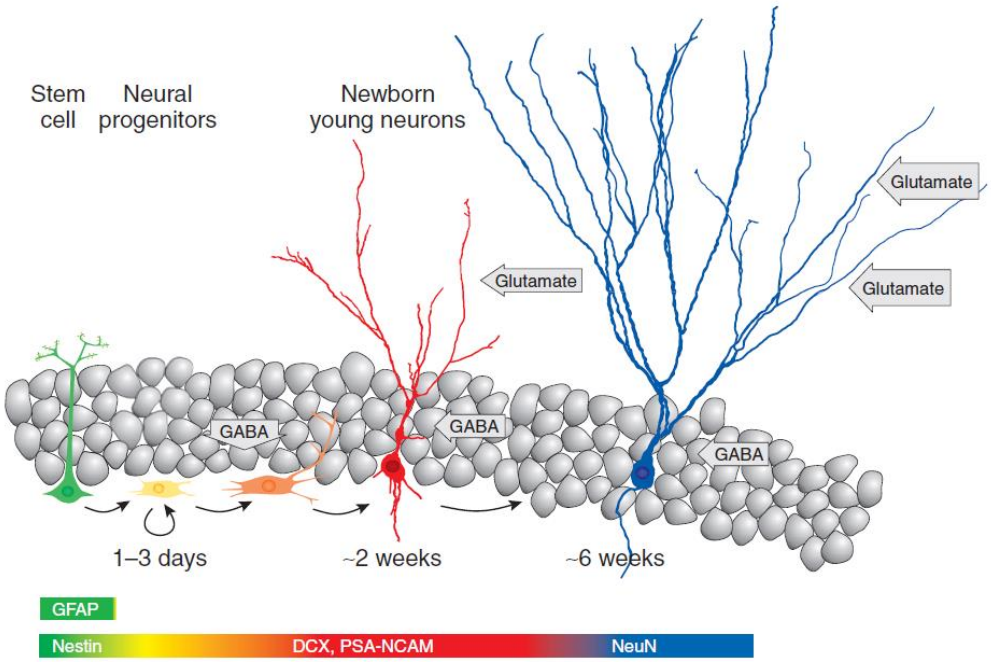


Figure 1. Development of newborn neurons from NSCs in the DG of adult hippocampus. Neural progenitors are derived from NSCs, which are characterized by the expression of glial fibrillary acidic protein (GFAP) and nestin. The progenitors continue to divide for a few more days and give rise to neurons and glia. The neurotransmitter GABA exhibits excitatory function in the young neurons and gradually becoming inhibitory afterward. At the same time, the young cells start to form glutamatergic excitatory synapses. Different developmental markers such as DCX and NeuN are used to distinguish the newly generated immature and mature neurons, respectively. Modified from Bischofberger, 2007.

colleagues ended a century-long controversy over the existence of adult neurogenesis in mammalian brain (Gage et al., 1995; Palmer et al., 1995; Eriksson et al., 1998). Currently, adult neurogenesis and NSCs are among the most actively investigated research topics in the field of neuroscience.

1.1.2 Adult Neural Stem Cells

NSCs are multipotent cells residing in neurogenic niches within the brain which, by definition, possess the capabilities to differentiate into all types of neural cells and to proliferate in the nervous system, as well as to maintain the stem cell population by self-renewal (**Figure 2**; Gage, 2000). Since the discovery of adult neurogenesis, the NSC research has earned immense interests from the field of neuroscience for the therapeutic potential NSCs pose in attempts to treat neurodegeneration and to counter aging over the past decades. There have been progressive advancements in our understanding of NSC biology in relation to the development, regulation, and function of newly-generated neurons in adult brain over the recent years.

Despite NSCs have reportedly been derived from a different adult brain regions, it is generally perceived that adult NSCs are present specifically in the subgranular zone (SGZ) of the hippocampal dentate gyrus and the subventricular zone (SVZ) of the lateral ventricle wall (**Figure 3**; Gage, 2000; Ming and Song, 2005). NSCs and neurogenesis in the SGZ function to modulate brain plasticity and are imperative for spatial learning and memory (Snyder et al., 2005). Although the niche architecture in the SVZ and SGZ is structurally distinct, there are shared features, including their cellular niche components and extracellular niche signals that regulate behavior of adult NSCs and their development. For instance, the microenvironments of SGZ and SVZ contain microglia, astrocytes, and vasculature specially designed to permit, regulate, and support adult neurogenesis (Aimone et al., 2014). Since this thesis aims to present experimental findings based on adult hippocampal NSCs, the detailed descriptions of studies in NSCs in the SVZ is omitted hereafter and the following sections will focus on the biology of NSCs originated from the dentate gyrus.

One of the intriguing features of neurogenesis in the SGZ is that, unlike most somatic tissues the dentate gyrus displays an increase in its volume over the adulthood through continued addition of newborn neurons generated from NSCs. A large number of newborn neurons generated from adult NSCs populates in neurogenic niches have been shown to integrate into pre-existing neural circuits, enabled by their uniquely enhanced plasticity during specific period of time windows (Cameron and McKay, 2001; Ge et al., 2007). Since the dentate gyrus of hippocampus plays

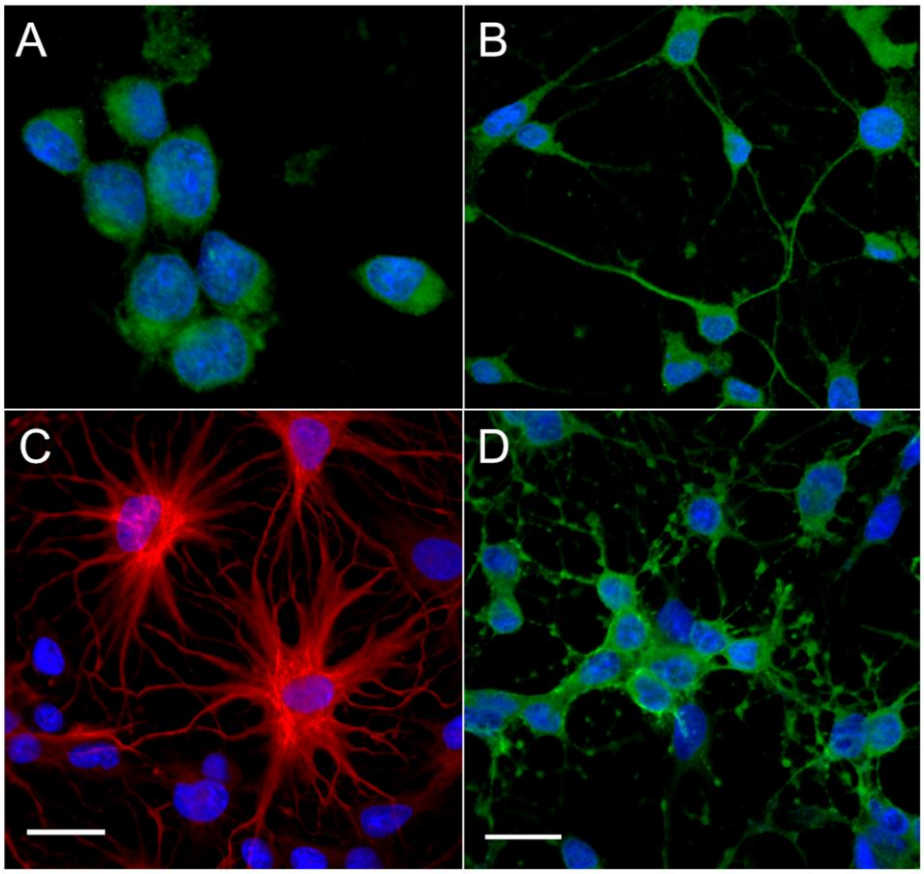


Figure 2. Immunofluorescent staining of neurons, astrocytes, and oligodendrocytes differentiated from adult rat HCN cells. (A): Undifferentiated HCN cells were immunostained with anti-Nestin. (B-D): HCN cells were differentiated into neurons (B), astrocytes (C), or oligodendrocytes (D), immunostained with anti-β-III tubulin, anti-GFAP, or anti-Oligodendrocytes, respectively. Scale bars, 20 µm.

a critical function in the formation of spatial and episodic memories, early researches in adult hippocampal neurogenesis have focused to explore whether the integration of newly-generated neurons in endogenous neural circuits may translate into behavioral or cognitive outcomes (Eichenbaum et al., 1999). For instance, irradiation-induced deficiency of neurogenesis in the rat dentate gyrus prevented the proliferation of subgranular cells and resulted in drastically reduced scores in spatial learning and memory tests (Snyder et al., 2005). Therefore, adult neurogenesis in the SGZ from NSCs is essential for enrichment of neurons and hippocampal growth, posing a promising contribution to formation of new memory and to counter the loss of memory via modulation of brain plasticity during adulthood. However, due to highly sensitive natures of adult neurogenesis to environmental cues, physiological stimuli and neuronal activity (Ma et al., 2009; van Praag et al., 1999; Kempermann et al., 1997), few scientific challenges must be overcome to enable the seamless translations from generation of neurons to functional integration into the endogenous circuits. Direct functional evidence in NSCs and newly generated neurons as to manifest in behavior and cognition still remains to be elucidated.

1.2 Programmed Cell Death

Proper cell death has as an important role as cell survival and proliferation in human health and disease. Cell death is not an undesirable incident, but a series of tailored events that constitutes essential part of normal life. Cell death is a cell-intrinsic suicide mechanism, strictly regulated by diverse cellular signals (Danial and Korsmeyer, 2004). The reserved nature of cell death process through the stages of initiation, execution and termination has led to the neologism, “programmed cell death (PCD)” which was first used back in 1960’s (Lockshin and Williams, 1965). Subtle disturbance in a delicate balance between cell survival and death – either through excessive or too little cell death – underlies a variety of human diseases such as cancer, autoimmune diseases, and neurodegeneration.

Given the importance of PCD, it is not surprising that cell death is a very complicated process with multiple pathways. In general, PCD can be classified into three types – apoptosis, autophagic cell death, and necrosis – mainly according to morphological criteria along with other characteristics (**Figure 4**; Clarke, 1990).

1.2.1 Programmed Cell Death: Classifications and Features

Apoptosis (meaning "*falling off*" in Greek) is the most thoroughly investiagted form of PCD that occurs in multicellular organisms. Since first used in the milestone article by Kerr, Wyllie, and Currie in 1972 to describe the

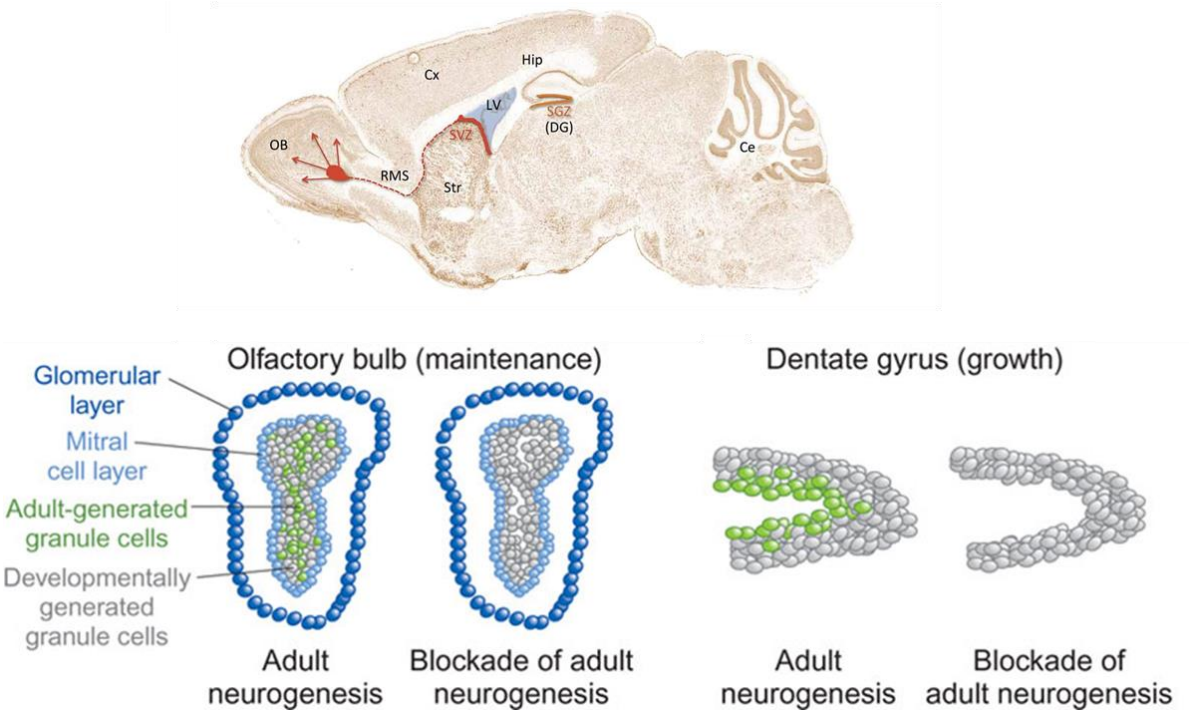


Figure 3. Adult neurogenesis in SVZ and SGZ. In the adult brain, neurogenesis perpetually occurs in two regions: the SVZ of the lateral ventricle (newborn neurons migrate along the rostral migratory stream to populate the olfactory bulb) and the SGZ of the hippocampus (new neurons migrate to the inner granule cell layer of the dentate gyrus. When neurogenesis in SVZ and SGZ is blocked, extensive depletion of granule cells in the olfactory bulb and dentate gyrus, are observed, respectively. Modified from Frankland & Miller, 2008; Batiz et al., 2016.

process of natural cell death occurring under normal physiological condition and during development (Kerr et al., 1972), there has been an explosive growth in our knowledge regarding apoptosis, specifically its role in tissue development and diseases. Cells experiencing apoptosis possess morphological characteristics of cell shrinkage, membrane blebbing, chromatin condensation, dissipation of mitochondrial membrane potential, changes in plasma membrane lipid configurations, and nuclear DNA fragmentation. Apoptotic cells are packed into apoptotic bodies and cleared by neighboring phagocytic cells to prevent inflammation. Upon initiation, the highly regulated processes of apoptosis cannot be halted. In general, apoptosis occurs by either death receptor-mediated *extrinsic* pathway or mitochondria-mediated *intrinsic* pathway. In extrinsic pathway, death receptors recruit procaspase-8 and several adaptor molecules upon binding of the extracellular ligands. Procaspase-8 undergoes autoproteolytic activation and serves as the initiator caspase by triggering the activation of downstream executioner caspases, such as caspase-3 and -7 (Li and Yuan, 2008). The intrinsic apoptosis pathway involves the mitochondria, which integrate and amplify cell death signals by release of pro-apoptotic molecules such as cytochrome c and apoptosis-inducing factor (14). Upon released into cytosol, cytochrome c activates caspase-9 which in turn activates the downstream executioner caspases (Li et al., 1997). Regardless of pathways, apoptosis involves the downstream executioner proteases which is the major morphological and biochemical manifestation of apoptotic cells. Biochemically, caspase activation and nuclear DNA fragmentation are widely used to define apoptosis (Galluzzi et al., 2007). It is also noteworthy to mention that although caspase activation is popularly used as a surrogate marker of apoptosis, cells can opt to undergo an alternative, caspase-independent cell death, such as AIF-dependent cell death (Zhu et al., 2003).

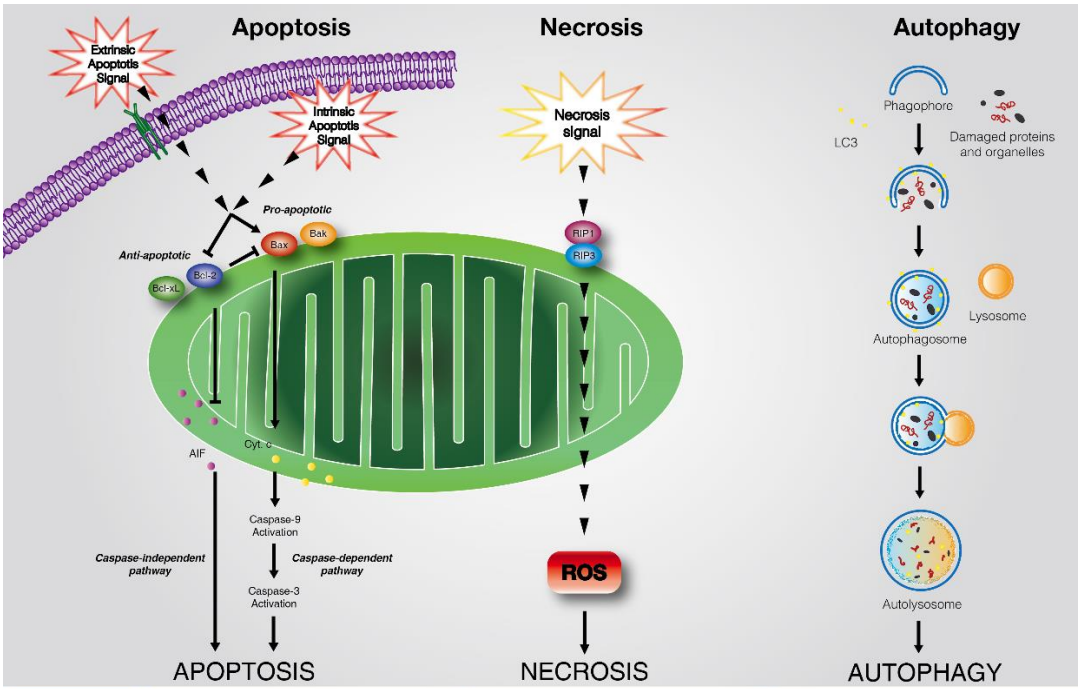
In brief, autophagy is a cellular catabolic process by which cytosolic constituents including proteins and subcellular organelles are sequestered in double-membrane vesicles called autophagosomes and degraded by lysosomal hydrolases after fusion of autophagosomes with lysosomes (Shintani and Klionsky, 2004). Autophagy in a normal physiological state plays a cytoprotective role by eliminating damaged organelles and proteins; but autophagy has also shown to contribute to cell death if overwhelmed or induced excessively (Kourtis and Tavernarakis, 2009). Despite the wide occurrence of autophagy in cells undergoing apoptosis or necrosis, possibly suggesting a mechanistic interplay between autophagy and other modes of PCD, the interrelation between autophagy and PCD appears to vary by cell type and stimulus (Fimia and Piacentini, 2010). ACD is an intriguing phenomenon to researchers because it is a paradoxical event where autophagy (meaning “eating itself” in Greek), which is generally known to exert cytoprotection through engulfment of cytoplasmic components to maintain cellular energy level, leads a cell to its

own destruction. Cells undergoing autophagic cell death (ACD) display increased autophagic flux in the absence of apoptotic markers. Recent evidence of the presence of autophagic vacuoles in dying cells gave a rise to ACD (Kourtis and Tavernarakis, 2009). However, precaution should be taken since the presence of an increased number of autophagic vacuoles in dying cells does not necessarily mean that autophagy has a primary role in cell death (Boya et al., 2005). Because the causal relationship between autophagy and cell death has not been firmly established in many reports, "cell death *through* autophagy" (autophagy can participate in execution of cell death and manipulation of autophagic flux should efficiently affect cell death rate) sometimes has not been distinguished from "cell death *with* autophagy" (autophagy may be an irrelevant epiphenomenon or a futile attempt of dying cells to survive without causal connection with cell death). This confusion has led some to argue against the existence of ACD (Kroemer and Levine, 2008). However, despite these controversies, accumulating evidence supports the indispensable existence of ACD (Clarke and Puyal, 2012; Shen and Codogno, 2011). Recently, Shen and Codogno have proposed a new set of criteria for ACD and listed several experimental situations where ACD meeting these criteria exists (Shen and Codogno, 2011). The new set of qualification for ACD are as follows: i) cell death occurs without the involvement of apoptosis; ii) there is an increase of autophagic flux, as well as an increase of the autophagic markers, in the cells undergoing ACD; and iii) both pharmacological suppression and genetic suppression of autophagy are able to prevent cell death.

Traditionally considered as a passive form of cell death, necrosis is characterized by the swelling of several subcellular organelles and the rupture of the plasma membrane in the dying cell, which subsequently results in the release of cytoplasmic contents and inflammation. Necrotic cell death can be distinguished from apoptosis by the alteration in nuclear morphology while lacking the characteristics of apoptosis, namely chromatin condensation and DNA fragmentation. In contrast to the initial belief that necrosis occurs "by accident," several studies demonstrated evidence of "programmed" necrosis (Han et al., 2008). Programmed necrotic cell death is only observed under conditions in which apoptosis is blocked. Supporting evidence revealed that the apoptosis process suppresses necrosis through caspase-mediated cleavage and the inactivation of proteins required for programmed necrosis (Ha and Snyder, 1999).

1.2.2 Programmed Cell Death in the Brain

Over past few decades, despite the rigorous efforts to understand PCD in the adult brain, it still remains to be



	Apoptosis	Necrosis	Autophagy
Key morphological features	Chromatic condensation Blebbing Cell shrinkage	Cell swelling Dilation of subcellular organelles Loss of membrane integrity	Degradation of organelles Decrease in cell size Double-membraned autophagic vesicles
Biochemical hallmarks	Caspase activation Dissipation of mitochondrial transmembrane potential (ΔΨm) DNA degradation	RIP1/RIP3 activation Overgeneratino of ROS Inflammatory (often, pro-inflammatory)	LC3 Organelle-specific degradation
Key regulators	Bcl-2 family proteins Caspases Death receptors AIF	RIP kinases PARP Calcium-regulated proteins Death receptors	PI3K mTOR Lysosomal hydrolases

Figure 4. Types and characteristics of PCD. Sequential induction of apoptotic (left), necrotic (middle), and autophagic (right) PCD in mammalian cells is shown with the major players of each pathway. Key morphological alterations, biochemical characteristics, and main regulators are listed in the table (bottom). Modified from Chung & Yu, 2013.

elucidated. Both *postmortem* and *in vivo* studies of PCD in the adult brain are greatly limited to observation-based reports as the assessment of PCD in the adult brain involves diverse interplays occurring at cellular and tissue levels. For instance, efficient clearance of dead cells in the brain by immune cells such as microglia, known as the "brain macrophages," provides brain scientists very small amount of, if any, valid samples – unremoved dead cells in the brain – to examine (Kreutzberg, 1996). Even with the methodological limitations and technical difficulties entailed to explore PCD in the adult brain *postmortem* or *in vivo*, the prize is too grand to pass as better understanding of cell death in pathological and aging brains can provide ultimate solution to neurological diseases and dementia. Alternatively, development of various *in vitro* models have been widely adopted to study PCD in pathophysiological states at molecular and cellular levels. Because this thesis is mainly concerned with survival and death of NSCs, it is noteworthy to address the significance of PCD in NSCs. Due to their ability to self-renew, proliferate and differentiate into neurons, astrocytes, and oligodendrocytes, NSCs are important in normal brain development and function, in regulation of brain plasticity, and in maintenance of tissue homeostasis in the brain (Chung and Yu, 2013). Hence, the size of NSC pool needs to be finely regulated through cell death to ensure the appropriate development and function of the brain.

1.3 Autophagy

Autophagy is a physiological process that catabolizes cytoplasmic materials including long-lived proteins, macromolecules and organelles through autophagosomes and lysosomes. Over 36 autophagy-related (Atg) genes have been identified to regulate execution of autophagy in finely-synchronized manner (**Figure 5**). Upon induction of autophagy, Atg protein complexes ULK (ULK1-Atg13-FIP200-Atg101) and class III PI3 kinase (Beclin1-Atg14-Vps34-Vps15) are activated and recruited to the sites of autophagosome formation site. Conjugation of the Atg12-Atg5-Atg16L1 complex and microtubule-associated protein light chain 3 (LC3) to the lipid phosphatidylethanolamine (PE) on the surface of autophagosomes proceeds to elongation and completion of autophagosomes (Itakura and Mizushima, 2010; Yang and Klionsky, 2010). After the fusion with the lysosomes, the substrates inside autophagosomes are subsequently degraded.

Long-lived proteins or damaged organelles are subject to degradation, and the resulting products are reused to provide metabolic intermediates. Therefore, autophagy helps to reduce cellular stress and maintain cellular homeostasis and cell viability. In contrast, the self-destructive role of autophagy has implicated autophagy

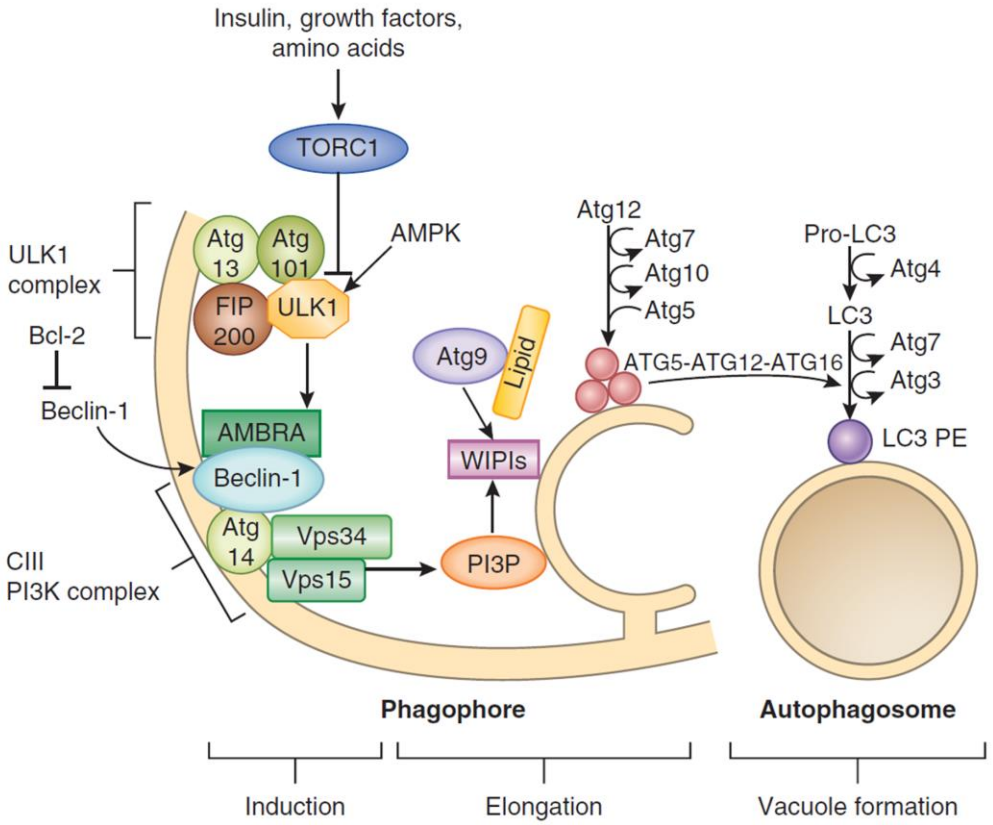


Figure 5. Molecular network of autophagy induction. Autophagosome formation can be initiated via mTOR inhibition resulting in the phosphorylation of ULK1 at sites that activate it and catalyze phosphorylation of other components of the ULK complex (composed of ULK1, ULK2, Atg13, FIP200 and Atg101). ULK1 in turn phosphorylates the PI3K CIII complex I (Vps34, Vps15, Atg14, and beclin-1), to move to the isolation membrane. Atg5 and Atg12 are conjugated to each other in the presence of Atg7 and Atg10, and the Atg5-Atg12 complex on the isolation membrane induces the conjugation of phosphatidylethanolamine (PS) and LC3, which facilitates closure of the isolation membrane. Atg4 removes LC3-II from the outer surface of autophagosomes, and LC3 inside is eventually degraded when the fusion between autophagosome and lysosomes occurs. Modified from Nixon, 2013.

mechanisms in cell death pathways (Baehrecke, 2005; Meijer and Codogno, 2004).

1.3.1 Autophagic Cell Death: Insulin Withdrawal Model in Hippocampal Neural Stem Cells

Currently, two established models of mammalian ACD qualified for the newly-proposed criteria (see 1.2.1 Programmed Cell Death: Classifications and Features) utilize neuronal cells (Uchiyama et al., 2009; Yu et al., 2008). Stemming from our first breakthrough report on induction of ACD in hippocampal neural stem (HCN) cells by insulin withdrawal (**Figure 6**; Yu et al., 2008; Baek et al., 2009), we have taken advantage of the given model to thoroughly investigate the underlying molecular machanisms and the functional implication of ACD in NSCs. Research in brain autophagy is especially interesting since the brain is specially protected against nutrient deficiency by a constant supply of nutrients from other organs even under starvation conditions (Mizushima et al., 2004). The high degree of resistance against nutrient deprivation in the brain does not indicate that autophagy is absent or inactive in the brain; rather it demonstrates that constitutive activity of autophagy proceeds at a basal rate in the brain. Therefore, abnormally elevated level of autophagy in brain regions represents an occurrence of critically dysregulated or pathological states in the brain.

The mechanisms underlying cell death of NSCs in response to degenerating stressors remain largely unknown, although the tremendous interest has risen in exploring the NSC biology. One of the best understood signals of NSC proliferation is growth factors. Growth factors play an important role in regulating the size of the NSC pool by directly stimulating proliferation or cell cycle kinetics or modulating cell death pathways. To study how a given growth factor affects the proliferation of NSCs and induces cell death following withdrawal, we investigated the cell death mode of HCN cells following insulin withdrawal. Insulin plays a key role in the hippocampal function and HCN cells are dependent on insulin signaling for proliferation (Machida et al., 2012). The insulin signaling enhances cell survival through the activation of PI3K and Akt, which inhibits glycogen synthase kinase-3β (GSK-3β) and disinhibits mammalian target of rapamycin (mTOR) at normal state. Upon insulin withdrawal, HCN cells undergo ACD through the inactivation of mTOR (Yu et al., 2008) which has a leading role in triggering autophagy via inhibition of the PI3K/Akt, "the insulin signaling," pathway. Insulin withdrawal increased autophagy flux and cell death, which could be attenuated upon the genetic suppression of autophagy. On the other hand, the promotion of autophagy using the mTOR inhibitor rapamycin increased cell death. HCN cells have intact apoptotic machinery and undergo typical

apoptosis, as shown by staurosporine-induced cell death. Nevertheless, insulin-deprived HCN cells did not exhibit apoptotic hallmarks, such as caspase-3 activation or nucleosomal DNA

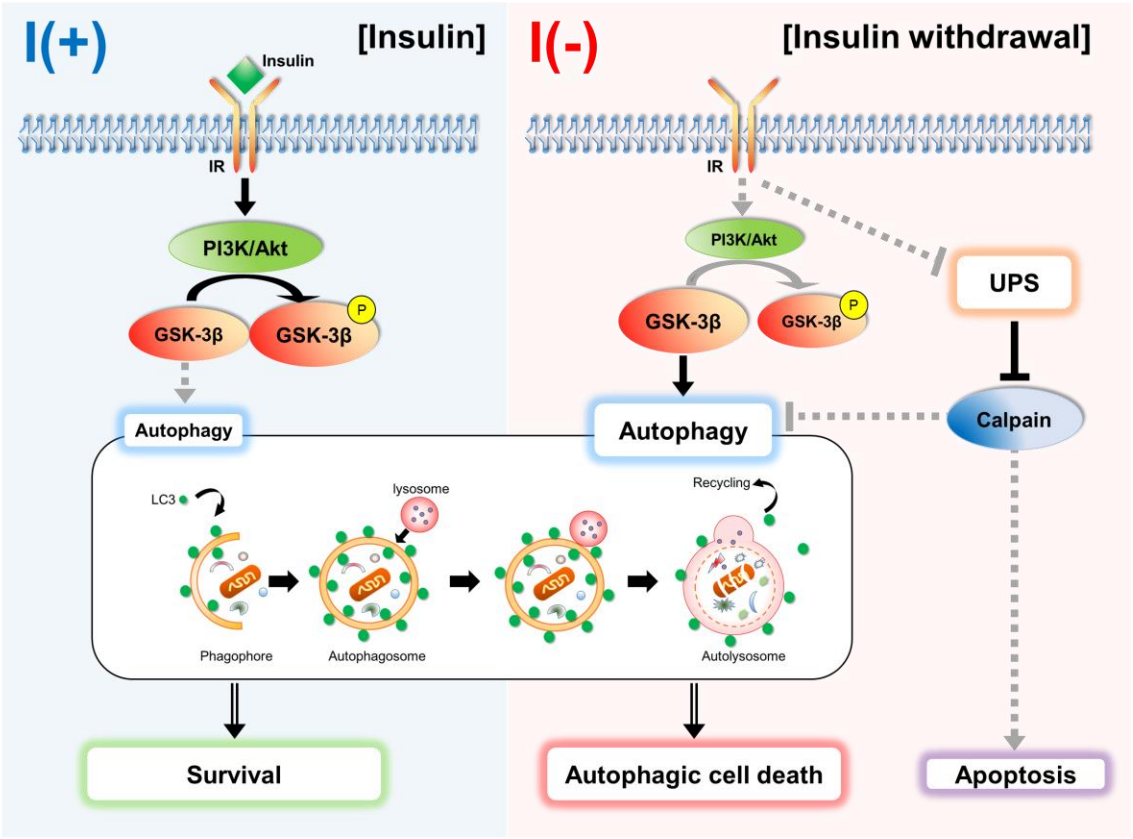


Figure 6. Insulin signaling pathways in insulin withdrawal model of ACD. The insulin signaling cascade is initiated upon insulin binding to insulin receptor (IR). This leads to the recruitment and activation of phospho-inositol-3 kinase (PI3K), and subsequent activation of Akt. Phosphorylation of Akt activates downstream molecules in the insulin signaling cascade including GSK-3β. In a normal state, activated Akt leads to inactivation of GSK-3β. In contrast, excessive autophagy is induced by the increased activation of GSK-3β that results from inactivation of PI3K and Akt upon insulin withdrawal. In addition, insulin withdrawal also upregulates UPS, leading to the degradation of calpain. This maintains the level of calpain activity below the threshold required to inhibit autophagy and induce apoptosis, switching the mode of PCD to ACD, rather than apoptosis, in HCN cells. Modified from Hong et al., 2016.

fragmentation, indicating the non-apoptotic nature of HCN cell death induced by insulin withdrawal. A correlation of cell death rate with the level of autophagic flux and the absence of apoptosis strongly suggests the primary role of autophagy in cell death. Hence, fulfilling the criteria listed above, insulin withdrawal-induced autophagic death in HCN cells represents a valid genuine model of mammalian ACD. Thus far, we have identified regulatory molecules and their functional roles in ACD using the model (Yu et al., 2008; Baek et al., 2009; Chung et al., 2015; Ha et al., 2015; Chung et al., 2016).

The other model of ACD besides insulin withdrawal model of ACD in HCN cells utilizes hypoxia/ischemia-induced neuronal cell death in both neonatal and adult mouse brains (Uchiyama et al., 2009). Conditional knockout mice (Atg7flox/flox: nestin-Cre) with specific deletion of Atg7 in the CNS tissue showed nearly complete resistance to hypoxia/ischemia-induced cell death in the hippocampus, suggesting the possibility of ACD (Uchiyama et al., 2009; Koike et al., 2008). However, identifying this paradigm as a genuine model of ACD should not be concluded hastily since this hypoxia/ischemia-induced cell death in the hippocampus also accompanied the activation of caspase-3, as well as autophagic markers (Koike et al., 2008).

1.3.2 Autophagy in Neurodegeneration

As described above, autophagy is a lysosomal degradative process which functions to recycle obsolete cellular constituents and eliminate damaged organelles. Because neurons have particularly large cytoplasmic volume due to their distinct morphological extensions of dendrites and axons, the clearance of long-lived proteins and dysfunctional organelles playing essential role in the maintenance of cellular homeostasis and thereby the failure of which underlies diverse human pathologies (**Figure 7**). Therefore, neurons are unusually reliable in autophagy to remove these obsolete cellular constituents, which also implies that defect in proteolytic clearance of autophagy substrates in neurons may pose highly destructive effects. Indeed, accumulation of protein aggregates due to defective autophagy competence underlies pathology of diverse diseases (Boland et al., 2008). Likewise, mutations of genes involved in autophagy pathway have been identified to contribute pathogenesis of, particularly, late-onset diseases such as familial Alzheimer's disease, amyotrophic lateral sclerosis, and familial Parkinson's disease (Nixon, 2013).

For the purpose of this thesis, we have focused on the contribution of pathological autophagy in Alzheimer's disease (AD). Characterized by progressive neuronal death, molecular mechanisms underlying AD remains elusive for over 100 years. A causative role of autophagy failure has been strongly implicated in neuronal death observed in

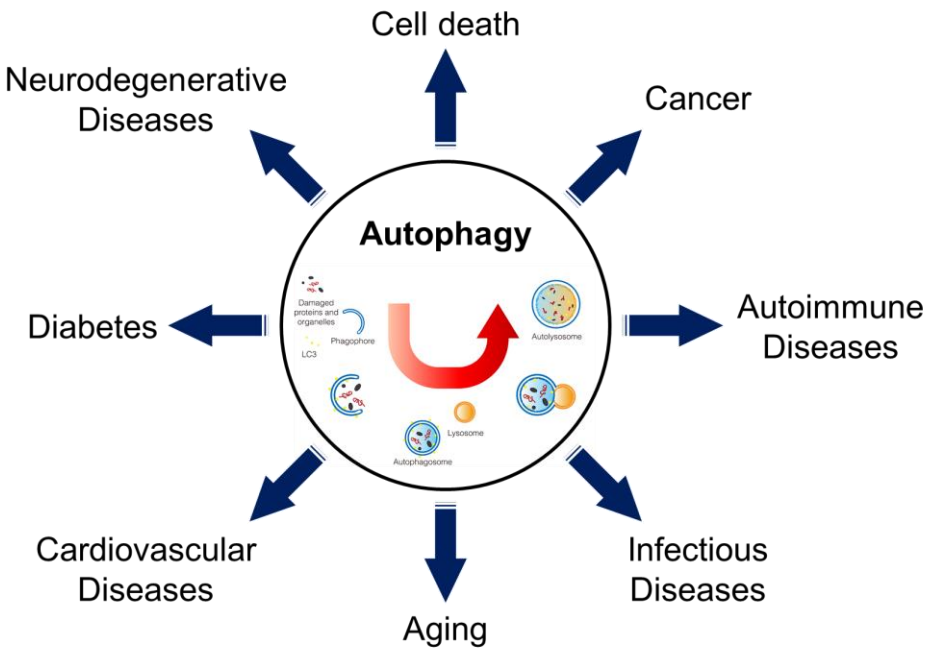


Figure 7. Autophagy in human diseases. Protein degradation, particularly through autophagy and UPS, is crucial in maintenance of normal cellular physiology. Autophagy generally functions as a cytoprotective mechanism to prevent various diseases, and dysfunctional autophagy leads to pathology. However, in certain contexts – for example, if induced excessively – autophagy can be deleterious.

AD since accumulation of autophagic vacuoles in degenerating neurons in AD has become the hallmark of AD pathology. In the calpain hypothesis underlying AD pathology, calpain is the culprit for autophagic-lysosomal failure. The calpain-mediated cleavage of Hsp70, a protectant against cellular stress, causes lysosomal rupture with cytoplasmic release of cathepsin (Mosser et al., 2000). Moreover, lysosomal membrane permeabilization (LMP), which underlies the neuronal cell death due to failed autophagy-mediated clearance of cellular waste, is another heavily implicated lysosomal event in Alzheimer's disease (Boya and Kroemer, 2008). One important mediatory molecule of LMP in neuronal cell death is calpain. Abnormal hyperactivity of calpains, which negatively regulate autophagy induction, are implicated in pathogenesis of Alzheimer's disease. The calpain hypothesis about the implication of calpain in neuronal cell death is supported by accumulating evidence from an abnormal increase in proteolytic activity of calpain in the brains of patients suffering from neurodegenerative disorders compared with control brains (Camins et al., 2009).

In conclusion, accumulating evidence supporting a defective autophagy in pathogenesis of several major neurodegenerative diseases is becoming a compelling fact, especially in relation to its involvement in proteostasis and metabolic regulation. Currently, various therapeutic strategies for generation of drugs to modulate specific stages of autophagy are under development for ultimate purpose to end neurodegeneration.

CHAPTER 2: General Materials and Common Techniques

2.1 Cell Culture

HCN cells were isolated as reported originally (Palmer et al., 1997). Cells were cultured in chemically defined serum-free medium containing Dulbecco's Modified Eagle's Medium/F-12 supplemented with N2 components. Insulin was omitted to prepare insulin-deficient medium. Insulin-containing and insulin-deficient media are denoted as I(+) and I(-), respectively, in this study.

Cells were cultured in chemically defined serum-free medium containing Dulbecco's Modified Eagle's Medium/F-12 supplemented with N2 components. Insulin was omitted to prepare insulin-deficient medium. Insulin-containing and insulin-deficient media are denoted as I(+) and I(-), respectively in this study. All plastic and glassware for HCN cell culture were coated with poly-L-ornithine (10 µg/ml for plastic plates and 50 µg/ml for glass; Sigma-Aldrich) and mouse laminin (5 µg/ml; BD Pharmingen). In brief, cells were cultured in chemically defined serum-free medium containing Dulbecco's modified Eagle's medium (Invitrogen) supplemented with Ham's F-12 medium (Invitrogen), 1 mM L-glutamine (Invitrogen), 100 µg/ml streptomycin, 100 U/ml penicillin (Invitrogen), 20 ng/ml bFGF (Invitrogen), and our own-made N2 supplement, which includes 5 mg/l insulin (Sigma-Aldrich), 16 mg/l putrescine dihydrochloride (Sigma-Aldrich), 100 mg/l transferrin (Sigma-Aldrich), 30 nM sodium selenite (Sigma-Aldrich), and 20 nM progesterone (Sigma-Aldrich). Medium was complemented with sodium bicarbonate (1.27 g/l) and adjusted to pH 7.2. Insulin was omitted to prepare insulin-free medium. The cells were normally passaged at a dilution of 1:5. To determine the effect of insulin withdrawal on HCN cell growth, the cells were cultured in insulin-containing complete medium and insulin-free medium, in parallel.

2.2 Western Blot Analysis

HCN cells were harvested at the indicated time points and then lysed in radioimmunoprecipitation assay buffer (R0278; Sigma-Aldrich) containing 1× protease inhibitors cocktail (87786; Thermo Scientific) and 1× phosphatase inhibitors cocktail (78420; Thermo Scientific) for 30 min on ice. Following centrifugation (12,000 g, 10 min), protein concentrations of the lysates were measured using the BCA protein assay reagent (23224; Thermo Scientific). Typically, 15-20 µg protein per well was loaded for Western blotting analysis. The proteins were electrotransferred to polyvinylidene fluoride membrane with a semi-dry electrophoretic transfer cell (Bio-Rad, Richmond). Membranes

were blocked for 1 h at room temperature in a blocking solution consisting of 5 % nonfat dry milk, 0.1 % Tween 20, and phosphate-buffered saline (PBS), pH 7.4. The membranes were then incubated overnight with the primary antibodies diluted according to the manufacturers' recommendations. The membranes were washed three times for 10 min each, followed by 1 h incubation at room temperature with peroxidase-conjugated secondary antibodies diluted in blocking solution. After washing, the membranes were then processed for analysis using a chemiluminescence detection kit (34080; Thermo Scientific).

2.3 Immunohistochemistry (IHC)

All procedures for the care and use of laboratory animals were approved by the Institutional Animal Care and Use Committee (IACUC) at Daegu Gyeongbuk Institute of Science and Technology (DGIST). The 8-week-old rats were perfused with 1× PBS and 4 % PFA. After dissection, the post-fixation was performed with 4 % PFA for 12 h. The brain was immersed in 30 % sucrose in PBS at 4 ℃ until it sank. The brain was embedded in OCT compound and frozen on dry ice. The brain was cut into 40 μm serial fee-floating coronal sections. The brain sections were washed with 1× PBS three times for 10 min. Blocking was performed for 1 h into 5 % normal donkey serum (Jackson ImmunoResearch) with 1 % BSA and 0.3 % Triton X-100. The brain sections were incubated with primary antibody diluted in 5 % NDS and 0.3 % Triton X-100 for 18 h at 4 ℃. After washing with 1× PBS, the brain sections were incubated with secondary antibody diluted in 5 % NDS and 0.3 % Triton X-100 for 2 h at room temperature in dark. Hoechst 33342 (Invitrogen; 1/10000 dilution) was stained for 10 min at room temperature. After sufficient washing, the brain sections were mounted on slide glass. The imaging was processed under laser scanning microscopes 700 (Carl Zeiss).

2.4 Immunocytochemistry (ICC)

Cells were fixed with 4 % paraformaldehyde and permeabilized with 0.1 % Triton X-100 in PBS. Following blocking with 0.5 % bovine serum albumin (Sigma-Aldrich; A6003), the fixed cells were incubated overnight with primary antibodies at 1:100 to 1:250 dilution, followed by incubation with anti-rabbit Alexa 488 secondary antibody (711-545-152; Jackson-ImmunoResearch). ICC samples were visualized under Zeiss LSM 700 confocal laser scanning microscope (Carl Zeiss) and collected images were further analyzed using ImageJ software (NIH).

2.5 Real-Time Quantitative PCR

Total RNA was isolated from cells using the ImProm-II Reverse Transcriptase kit (A3803; Promega) and reverse-transcribed into cDNA. Real-time quantitative PCR was performed with the CFX96 Real-Time System (Bio-Rad) and iTaq Universal SYBR Green Supermix (Bio-Rad). Results were analyzed using MS Excel 2013 (Microsoft). β-Actin was used as the reference gene for normalization. Primers used for Real-time quantitative PCR are listed in Table 1.

2.6 Cell Death Assay

HCN cells were seeded in a 96-well plate at a density of 5×10^4 cells per cm^2. Cell death was assayed using Hoechst 33342 (H3570; Invitrogen) and propidium iodide (P4170; Sigma-Aldrich) staining. In the treatment, each reagent did not exceed 0.5% of the total volume of medium to prevent potential cytotoxicity. Cells were imaged for further analysis under a fluorescence microscope (Axiovert 40 CFL; Carl Zeiss). Collected images were further analyzed using ImageJ software (NIH, Bethesda).

2.7 Transfection for Delivery of DNAs or siRNAs

For delivery of DNA constructs, HCN cells were first cultured overnight in 6-well plates at a density of 1×10^5 cells/well in medium without penicillin/streptomycin. On the following day, Lipofectamine 2000 (11668027; Invitrogen)-mediated transfection of plasmids is performed. Cells were replated in a 6-well plate at 24 h post-transfection and insulin withdrawal or treatment of reagents were applied according to experimental designs.

Nucleofection-mediated transfection was used to deliver siRNAs into HCN cells. Cells were transfected with siRNAs (50-200 nM) using a Nucleofector Kit (V4XP-4024; Lonza) according to the manufacturer's instructions. Typically, 1 5×10^6 cells were suspended in an appropriate volume of Nucleofector Kit solution per reaction and transfection was performed using a 4D-Nucleofector (Lonza). After the nucleofection, the cells were collected in preheated media and seeded in plates according to the experimental designs.

2.8 Flow Cytometry

Cells were harvested in cold PBS and centrifuged. After discarding the supernatant, cells were resuspended and incubated in 1× Annexin V-binding buffer for 15 min. Then, 5 μL of FITC Annexin V (556420; BD Biosciences) was

added to 100 µL of cell suspension and incubated for another 15 min. The Annexin V-stained cells were subjected to analysis using a Gallios flow cytometer (Beckman Coulter) according to the manufacturer's instructions. Acquired data were further analyzed using the Kaluza software (Beckman Coulter).

2.9 Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM) with the results of at least three independent experiments. Statistical significance was determined using the paired t-test for two-group experiments. For comparison of experiments with three or more groups, one-way analysis of variance (ANOVA) and Turkey's test were used. Differences were considered statistically significant when $p < 0.05$.

CHAPTER 3: Calpain Determines the Propensity of Adult HCN Cells to ACD Following Insulin Withdrawal

3.1 Introduction

Neural stem cells (NSCs) possess multipotency to differentiate into each type of neural lineage in the nervous system, and the self-renewing capability to proliferate and maintain stem cell populations (Gage, 2000). Because a single neural stem or progenitor cell can undergo exponential expansion, the size of the NSC pool is under tight control through programmed cell death (PCD) during brain development and adult neurogenesis to maintain the high integrity of the neurogenic population (de la Rosa and de Pablo, 2000; Gilmore et al., 2000). Any impairment in this form of NSC quality control may render them highly susceptible to disastrous consequences, such as neurodegeneration. Thus, gaining deeper insight into the PCD of NSCs may provide key guidance in developing efficient cellular therapies for the treatment of neurodegenerative diseases.

We have previously demonstrated that adult hippocampal neural stem (HCN) cells undergo autophagic cell death (ACD) following insulin withdrawal (Baek et al., 2009; Yu et al., 2008). Autophagy ("*self-eating*" in Greek) is a cellular process for the catabolism of a cell's own cytosolic contents (Klionsky, 2004). Double-membrane vesicles called autophagosomes encircle and digest autophagy substrates using lysosomal hydrolases after fusion with lysosomes (Dunn, 1994). Autophagy removes long-lived proteins, protein aggregates, and damaged organelles and converts them to metabolic intermediates, thereby relieving cellular stresses and fulfilling pro-survival function (Shintani and Klionsky, 2004). However, prolonged autophagy can cause cell death, leading to ACD (Tsujimoto and Shimizu, 2005). Due to well-established role of autophagy as a cyto-protective and adaptive response to adverse cellular conditions, the mere display of autophagic markers in dying cells is not sufficient to claim autophagy as the causative mode of cell death. Whether autophagy plays a central role in cell death (cell death through autophagy), or autophagy is a failed survival-related reaction (cell death with autophagy) needs to be clarified. Thus, Shen and Codogno recently suggested a set of criteria for ACD: i) the occurrence of cell death without signs of apoptosis, ii) an increase in autophagic flux, and iii) the prevention of cell death by pharmacological or genetic suppression of autophagy (Shen and Codogno, 2011). The autophagic death of insulin-deprived HCN cells showed no signs of apoptosis (no chromosomal DNA fragmentation or caspase activation), an increase in autophagy flux, and was blocked upon genetic suppression of autophagy by knockdown of Atg7, and therefore met all of the aforementioned criteria of

ACD (Baek et al., 2009; Yu et al., 2008). Cell death in insulin-deprived HCN cells occurred in a time-dependent manner, reaching over 50% cell death at day 3. Moreover, the rate of cell death increased proportionally in close correlation with the degree of autophagy induction. Of note, HCN cells underwent apoptosis in response to typical apoptotic inducers, such as staurosporine (Baek et al., 2009; Yu et al., 2008). Therefore, the lack of apoptosis induction in insulin-deprived HCN cells was not due to any defect in apoptotic machinery, but rather was a cell-intrinsic feature and advocates the central role of autophagy in cell death. To date, HCN cell death following insulin withdrawal has been regarded as a bona fide model of ACD in mammalian systems (Clarke and Puyal, 2012; Shen and Codogno, 2011)

Calpain is a family of cytosolic calcium (Ca^{2+})-activated cysteine proteases ubiquitously expressed in mammals (Liu et al., 2008). By cleaving various protein substrates, calpain can participate in essential intracellular events including autophagy and apoptosis. Calpain 1 and 2, the most characterized isoforms of the calpain family, are also known as μ (micro)- and m (milli)-calpain, respectively, reflecting their different Ca^{2+} concentration requirements for activation. As the name suggests, calpain 1 (μ -calpain) is activated by micromolar concentrations of Ca^{2+}, whereas calpain 2 (m-calpain) requires millimolar concentrations of Ca^{2+} for activation (Liu et al., 2008). Calpain can play positive or negative roles in modulating autophagy (Cheng et al., 2008; Demarchi et al., 2006; Madden et al., 2007; Yousefi et al., 2006). Therefore, the outcomes of calpain activation in terms of autophagy may be different depending on the cell types and stress context.

We sought to elucidate the molecular mechanisms that drive the mode of cell death towards ACD rather than apoptosis in HCN cells following insulin withdrawal. Here, we report that low calpain activity underlies the propensity of HCN cells to ACD following insulin withdrawal. Interestingly, conditions leading to high calpain activity induced a complete ACD-to-apoptosis switch under the same insulin withdrawal condition. Furthermore, we delineate here the involvement of the ubiquitin-proteasome system (UPS) in the demise of HCN cells. To the best of our knowledge, this is the first report on the cell death mode switching mechanism in NSCs. The results of our study provide novel insight into the complex mechanisms interconnecting autophagy and apoptosis and their roles in the survival and death of NSCs.

3.2 Materials and Methods

3.2.1 Antibodies and Reagents

The antibodies and reagents used were as follows: calpain 1 (#2556; Cell Signaling Technology), calpain 2 (#2539; Cell Signaling Technology), LC3B (L7543; Sigma-Aldrich), SQSTM1/p62 (#5114S; Cell Signaling Technology), β-actin (#4967S; Cell Signaling Technology), ubiquitin (Z0458; Dako), and cleaved caspase-3 antibodies (#9661; Cell Signaling Technology). Bafilomycin A1 (BafA1; B1793; Sigma-Aldrich;), calpeptin (BML-PI101; Enzo Life Sciences), Z-VAD-FMK (FMK001; R&D Systems), necrostatin-1 (BML-AP309; Enzo Life Sciences) and lactacystin (L6785; Sigma-Aldrich) were diluted in dimethyl sulfoxide at appropriate concentrations.

3.2.2 Plasmids and siRNAs

pRK5-calpain 1 and -calpain 2 plasmids were constructed by subcloning human calpain 1 (SC116897; OriGene) and calpain 2 (SC119079; OriGene) genes into the pRK5 vector obtained from Addgene (17612). For siRNA-mediated transient knockdown experiments, HCN cells were transfected with SMARTpool small interfering RNAs against rat calpain 1 (L-092181-02-0005; Dharmacon), calpain 2 (L-092084-02-0005; Dharmacon), or a control non-targeting siRNAs (D-001810-01-20; Dharmacon). HCN cells were transfected with plasmid DNAs (5-20 μg) or siRNAs (50-200 nM) using a Nucleofector Kit (V4XP-4024; Lonza) according to the manufacturer's instructions. Typically, 1×10^6 cells were suspended in an appropriate volume of Nucleofector Kit solution per reaction and transfection was performed using a 4D-Nucleofector (Lonza). After the nucleofection, the cells were collected in preheated media and seeded in plates according to the experimental designs.

3.2.3 Immunocytochemistry

Immunocytochemistry (ICC) was performed for detection of calpain 2 protein and quantification of LC3 and ubiquitin puncta, following the procedures described in Chapter 2 (2.4 Immunocytochemistry). To confirm the non-lysosomal localization of calpain 2, LysoTracker (L-7528; Molecular Probes) was used at 1 μM for 1 h.

3.2.4 Intracellular Calcium Imaging

Cells were cultured in 12-well plates at the density of 1×10^5 cells/well in I(+) or I(-) medium for 24 h and stained with 3 μM of Fluo-3 AM reagent (F-23915; Molecular Probes) at 37 ℃ for 40 min. After labeling, the medium was

replaced with the transparent DMEM/F-12 containing no phenol red (11039; Invitrogen) and cells were visualized under Zeiss LSM 7 Live microscope (Carl Zeiss) using a ×20 objective. Collected images were analyzed using the NIH ImageJ software.

3.2.5 Calpain Activity Assay

Calpain-Glo Protease Assay (G8502; Promega) was used according to the manufacturer's instructions to measure the enzyme activity of calpain. HCN cells were seeded onto 24-well plates (1×10^4 cells/well). Freshly prepared dilution of the Calpain-Glo Reagent stock solution was added to cells and luminescence was recorded with a plate-reading luminometer (SpectraMax L; Molecular Devices). All experiments were carried out in triplicate, and the luminescence values were normalized by protein concentrations of each condition after blank values were subtracted.

3.3 Results

3.3.1 Calpain 1 and 2 are Differentially Expressed in HCN Cells

Because calpain 1 and 2 are the most ubiquitously expressed calpain isoforms in the brain, we first assessed calpain 1 and 2 expression levels in HCN cells. Adult rat HCN cells used in the current investigation were freshly isolated based on the published protocols and are bona fide NSCs, as revealed by nestin-positive immunostaining and their intact differentiation competence similar to the original report (data not shown; Palmer et al., 1997). Calpain 2 protein was readily detectable in HCN cells with an expression level comparable to those measured in widely used non-neural HEK293 and neural SH-SY5Y cell lines (**Figure 8A**). However, the level of calpain 1 was very low and barely detectable. Likewise, the calpain 2 mRNA level was more than 150-fold times greater than that of calpain 1 in HCN cells (**Figure 8B**).

Next, we characterized the changes in calpain expression levels following insulin withdrawal. Due to very low expression level, we were unable to compare calpain 1 protein levels between HCN cells cultured in I(+) and I(-) media (**Figure 8C**). The expression of calpain 2 protein was reduced in insulin-deprived HCN cells (Fig. 1C). However, the level of calpain 2 mRNA was modestly elevated, indicating that calpain 2 may undergo protein degradation following insulin withdrawal (**Figure 8D**). The transcriptional upregulation was not observed for calpain 1 (data not shown). Subcellular localization of calpain 2 was examined by immunocytochemistry in both I(+) and I(-) conditions. Consistent with immunoblotting analysis (**Figure 8C**), fluorescence intensity of calpain 2 proteins was visibly greater in I(+) condition compared to I(-) condition (**Figure 8E**). Collectively, these data reveal a predominant expression of the calpain 2 subtype and its post-translational change upon insulin withdrawal in HCN cells.

3.3.2 Calpain 2 Inhibition Potentiates Autophagic Death of I(-) HCN Cells

As there have been conflicting reports on the role of calpain in the regulation of autophagy, we assessed whether calpain was involved in the regulation of ACD in HCN cells using a calpain inhibitor, calpeptin. Calpeptin treatment in HCN cells cultured in I(+) medium had no effect on cell viability or autophagy level (data not shown). However, the addition of calpeptin further potentiated autophagy, as indicated by an increased level of biochemical marker of autophagy, type II of LC3 (LC3-II) in the I(-) condition (**Figure 9A**). The LC3 protein undergoes a cleavage at its C-terminus resulting in cytosolic LC3-I, which is recruited to autophagosome membranes upon autophagy induction and

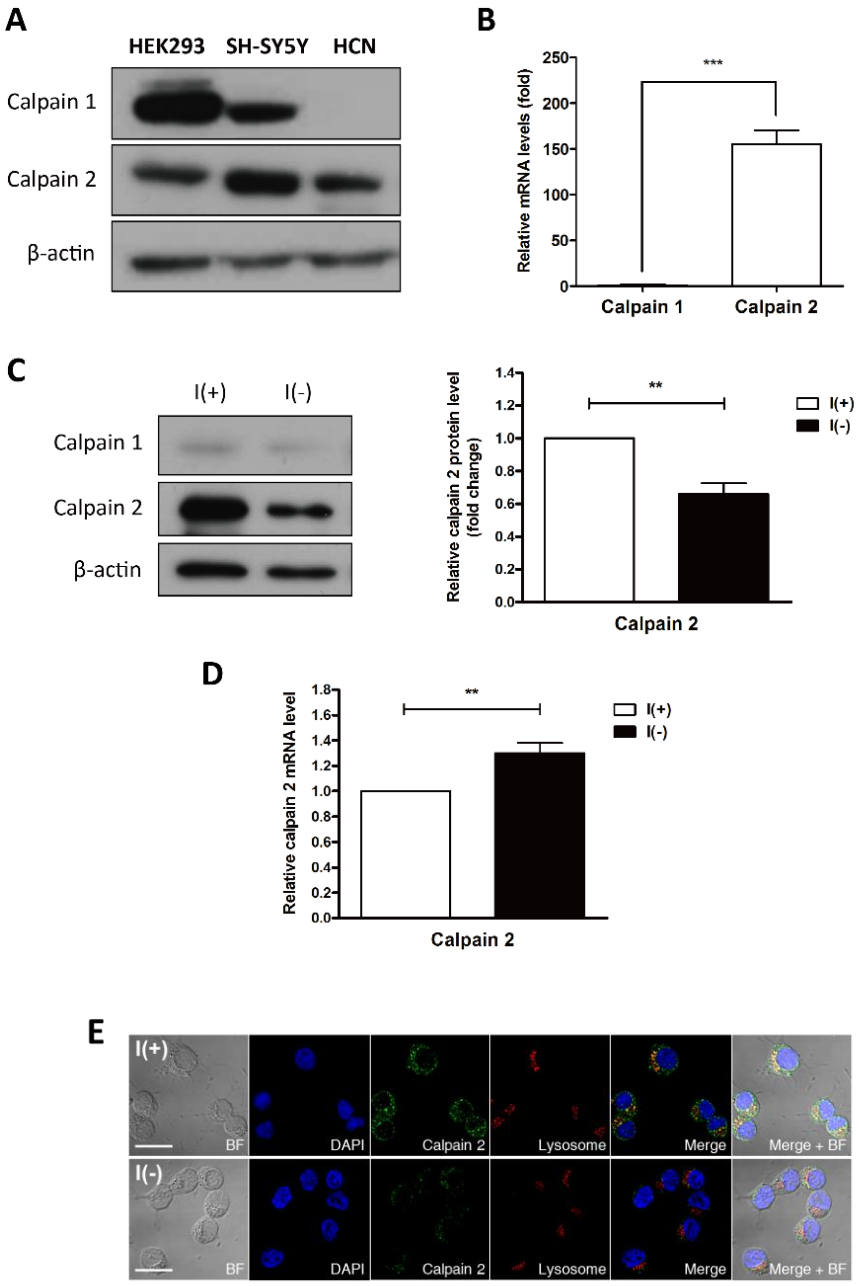


Figure 8. Calpain 1 and 2 are expressed distinctly in adult rat HCN cells. (A): A comparison of the expression levels of calpain 1 and 2 proteins in HCN cells with widely used non-neural HEK293 and neural SH-SY5Y cells. (B): A comparison of the calpain 1 and 2 mRNA levels in HCN cells cultured in insulin-containing condition by qRT-PCR analysis. Relative mRNA levels are represented as the mean ± SEM, ***$p < 0.001$. (C): A change in the calpain 1 and 2 protein levels 24 h after insulin withdrawal. Insulin-containing and insulin-deficient media are denoted as I(+) and I(-), respectively in this study. Right graph, quantification of the results of nine independent experiments. The signal intensity of calpain 2 was quantified using the ImageJ software and normalized to β-actin. The bars represent the mean ± SEM, **$p < 0.01$. (D): A change in the calpain 2 mRNA levels 24 h following insulin withdrawal. mRNA levels were assessed by qRT-PCR and are expressed as relative fold. Bars represent the mean ± SEM, **$p < 0.01$. (E): Immunofluorescence images of calpain 2 proteins in HCN cells. Bright field images are denoted as BF. Scale bars, 20 μm.

converted to LC3-II through conjugation with phophatidylethanolamine. Thus, both the level of lipid-conjugated LC3-II and the rate of conversion from LC3-I to LC3-II are generally used to assess the level of autophagy (Klionsky et al.,2012). The calpeptin-induced potentiation of autophagy was accompanied by an increased rate of cell death (**Figure 9B**). The inhibition of calpain enzyme activity by calpeptin was confirmed by calpain activity assay (**Figure 9C**).

Additionally, we also silenced calpain 2 genetically using a calpain 2-targeting siRNA. Calpain 2-depleted HCN cells exhibited an increase in the conversion of LC3-I to LC3-II following insulin withdrawal (**Figure 9D**) and the rate of cell death increased proportionally with respect to the increase in LC3-II (**Figure 9E**). Similar to the ineffectiveness of calpeptin noted in the I(+) condition, the calpain 2 knockdown had little effect on autophagy or the rate of cell death in HCN cells cultured in the I(+) medium (data not shown). The siRNA-mediated knockdown yielded ~40% decrease of calpain 2 transcripts compared to control groups (data not shown).

As reported in the previously published studies, the rate of ACD in insulin-deprived HCN cells correlates with the degree of autophagy induction (Baek et al., 2009; Yu et al., 2008). Likewise, pharmacological and genetic inactivation of calpain boosted the autophagy level further and caused more cell death in insulin-deprived HCN cells than insulin withdrawal alone. Despite the increased level of cell death, the activation of caspase-3 was still not observed in calpeptin-treated or calpain 2-depleted HCN cells, consistent with the notion of ACD (data not shown). However, silencing of calpain 1 which is endogenously expressed at minimal level fail to induce either autophagy or the rate of cell death in HCN cells (**Figure 10A,B**). Taken together, these data suggest that calpain is a negative regulator of autophagy and the low calpain activity due to the basal absence of calpain 1 and the degradation of calpain 2 may be related to the prevalence of ACD in insulin-deprived HCN cells.

3.3.3 Ectopic Expression of Calpain 1 Induces Apoptosis in HCN Cells Following Insulin Withdrawal

The decreased level of calpain 2 following insulin withdrawal (**Figure 8C**) suggested a possible downturn of the autophagic death of HCN cells when calpain 2 levels were rescued to a basal level or higher. However, the ectopic expression of calpain 2 failed to yield a significant level of over-expression (data not shown), presumably due to continuous degradation of calpain 2 in the I(-) condition. Therefore, we explored the alternative possibility that ectopic expression of calpain 1 could disrupt the endogenous propensity to ACD in insulin-deprived HCN cells, as calpain 1 is more readily activated in response to an increase in intracellular Ca^{2+} levels, but is barely expressed in HCN cells. Whereas overexpression of calpain 1 in the I(+) condition hardly affected the autophagy level or cell death rate (data

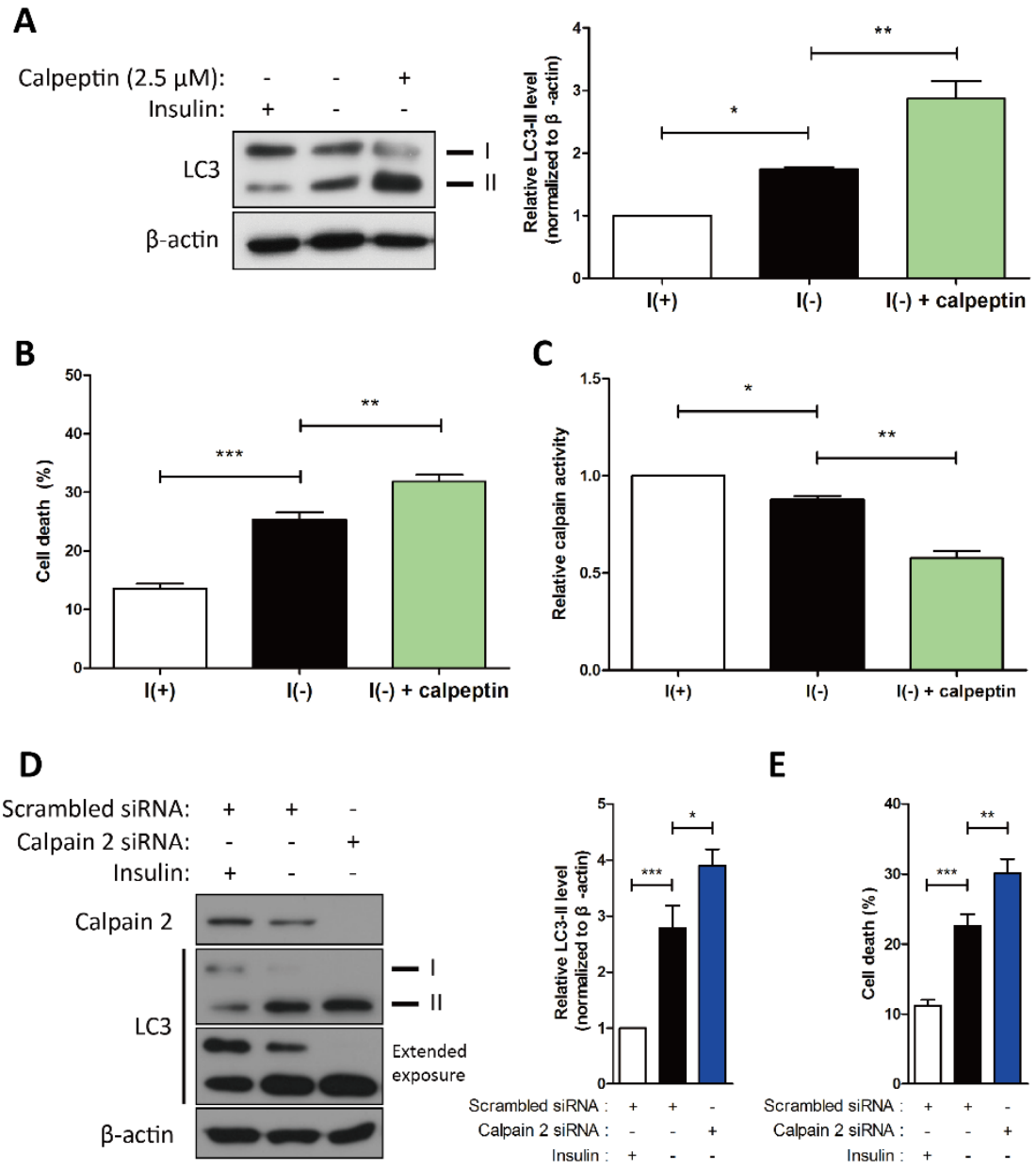


Figure 9. Calpain inhibition augments autophagic death of I(-) HCN cells. (A): Western blot analysis of LC3 in HCN cells cultured in I(-) medium with or without calpeptin (2.5 µM) for 24 h. Right graph, quantification of the results of four independent experiments. The signal intensity of LC3-II was quantified using the ImageJ software and normalized to β-actin. Bars represent the mean ± SEM; *p < 0.05 and **p < 0.01. (B): Measurement of cell death in insulin-deprived HCN cells treated with calpeptin (2.5 µM) for 24 h. (C): Enzymatic activity of calpain was assessed in calpeptin-treated HCN cells. Bars represent the mean ± SEM; *p < 0.05 and **p < 0.01. (D): Analysis of calpain 2-depleted HCN cells. Calpain 2-silenced HCN cells were cultured in I(-) medium for 24 h and subjected to Western blotting analyses. Right graph, quantification of the results of seven independent experiments. The signal intensity of LC3-II was quantified using the ImageJ software and normalized to β-actin. The bars represent the mean ± SEM; *p < 0.05 and ***p < 0.001. (E): Measurement of cell death in calpain 2-depleted HCN cells following insulin withdrawal for 24 h. The bars represent the mean ± SEM; **p < 0.01 and ***p < 0.001.

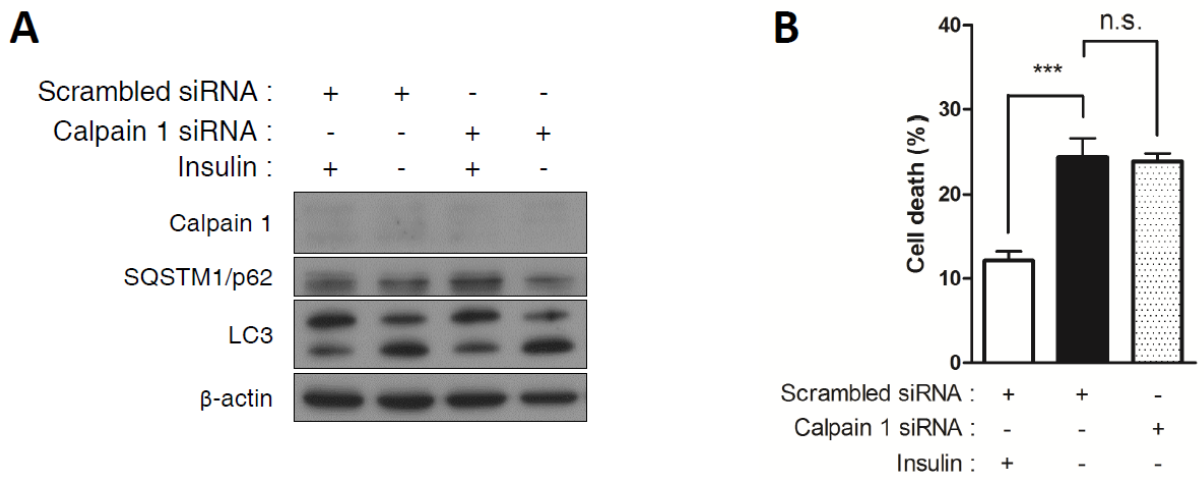


Figure 10. Knockdown of calpain 1 does not exhibit autophagy-related alterations in HCN cells. (A) Autophagy markers SQSTM1/p62 and LC3 were assessed using western blot analylsis. (B) Rate of cell death was measured using PI and Hoechst 33342 dyes and represented as bars with the mean ±SEM, ***$p < 0.001$.

not shown), over-expression of calpain 1 in insulin-deprived HCN cells caused more cell death than insulin withdrawal alone (**Figure 11**). Of interest, immunoblotting analyses revealed caspase-3 activation, as well as a significant reduction in the LC3-II level, suggesting the suppression of autophagy and the induction of apoptosis (**Figure 11B**). To ascertain the induction of apoptosis by calpain 1 overexpression in insulin-deprived HCN cells, we adopted another apoptosis assay that involved Annexin V-staining and flow cytometry analysis. The Annexin V dye binds to phosphatidylserine (PS), which is an inner-membrane phospholipid that becomes exposed on the outer surface of the plasma membrane during apoptosis. HCN cells cultured in I(-) medium were barely stained by Annexin V and its ratio was similar to HCN cells cultured in I(+) medium, again demonstrating a lack of apoptotic signs (**Figure 11C**). In contrast, calpain 1 over-expression substantially increased the number of Annexin V-stained HCN cells following insulin withdrawal, and the percentage of Annexin V-positive cells amounted to the overall level of cell death (**Figure 11A,C**). These data suggested an outright switch in the mode of PCD from the default ACD to apoptosis. Collectively, our data indicate that the ectopic expression of calpain 1 alters the mode of cell death from ACD to apoptosis in insulin-deprived HCN cells.

3.3.4 Degradation of Calpain 2 Is Achieved via UPS, Not Autophagy

Because the mRNA level of calpain 2 was increased to a moderate degree in the I(-) condition (**Figure 8C**), we presumed that the diminished level of calpain 2 protein in insulin-deprived HCN cells was likely due to enhanced protein degradation. To explore the possible degradation of calpain 2 via autophagy, we blocked the autophagic flux using bafilomycin A (BafA1), which prevents maturation of autophagic vacuoles by inhibiting autophagosome-lysosome fusion (Yamamoto et al., 1998). Treatment with BafA1 (30 nM) for 3 h effectively blocked the autophagic flux, resulting in large accumulations of LC3-II and SQSTM1/p62 proteins in the I(-) condition. SQSTM1/p62 is another autophagy marker which interacts with both LC3 and ubiquitinated substrates to deliver them to the autophagy system (Bjorkoy et al., 2006). Because p62 becomes incorporated and degraded in the process of autophagy, an increased autophagic flux correlates with a decreased level of p62. However, impairment of autohagic flux by BafA1 blocks turnover of LC3 and p62, resulting in their accumulation. Compared with a high level of LC3-II and p62 upregulation, calpain 2 accumulation was not substantial after BafA1 treatment, suggesting that autophagy may not be the major pathway for calpain 2 degradation (**Figure 12**).

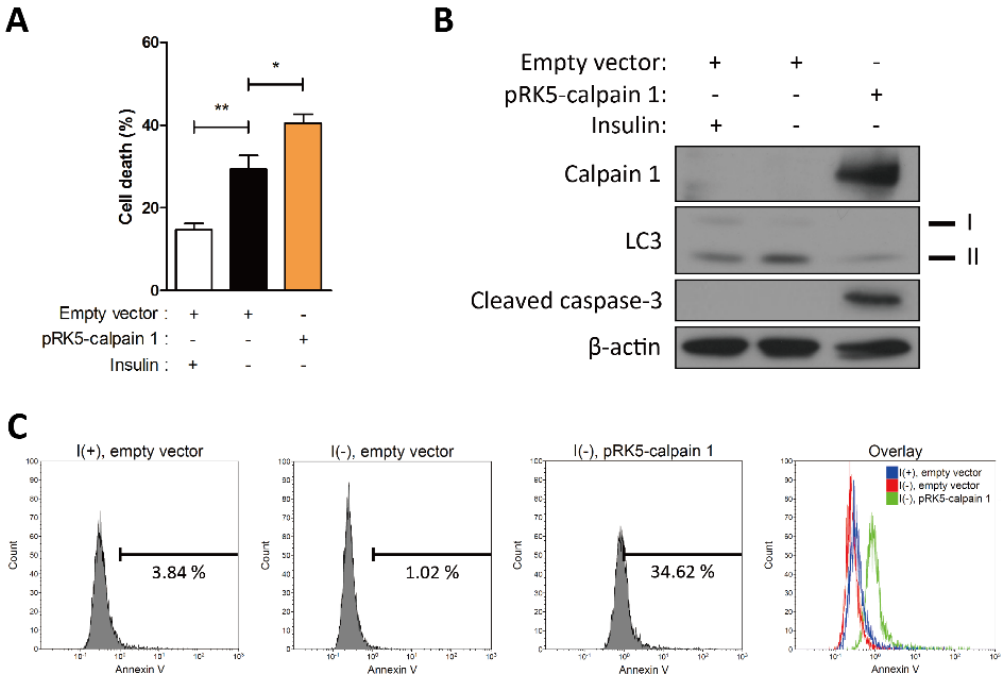


Figure 11. Calpain 1 overexpression in I(-) HCN cells switches the mode of cell death from ACD to apoptosis. (A): Rate of cell death in calpain 1-overexpressing HCN cells. Cell death was measured 24 h after insulin withdrawal and presented as mean ± SEM. *$p < 0.05$ and **$p < 0.01$. (B): Western blot analysis of calpain 1-overexpressing HCN cells. HCN cells were transfected with pRK5-calpain 1 or control vector and analyzed 24 h after insulin withdrawal. (C): Apoptosis in calpain 1-overexpressing HCN cells assessed by Annexin V staining. Annexin V-stained cells were fixed and subjected to flow cytometry analysis 24 h after insulin withdrawal.

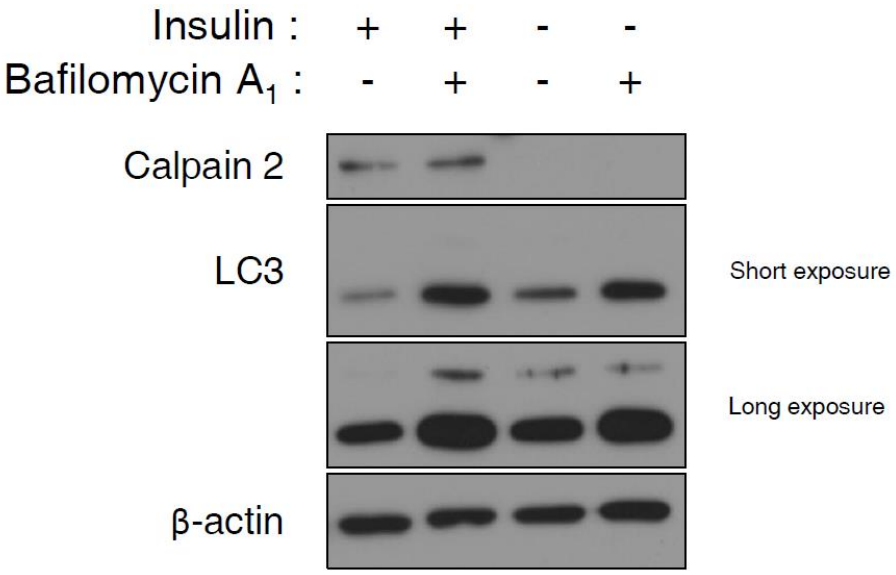


Figure 12. Calpain 2 does not undergo protein degradation via autophagy. Western blotting analysis revealed calpain 2 proteins did not accumulate following treatment of Bafilomycin A_1 while LC3 proteins robustly accumulated.

Together with autophagy, the UPS is a major intracellular degradation pathway (Pickart, 2001). Thus, we applied a proteasome inhibitor, lactacystin, to HCN cells cultured in I(-) medium to examine whether UPS-mediated degradation of calpain 2 accounted for its decrease. Lactacystin is a highly specific proteasome inhibitor which targets the N-terminal threonine of subunit X in the mammalian 20S proteasome (Bogyo et al., 1997). In most published studies, lactacystin has been used at concentrations of 10 μM or higher. However, in insulin-deprived HCN cells, treatment with lactacystin at 1 μM for 3 h was enough to block calpain 2 degradation efficiently (data not shown). The levels of calpain 2 proteins were elevated substantially such that they were comparable to that of insulin-containing HCN cells (**Figure 13A**). These findings suggest that the UPS is the main route for the degradation of calpain 2 following insulin withdrawal. Proteasome inhibition was verified by the accumulation of ubiquitinated proteins, as shown by immunoblotting analysis and immunostaining with an anti-ubiquitin antibody (**Figure 13B,C**). Together, our data suggest that the calpain 2 level is altered primarily through UPS rather than autophagy.

3.3.5 Proteasome Inhibition Elevates the Concentration of Intracellular Calcium and Activates Calpain in I(-) HCN Cells

Because proteasome inhibition blocked calpain 2 degradation, we examined whether calpain activity was upregulated after treatment with lactacystin. The calpain activity was lower in I(-) HCN cells than in I(+) HCN cells. Intriguingly, the enzymatic activity of calpain in I(-) HCN cells treated with lactacystin was increased notably (**Figure 14A**). Because calpain 2, the predominant calpain isoform in HCN cells, requires millimolar levels of intracellular Ca^{2+} for its activity, we hypothesized that the robust increase of calpain activity following proteasome inhibition was likely accompanied by a surge of intracellular Ca^{2+}. Thus, we measured the intracellular Ca^{2+} levels in HCN cells using the fluorescent Ca^{2+} indicator Fluo-3 AM (**Figure 14B**). Consistent with our hypothesis, the level of intracellular Ca^{2+} in I(-) HCN cells showed a marked rise following lactacystin treatment as compared with the level observed in I(-) HCN cells (**Figure 14C**). I(-) HCN cells also showed an increase in the intracellular Ca^{2+} concentration, but to a much lesser extent than co-treatment with lactacystin, suggesting that a modest increase in intracellular Ca^{2+} levels in I(-) HCN cells may not be sufficient to induce the potent activation of calpain 2, due to its high Ca^{2+} concentration requirement for activation. Additionally, the UPS constantly degrades calpain 2, further restraining its activation. These data demonstrate that proteasome inhibition induces a surge of the intracellular Ca^{2+} level, a concomitant accumulation of calpain 2, and a subsequent increase in calpain activity in insulin-deprived HCN cells.

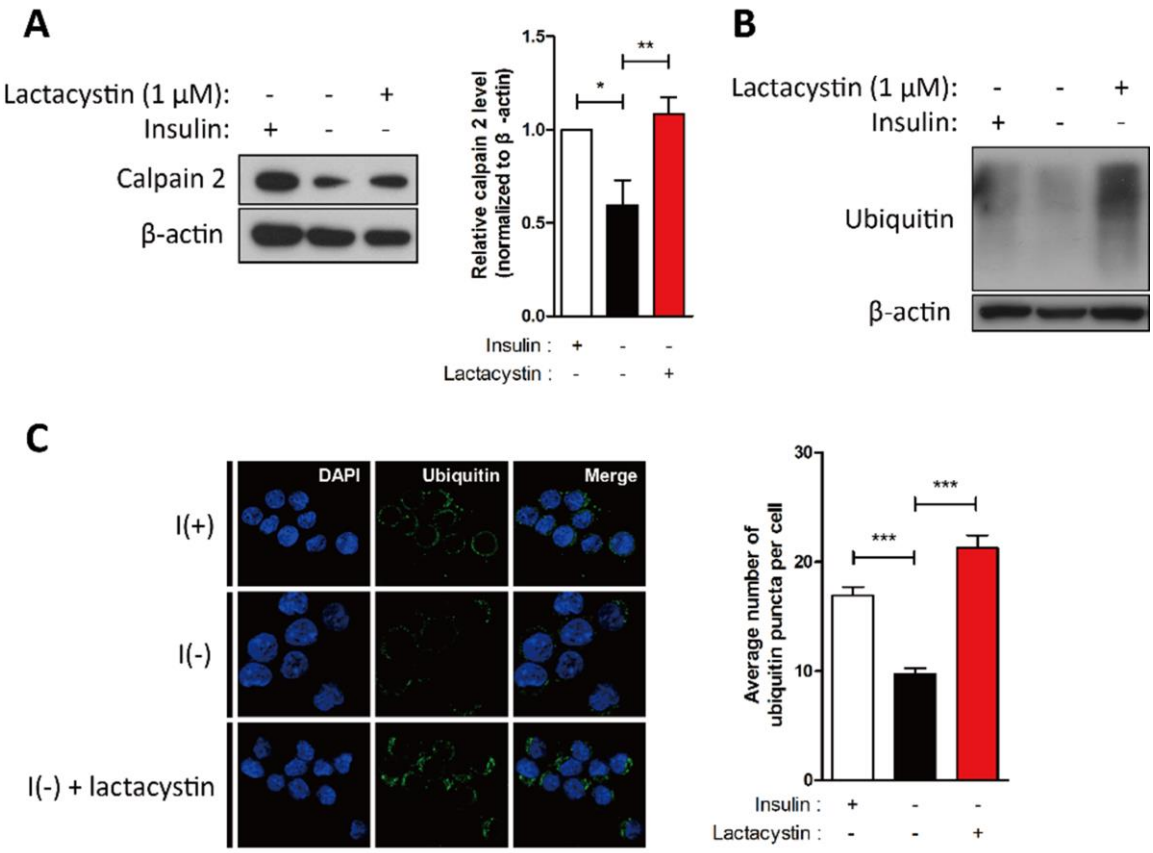


Figure 13. Calpain 2 undergoes proteasome-dependent protein degradation in I(-) HCN cells. (A): Accumulation of calpain 2 upon proteasome inhibition. HCN cells were cultured in I(-) medium with or without lactacystin for 24 h. Right graph, quantification of the results of five independent experiments. The signal intensity of calpain 2 was quantified using the ImageJ software and normalized to β-actin. The bars represent the mean ± SEM, *$p < 0.05$. (B): Accumulation of ubiquitin in proteasome-inhibited HCN cells. HCN cells were cultured in I(-) medium with or without lactacystin (1 μM) for 24 h and subjected to Western blotting. (C): Immunofluorescence images of accumulated ubiquitin in proteasome-inhibited HCN cells. DAPI-stained cells were labeled with ubiquitin antibody and examined under a confocal microscope to monitor the formation of ubiquitin-positive puncta 24 h after treatment. Right graph, quantification of the average numbers of ubiquitin-positive puncta in HCN cells were quantified and are presented as mean ± SEM. ***$p < 0.001$.

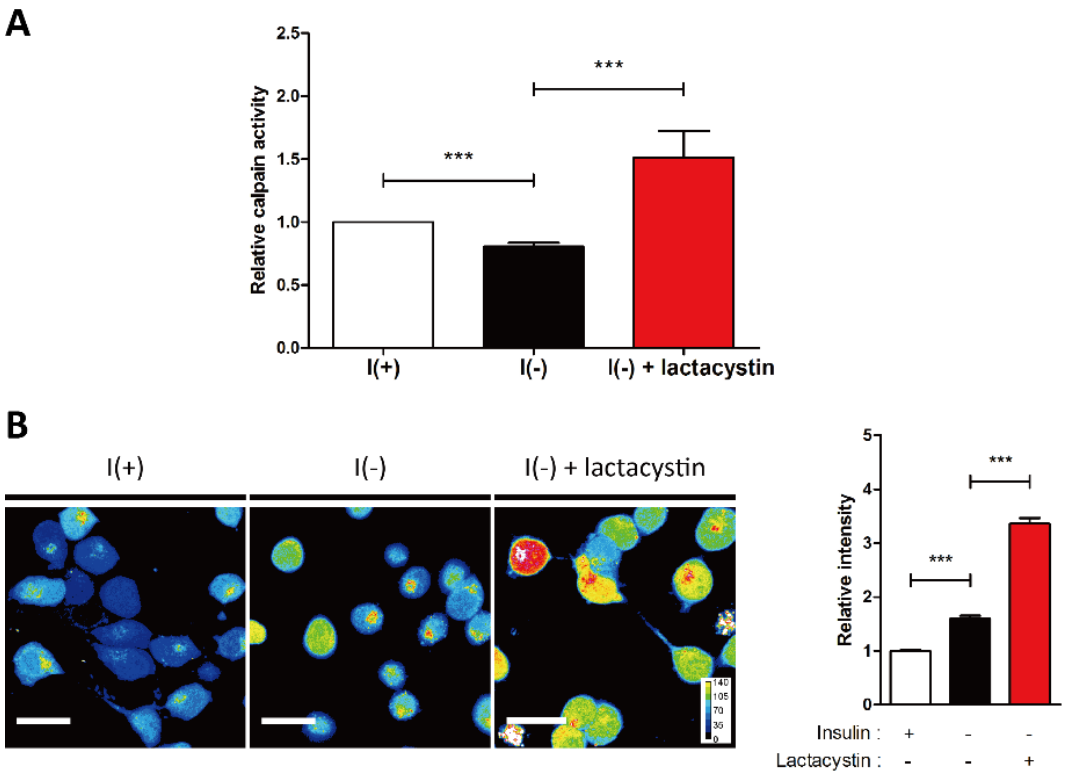


Figure 14. Proteasome inhibition elevates the concentration of intracellular calcium and activates calpain in I(-) HCN cells. (A): Calpain activity assay. HCN cells were cultured in I(-) media with or without lactacystin for 24 h. The bars represent the mean ± SEM, *p < 0.05, ***p < 0.001. (B): Immunofluorescence-based detection of intracellular calcium in HCN cells. HCN cells were cultured in I(-) media with or without lactacystin for 24 h and labeled with Fluo-3 AM calcium dye (3 µM). Fluorescence signal was visualized under a live confocal microscope. Scale bars, 20 µm. Right graph, quantification of the fluorescence intensity of Fluo-3 AM. Relative intensity was quantified using the ImageJ software and presented as mean ± SEM. ***p < 0.001.

3.3.6 Lactacystin Switches the Default Autophagic Death of I(-) HCN Cells to Apoptosis.

Several studies have demonstrated a degree of interplay between the autophagy-lysosomal and ubiquitin-proteasome pathways for the maintenance of cellular homoeostasis (Korolchuk et al., 2010). The induction of autophagy after UPS impairment is commonly perceived as a compensatory mechanism to prevent the accumulation of ubiquitinated proteins (Ding et al., 2007; Pandey et al., 2007; Zhu et al., 2010). However, our data suggested that lactacystin-mediated proteasome inhibition caused calpain activation. Since calpain repressed autophagy in the insulin withdrawal model of ACD in HCN cells, we, therefore, posited that proteasome inhibition by lactacystin would lead to an interruption of autophagy flux, and thus alteration of ACD instead of compensatory upregulation of autophagy and furtherance of ACD in HCN cells. First, the cell death rate was measured following lactacystin treatment in insulin-deprived HCN cells. Proteasome inhibition by lactacystin induced a significantly higher level of cell death than insulin withdrawal alone (**Figure 15A**). While the pan-caspase inhibitor Z-VAD failed to protect HCN cells from insulin withdrawal-induced cell death, the addition of Z-VAD abolished the cell death induced by lactacystin, demonstrating that proteasome inhibition-mediated death in I(-) HCN cells was a caspase-dependent apoptotic event (**Figure 15A**). Apparent activation of caspase-3 was observed in lactacystin-treated HCN cells which, without lactacystin administration, would normally experience pure autophagic death upon insulin removal (**Figure 15B**). Using flow cytometry, we further confirmed the apoptotic death of HCN cells following lactacystin treatment by detecting Annexin V-stained cells (**Figure 15C**). However, lactacystin treatment caused a reduction in the LC3-II level and a restoration of p62 level (**Figure 15D**). Decreased autophagic activity was also evidenced by a decline in the average number of LC3 puncta per cell, similar to the observed reduction of the LC3-II protein level (**Figure 15E**). Therefore, these data indicated that inhibition of proteasome activity caused suppression of autophagy rather than compensatory enhancement of autophagy. Furthermore, the increased rate of cell death despite the inhibition of ACD by lactacystin in I(-) HCN cells is because of induction of apoptosis. Necroptosis, another form of PCD was not involved in the lactacystin-induced switch in the mode of cell death, as the rate of cell death of I(-) HCN cells co-treated with lactacystin was not affected by necrostatin-1, a chemical inhibitor of necroptosis (**Figure 16**). These results indicate that proteasome inhibition by lactacystin completely switches the mode of cell death from autophagy to apoptosis in insulin-deprived HCN cells.

In conclusion, the functional role of calpain in NSCs remained relatively unknown to date. Our results indicate

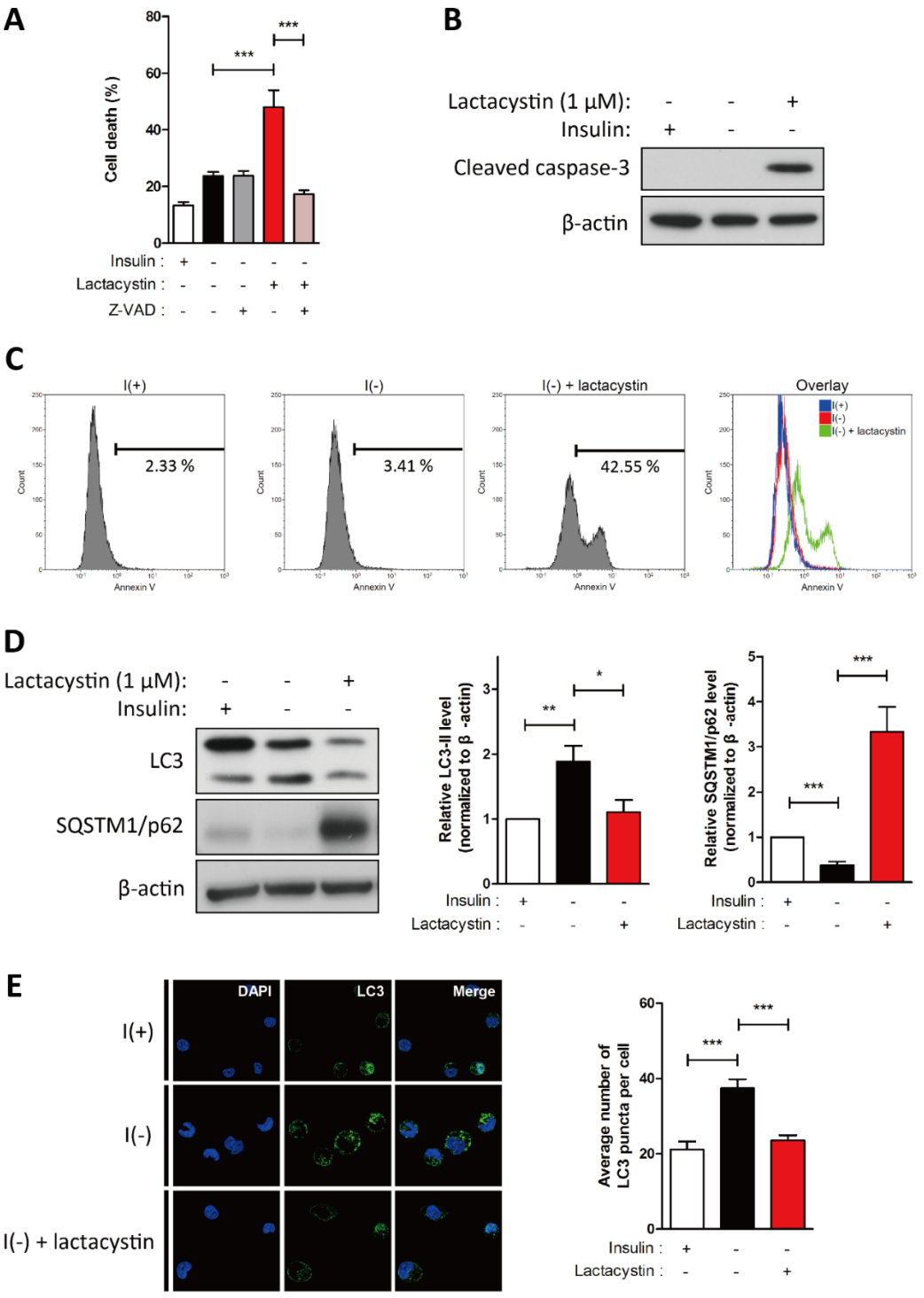


Figure 15. Lactacystin switches the default ACD to apoptosis in I(-) HCN cells. (A): Increased rate of cell death by lactacystin in insulin-deprived HCN cells. HCN cells were cultured in I(-) medium with or without lactacystin for 24 hours. The pan-caspase inhibitor Z-VAD was used to block the caspase-dependent death of HCN cells treated with lactacystin. The rates of cell death are the quantification of the results from three independent experiments with three replicates per experiment and presented as mean ± SE. *, $p < .05$ ***, $p < .001$, n.s., not significant. (B): Immunoblot analysis of caspase-3 activation in lactacystin-treated HCN cells. HCN cells were cultured in I(-) medium with or without lactacystin for 24 hours. Caspase-3 activation was demonstrated by Western blotting with an antibody against

the cleaved form of caspase-3. (C): Flow cytometry analysis of apoptotic cell death in lactacystin-treated HCN cells. HCN cells were cultured in I(-) medium with or without lactacystin for 24 hours. Annexin V-stained cells were fixed and subjected to flow cytometry analysis. (D): Western blot analysis of an altered level of autophagy in insulin-deprived HCN cells cotreated with lactacystin. HCN cells were cultured in I(-) medium with or without lactacystin for 24 hours and autophagy marker proteins LC3 and SQSTM1/p62 were analyzed. Right graphs, quantification of the eight independent experiments. The signal intensities of LC3-II and SQSTM1/p62 were quantified using the ImageJ software and normalized to β-actin. The bars represent mean ± SE. *, $p < .05$; **, $p < .01$; ***, $p < .001$. (E): Immunofluorescence images of LC3 in HCN cells cultured in I(-) medium with or without lactacystin for 24 hours. HCN cells were stained with DAPI and LC3 antibody and visualized under a confocal microscope. Scale bars = 20 μm. The average number of LC3-positive puncta per cell ($n = 336 – 379$ per condition) was quantified using ImageJ and presented as mean ± SE. ***, $p < .001$.

that low calpain activity results in a preference for ACD over apoptosis in insulin-deprived HCN cells. Furthermore, we delineate here the active involvement of the UPS in the demise of HCN cells and provide evidence that calpain and UPS are regulatory modules for cross-talk between apoptosis and ACD in HCN cells. The results of our study will unveil the sophisticated mechanisms underlying the interplay and switch between different modes of PCD in NSCs during neural development, neurogenesis, and diseases of the nervous system.

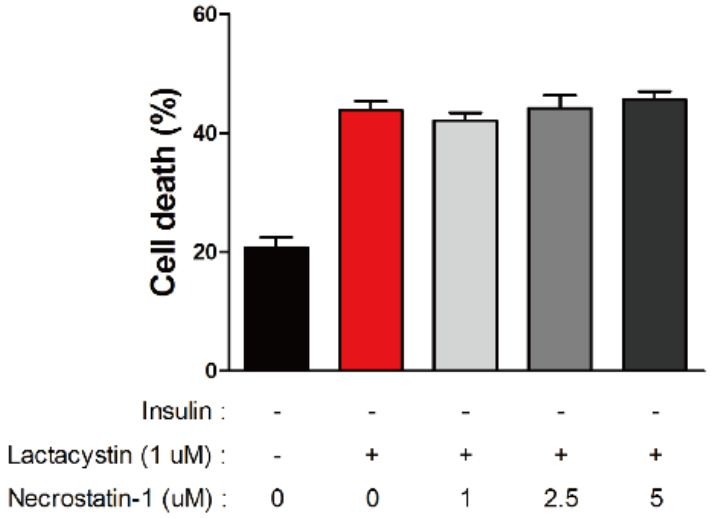


Figure 16. Lactacystin-mediated switch of cell death mode in I(-) HCN cells does not involve necrosis. Treatment with necrostatin-1 at the different concentrations failed to reduce the rate of lactacystin-induced apoptotic death of HCN cells cultured in I(-).

3.4 Discussion

The results of our study have important implications on several levels. Our novel discovery establishes calpain as a key negative regulator of ACD and a molecular switch to apoptosis in the demise of HCN cells. The differential expression pattern of calpain 1 and 2, together with the dynamic changes in the calpain 2 protein level and intracellular Ca^{2+} concentration, may underlie this role of calpain. Also, our study unveiled an intriguing, non-complementary interplay between autophagy and UPS for interconnection between autophagy and apoptosis via calpain in HCN cells. As for the physiological implications of the current study, recent observations of defective insulin signaling and impaired insulin transport in the brain, especially in the hippocampus of patients with Alzheimer's disease (AD; de la Monte, 2009; Talbot et al., 2012) suggest the potential relevance of autophagy-dependent death of insulin-deprived HCN cells in the pathogenesis of AD. Despite extensive NSC research conducted over the past few decades, the mechanisms underlying the programmed death of NSCs has remained under-studied. The insulin withdrawal model in HCN cells may provide a unique opportunity to decipher the molecular mechanisms of PCD in NSCs.

Of note, Santos et al. reported the distinct regulatory functions of calpain 1 and 2 in NSCs derived from the fetal mouse forebrain, as calpain 1 repressed neural differentiation while calpain 2 promoted glial differentiation (Santos et al., 2012). Although this finding may be not directly applicable to HCN cells due to different cellular origins and expression profiles of calpain family members, it further validates the existence of the differential cellular functions of calpain 1 and 2 in NSCs. Another study by Machado et al. investigated the role of calpain in adult neurogenesis, in which the deletion of calpastatin, the endogenous calpain inhibitor, impaired both NSC proliferation and neuroblast migration while calpain inhibition increased NSC proliferation, migration speed and distance in the neurogenic regions (Machado et al., 2015).

Manifestations of autophagic characteristics in dying cells have been observed since the 1960s (Schin and Clever, 1965). However, it was not until recently that ACD began to gain attention. Compared with the well-known biochemical mechanisms and physiological roles of apoptosis, the role of ACD and relevant mechanisms have remained elusive. Nevertheless, it is generally accepted that autophagy can promote or antagonize apoptosis. However, the key factors determining the outcome of the relationship between autophagy and apoptosis for cell survival or death are not well understood. One way autophagy affects apoptosis is through degradation of essential apoptotic molecules, such as caspase-8 or -9 through autophagy (Hou et al., 2010; Jeong et al., 2011). On the other hand, apoptosis can also change autophagic flux by targeting important autophagy molecules, such as Beclin 1 or Atg5 for proteolytic cleavage

(Luo and Rubinsztein, 2010; Yousefi et al., 2006). In this regard, calpain is interesting because calpain protease activity can promote or suppress autophagy. Calpain is a well-known cell death protease under various pathophysiological conditions in the brain (Crocker et al., 2003; Gafni and Ellerby, 2002; Saito et al., 1993). Prior reports suggested a requirement of calpain for the facilitation of autophagy (Cheng et al., 2008; Demarchi et al., 2006). However, the results from several other studies have demonstrated negative control of autophagy by calpain. Madden et al. reported that calpain inhibition, in combination with caspase-8 suppression, induced autophagy in the Z-VAD-induced death of L929 fibroblast cells (Madden et al., 2007), although they did not specify which isoform of calpain played the critical role in the inhibition of ACD. ACD in L929 cells required prior inhibition of caspases, especially caspase-8. In our study, insulin-deprived HCN cells did not display signs of apoptosis or require caspase inhibition for the progress of ACD, suggesting the existence of an innate system that places apoptosis on hold in HCN cells. This is not due to any defect in the apoptotic capabilities of HCN cells, as they were derived from adult wild-type rat hippocampal tissues and undergo typical apoptosis in response to staurosporine (Baek et al., 2009; Yu et al., 2008), etoposide, and oxidative stress (data not shown). Identification of the as-yet-unknown endogenous antagonizing pathway(s) to block apoptosis would be of great interest, and much of our research efforts have been concentrated on addressing this question.

The Ca^{2+} concentration required for the proteolytic activities of calpain 2 is much higher than that of calpain 1. Therefore, although we did not measure the exact concentration of intracellular Ca^{2+}, it was clear that the increase in intracellular Ca^{2+} concentration subsequent to insulin withdrawal was not sufficient to activate calpain 2. On the contrary, lactacystin-mediated proteasome inhibition induced a robust increase in the intracellular Ca^{2+} concentrations in insulin-deprived HCN cells, and subsequent activation of calpain. Further studies will be required to define the key regulatory elements targeted by calpain for the inhibition of autophagy and potent induction of apoptosis in the regulation of HCN cell death.

The non-complementary interaction between autophagy and UPS in insulin-deprived HCN cells is also interesting. Compromised cellular clearance due to UPS inhibition would be expected to be compensated for by an increase in the autophagy level. However, proteasome inhibition did not lead to the complementary enhancement of autophagic flux, but rather inhibited ACD in insulin-deprived HCN cells. Intriguingly, this inhibition of ACD did not confer protection; instead, it exacerbated overall cell demise, resulting in a higher cell death rate than insulin withdrawal alone. The increased level of cell death was characterized by caspase-3 activation and Annexin V staining,

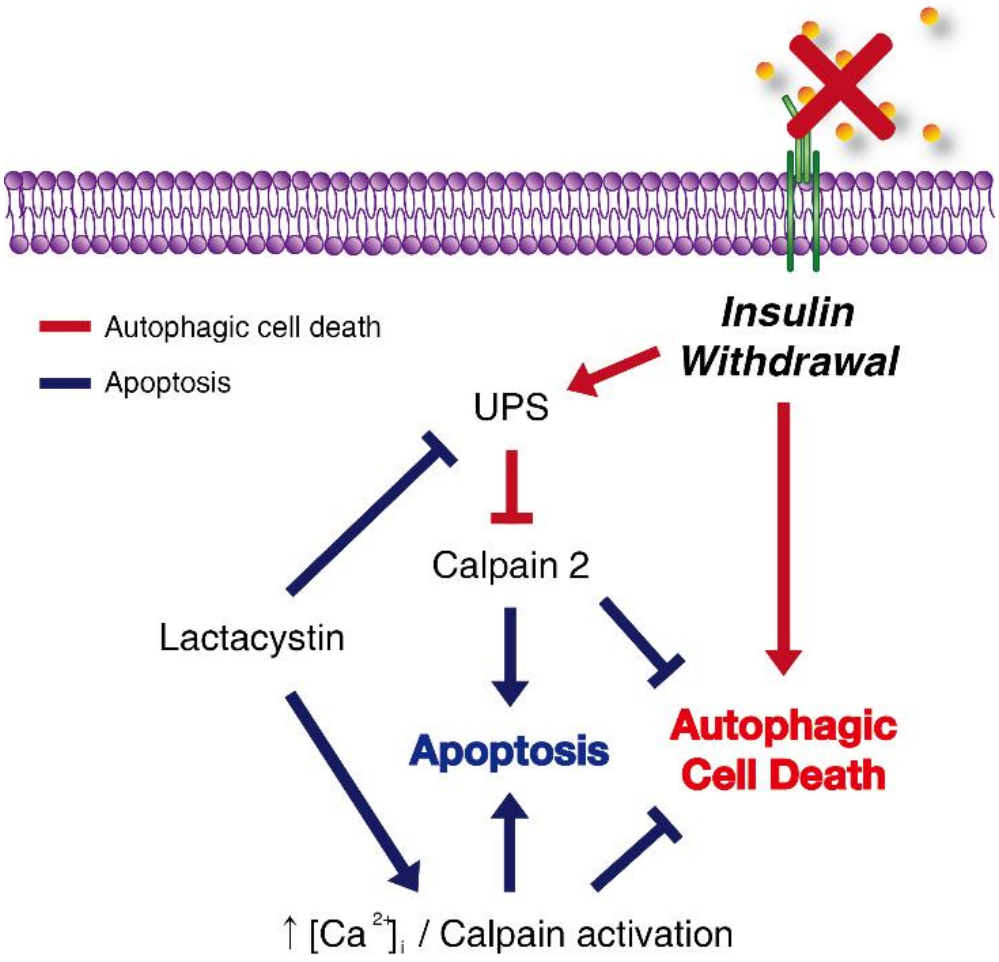


Figure 17. Schematic diagram illustrating the switch of PCD mode between autophagy and apoptosis via calpain. Insulin withdrawal activates autophagy and subsequent cell death in HCN cells. Simultaneously, the degradation of calpain 2 by the UPS maintains low calpain activity and sets a preference of ACD over apoptosis in HCN cells. In contrast, the conditions giving rise to high calpain activity, such as ectopic expression of calpain 1 or inhibition of UPS incurring a surge of intracellular Ca^{2+} and blocking of calpain degradation, is sufficient to induce apoptosis while suppressing autophagy in HCN cells.

indicating induction of apoptosis. Consistent with our hypothesis of calpain as a switch from ACD to apoptosis, we were able to observe high calpain activity following UPS inhibition in insulin-deprived HCN cells. The enhanced activation of calpain was apparently due to a blockade of calpain 2 degradation and a surge in the intracellular Ca^{2+} concentration.

In short, HCN cells seem to maintain a subtle balance of intrinsic calpain activity within a certain range. Our study revealed predominant expression of calpain 2, with a near absence of the more sensitive calpain 1 in HCN cells. In insulin-deprived HCN cells, calpain (most likely calpain 2) activity is kept below a certain level, thereby preventing apoptosis induction while allowing the progress of autophagy as a default mode of cell death. Greater repression of calpain activity tilts the mode of cell death further towards autophagy. In contrast, high calpain activity switches ACD completely to apoptosis. Based on these results, we propose a working model for the regulation of ACD in HCN cells (**Figure 17**). Following insulin withdrawal, there is a modest increase in the intracellular Ca^{2+} concentration. However, the level of calpain activity is kept low through the constant degradation of calpain 2 by UPS and an insufficient rise in the intracellular Ca^{2+} level. Upon proteasome inhibition, a marked increase in intracellular Ca^{2+} level occurs, as well as blocking of calpain 2 degradation. This leads to a robust increase in calpain activity and a complete ACD-to-apoptosis switch in insulin-deprived HCN cells. Ectopic expression of calpain 1 also switches the default ACD to apoptosis, consistent with our hypothesis of calpain as a molecular switch between autophagic and apoptotic programmed cell death in HCN cells.

CHAPTER 4: Mediation of ACD by RyR3 in Adult HCN Cells

4.1 Introduction

A subtle balance between cell survival and death is maintained through intricate networks of molecular signaling machinery. Ca^{2+} is a critical intracellular signal that regulates many cellular processes in development stages of embryo, neuronal proliferation and cognitive ability in the brain (Webb and Miller, 2003; Rosenberg and Spitzer, 2011; Bading, 2013). Due to its versatile nature, molecular mechanisms of Ca^{2+}-dependent signaling exist in all types of mammalian cells, and its malfunction fails networks of intracellular signals that maintains cellular homeostasis and cell survival. For instance, Ca^{2+} possesses dual roles in determination of cell fate including both the sustenance of cell viability (Yano et al., 1998) and the activation of cell death machinery (Alberdi et al., 2010), depending on cellular contexts. In the field of programmed cell death (PCD), Ca^{2+} regulation of PCD has been widely investigated in various pathological contexts. Accumulating evidence suggests that an intimate relationship between the perturbed intracellular Ca^{2+} distribution and PCD underlies the pathophysiology of various neural diseases, such as Alzheimer's (Guo et al., 1996) and Parkinson's (Gandhi et al., 2009). The importance of Ca^{2+} regulation in neuronal cell death has led to the Ca^{2+} hypothesis in the pathogenesis of Alzheimer's disease (AD), first proposed by Khachaturian in 1980s (Khachaturian, 1987; 1994). The Ca^{2+} hypothesis states that the persistent upregulation of Ca^{2+} signaling in the hippocampus is responsible for neuronal death and progressive decline in cognition and memory (Guo et al., 1996; Palop et al., 2003; Du et al., 2008) commonly observed in AD.

Apoptosis, necrosis, and autophagic cell death (ACD) are three major modes of PCD which are classified by distinct morphological and biochemical features along with key molecular regulators (Edinger and Thompson, 2004). Despite the unique characteristics among the modes of PCD, Ca^{2+} is important in all three, mostly functioning as an upstream trigger of cell death pathways (Zhivotovsky and Orrenius, 2011). In particular, ryanodine receptors (RyRs) and inositol 1,4,5-triphosphate receptors (IP_3Rs), the main Ca^{2+} release channels in endoplasmic reticulum (ER), have been implicated in Ca^{2+}-mediated regulation of PCD (**Figure 18**; Mori et al., 2005; Kasri et al., 2006). Compared to apoptosis and necrosis, the exact regulatory function of intracellular Ca^{2+} in ACD has not yet been comprehensively elucidated.

Regulation of autophagy by intracellular Ca^{2+} has been shown to occur in several cell lines and pathological models. Autophagy, meaning "self-eating" in Greek, is a cellular process responsible for degradation of cytosolic

proteins and subcellular organelles in the lysosomes (Klionsky, 2004). Target constituents and organelles are sequestered in double-membrane vesicles termed autophagosomes and degraded by lysosomal enzymes upon fusion

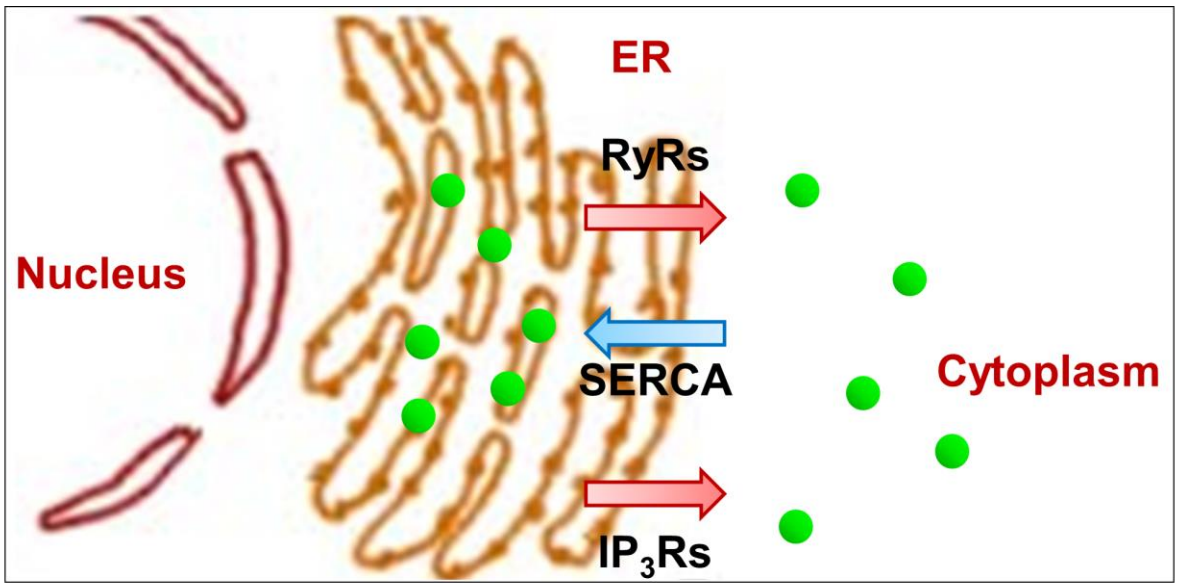


Figure 18. Illustration of intracellular Ca^{2+} dynamics between the ER and the cytosol. The equilibrium of the ER Ca^{2+}-content is maintained by influx of cytosolic Ca^{2+} via sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPases (SERCA) and efflux of ER Ca^{2+} via inositol-1,4,5-phosphate- (IP3Rs) and ryanodine-receptors (RyRs) out of the ER.

of autophagosomes and lysosomes. Autophagy is generally known as a cytoprotective process which converts long-lived proteins or damaged organelles into metabolic intermediates to counter cellular stress and maintain cellular homeostasis (Dunn, 1994). However, as in the case of ACD, autophagy can function as the primary mode of cell death (Yu et al., 2008; Clarke and Puyal, 2012). ACD is defined as a non-apoptotic PCD by or through autophagy as its name suggests (Shen and Codogno, 2011). Interestingly, debate remains as to the exact function of intracellular Ca^{2+} in control of autophagy; two opposite views exist based on conflicting reports suggesting both stimulatory and inhibitory roles for Ca^{2+} in autophagy (Criollo et al., 2007; Hoyer-Hansen et al., 2007; Gao et al., 2008; Harr et al., 2010).

We have previously established the cellular model of ACD in primary cultured adult hippocampal neural stem/progenitor (HCN) cells following insulin withdrawal (Yu et al., 2008). Several molecular mechanisms underlying interactions between apoptosis and autophagy, and regulation of PCD in neural stem cells (NSCs) were identified utilizing the insulin withdrawal model of ACD (Yu et al., 2008; Baek et al., 2009; Chung et al., 2015; Ha et al., 2015). NSCs, by definition, feature the multipotency to proliferate and differentiate into different types of neural lineage in the nervous system, and the self-renewal capability to maintain the stem cell population (Gage, 2000). As such, HCN cells have intact differentiation competence as bona fide neural stem/progenitor cells (data not shown) with the homogenous expression of neural stem/progenitor marker, nestin (Yu et al., 2008). PCD functions as a rigid quality control mechanism to eliminate faulty or superfluous cells and thereby maintain the integrity and size of the NSC population (Lindsten et al., 2003). The unique properties of NSCs ensure generation of normal tissues in the brain during development and even in adult stages (Oppenheim, 1991; Biebl et al., 2000). Conversely, abnormal functions in NSC physiology may render them largely susceptible to pernicious consequences. For instance, dysregulation in cell cycle, neuronal differentiation, or cell death of NSCs may result in neuronal loss through neurodegeneration and may eventually deteriorate higher cognitive functions (Yamasaki et al., 2007). Therefore, understanding the mechanisms governing survival and death of NSCs is pivotal for the development of therapeutic designs utilizing endogenous NSCs, especially in regard to counter aging and neurodegenerative diseases.

Insulin withdrawal drove the mode of cell death towards ACD in HCN cells despite their intact apoptotic capabilities (Yu et al., 2008; Ha et al., 2015). Of particular interest, we observed a rise in intracellular Ca^{2+} level in insulin-deprived HCN cells (denoted as I(-) HCN cells with their counterpart grown in insulin-containing normal condition as I(+) HCN cells, hereafter) (Chung et al., 2015). Since high intracellular Ca^{2+} level can promote or suppress

autophagy induction depending on cell types and stress context (East and Campanella, 2013), we wondered whether high intracellular Ca^{2+} level is related to the default ACD in I(-) HCN cells. To test this idea, we targeted RyRs and IP_3Rs, two well-known ER Ca^{2+} channels as the potential route of intracellular Ca^{2+} rise. Here, we observed that a rise in intracellular Ca^{2+} levels occurred mainly through type 3 RyRs (RyR3) rather than IP_3Rs, and this rise augmented ACD in HCN cells. Our findings can provide a novel insight into the Ca^{2+}-mediated regulation of PCD in NSCs and the potential role of RyR3 as a novel molecular target for treatment of neurodegenerative diseases by stem cell therapies.

4.2 Materials and Methods

4.2.1 Pharmacological Reagents

The pharmacological reagents used were prepared at the indicated stock concentrations as follows: Caffeine (C0750; Sigma-Aldrich) was prepared in I(-) medium at 75 mM. IP_3 (60960; Cayman Chemical) was diluted in PBS at 5 mM and dantrolene (14663-23-1; Sigma-Aldrich) was liquefied in dimethyl sulfoxide at 20 mM.

4.2.2 Immunofluorescence-based Ca^{2+} Imaging

HCN cells were first cultured overnight in 6-well plates at a density of 1×10^5 cells/well in medium without penicillin/streptomycin. On the following day, Lipofectamine 2000 (11668027; Invitrogen)-mediated transfection of pCMV R-CEPIA1er (58216; Addgene) was performed according to the manufacturer's instruction. Cells were replated on 18 mm round glass coverslips placed in a 12-well plate at 24 h post-transfection and insulin withdrawal or treatment of reagents were applied. At 48 h post-transfection, imaging procedures were proceeded accordingly following each experimental design. For labeling intracellular Ca^{2+}, cells were incubated with Fluo-4 AM reagent (F14201; Molecular Probes) at 3 μM for 40 min at 37 ℃. ER Ca^{2+} was assayed by detection of Ca^{2+}-measuring organelle-entrapped protein indicator 1 in the ER (CEPIA1er). To monitor intracellular Ca^{2+} and ER Ca^{2+} simultaneously, as depicted in **Figures 19A, 21G and 23C**, HCN cells transfected with pCMV R-CEPIA1er construct were stained with Fluo-4 AM at 48 h post-transfection. For selective analysis of ER Ca^{2+} expressions in autophagy-induced HCN cells, co-transfection of pCMV R-CEPIA1er and pEGFP-LC3 (21073; Addgene) constructs were performed as shown in **Figure 24A**. Images were obtained with a confocal microscope (LSM700; Carl Zeiss)

ImageJ (NIH) was used to determine the fluorescence intensities of Fluo-4 AM and CEPIA1er signals. Collected images were compiled after being adjusted for brightness and contrast using ImageJ. Background of each summed image was subjected to threshold filtering, and thereby the pixels brighter than a given threshold background value were considered as the signals attributable to the fluorescent Ca^{2+} indicators.

4.2.3 Autophagic Flux Assay

The HCN cells expressing mRFP-GFP-LC3 tandem construct were plated on glass coverslips in 12-well plates at a density of 2.0×10^5 cells/ml. The HCN cells were fixed in 4 % paraformaldehyde solution for 5 min at room temperature. After washing with PBS twice, the cells were mounted on slides with Mount solution (S3023; Dako) and

images were obtained with a confocal microscope (LSM700; Carl Zeiss). For delivery of mRFP-GFP-LC3 tandem construct, we followed the transfection procedure described above. The mRFP-GFP-LC3 construct was a kind gift from Dr. Eun-Kyoung Kim (DGIST).

Generation of CRISPR/Cas9-mediated RYR3 Knockout HCN Cells

Guide RNAs for gene inactivation of RyR3 were designed and purchased from ToolGen. HCN cells were transfected with Cas9- and gRNA-encoding plasmids using a Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. Homogeneity of RYR3 knockout was achieved by hygromycin selection (Cat. # ant-hg-1; InvivoGen) at 24 h post- transfection, followed by a complete medium change.

4.3 Results

4.3.1 ER-to-Cytosol Ca^{2+} Efflux is Increased Following Insulin Withdrawal in HCN Cells

Our prior studies on autophagic death of insulin-deprived adult rat HCN cells have identified multiple upstream regulators of autophagy and PCD (Yu et al., 2008; Baek et al., 2009; Chung et al., 2015; Ha et al., 2015). The discovery of calpain as a molecular determinant of PCD modes has hinted that intracellular Ca^{2+} signaling may underlie the interconnection of cell death pathways upon insulin removal in HCN cells (Chung et al., 2015). Through live-imaging and Western blot analyses we found that absence of insulin gradually induces autophagic response in HCN cells as early as 2 h after insulin withdrawal (**Figures 19A,B,C**). To monitor changes in Ca^{2+} dynamics, we measured the intracellular and ER-specific Ca^{2+} levels in I(-) HCN cells using the intracellular Ca^{2+} indicator Fluo-4 AM and a genetically encoded ER Ca^{2+} indicator CEPIA1er, respectively (**Figure 19D**). While the intracellular Ca^{2+} level in insulin-deprived HCN cells was two-fold greater than control cells, the fluorescence intensity of CEPIA1er revealed that ER Ca^{2+} levels were halved following insulin withdrawal (**Figures 19E,F**). We next tested whether autophagy regulation in HCN cells is mainly achieved by intracellular Ca^{2+} dynamics or involves extracellular Ca^{2+} influx. A specific Ca^{2+} chelator EGTA was added to restrict availability of extracellular Ca^{2+} from HCN cells. We found that presence of EGTA for 6 h affected neither autophagy induction (**Figure 19G**) nor the subsequent cell death in both I(+) and I(-) conditions (data not shown), indicating that autophagy in HCN cells is modulated mainly through intracellular Ca^{2+} events. Autophagy induction was assessed based on the increased modification of LC3-I into LC3-II. Conversion of LC3-I (18 kDa) to LC3-II (16 kDa) occurs after lipidation in autophagosomes, therefore an increase of LC3-II indicates the increase in the number of autophagosomes (Ichimura et al., 2000).Accordingly, LC3 has been well established as a marker of autophagic flux in I(-) HCN cells in our previous reports (Yu et al., 2008; Baek et al., 2009; Chung et al., 2015; Ha et al., 2015).

4.3.2 RyR3 is the Major RyR Isoform Expressed in HCN Cells and its Expression is Elevated Following Insulin Withdrawal

Since insulin withdrawal-induced autophagy in HCN cells accompanies altered levels of ER Ca^{2+}, we first characterized expression patterns of RyRs and IP_3Rs, the main Ca^{2+} release channels in ER membranes. Real-time quantitative PCR analysis revealed that genes encoding type 3 RyR (RYR3) and type 2 IP_3R (ITPR2) were the most abundantly expressed RyR and IP_3R transcripts in HCN cells at the basal state, respectively (**Figure 20A**).

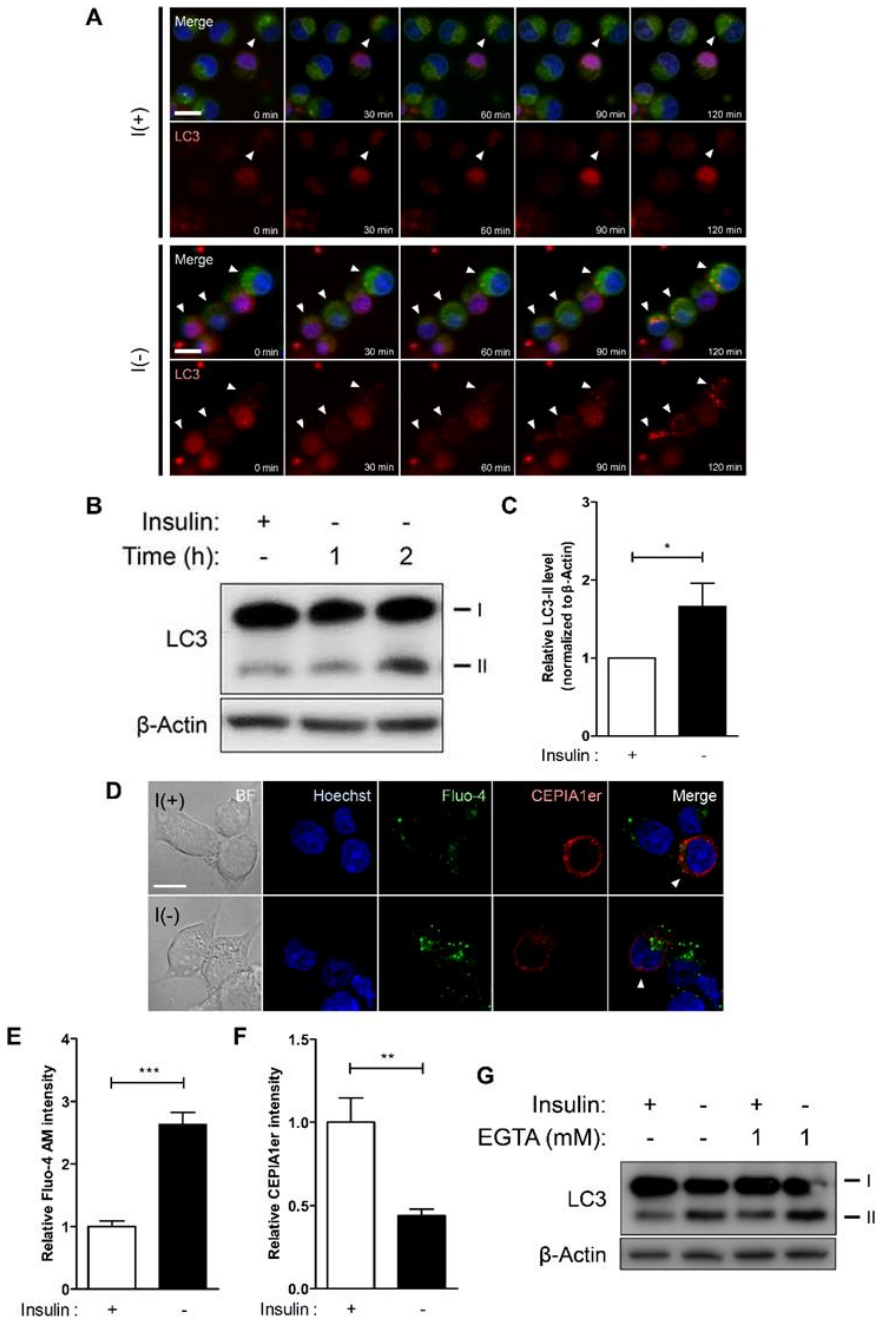


Figure 19. Insulin withdrawal increases intracellular Ca^{2+} levels without the involvement of extracellular Ca^{2+} in HCN cells. (A-C): Time-course of autophagic induction upon insulin withdrawal was assessed in HCN cells. (A) Live-imaging analysis of RFP-LC3-expressing HCN cells stained with Lysotracker Green. Cells indicated by arrowheads show LC3 puncta co-localized with lysosomes. Scale bar, 10 μm. (B) Western blot analysis of HCN cells following insulin withdrawal for 1 h and 2 h. The signal intensity of LC3-II was quantified in (C) using the ImageJ software and normalized to β-actin (n = 4). (D-F): Levels of ER Ca^{2+} in HCN cells were assessed using Fluo-4 AM and pCMV R-Ca^{2+}-measuring organelle entrapped protein indicator 1 in the ER (CEPIA1er), respectively. (D) Immunofluorescence analysis of Fluo-4 AM and pCMV R-CEPIA1er in HCN cells under I(+) and I(-) conditions for 24 h. Cells subjected to analysis are indicated by arrowheads. Scale bar, 10 μm. Fluorescence intensities of Fluo-4 AM (E) and CEPIA1er (F) in HCN cells (n = 30 for I(+), 36 for I(-)). (G): Western blot analysis of a biochemical marker of autophagy LC3 in HCN cells cultured in I(+) and I(-) conditions with or without EGTA (1 mM) for 6 h. The bars represent the mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001.

Interestingly, insulin withdrawal for 24 h enhanced the expression of RYR1 and RYR3 with more significant increase of RYR3 while genes encoding IP_3Rs were unaffected (**Figures 20B,C**). Given the abundance of RYR3 and its substantial upregulation following insulin withdrawal, these data suggest a potential role of RyR3 in the modulation of autophagy in HCN cells.

4.3.3 A RyR Agonist Caffeine Further Promotes ACD in I(-) HCN Cells

Dramatic elevation of RyR transcripts observed in I(-) HCN cells compared to control I(+) cells prompted us to investigate the functional role of RyRs with respect to autophagy activation. Stemming from our results in **Figure 20**, we hypothesized that RyRs are more profoundly associated with insulin withdrawal-induced ACD in HCN cells than IP_3Rs. As expected, treatment with the RyR agonist caffeine enhanced autophagic activity shown by the heightened level of the autophagy marker LC3-II in I(-) HCN cells experiencing ACD (**Figures 21A,B**). A reduction in the levels of SQSTM1/p62, an adaptor protein which recognizes and loads cargo proteins or organelles subjected for autophagic degradation into autophagosomes, also reflects the promotion of autophagy by caffeine (**Figures 21A**). The increased rate of cell death in caffeine-treated cells demonstrates that the furtherance of autophagy-dependent cell death mechanism remains intact in HCN cells (**Figures 21C**). However, an IP_3R agonist IP_3 did not increase autophagic activity (**Figures 21D,E**). Likewise, treatment with IP_3 failed to increase the rate of cell death in I(-) HCN cells (Figure 3F). To examine whether the caffeine-induced potentiation of ACD involves ER-to-cytosol Ca^{2+} transfer, we measured the signal intensities of Fluo-4 AM and CEPIA1er following the presented experimental scheme (**Figures 21G,H**). As expected, addition of caffeine in I(-) HCN cells led to greater elevation and reduction in fluorescence intensities of Fluo-4 AM and CEPIA1er compared with I(-) alone, respectively (**Figures 21I,J**). Thus, addition of caffeine further enhanced ACD in I(-) HCN cells by accelerating the release of Ca^{2+} from the ER.

4.3.4 ACD Induction by Caffeine is Precluded in Autophagy-Defective HCN Cells Depleted of Atg7

Due to accelerated autophagic activity and the subsequent cell death induced by caffeine in I(-) HCN cells, we characterized its effect in the absence of autophagy to confirm the requirement of autophagy for the action of caffeine. Autophagy-related genes (Atg) initiate autophagosome formation through Atg12-Atg5 and LC3-II complexes (Mizushima et al., 1998). Atg7 is required for Atg12-Atg5 conjugation and LC3 lipidation (Ohsumi, 2001) and we generated Atg7 stable knockdown HCN cells by genetically suppressing Atg7 expression with the lentivirus

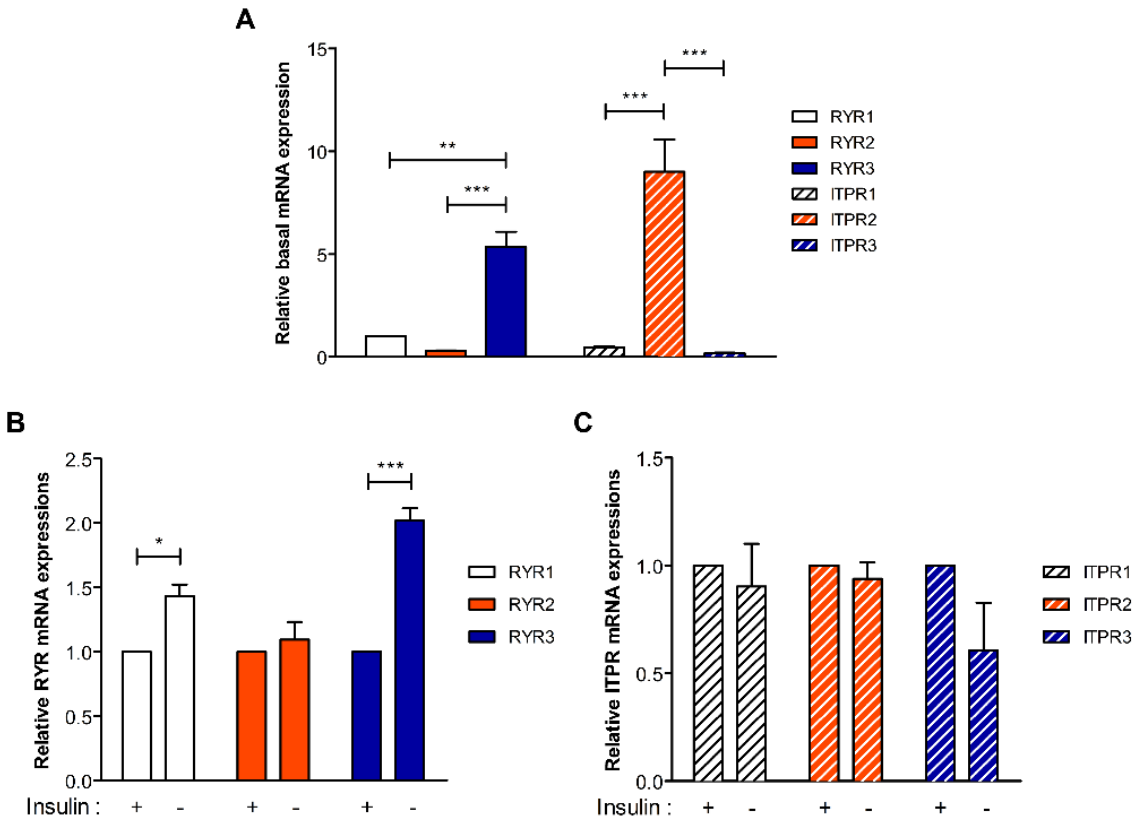


Figure 20. RyR3 is the major isoform in HCN cells and its expression is upregulated following insulin withdrawal. (A): Real-time quantitative PCR analysis of basal expression levels of RyR and IP_3R genes denoted as RYR and ITPR, respectively, after normalization to β-actin. (B,C): A change in the mRNA levels of RYR (B) and ITPR (C) 24 h following insulin withdrawal. Each set of experiment was conducted in triplicate per experiment (n = 4). The bars represent the mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001.

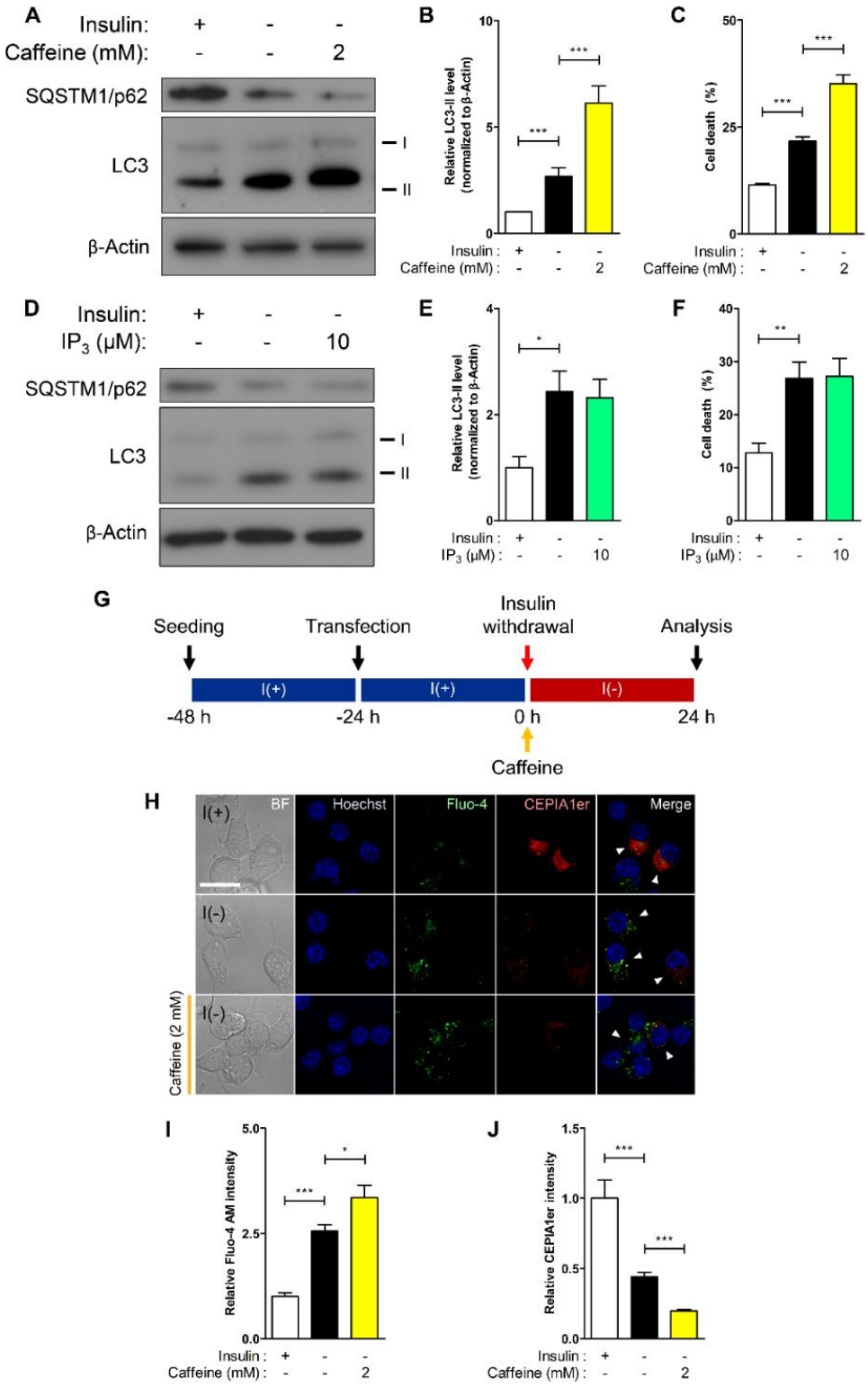


Figure 21. RyR agonist caffeine potentiates ACD in I(-) HCN cells. (A-C): Effect of caffeine on autophagy and cell death was analyzed in HCN cells. (A) Western blot analysis of LC3 and SQSTM1/p62 in I(-) HCN cells treated with caffeine for 24 h. The signal intensity of LC3-II was quantified in (B) and normalized to β-actin (n = 7). (C) Rate of cell death measured in parallel sets of experiments as conducted in (B). (D-F): Analysis of IP_3 effect on autophagy

and cell death in I(-) HCN cells treated with IP_3 for 24 h. (D) Western blot analysis of LC3 and SQSTM1/p62 in I(-) HCN cells treated with IP_3. The signal intensity of LC3-II was quantified in (E) and normalized to β-actin (n = 7). (F) Rate of cell death measured in parallel sets of experiments conducted in (E). (G-J) Levels of intracellular and ER Ca^{2+} in I(-) HCN cells treated with caffeine for 24 h were measured by Fluo-4 AM and pCMV R-CEPIA1er, respectively. (G): An experimental scheme for immunofluorescence-based analysis of Ca^{2+} in HCN cells. (H): Immunofluorescence images of Fluo-4 AM and pCMV R-CEPIA1er in caffeine-treated I(-) HCN cells compared to I(-) alone or I(+) cells. Cells subjected to analysis are indicated by arrowheads. Scale bar, 10 μm. (I,J): Fluorescence intensities were quantified (n = 40 for I(+), 36 for I(-), 47 for I(-)/Caffeine). The bars represent the mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001.

expressing Atg7-targeting shRNA. As shown in **Figure 22A**, following insulin withdrawal, autophagy — depicted by LC3-II levels — was reduced in shAtg7-transduced HCN cells compared to cells transduced with control shScramble. Interestingly, 2 mM caffeine treatment did not significantly elevate cell death in autophagy-defective HCN cells while the rate of cell death in I(-) scramble controls was robustly increased (**Figure 22B**). Caffeine treatment in I(-) cells did not involve activation of caspase-dependent apoptosis, indicating that caffeine-induced cell death is a continuance of a genuine ACD in I(-) HCN cells (**Figure 22A**).

4.3.5 Autophagy is Diminished by Pharmacological or Genetic RyR Inhibition in I(-) HCN Cells

Our results on potentiation of autophagic death of I(-) HCN cells through stimulation of Ca^{2+} release by the RyR agonist caffeine provoked us to test whether inhibition of RyR reverses the autophagic events in I(-) HCN cells. To examine the effect of RyR inhibition on autophagy, we treated I(-) HCN cells with dantrolene, a RyR antagonist (Kobayashi et al., 2005). RyR inhibition by dantrolene effectively prevented Ca^{2+} release from the ER, as shown by the reversal of the changes in fluorescence intensities of Fluo-4 AM and CEPIA1er observed in I(-) HCN cells (**Figures 21A,B,C**). Thereby, dantrolene significantly reduced the level of LC3-II proteins in a concentration-dependent manner (**Figures 23D,E**).

Of the RyR isoforms in HCN cells, RyR3 is the most prominent, and following insulin withdrawal, the mRNA of this isoform showed the largest increase as well (**Figures 20A,B**). Along with the altered expression of RyR3 transcripts, the observation that autophagy is modulated by RyR agonist and antagonist suggests that ACD may be regulated via RyR3-mediated intracellular Ca^{2+} signaling in HCN cells. Thus, we generated RYR3 knockout (RYR3KO) HCN cells utilizing CRISPR/Cas9-mediated gene inactivation. Consistent with our hypothesis, the prominence of autophagy — as measured by LC3-II — was substantially decreased in I(-) HCN cells absent of RyR3 (**Figure 24B**). Furthermore, we used BafA1, a specific inhibitor of late phase autophagy that acts by preventing formation of autolysosomes, to further verify the attenuated autophagic flux in RYR3KO (**Figure 24A**). Robust accumulation of LC3-II proteins due to the inhibition of autophagic flux by BafA1 indicates a rapid conversion of LC3-I to LC3-II, reflective of high autophagic flux (Yamamoto et al., 1998). The accumulation of LC3-II in RYR3KO I(-) HCN cells by BafA1 treatment was substantially diminished compared to BafA1-treated I(-) control cells (**Figure 24B**). Consistent with the reduced autophagic flux in RYR3KO HCN cells, RyR3 depletion significantly reduced the insulin withdrawal-induced cell death in HCN cells up to 72 h (**Figure 24C**).

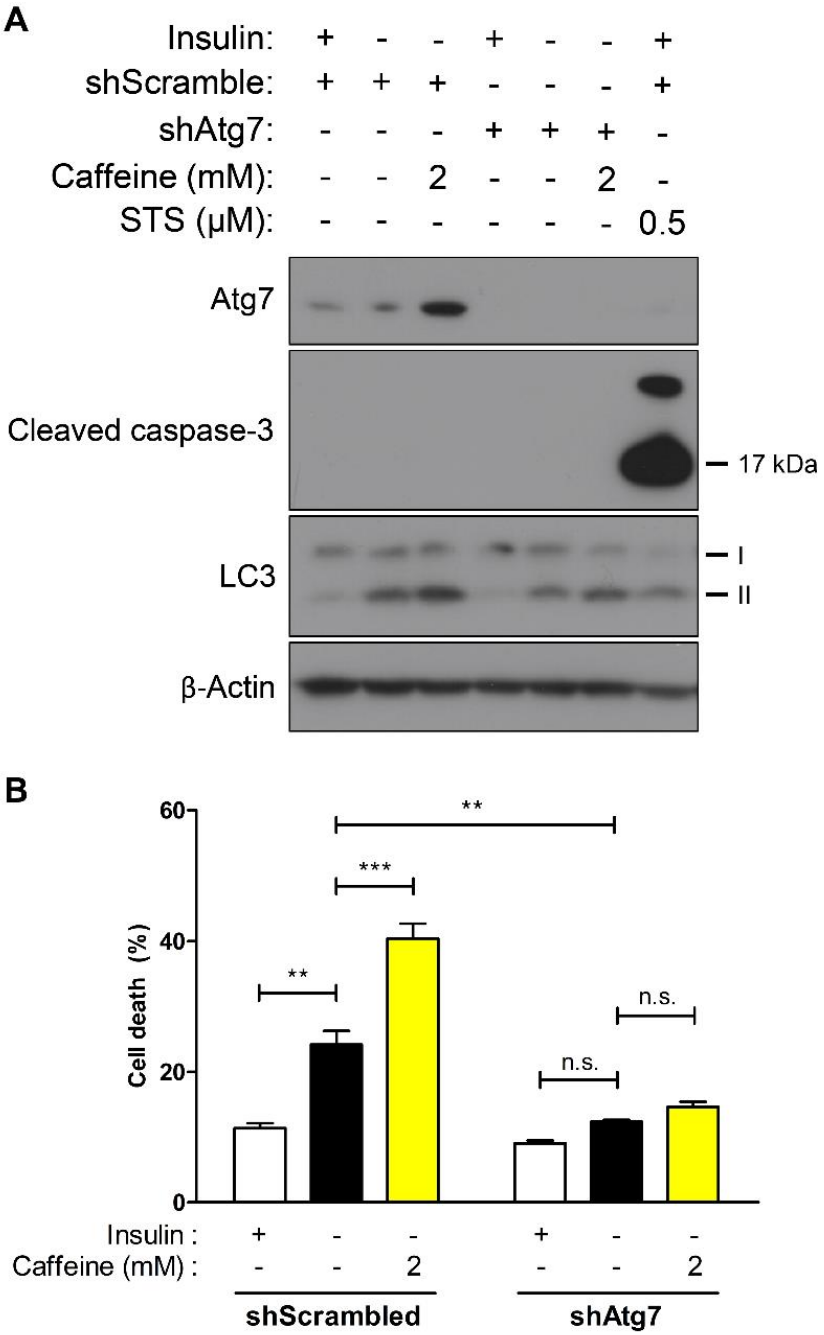


Figure 22. Caffeine-induced potentiation of ACD is prevented in Atg7 knockdown HCN cells. (A,B): Effect of caffeine on autophagic activity was assessed in autophagy-defective HCN cells generated by shRNA-mediated suppression of Atg7 gene. (A) Western blot analysis of autophagy- and apoptosis-related proteins after caffeine treatment for 24 h in I(-) HCN cells depleted of Atg7. Staurosporine (STS) was treated for 10 h before cell harvest to induce caspase-3 activation as a positive control of apoptosis. (B) Cell death analysis in caffeine-treated Atg7 knockdown HCN cells at 24 h. Each experiment set was performed in triplicate per experiment (n = 4). The bars represent the mean ± SEM; **p < 0.01, ***p < 0.001; n.s., non-significant.

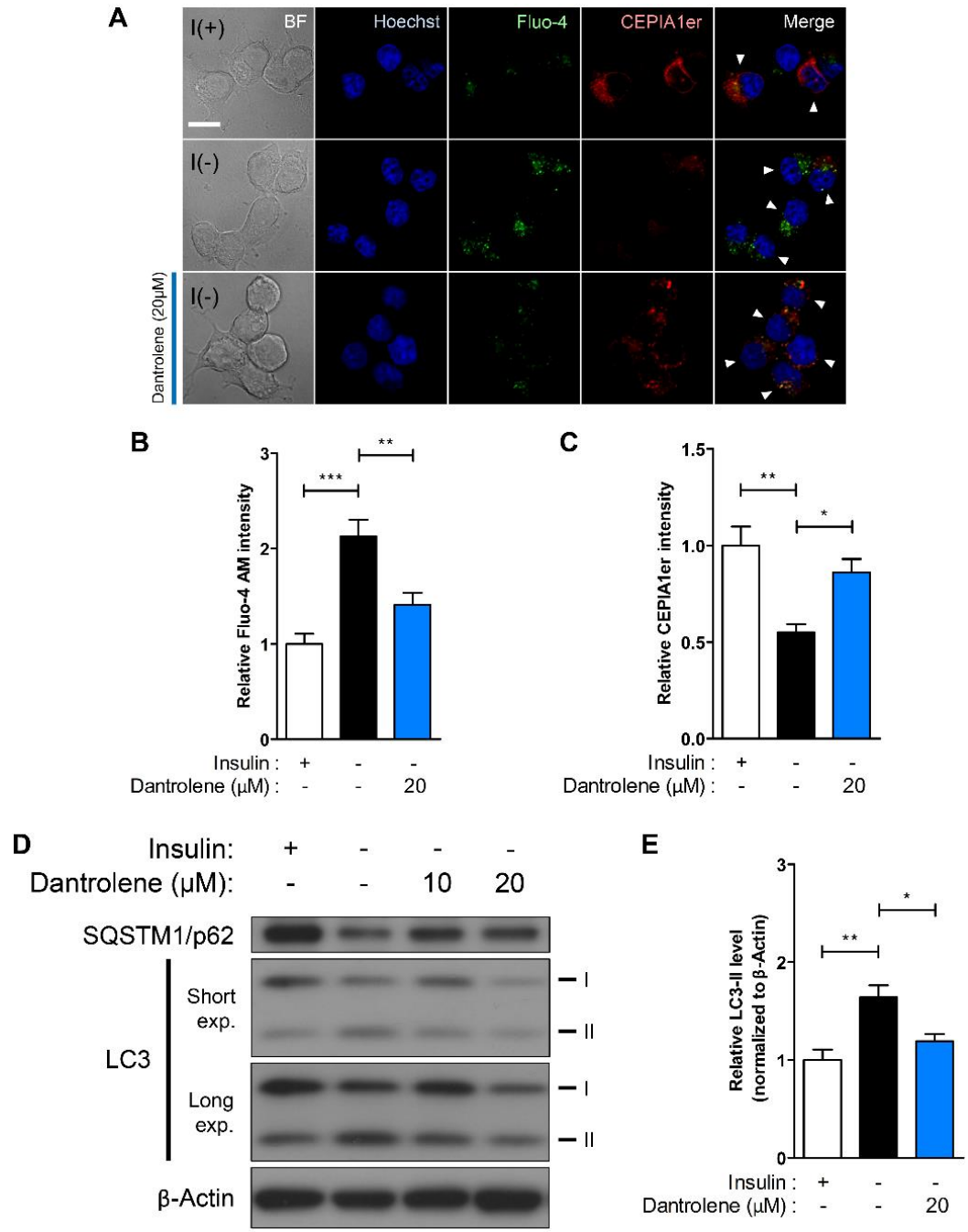


Figure 23. RyR inhibition suppresses ACD in HCN cells. (A-E): Treatment with the RyR antagonist dantrolene inhibits autophagic activity in HCN cells. (A) Immunofluorescence-based detection of Fluo-4 AM and pCMV R-CEPIA1er in I(-) HCN cells treated with dantrolene. Cells subjected to analysis are indicated by arrowheads. Scale bar, 10 μm. Fluorescence intensities of Fluo-4 AM (B) and CEPIA1er (C) in HCN cells were quantified (n = 22 for I(+), 30 for I(-), 27 for I(-)/Dantrolene). (D) Western blot analysis of LC3 and SQSTM1/p62 in I(-) HCN cells treated with dantrolene for 6 h. The signal intensity of LC3-II was quantified in (E) using the ImageJ software and normalized to β-actin (n = 6). The bars represent the mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001.

4.3.6 Knockout of RyR3 Gene Occludes ER Ca^{2+} Release and Thereby Prevents ACD in I(-) HCN Cells

Our results thus far indicate that RyR3 is essential for the regulation of ACD of HCN cells following insulin withdrawal. To further corroborate our hypothesis that a RyR3-mediated rise in intracellular Ca^{2+} level is a primary mechanism inducing autophagy in I(-) HCN cells, we measured Ca^{2+} levels in HCN cells lacking RyR3. Since it was of our specific interest to test RyR3-mediated ER Ca^{2+} regulation of autophagy, we co-assayed for the autophagy marker LC3 instead of Fluo-4 AM along with CEPIA1er for immunofluorescence-based analysis. Puncta formation of fluorescently tagged-LC3 has been commonly used to visualize the cells undergoing autophagy (Chung et al., 2015; Ha et al., 2015). Therefore, co-transfection of pCMV R-CEPIA1er and pEGFP-LC3 constructs allows selective analysis of ER Ca^{2+} levels in autophagy induced-HCN cells at the single cell level. (**Figure 25A**). Quantification of CEPIA1er fluorescence intensity in EGFP-LC3-positive HCN cells revealed no significant ER Ca^{2+} changes provoked by caffeine in RYR3KO HCN cells while caffeine promoted ER Ca^{2+} release in I(-) Cas9 control cells (**Figure 25B**).

Lastly, we explored whether RYR3KO HCN cells resistant to insulin withdrawal-induced ACD were also impervious to caffeine-mediated induction of autophagy. Western blot analysis revealed that induction of autophagy following caffeine treatment was only visible in Cas9 I(-) control HCN cells, but not in RYR3KO I(-) cells (**Figure 25C**). Likewise, caffeine potentiated ACD in control I(-) cells, but not in RYR3KO I(-) HCN cells, consistent with our results from **Figure 24** (**Figure 25D**). Since cleaved caspase-3, which reflects the activation of apoptosis, was not detected in either control or RYR3KO HCN cells, it is plausible to conclude that the altered rate of cell death by caffeine was due to ACD, further implicating the prominence of RyR3 in regulation of autophagy in HCN cells.

To more precisely monitor changes in autophagic flux by caffeine treatment and RyR3 gene inactivation, we utilized monomeric RFP-GFP tandem fluorescent-tagged LC3 (mRFP-GFP tandem LC3). The mRFP-GFP tandem LC3 assay is based on lysosomal quenching of GFP fluorescence but not mRFP fluorescence in cellular compartments with low pH such as inside the lysosome (Bampton et al., 2005). By assessing the percentage of mRFP-only positive LC3 puncta out of total fluorescent-tagged LC3 puncta, we morphologically traced autophagosome maturation and thereby measured autophagic flux in HCN cells (**Figure 26A**). Cas9 control cells under I(-) condition exhibited significantly greater percentage of mRFP-only positive LC3 puncta compared to I(+) condition; moreover, caffeine further increased the percentage of mRFP-only LC3 punta as well as the total number of LC3 puncta (**Figure 26B**). These data indicated increased autophagy flux and maturation of autophagic vacuoles into autolysosomes by insulin withdrawal and caffeine treatment. On the contrary, there were no apparent increases in either percentage of mRFP-

only LC3 puncta or total LC3 punta numbers in RYR3KO HCN cells, suggesting defective autophagy induction in the absence of RyR3 despite insulin withdrawal or caffeine treatment (**Figures 26A,B**).

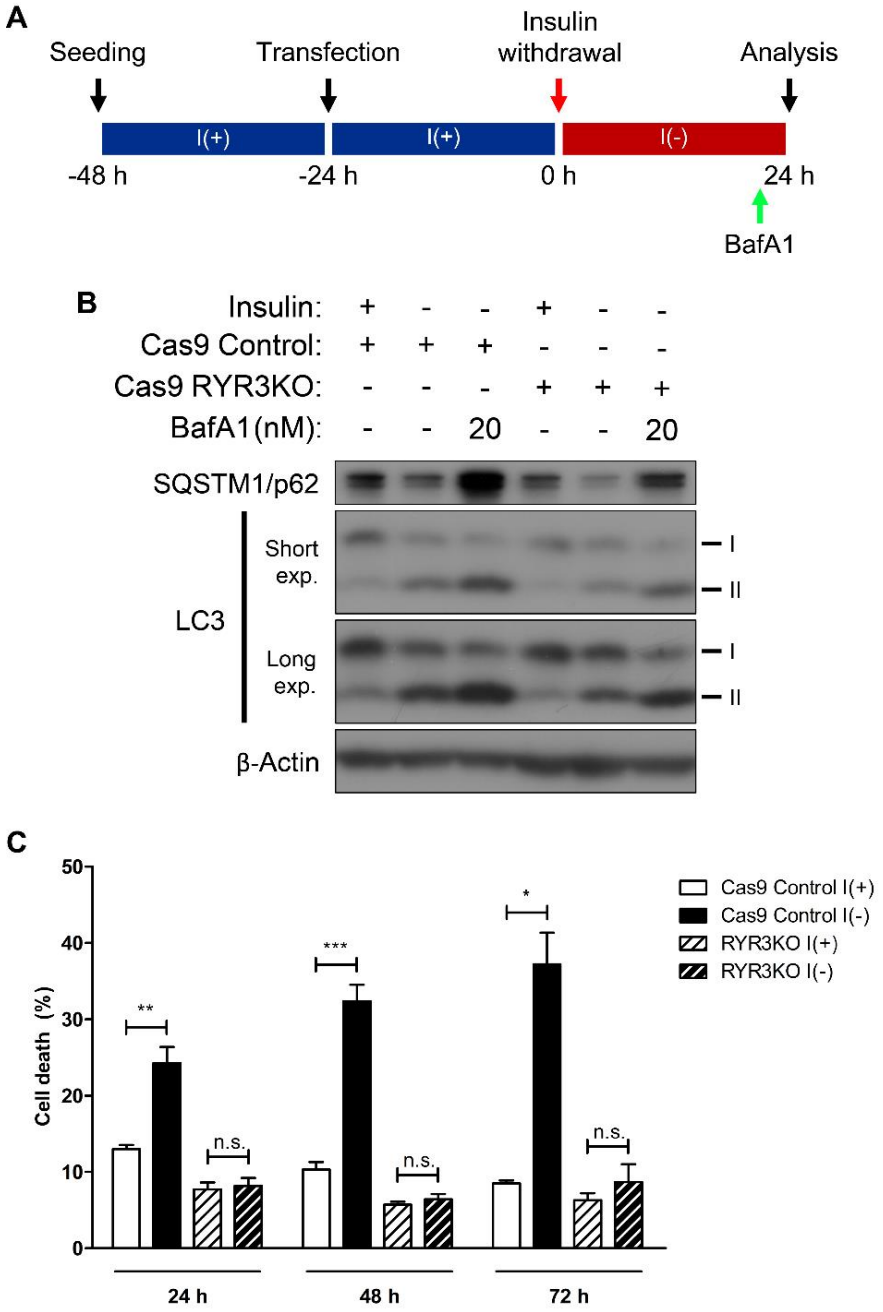


Figure 24. Genetic suppression of RyR3 diminishes autophagic flux in I(-) HCN cells. (A-C) Effect of CRISPR/Cas9-induced RYR3 knockout on ACD in I(-) HCN cells. (A) An experimental scheme for inhibition of autophagic flux by BafA1 treatment. (B) Characterization of autophagic flux by immunoblotting of LC3 and SQSTM1/p62 in RYR3KO I(-) HCN cells treated with BafA1. Cells were treated with BafA1 for 2 h at given concentration before harvest. (C) Rate of cell death in RYR3KO HCN cells under I(+) and I(-) conditions for 24 h, 48 h, and 72 h; each experiment performed in triplicate per experiment (n = 4). The bars represent the mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001; n.s., non-significant.

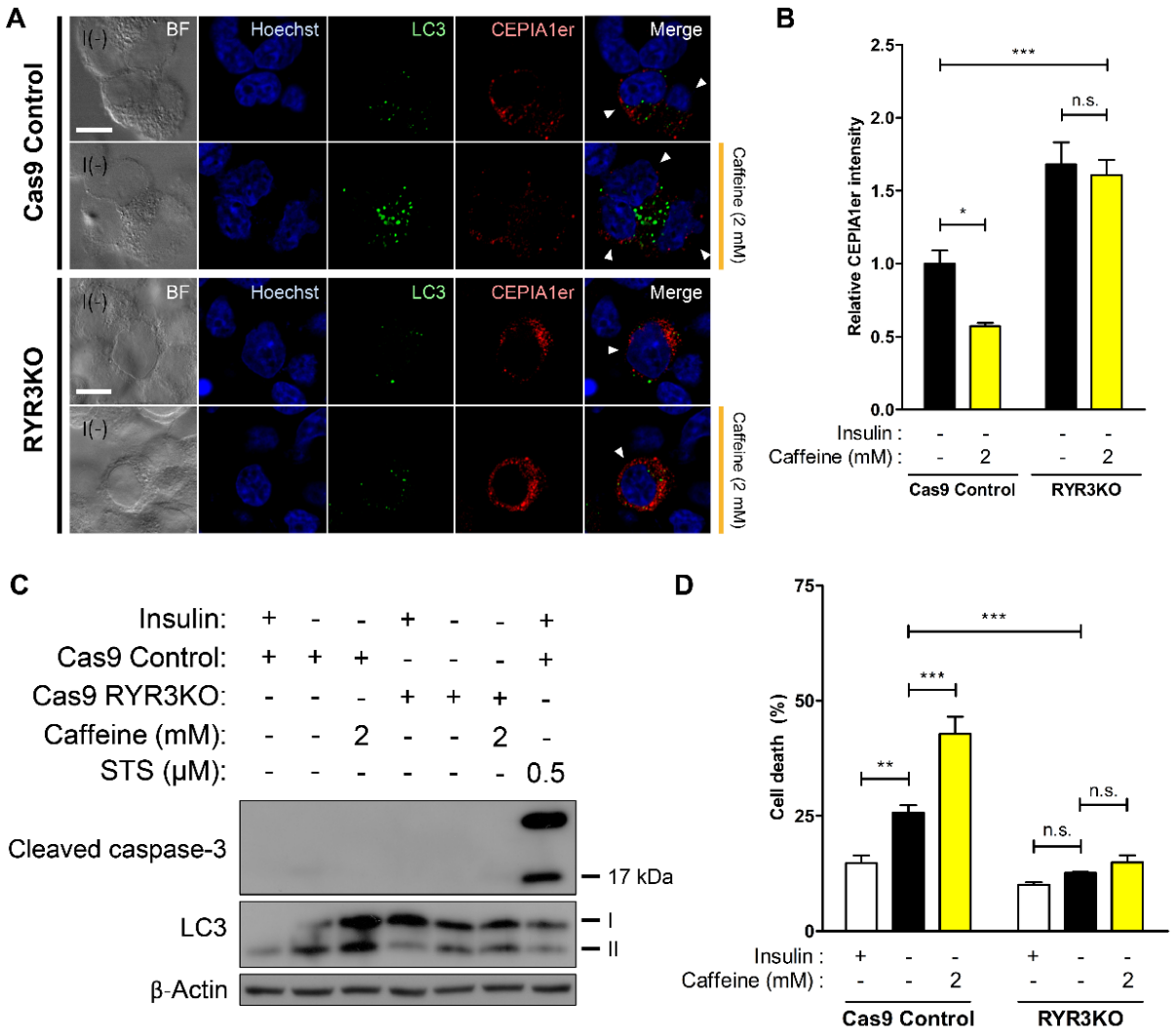


Figure 25. ER Ca^{2+} efflux is significantly inhibited in I(-) RYR3KO HCN cells. (A,B) Caffeine was used to provoke ER-to-cytosol transfer of Ca^{2+} in control I(-) and RYR3KO I(-) HCN cells. (A) Immunofluorescence images of pCMV R-CEPIA1er in HCN cells experiencing autophagy depicted by puncta formation of EGFP-LC3 24 h following insulin withdrawal. Cells subjected to analysis are indicated by arrowheads. Scale bar, 10 μm. Fluorescence intensity of CEPIA1er (B) selectively in EGFP-LC3-positive HCN cells was quantified (n = 36 for Cas9 Control I(-), 41 for Cas9 Control I(-)/Caffeine, 32 for RYR3KO I(-), 43 for RYR3KO/Caffeine). The bars represent the mean ± SEM; *p < 0.05, ***p < 0.001; n.s., non-significant. (C,D) Caffeine failed to facilitate ACD of I(-) HCN cells in the absence of RyR3. (C) Western blot analysis of cleaved caspase-3 and LC3 in I(-) RYR3KO HCN cells treated with caffeine. (D) Cell death analysis in RYR3KO HCN cells treated with caffeine for 24 h. Each experiment set was performed in triplicate per experiment (n = 6). The bars represent the mean ± SEM; **p < 0.01, ***p < 0.001; n.s., non-significant.

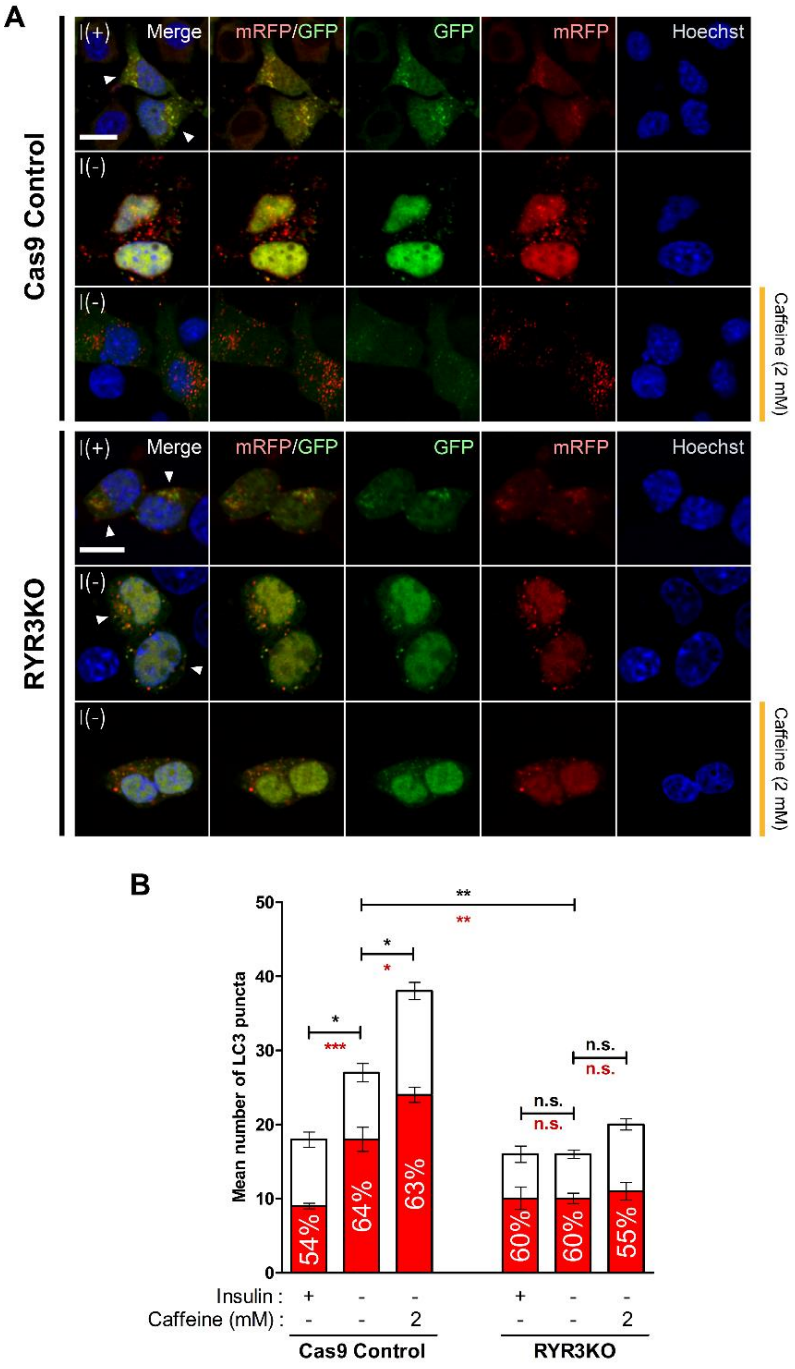


Figure 26. Autophagic flux in RYR3KO HCN cells is unaltered upon insulin withdrawal or caffeine treatment. (A,B) Caffeine failed to induce autophagic flux of I(-) HCN cells in the absence of RyR3. (A) Immunofluorescence analysis of HCN cells transfected with mRFP-GFP-LC3 tandem construct. Images were analyzed 24 h following insulin withdrawal with or without caffeine treatment. Cells subjected to analysis are indicated by arrowheads. Scale bar, 10 μm. (B) Quantification of mRFP- and GFP-LC3 puncta in RYR3KO HCN cells treated with caffeine compared to controls (n = 22 for Cas9 Control I(+), 30 for Cas9 Control I(-), 27 for Cas9 Control I(-)/Caffeine, 29 for RYR3KO I(+), 34 for RYR3KO I(-), 25 for RYR3KO/Caffeine). The bars represent the mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001; n.s., non-significant. The black and red asterisks indicate the statistical significance in the comparison of total and mRFP puncta numbers, respectively.

4.4 Discussion

PCD in multipotent NSCs is of immense importance due to its roles in ensuring appropriate brain development and function through the fine regulation of the size and integrity of NSC pools. Many reports have documented the pathophysiology of neurodegeneration by aberrant regulation of the subtle balance between cell survival and death in the brain (Vila and Przedborski, 2003; Krantic et al., 2005). However, the mechanisms underlying PCD of NSCs have been relatively under-investigated compared to a series of extensive studies on neuronal cell death conducted for the past several decades. To this end, albeit investigated at the cellular level, our insulin withdrawal model of ACD in HCN cells has physiological implications especially in relation to AD. Recent studies have delineated that impaired insulin signaling and resistance to insulin or glucose uptake in the brain are strongly associated with the pathogenesis of AD (Talbot et al., 2012; De Felice et al., 2014; Willette et al., 2015). This compelling clinical evidence implicates the autophagy-related cellular processes including ACD due to dysregulated insulin signaling in pathogenic development of AD.

Our previous discovery on calpain as a key negative regulator of ACD and a molecular switch of PCD modes in HCN cells suggests a critical role for intracellular Ca^{2+} in the regulation of programmed death of NSCs (Chung et al., 2015). In this study, we elucidated RyR3-mediated intracellular Ca^{2+} dynamics underlies ACD of HCN cells following insulin withdrawal. A distinct pattern of ER Ca^{2+} change denoted by CEPIA1er, along with the increased intracellular Ca^{2+} levels in I(-) HCN cells, hinted to the possible existence of molecular regulators of intracellular Ca^{2+} dynamics which respond upon insulin withdrawal and subsequently govern autophagy induction. Through real-time quantitative PCR analysis, we assessed the expression levels of RyR and IP_3R genes, denoted as RYR and ITPR, respectively, the genes encoding for the main ER Ca^{2+} release channels (**Figure 20**). RYR3 and ITPR2 were identified to be the major isoforms of RyR and IP_3R, respectively. Intriguingly, only the expression of RyR3 transcripts showed roughly a two-fold increase in response to insulin withdrawal for 24 h while IP_3R2 gene expression was unaltered, suggesting a more pronounced involvement of RyR3 in insulin withdrawal-induced autophagy in HCN cells. Furthermore, RyR activation by caffeine, but not IP_3, elevated ACD in I(-) HCN cells. A series of subsequent experiments utilizing RYR3KO HCN cells further corroborated our notion on the selective modulation of autophagy in HCN cells via RyR3-mediated ER Ca^{2+} efflux.

Lack of acceleration in autophagic flux following caffeine treatment in RYR3KO I(-) HCN cells were supported by both Western blot and fluorescence-based analyses. Assessment of autophagy flux using a tandem fluorescent-

tagged LC3 may warrant caution, because of the occasional aberrant, prolonged stability of GFP in acidic pH (Bampton et al., 2005; Mizushima, 2009). Thus, our imaging analysis was focused on the percentage of mRFP-only positive LC3 puncta out of total fluorescent-tagged LC3 puncta to represent the relative number of lysosome-localized LC3 puncta (**Figure 26B**).

Our finding on RyR3 regulation of PCD in the absence of insulin signaling is supported by similar results from other studies. Dysregulation of intracellular Ca^{2+} is an underlying component of various neurodegenerative diseases, and recent evidence implicates RyR in the pathology of AD, further highlighting the functional role of RyRs in the aberrant physiological conditions (Supnet et al., 2010; Bruno et al., 2012; Oules et al., 2012; Wu et al., 2013).

Under controlled in vitro setting, we were able to examine the regulation of RyR3 in autophagy of HCN cells in the absence of other types of PCD; however, it is unlikely that only a single mode of cell death is activated in the affected brain region under neuropathological situations. Clinical evidence on co-existence of autophagy and apoptosis have been reported in the brains of patients with Alzheimer's and Parkinson's diseases (Anglade et al., 1997; Stadelmann et al., 1999). Indeed, crosstalk between autophagy and other PCD modes, mostly apoptosis, has been documented (Eisenberg-Lerner et al., 2009; Chung and Yu, 2013; Chung et al., 2015). Admittedly, the association between RyR3-mediated Ca^{2+} signaling and ACD induction in HCN cells should be perceived as one of several cellular events that could occur in vivo. Nevertheless, insulin withdrawal-induced death of HCN cells is regarded as a genuine model of ACD (Shen and Codogno, 2011; Clarke and Puyal, 2012) and can be utilized to study the molecular mechanisms of autophagy and its interrelation with other modes of PCD.

To our knowledge, this is the first report suggesting that RyR3 is the major isoform expressed in NSCs in the hippocampus. Interestingly, mRNA levels of RyR2 were reportedly shown to be substantially greater than other RyR isoforms in mouse hippocampal neurons (Wu et al., 2013). Moreover, a reduced insulin signaling was implicated in neuroprotective autophagy activation in cortical neurons (Young et al., 2009). Connecting these findings, the distinctly pronounced expression pattern of RyR3 may render NSCs to undergo PCD by autophagy instead of neuroprotection. A previous study by Balschun et al. has delineated impairment of synaptic plasticity in the hippocampi of mice lacking RyR3 (Balschun et al., 1999). Combining our observation of pro-survival effect by RYR3 knockout in NSCs and a seemingly harmful effect in neurons by RyR3 deletion suggest a distinct, cell type-specific role for RyR3. The exact functional implication of differential expression of RyR isoforms in NSCs and neurons remains an enthralling research topic to be investigated. In summary, we unveiled the unique regulatory function of RyR3-mediated intracellular Ca^{2+}

on ACD in HCN cells (**Figure 27**). Our results may provide a profound insight into understanding the intricate mechanisms underlying survival and death of NSCs during developmental stages, neurogenesis, and even pathophysiological contexts.

Lack of acceleration in autophagic flux following caffeine treatment in RYR3KO I(-) HCN cells were supported by both Western blot and fluorescence-based analyses. Assessment of autophagy flux using a tandem fluorescent-tagged LC3 may warrant caution, because of the occasional aberrant, prolonged stability of GFP in acidic pH (Bampton et al., 2005; Mizushima, 2009). Thus, our imaging analysis focused on the percentage of mRFP-positive LC3 puncta out of total fluorescent-tagged LC3 puncta to represent the relative number of lysosome-localized LC3 puncta (**Figure 26B**).

Our finding on RyR3 regulation of PCD in the absence of insulin signaling is supported by similar results from other studies. Dysregulation of intracellular Ca^{2+} is an underlying component of various neurodegenerative diseases, and recent evidence implicates RyR in the pathology of AD, further highlighting the functional role of RyRs in the aberrant physiological conditions (Bruno et al., 2012; Oules et al., 2012; Supnet, Noonan, Richard, Bradley, & Mayne, 2010; Wu, Yamaguchi, Lai, & Shen, 2013).

Under controlled in vitro setting, we were able to examine the regulation of RyR3 in autophagy of HCN cells in the absence of other types of PCD; however, it is unlikely that only a single mode of cell death is activated in the affected brain region under neuropathological situations. Clinical evidence on co-existence of autophagy and apoptosis have been reported in the brains of patients with Alzheimer's and Parkinson's diseases (Anglade et al., 1997; Stadelmann et al., 1999). Indeed, crosstalk between autophagy and other PCD modes, mostly apoptosis, has been documented (Chung et al., 2015; Chung & Yu, 2013; Eisenberg-Lerner, Bialik, Simon, & Kimchi, 2009). Admittedly, the association between RyR3-mediated Ca^{2+} signaling and ACD induction in HCN cells should be perceived as one of several cellular events that could occur in vivo. Nevertheless, insulin withdrawal-induced death of HCN cells is regarded as a genuine model of ACD (Clarke & Puyal, 2012; Shen & Codogno, 2011) and can be utilized to study the molecular mechanisms of autophagy and its interrelation with other modes of PCD.

To our knowledge, this is the first report suggesting that RyR3 is the major isoform expressed in NSCs in the hippocampus. Interestingly, mRNA levels of RyR2 were reportedly shown to be substantially greater than other RyR isoforms in mouse hippocampal neurons (Wu et al., 2013). Moreover, a reduced insulin signaling was implicated in neuroprotective autophagy activation in cortical neurons (Young, Martinez, & La Spada, 2009). Connecting these

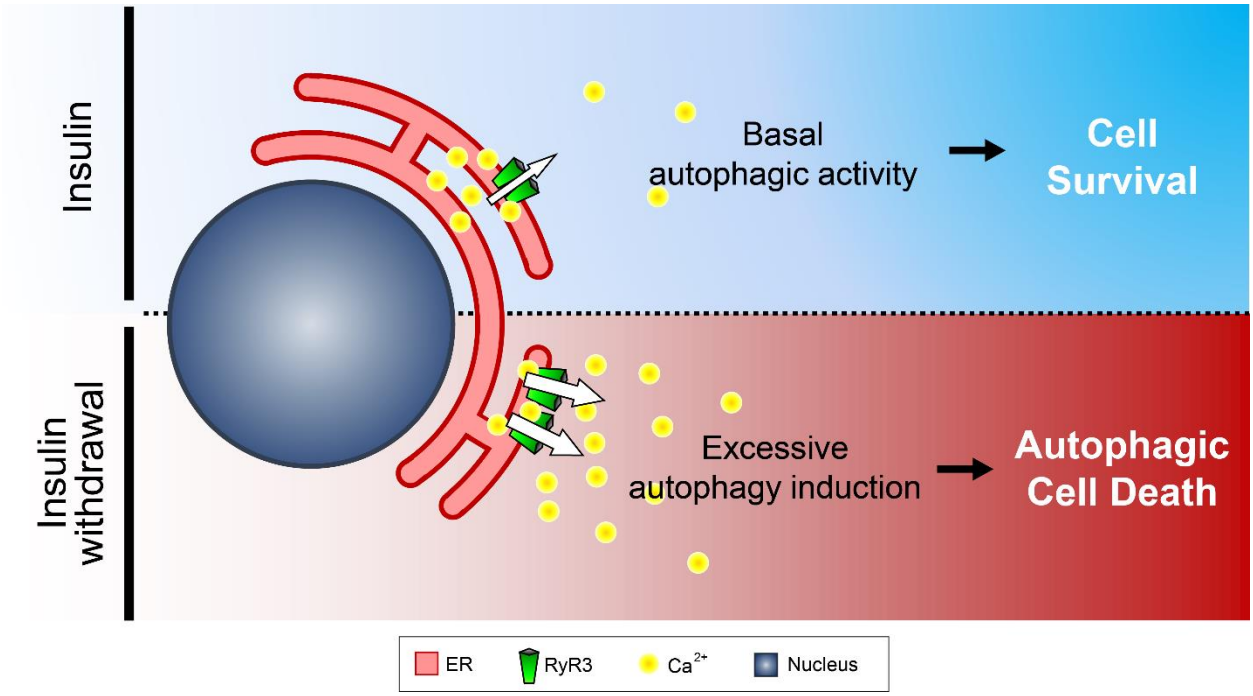


Figure 27. Schematic diagram illustrating the role of ER-to-cytosol Ca^{2+} in regulation of survival and death of NSCs in insulin withdrawal model. Presence of insulin constitutively ensures proper distribution of intracellular Ca^{2+} which maintains homeostasis of HCN cells. Inactivation of the pro-survival insulin signaling pathway by insulin withdrawal leads to increased expression and activation of RyR3 which subsequently discharge Ca^{2+} from the ER, the major intracellular Ca^{2+} store. Increase in cytosolic Ca^{2+} subsequently triggers a signaling cascade that induces ACD in HCN cells.

findings, the distinctly pronounced expression pattern of RyR3 may render NSCs to undergo PCD by autophagy instead of neuroprotection. A previous study by Balschun et al. has delineated impairment of synaptic plasticity in the hippocampi of mice lacking RyR3 (Balschun et al., 1999). Combining our observation of pro-survival effect by RYR3 knockout in NSCs and a seemingly harmful effect in neurons by RyR3 deletion suggest a distinct, cell type-specific role for RyR3. The exact functional implication of differential expression of RyR isoforms in NSCs and neurons remains an enthralling research topic to be investigated. In summary, we unveiled the unique regulatory function of RyR3-mediated intracellular Ca^{2+} on ACD in HCN cells (**Figure 27**). Our results may provide a profound insight into understanding the intricate mechanisms underlying survival and death of NSCs during developmental stages, neurogenesis, and even pathophysiological contexts.

CHAPTER 5: A Novel Function of Presenilin-2 in Regulation of Autophagic Death of HCN Cells

5.1 Introduction

Alzheimer's disease (AD) is the most common form of dementia in the world; yet, the pathogenesis of AD is still largely mysterious. The majority of basic research on AD is concentrated on rare genetic mutant forms of familial AD (fAD) which only accounts for between 2-3% of all AD cases. Most of fAD cases are caused by point mutations in genes encoding amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2). PS1 and PS2 are two homologous proteins (67% identity) that are essential components of the γ-secretase complex which generates the amyloid β peptides (Aβ; Goedert & Spillantini, 2006). Both presenilins are multi-spanning transmembrane protein with a predicted 9 transmembrane domains (**Figure 28**). Heterogeneous proteolytic processing at hydrophilic loop domain generates N-terminal and C-terminal fragments. While autosomal dominant inheritance of mutations in the PS1 gene is more common cause of familial early onset AD, fAD-associated mutations are also present in PS2, although they exhibit far less prevalence than in PS1 (Sherrington et al., 1995; Levy Lahad et al., 1995).

Accumulating evidence suggests that a perturbation of cellular Ca^{2+} homeostasis is strongly implicated in fAD. Specifically, researchers have targeted presenilins to understand the causative role for Ca^{2+} dysregulation in fAD pathogenesis since presenilins are known to play a key role in modulation of ER Ca^{2+} (Bojarski et al., 2008; Zampese et al, 2011). Two compelling evidence that support the notion are as follows: i) several fAD-linked presenilin mutants exhibit altered expression or sensitivity of ER Ca^{2+} release channels, namely RyRs and IP_3Rs (Zampese et al., 2009); ii) unlike wild-type presenilins, fAD-linked mutants fail to form low-conductance Ca^{2+} leak channels in the ER membrane. However, the second evidence above faces challenging results from other groups who have reported the hyperactivity of ER Ca^{2+} release channels upon overexpression of fAD-linked presenilin mutants (Cheung et al., 2008; Cheung et al., 2010). Thus, even with the indisputable role of presenilins in Ca^{2+} homeostasis, controversy remains unsolved. Interestingly, majority of reports on the Ca^{2+}-related function of presenilins is conducted in neuronal models.

Recently, we have reported that ER-to-cytosol transition of Ca^{2+} via RyRs underlies the mediation of ACD in HCN cells (Chung et al., 2016). Based on our microarray analysis, we have detected a significant elevation of PS2 gene expression in HCN cells upon induction of ACD by insulin withdrawal. Due to near-absent prior knowledge on function of presenilins in NSCs, coupled with our array-based screening results, PS2 appears an ideal target molecule

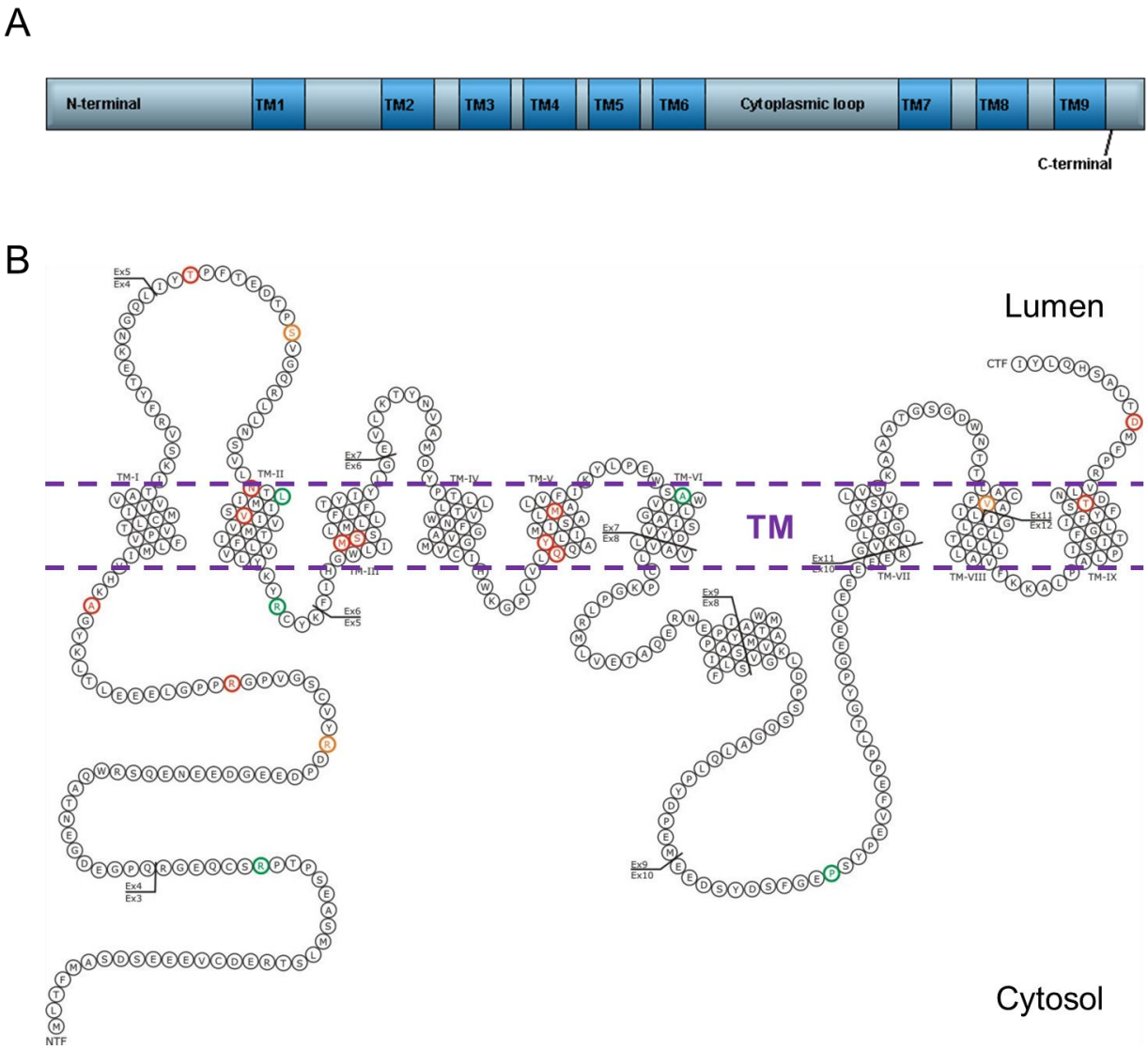


Figure 28. Illustration of Presenilin-2 structure. (A): PS2 protein domains, bright blue boxes are transmembrane domains (TM). (B): A diagram of PS2 gene map with identified mutations linked to familial AD. Modified from http://atlasgeneticsoncology.org (A), and http://www.molgen.vib-ua.be/ADMutations (B).

to examine its role in cell death, Ca^{2+} dynamics, and autophagy. Indeed, our findings indicate that PS2 is a positive mediator in ACD and plays a crucial function in NSC biology, both likely via alteration of Ca^{2+}. To our knowledge, this is the first thorough study on presenilin 2 in NSCs in context of PCD and autophagy, and our finding may provide valuable information for stem cell-based therapies for AD.

5.2 Materials and Methods

5.2.1 Antibodies and Reagents

The antibodies and reagents used were as follows: presenilin-1 (#5643; Cell Signaling Technology), presenilin-2 (#9979; Cell Signaling Technology), LC3B (L7543; Sigma-Aldrich), SQSTM1/p62 (#5114S; Cell Signaling Technology), β-actin (#4967S; Cell Signaling Technology), and cleaved caspase-3 antibodies (#9661; Cell Signaling Technology). Bafilomycin A1 (BafA; B1793; Sigma-Aldrich;), was diluted in dimethyl sulfoxide at appropriate concentrations.

5.2.2 Plasmids and siRNAs

pRK5-presenilin 1 and –presenilin 2 plasmids were constructed by subcloning human presenilin 1 (NM_000021; OriGene) and preseniln 2 (RC202921; OriGene) genes into the pRK5 vector obtained from Addgene (17612). For calpain knockdown experiments, HCN cells were transfected with SMARTpool small interfering RNAs against rat PSEN1 (L-090837-02-0005; Dharmacon), PSEN2 (L-095225-02-0005; Dharmacon), or a control non-targeting siRNAs (D-001810-01-20; Dharmacon). HCN cells were transfected with plasmid DNAs (5-20 μg) or siRNAs (50-200 nM) using a Nucleofector Kit (V4XP-4024; Lonza) according to the manufacturer's instructions. Typically, 1 $\times 10^6$ cells were suspended in an appropriate volume of Nucleofector Kit solution per reaction and transfection was performed using a 4D-Nucleofector (Lonza). After the nucleofection, the cells were collected in preheated media and seeded in plates according to the experimental designs.

5.2.3 Organotypic Hippocampal Slice Culture

Organotypic hippocampal slices were prepared according to the method described by Simoni and Yu (De Simoni and Yu, 2006) slight modifications due to experimental designs. Adult female Sprague Dawley rats (7–8 weeks; Koatech) were decapitated following all institutional and ethical regulations regarding animal handling. Brains were rapidly removed and placed into a ice-cold slicing medium composed of 0.02 mM HEPES in Earle's Balanced Salt Solution (EBSS; Welgene). Hippocampi were dissected out in fresh chilled slicing medium. Extracted hippocampi were then placed on the Teflon stage of a manual tissue slice chopper (Leica, Wetzlar) for coronal sectioning at 350 μm. Four hippocampal slices were seeded onto each Millicell culture plate insert (Millipore) in 6-well plates. After a 2-day stabilization period, hippocampal slices were deprived of insulin for 48 h prior to further experimental use.

5.2.4 Generation of CRISPR/Cas9-mediated Presenilin Knockout HCN Cell Lines

Guide RNAs for gene inactivation of PS1 and PS2 were designed and purchased from ToolGen. HCN cells were transfected with Cas9- and gRNA-encoding plasmids using a Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. Homogeneities of PS1 and PS2 knockout cells were achieved by hygromycin selection (Cat. # ant-hg-1; InvivoGen) at 24 h post- transfection, followed by a complete medium change.

5.3 Results

5.3.1 Presenilin-2 Exhibits Distinct Expression Pattern in HCN Cells

First, we were compelled to confirm the expression of PS2 in HCN cells with its expression in hippocampal neurons as positive control. Through western blot analysis, we detected relative lower level of PS2 proteins in HCN cells compared to hippocampal neurons and total lysate of the hippocampus (**Figure 29A**). Interestingly, the PS2 level in HCN cells appeared distinctly lower even in comparisons with non-neural cell lines. Immunofluorescene imaging analysis in organotypic slice cultures of adult rat hippocampi further supported the marginal expression of PS2 in NSCs in the dentate gyrus (**Figure 29B**).

5.3.2 Expression of Presenilin-2 in HCN Cells is Significantly Upregulated upon Insulin Withdrawal

Because it was of our foremost interest to identify PS2 as a key molecular regulator of autophagy and cell death in HCN cells, we utilized our well-established insulin withdrawal model of ACD. Consistent with our microarray data (data not shown), PS2 was upregulated in I(-) condition, characterized by the increased LC3-II (**Figure 29C**). qRT-PCR analysis was performed to compare the mRNA level of PS2 in I(-) to I(+) condition, revealing the similar pattern in its expression (**Figure 29D**). Because only presenilin-2, and not presenilin-1, was identified as a significantly upregulated gene in microarray-based analysis, we assessed the mRNA levels of PS1 and PS2 across I(+) and I(-) conditions. Interestingly, expression of presenilin-1 at protein level (data not shown) and mRNA level was unaltered in HCN cells experiencing ACD (**Figure 29E**). To bolster our *in vitro*-centered observation of PS2 upregulation in the absence of insulin, we conducted immunofluorescene imaging analysis in organotypic slice cultures of adult rat hippocampi under I(+) and I(-) to replicate our *in vitro* model in *ex vivo* paradigm, which likewise revealed identical results (**Figure 29E**).

5.3.3 Genetic Depletion of PS2 Prevents Induction of ACD by Insulin Withdrawal in HCN Cells

To elucidate whether PS2 upregulation observed in I(-) condition is by its causative regulatory function in mediation of ACD (or autophagy) or simply a cellular response to insulin deprivation, we performed siRNA-induced knockdown of PS2 in HCN cells. HCN cells with transient knockdown of PS2 exhibited significant reduction in the levels of PS2 and the autophagy marker protein LC3 (**Figures 30A-C**). Inconsistent with the set of results, autophagy substrate protein p62 – which is also degraded by UPS via ubiquitination – unexpectedly revealed a diminishing

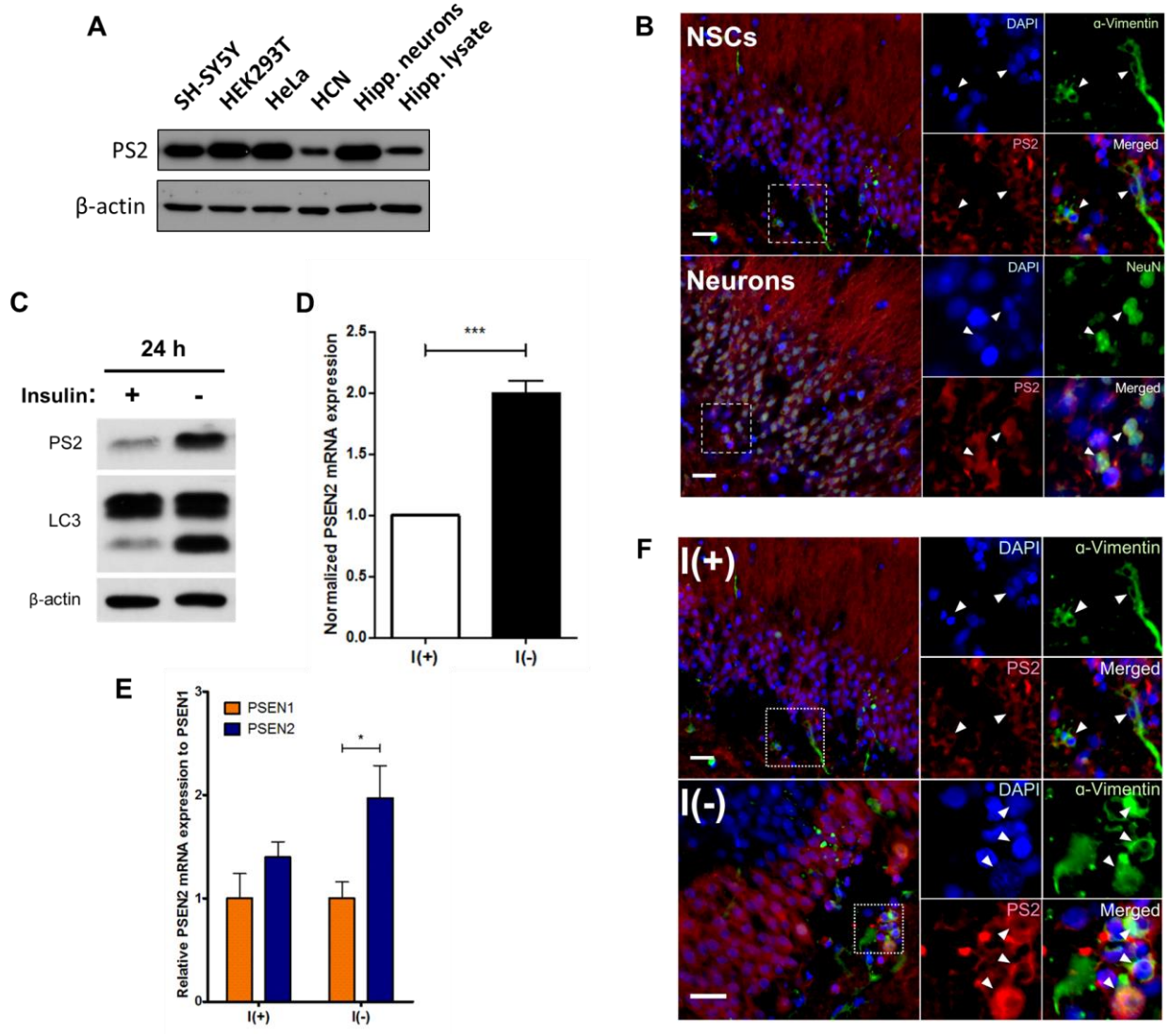


Figure 29. Presenilin-2 exhibits distinct expression pattern in HCN cells. (A): Comparison of PS2 expression in HCN cells versus non-neural cell lines, hippocampal neurons and hippocampal tissue was made by western blot analysis. (B): PS2 expression levels were observed in α-Vimentin-positive NSCs and NeuN-positive neurons in the dentate gyrus of adult rat hippocampus through *ex vivo* organotypic hippocampal slice culture. Arrow indicates the representative PS2-expressing cells. Scale bar, 20 μm. (D): Western blot analysis revealed increase in PS2 level along with the characteristic induction of LC3-II in I(-). (D,E): qRT-PCR analysis was performed to compare the mRNA level of PS2 in I(-) to I(+). Comparison between mRNA levels of PS1 and PS2 was also made across I(+) and I(-) conditions. Each set of experiment was conducted in triplicate per experiment (n = 4). The bars represent the mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001. (F): PS2 expression levels were observed in α-Vimentin-positive NSCs in the dentate gyrus of adult rat hippocampus under I(+) and I(-) conditions through *ex vivo* organotypic hippocampal slice culture. Arrow indicates the representative PS2-expressing cells. Scale bar, 20 μm.

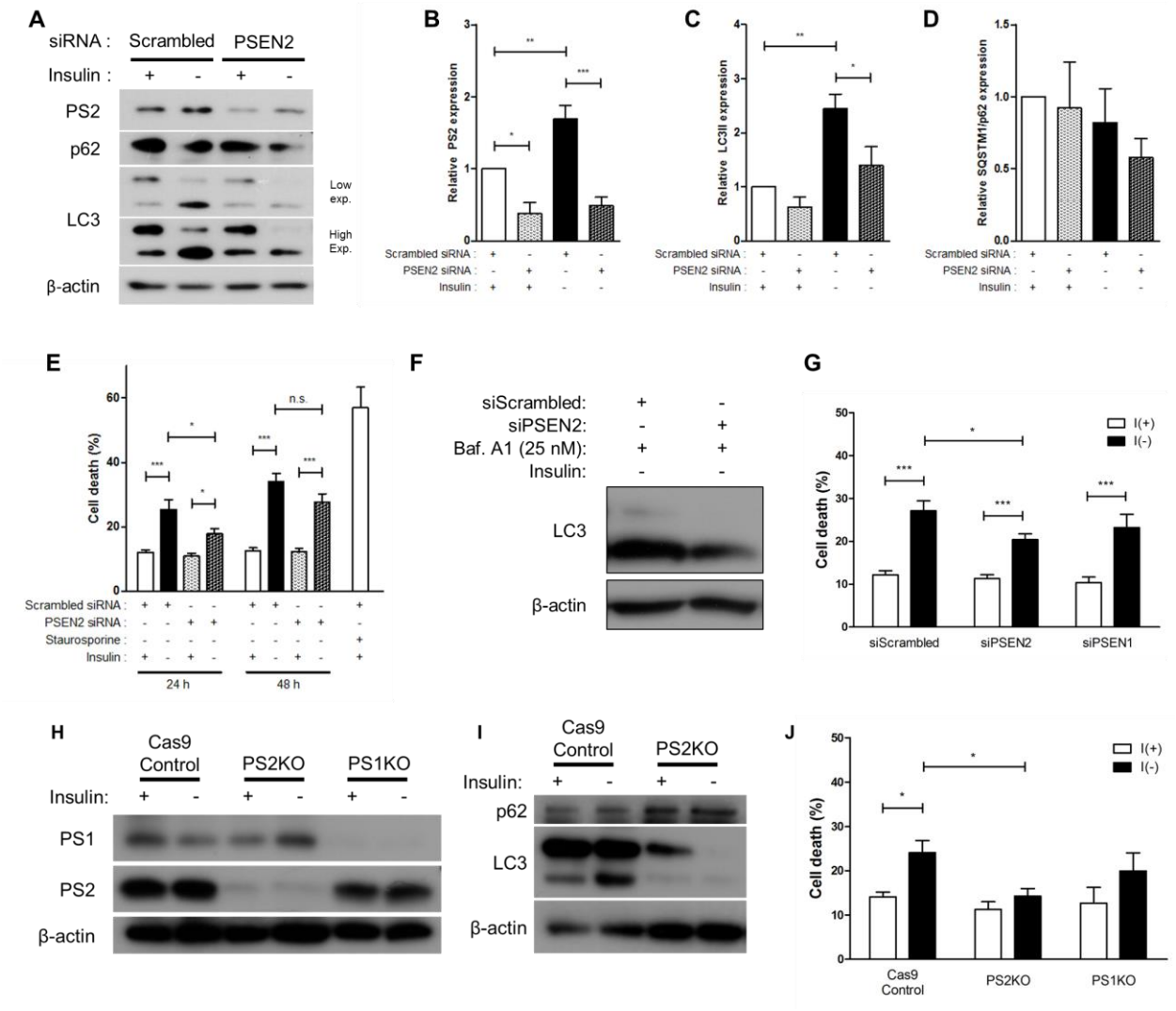


Figure 30. Genetic silencing of PS2 prevents induction of ACD in HCN cells. (A-D): Western blot analysis and quantification of PS2 and autophagy markers in siRNA-mediated PS2 knockdowns HCN cells in I(+) and I(-) conditions. (E): Rate of cell death was measured in PS2 knockdowns HCN cells in I(+) and I(-) conditions.. Each set of experiment was conducted in triplicate per experiment (n = 4). The bars represent the mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001. (F): Autophagy flux was assessed in insulin-deprived PS2 knockdowns HCN cells. (G): Cell death assay was performed in PS1 and PS2 knockdown HCN cells in both I(+) and I(-) conditions. The bars represent the mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001. (H-J): Analysis of CRISPR/Cas9-mediated PS2 knockout HCN cells.Western blot analysis of PS2 and PS1 in PS2KO and PS1KO HCN cells, respectively. (B): Level of LC3 in PS2KO HCN cells was assessed by western blot. (C): Rate of cell death of PS2KO and PS1KO HCN cells in I(+) and I(-) conditions was quantified. Each set of experiment was conducted in triplicate per experiment (n = 4). The bars represent the mean ± SEM; *p < 0.05.

pattern (**Figures 30D**). In addition, extended duration of insulin deprivation (i.e., 48 h) showed a decreasing trend in the rate of cell death in PS2-silenced HCN cells but did not reach statistical significance (**Figure 30E**), exhibiting the limitation of transient knockdown system. Since reduction of LC3-II with depleting pattern of p62 can be interpreted as an acceleration in autophagy flux, we used Baf.A1, a blocker of late-phase autophagy, for better assessment of autophagy flux in PS2 knockdown HCN cells. Upon treatment of bafilomycin A1, autophagy substrates subject to hydrolytic degradation remain accumulated. In PS2 knockdown HCN cells undergoing insulin withdrawal-induced ACD, LC3-II proteins were accumulated at relatively lower degree than the control when BafA1 was treated (**Figure 30F**), justifying our notion that PS2 is an active participant in mediation of ACD by insulin removal in HCN cells. Because presenilins are known to possess functional commonalities among the homologues – meaning that they can functionally compensate to some degrees – we were curious to see whether knockdown of PS1 can also prevent I(-) HCN cells from experience ACD. Unlike PS2, PS1 knockdown failed to significantly decrease the rate of insulin withdrawal-induced cell death (**Figure 30G**).

To enable further characterization of presenilins in HCN cells, we, we generated stable presenilin knockout HCN cell lines – both presenilin-1 knockout (PS1KO) and presenilin-2 knockout (PS2KO). CRISPR/Cas9-mediated gene deletion resulted in prominently more powerful depletion of presenilin proteins and the subsequent reductions in LC3 and cell death rates, as compared to the siRNA-mediated transient knockdown data (**Figures 30H-J**). Protein levels of PS2 and PS1 were significantly diminished in PS2KO and PS1KO HCN cells, respectively (**Figure 30H**). Consistent with our results from transiently silenced cells, PS2KO HCN cells exhibited reduction in LC3-II proteins with greater extent (**Figure 30I**). Consonantly, the rate of cell death in PS2KO cells in insulin-deprived condition was significantly lower than that of control cells (**Figure 30J**). PS1KO HCN cells showed a decreasing trend in induction of ACD upon insulin withdrawal but failed to reach statistical significance.

5.3.4 PS2 Expression Potentiates ACD in I(-) HCN Cells, but Not in Atg7-Deficient I(-) HCN Cells

As we witnessed the prevention of ACD induction in HCN cells depleted with PS2, it was our next mission to examine the opposite effect. To this end, we expressed HCN cells with human wild-type PS2, and observed the heightened induction of ACD in I(-) HCN cells with approximately 3-fold increased PS2 levels (**Figures 31A,B**). Consistent with the changes in biochemical markers, rate of cell death was significantly increased as well (**Figure 31C**). To ensure that our obtained results from both transient knockdown and stable knockout cells were indeed due

to silencing of PS2 gene, we performed a set of rescue experiment. The exogenously introduced PS2 protein was successfully able to rescue the loss-of-function phenotype – the reduced levels of autophagy markers and cell death – observed in PS2KO HCN cells under I(-) condition (**Figures 31D,E**). Interestingly, in Atg7 stable knockdown HCN cells generated by lentiviral expression of Atg7-targeting shRNA, both insulin withdrawal and PS2 expression failed to induce ACD (**Figures 31F,G**).

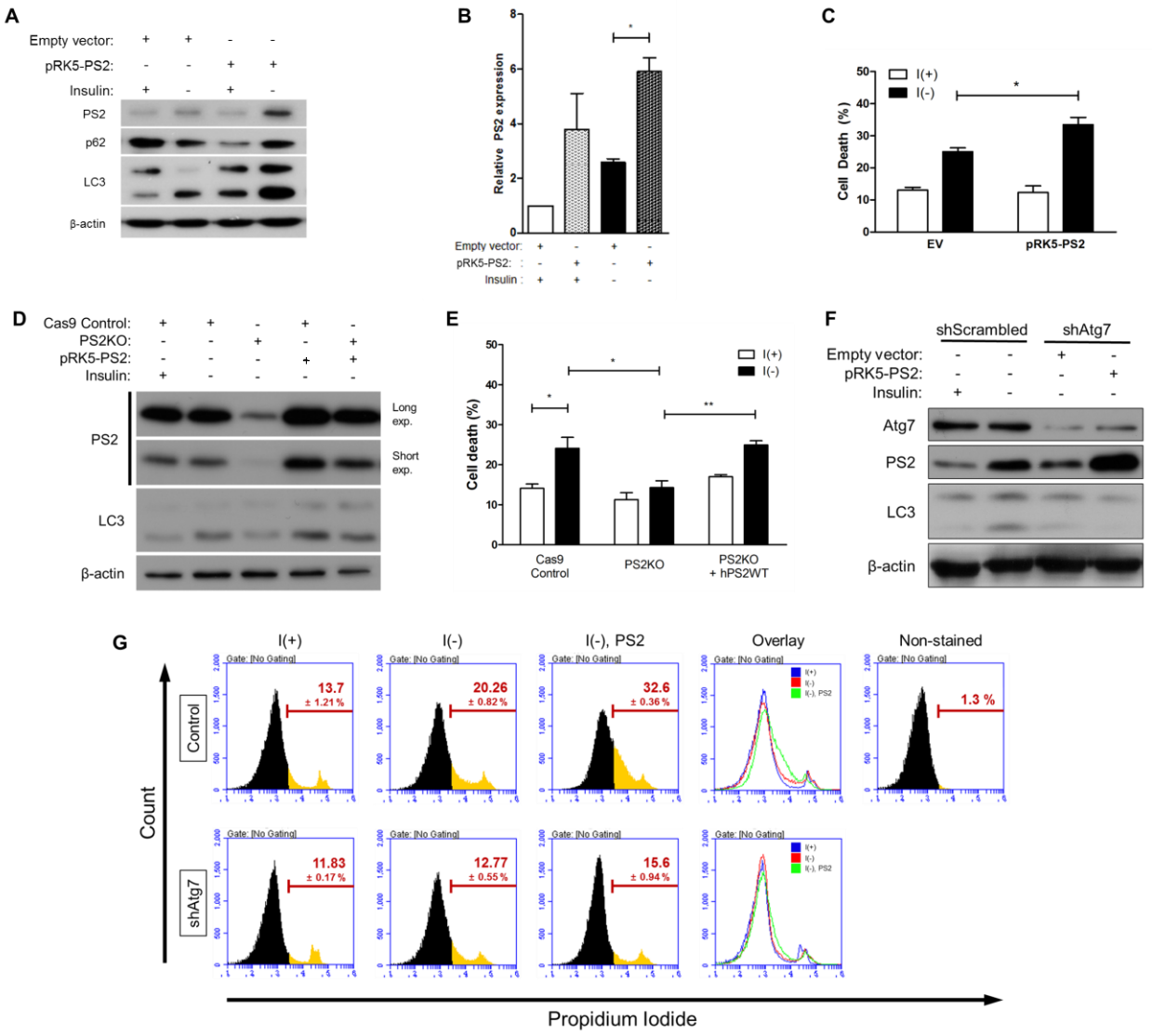


Figure 31. Ectopic expression of PS2 potentiates and rescues ACD in insulin-deprived HCN cells. (A,B): Western blot analysis and quantification of PS2 and autophagy marker proteins in PS2-overexpressed HCN cells. (C) Cell death assay was performed in PS2-expressing HCN cells under I(+) and I(-) conditions. Each set of experiment was conducted in triplicate per experiment (n = 4). The bars represent the mean ± SEM; *p < 0.05. (D,E): Human PS2 plasmid (hPS2WT) was transfected in PS2KO HCN cells to perform the rescue experiments. Rescue of PS2 and LC3 proteins by hPS2 was assessed by western blot and the rate of cell death was quantified under I(+) and I(-) conditions. Each set of experiment was conducted in triplicate per experiment (n = 4). The bars represent the mean ± SEM; *p < 0.05,**p<0.01. (F,G): Analysis of ACD in Atg7 knockdown HCN cells overexpressed with PS2 by western blot and flow cytometry.

5.4 Discussion

We have previously reported that efflux of Ca^{2+} from the ER underlies induction of autophagy upon insulin withdrawal in HCN cells (Chung et al., 2016). In the study, RyR3 was identified as the specific ER Ca^{2+}-releasing channel that enables the activation of autophagy and subsequent cell death. Interestingly, other isoforms of RyR, likely due to their low expressions, and another ER Ca^{2+}-releasing channel IP_3Rs appeared non-relevant in regulation of ACD in HCN cells. Given the importance of ER Ca^{2+} regulation established in our model system, PS2 – which has been suggested that fAD-linked presenilins function as leak channels of ER Ca^{2+} (Tu et al., 2006) – was an ideal molecular target to dissect its functional relevance in modulation of PCD in NSCs.

Our results, conducted thus far, provide an evident link between PS2 and autophagy in NSCs. Utilization of currently-available powerful genetic approaches, we were able to identify PS2 as a positive regulator of ACD in insulin-deprived HCN cells. Both siRNA-mediate transient knockdown of PS2 and CRISPR/Cas9-induced deletion of PS2 showed substantial downregulation of autophagy along with significant protection against insulin deprivation (**Figure 30**). Overexpression of PS2, conversely, upregulates autophgy and the subsequent death of HCN cells in insulin withdrawal condition (**Figure 31**). Rescue of autophagy levels and the rate of cell death by ectopic expression of PS2 in PS2-deleted HCN cells further strengthened the intact PS2 regulatory mechanism even in the absence of PS2, indicating PS1 likely does not functionally compensate autophagy in HCN cells. Moreover, expression of PS2 appears to show direct response in presence of other cytotoxic stressors that ultimately cause cell death (data not shown), implying the intimate relationship between PS2 and PCD in HCN cells. However, caution should be taken for development of NSC therapies based on PS2 as presenilins are also heavily implicated in regulation of neurogenesis (Handler, 2000) – for instance, enhancing survival of NSCs by PS2 modulation may negatively affect NSC ability to give rise to granular neurons. Indeed, our preliminary results suggest that differentiating capability in HCN cells is altered in absence of PS2 (data not shown).

Based on our observation from western blot analysis and *ex vivo* organotypic slice cultures, PS2 expression in NSCs appears significantly lower than its expression in neurons and other non-neural cell lines (**Figure 29**). ACD is widely perceived to occur when autophagy is excessively induced; however, the exact definition or threshold of the *excessive* induction of autophagy is vague at most. Because neurons have particularly large cytoplasmic volume due to their distinct morphological extensions of dendrites and axons, the clearance of long-lived proteins and dysfunctional organelles via autophagy is crucial in the maintenance of cellular homeostasis and thereby the failure

of which underlies diverse human pathologies (**Figure 7**). Unlike neurons, NSCs morphologically possess small cytoplasmic volume thereby more sensitively experience the changes in autophagy activation than neurons, leading to different physiological responses in NSCs and neurons.

To our knowledge, this is the first evidence of PS2 in regulation of autophagy and PCD in HCN cells. Admittedly, our discovery of PS2 as a positive regulator of ACD in HCN cells warrants further detailed study on the functional implication of PS2-mediated ACD in HCN cells to understand the sophisticated molecular mechanisms of NSC biology. Due to dearth of knowledge on function of presenilins in NSCs, our investigation in physiological role of PS2 in NSCs may provide novel insight into development of stem cell-based therapeutic strategy.

CHAPTER 6: General Discussion

Due to their potential for aiding development of novel therapeutic avenues for the treatment of neurodegenerative diseases, multipotent NSCs have always drawn significant amount of attention from both the scientific community and the public for decades. However, since the discovery of adult neurogenesis in mammals by Gage and his colleagues (Gage et al, 1995; Gage, 2000) the genuine interest in NSC research exploded in hopes of countering neurodegenerative diseases and aging throug NSC transplantation. However, great challenges still awaits, such as the hostile environment in the aging or diseased brain which, for instance, limits effectiveness of engrafted NSCs from functionally integrating into the endogenous neural circuits to generate newborn neurons. In order to best optimize the therapeutic potential of endogenous NSCs, understanding how NSCs respond to adverse cellular stresses and identifying the signaling molecules must be preceded. Basic science research in NSCs, therefore, possesses great importance as to provide valuable knowledge regarding the molecular mechanisms governing the survival and death of NSCs under pathological states. Despite extensive NSC research conducted over the past few decades, the mechanisms underlying the programmed death of NSCs has remained under-studied. The insulin withdrawal model in HCN cells may provide a unique opportunity to decipher the molecular mechanisms of PCD in NSCs.

The report on regulation of calpain in determination of PCD modes in HCN cells provides important implications on several levels (Chung et al., 2015). First, our finding establishes calpain as a key negative regulator of ACD and a molecular switch to apoptosis in HCN cells. The distinct expression pattern of calpain 1 and 2, in conjunction with the dynamic changes in the calpain 2 protein level and intracellular Ca^{2+} concentration, appear to underlie the role of calpain in the demise of HCN cells. Our findings elaborated an intriguing, non-complementary interplay between autophagy and UPS for interconnection between autophagy and apoptosis via calpain in HCN cells. Understanding how diverse manifestations of PCD are interrelated is of great significance because it is unlikely that only a single mode of cell death is activated in the affected brain region under neuropathological situations. Clinical evidence on co-existence of autophagy and apoptosis have been reported in the brains of patients with Alzheimer's and Parkinson's diseases (Anglade et al., 1997; Stadelmann et al., 1999). Indeed, crosstalk between autophagy and other PCD modes, mostly apoptosis, has been documented (Eisenberg-Lerner et al., 2009; Chung and Yu, 2013; Chung et al., 2015).

Manifestations of autophagic characteristics in dying cells have been observed since the 1960s (Schin and Clever, 1965). However, it was not until recently that ACD began to gain attention. Compared with the well-known biochemical mechanisms and physiological roles of apoptosis, the role of ACD and relevant mechanisms have

remained elusive. Nevertheless, it is generally accepted that autophagy can promote or antagonize apoptosis. However, the key factors determining the outcome of the relationship between autophagy and apoptosis for cell survival or death are not well understood. One way autophagy affects apoptosis is through degradation of essential apoptotic molecules, such as caspase-8 or -9 through autophagy (Hou et al., 2010; Jeong et al., 2011). On the other hand, apoptosis can also change autophagic flux by targeting important autophagy molecules, such as Beclin 1 or Atg5 for proteolytic cleavage (Luo and Rubinsztein, 2010; Yousefi et al., 2006). Our recent discovery of valosin-containing proteins (VCP) suggests that certain molecules like VCP can participate in signaling pathways of both autophagy and apoptosis (Yeo et al., 2016). In this regard, calpain is an interesting molecular target because calpain protease activity can promote or suppress autophagy. Prior reports suggested a requirement of calpain for the facilitation of autophagy (Cheng et al., 2008; Demarchi et al., 2006). However, the results from several other studies have demonstrated negative control of autophagy by calpain. Madden et al. reported that calpain inhibition, in combination with caspase-8 suppression, induced autophagy in the Z-VAD-induced death of L929 fibroblast cells (Madden et al., 2007), although they did not specify which isoform of calpain played the critical role in the inhibition of ACD. ACD in L929 cells required prior inhibition of caspases, especially caspase-8. In our study, insulin-deprived HCN cells did not display signs of apoptosis or require caspase inhibition for the progress of ACD, suggesting the existence of an innate system that places apoptosis on hold in HCN cells. This is not due to any defect in the apoptotic capabilities of HCN cells, as they were derived from adult wild-type rat hippocampal tissues and undergo typical apoptosis in response to staurosporine (Baek et al., 2009; Yu et al., 2008), etoposide, and oxidative stress (data not shown). Identification of the as-yet-unknown endogenous antagonizing pathway(s) to block apoptosis would be of great interest, and much of our research efforts have been concentrated on addressing this question.

Interestingly, lines of scientific evidence from other groups appear to support our findings, though apparent indirectness exists due to utilization of different models. Recently, the distinct regulatory functions of calpain 1 and 2 in NSCs was reported (Santos et al., 2012). In the study, investigators showed that calpain 1 repressed neural differentiation while calpain 2 promoted glial differentiation in NSCs derived from the fetal mouse forebrain. Although the finding may be not directly applicable to our results in HCN cells due to different cellular origins and expression profiles of calpain family members, it further validates the existence of the differential cellular functions of calpain 1 and 2 in NSCs. Another study by Machado et al. investigated the role of calpain in adult neurogenesis, in which the deletion of calpastatin, the endogenous calpain inhibitor, impaired both NSC proliferation and neuroblast

migration while calpain inhibition increased NSC proliferation, migration speed and distance in the neurogenic regions (Machado et al., 2015). This report, in particular, appears to provide physiological relevance to our discovery as their *in vivo* experiment revealed calpain activation from deletion of calpastatin aberrantly downregulated NSC proliferation, which to our understanding may have occurred due to calpain-induced apoptosis in NSCs. It is also noteworthy to ponder the possible enhancement of tissue homeostasis in the neurogenic region through ACD of selective NSCs that are highly vulnerable to external stresses since calpain inhibition, which according to our model induced autophagic death of NSCs, led to prominent well-being within the neurogenic niche in the dentate gyrus (Machado et al., 2015).Because distinct characteristics of different cell types exist in nature, it is not surprising to expect that application of certain experimental stimuli may induce different outcomes – which seemingly is the case with insulin deprivation in NSCs and neurons. Young et al. have reported that removal of insulin in neurons expectedly induces autophagy; however, such activation of autophagy entails neuroprotection against cytotoxicity which directly opposes our observations in HCN cells. These differing phenotypes between the cell types are likely due to genetically programmed expression of molecules distinct in each cell type. Because neurons have particularly large cytoplasmic volume due to their distinct morphological extensions of dendrites and axons, the clearance of long-lived proteins and dysfunctional organelles via autophagy is crucial in the maintenance of cellular homeostasis and thereby the failure of which underlies diverse human pathologies (**Figure 7**). Unlike neurons, NSCs morphologically possess small cytoplasmic volume thereby more sensitively experience the changes in autophagy activation than neurons, leading to different physiological responses in NSCs and neurons.

To our interest, one great example is RyRs – mRNA levels of RyR2 were reportedly shown to be substantially greater than other RyR isoforms in mouse hippocampal neurons (Wu et al., 2013), while our data suggests RyR3 is the major isoform in HCN cells (Figure 19). A previous study by Balschun et al. also supports our notion as it delineated impairment of synaptic plasticity in the hippocampi of mice lacking RyR3 (Balschun et al., 1999). Combining our observation of pro-survival effect by RYR3 knockout in NSCs and a seemingly harmful effect in neurons by RyR3 deletion, a distinct, cell type-specific role for RyR3, and likely calpains as well, does indeed exists. The exact functional implication of differential expression of RyR isoforms in NSCs and neurons remains an enthralling research topic to be investigated.

Lastly, I would like to close the discussion by addressing physiological implication of our model. Admittedly, overcoming the dearth of *in vivo* approach to support our results obtained in HCN cells is one of the challenges to

provide greater translational values in our study. However, as for the physiological implications of our *in vitro*-concentrated studies, recent observations of defective insulin signaling and impaired insulin transport in the brain, especially in the hippocampus of patients with Alzheimer's disease (AD; de la Monte, 2009; Talbot et al., 2012) suggest the potential relevance of autophagy-dependent death of insulin-deprived HCN cells in the pathogenesis of AD. Intriguingly, calpain and RyR3 are also heavily implicated in brain pathology. Disturbed activation of calpain, a well-known cell death protease, has been observed under various pathophysiological conditions in the brain (Crocker et al., 2003; Gafni and Ellerby, 2002; Saito et al., 1993). Furthermore, our finding on RyR3 regulation of PCD in the absence of insulin signaling is also supported by similar clinical observations from other studies. Dysregulation of intracellular Ca^{2+} is an underlying component of various neurodegenerative diseases, and recent evidence implicates RyR in the pathology of AD, further highlighting the functional role of RyRs in the aberrant physiological conditions (Supnet et al., 2010; Bruno et al., 2012; Oules et al., 2012; Wu et al., 2013). Thus, our results obtained from insulin withdrawal model of ACD in HCN cells seemingly provides translational implication to some degree.

References

Aimone, J. B., Li, Y., Lee, S. W., Clemenson, G. D., Deng, W., & Gage, F. H. (2014). Regulation and Function of Adult Neurogenesis: From Genes to Cognition. *Physiological Reviews*, 94(4), 991–1026.

Alberdi, E., Sanchez-Gomez, M.V., Cavaliere, F., Perez-Samartin, A., Zugaza, J.L., Trullas, R., Domercq, M. & Matute, C. (2010). Amyloid beta oligomers induce Ca^{2+} dysregulation and neuronal death through activation of ionotropic glutamate receptors. *Cell Calcium*, 47, 264-72.

Altman, J. (1962). Are new neurons formed in the brains of adult mammals? *Science*, 135(3509), 1127-8.

Altman, J. & Das, G.D. (1965a). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *The Journal of Comparative Neurology*, 124(3), 319-35.

Altman, J. & Das, G.D. (1965b). Post-natal origin of microneurones in the rat brain. *Nature*, 207(5000), 953-6.

Anglade, P., Vyas, S., Javoy-Agid, F., Herrero, M.T., Michel, P.P., Marquez, J., Mouatt-Prigent, A., Ruberg, M., Hirsch, E.C. & Agid, Y. (1997). Apoptosis and autophagy in nigral neurons of patients with Parkinson's disease. *Histology & Histopathology*. 12, 25-31.

Bading, H. (2013). Nuclear calcium signalling in the regulation of brain function. *Nature Reviews Neurosciece*. 14, 593-608.

Baehrecke, E. H. (2005). Autophagy: dual roles in life and death? *Nature Reviews Molecular Cell Biology*, 6:505-10.

Baek, S. H., Kim, E. K., Goudreau, J. L., Lookingland, K. J., Kim, S. W. & Yu, S. W. (2009). Insulin withdrawal-induced cell death in adult hippocampal neural stem cells as a model of autophagic cell death. *Autophagy*, 5(2), 277-9.

Balschun, D., Wolfer, D.P., Bertocchini, F., Barone, V., Conti, A., Zuschratter, W., Missiaen, L., Lipp, H.P., Frey, J.U. & Sorrentino, V. (1999). Deletion of the ryanodine receptor type 3 (RyR3) impairs forms of synaptic plasticity and spatial learning. *EMBO Journal*. 18, 5264-73.

Bampton, E.T., Goemans, C.G., Niranjan, D., Mizushima, N. & Tolkovsky, A.M. (2005). The dynamics of autophagy visualized in live cells: from autophagosome formation to fusion with endo/lysosomes. *Autophagy* 1, 23-36.

Batiz, L. F., Castro, M. A., Burgos, P. V., Velásquez, Z. D., Muñoz, R. I., Lafourcade, C. A., Troncoso, P. & Wyneken, U. (2016). Exosomes as Novel Regulators of Adult Neurogenic Niches. *Frontiers in Cellular Neuroscience*, doi.org/10.3389/fncel.2015.00501

Biebl, M., Cooper, C.M., Winkler, J. & Kuhn, H.G. (2000). Analysis of neurogenesis and programmed cell death reveals a self-renewing capacity in the adult rat brain. *Neuroscience Letters*. 291, 17-20.

Bischofberger, J. (2007). Young and excitable: new neurons in memory networks. *Nature Neuroscience*, 10, 273-5.

Boland, B., Kumar, A., Lee, S., Platt, F. M., Wegiel, J., Yu, H. & Nixon, R. A. (2008). Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease. *Journal of Neuroscience*, 28, 6926-37.

Bogyo, M., McMaster, J. S., Gaczynska, M., Tortorella, D., Goldberg, A. L. & Ploegh, H. (1997). Covalent modification of the active site threonine of proteasomal beta subunits and the Escherichia coli homolog HslV by a new class of inhibitors. *Proceedings of the National Academy of Sciences of the United States of America*, 94(13), 6629-34.

Bojarski, L., Herms, J. & Kuznicki, J. (2008). Calcium dysregulation in Alzheimer's disease. *Neurochemistry International*, 52, 621-33.

Boya, P., Gonzalez-Polo, R. A., Casares, N., Perfettini, J. L., Dessen, P., Larochette, N., Metivier, D., Meley, D., Souquere, S., Yoshimori, T., Pierron, G., Codogno, P., & Kroemer, G. (2005). Inhibition of macroautophagy triggers apoptosis. *Molecular and Cellular Biology*, 25, 1025-40.

Boya, P. & Kroemer, G. (2008). Lysosomal membrane permeabilization in cell death. *Oncogene* 27, 6434-51.

Bruno, A.M., Huang, J.Y., Bennett, D.A., Marr, R.A., Hastings, M.L. & Stutzmann, G.E. (2012). Altered ryanodine receptor expression in mild cognitive impairment and Alzheimer's disease. *Neurobiology of Aging*, 33, 1001 e1001-6. doi: 10.1016/j.neurobiolaging.2011.03.011.

Cameron, H.A. & McKay, R.D. (2001). Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. *Journal of Comparative Neurology*, 435(4), 406-17.

Camins, A., Crespo-Biel, N., Junyent, F., Verdaguer, E., Canudas, A. M. & Pallàs M. (2009). Calpains as a target for therapy of neurodegenerative diseases: putative role of lithium. *Current Drug Metabolism*, 10(5):433-47.

Cheng, Y., Qiu, F., Huang, J., Tashiro, S., Onodera, S. & Ikejima, T. (2008). Apoptosis-suppressing and autophagy-promoting effects of calpain on oridonin-induced L929 cell death. *Archives of Biochemistry and Biophysics*, 475(2), 148-55. doi: 10.1016/j.abb.2008.04.027

Cheung, K. H., Shineman, D., Müller, M., Cárdenas, C., Mei, L., Yang, J., Tomita, T., Iwatsubo, T., Lee, V. M. & Foskett, J.K. (2008). Mechanism of Ca2+ disruption in Alzheimer's disease by presenilin regulation of InsP3 receptor channel gating. *Neuron*, 58, 871–83.

Cheung, K. H., Mei, L., Mak, D. O., Hayashi, I., Iwatsubo, T., Kang, D. E. & Foskett, J.K. (2010). Gain-of-function enhancement of IP3 receptor modal gating by familial Alzheimer's disease-linked presenilin mutants in human cells and mouse neurons. *Science Signaling*, 23;3(114):ra22. doi: 10.1126/scisignal.2000818

Chung, K. M., & Yu, S. W. (2013). Interplay between autophagy and programmed cell death in mammalian neural stem cells. BMB Rep, 46(8), 383-390.

Chung, K. M., Park, H., Jung, S., Ha, S., Yoo, S. J., Woo, H., Lee, H. J., Kim, S. W., Kim, E. K., Moon, C. &Yu, S. W. (2015). Calpain determines the propensity of adult hippocampal neural stem cells to autophagic cell death following insulin withdrawal. *Stem Cells*, 33(10), 3052-3064.

Chung, K. M., Jeong, E. J., Park, H., An, H. K. & Yu, S. W. (2016). Mediation of autophagic cell death by type 3 ryanodine receptor (RyR3) in adult hippocampal neural stem cells. *Frontiers in Cellular Neuroscience*, 10, 116. doi: 10.3389/fncel.2016.00116

Clarke, P. G. (1990). Developmental cell death: morphological diversity and multiple mechanisms. *Anatomy and Embryology*, 181, 195-213.

Clarke, P. G., & Puyal, J. (2012). Autophagic cell death exists. *Autophagy*, 8(6), 867-9.

Clarke, E. (1992). Cajal's Degeneration and regeneration of the nervous system. *Medical History*, 36(4), 465.

Criollo, A., Maiuri, M.C., Tasdemir, E., Vitale, I., Fiebig, A.A., Andrews, D., Molgo, J., Diaz, J., Lavandero, S., Harper, F., Pierron, G., di Stefano, D., Rizzuto, R., Szabadkai, G. & Kroemer, G. (2007). Regulation of autophagy by the inositol trisphosphate receptor. *Cell Death & Differentiation*, 14, 1029-39.

Crocker, S. J., Smith, P. D., Jackson-Lewis, V., Lamba, W. R., Hayley, S. P., Grimm, E., Callaghan, S. M., Slack, R. S., Melloni, E., Przedborski, S., Robertson, G. S., Anisman, H., Merali, Z. & Park, D. S. (2003). Inhibition of calpains prevents neuronal and behavioral deficits in an MPTP mouse model of Parkinson's disease. *Journal of Neuroscience*, 23(10), 4081-91.

Daial, N. & Korsmeyer, S. J. (2004). Cell death: critical control points. *Cell*, 116, 205-19.

De Felice, F.G., Lourenco, M.V. & Ferreira, S.T. (2014). How does brain insulin resistance develop in Alzheimer's disease? *Alzheimer's Disease & Dementia*, 10, S26-32.

De Simoni, A. & Yu, L. M. (2006). Preparation of organotypic hippocampal slice cultures: interface method. *Nature Protocols*. 1(3), 1439-45. doi:10.1038/nprot.2006.228.

Demarchi, F., Bertoli, C., Copetti, T., Tanida, I., Brancolini, C., Eskelinen, E. L. & Schneider, C. (2006). Calpain is required for macroautophagy in mammalian cells. *Journal of Cell Biology*, 175(4), 595-605. doi: 10.1083/jcb.200601024

Ding, W. X., Ni, H. M., Gao, W., Yoshimori, T., Stolz, D. B., Ron, D. & Yin, X. M. (2007). Linking of autophagy to ubiquitin-proteasome system is important for the regulation of endoplasmic reticulum stress and cell viability. *American Journal of Pathology*, 171(2), 513-24.

Du, H., Guo, L., Fang, F., Chen, D., Sosunov, A.A., McKhann, G.M., Yan, Y., Wang, C., Zhang, H., Molkentin, J.D., Gunn-Moore, F.J., Vonsattel, J.P., Arancio, O., Chen, J.X. & Yan, S.D. (2008). Cyclophilin D deficiency attenuates mitochondrial and neuronal perturbation and ameliorates learning and memory in Alzheimer's disease. *Nature Medicine*, 14, 1097-1105.

Dunn, W. A., Jr. (1994). Autophagy and related mechanisms of lysosome-mediated protein degradation. *Trends in Cell Biology*, 4(4), 139-43.

Edinger, A.L. & Thompson, C.B. (2004). Death by design: apoptosis, necrosis and autophagy. *Current Opinions in Cell Biology*, 16, 663-9.

Eichenbaum, H., Dudchenko, P., Wood, E., Shapiro, M. & Tanila H. (1999). The hippocampus, memory, and place cells: is it spatial memory or a memory space? *Neuron*, 23, 209 –226.

Eisenberg-Lerner, A., Bialik, S., Simon, H.U. & Kimchi, A. (2009). Life and death partners: apoptosis, autophagy and the cross-talk between them. *Cell Death & Differentiation*, 16, 966-75.

Eriksson, P.S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A.M., Nordborg, C., Peterson, D.A. & Gage, F.H. (1998). Neurogenesis in the adult human hippocampus. *Nature Medicine*, 4(11), 1313-7. doi:10.1038/3305.

Frankland, P. W. & Miller, F. D. (2008). Regenerating your senses: multiple roles for neurogenesis in the adult brain. *Nature Neuroscience*, 11, 1124 - 1126

Gafni, J. & Ellerby, L. M. (2002). Calpain activation in Huntington's disease. *Journal of Neuroscience*, 22(12), 4842-9.

Gage, F.H., Ray, J. & Fisher, L.J. (1995). Isolation, characterization, and use of stem cells from the CNS. *Annual Review of Neuroscience*, 18, 159-92. doi:10.1146/annurev.ne.18.030195.001111.

Gage, F.H. (2000). Mammalian neural stem cells. *Science*, 287(5457), 1433-8.

Gandhi, S., Wood-Kaczmar, A., Yao, Z., Plun-Favreau, H., Deas, E., Klupsch, K., Downward, J., Latchman, D.S., Tabrizi, S.J., Wood, N.W., Duchen, M.R. & Abramov, A.Y. (2009). PINK1-associated Parkinson's disease is caused by neuronal vulnerability to calcium-induced cell death. *Molecular Cell*, 33, 627-638.

Gao, W., Ding, W.X., Stolz, D.B. & Yin, X.M. (2008). Induction of macroautophagy by exogenously introduced calcium. *Autophagy*, 4, 754-761.

Ge S., Yang C. H., Hsu, K. S., Ming, G. L. & Song, H. (2007). A critical period for enhanced synaptic plasticity in newly generated neurons of the adult brain. *Neuron*, 54, 559-66.

Gilmore, E. C., Nowakowski, R. S., Caviness, V. S., Jr. & Herrup, K. (2000). Cell birth, cell death, cell diversity and DNA breaks: how do they all fit together? *Trends in Neuroscience*, 23(3), 100-5.

Goedert, M. & Spillantini, M. G. (2006). A century of Alzheimer's disease. *Science*, 314, 777-81.

Guo, Q., Furukawa, K., Sopher, B.L., Pham, D.G., Xie, J., Robinson, N., Martin, G.M. & Mattson, M.P. (1996). Alzheimer's PS-1 mutation perturbs calcium homeostasis and sensitizes PC12 cells to death induced by amyloid beta-peptide. *Neuroreport* , 8, 379-83.

Ha, H. C., & Snyder, S. H. (1999). Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. *Proceedings of the National Academy of Sciences of the United States of America*, 96(24), 13978-82.

Ha, S., Ryu, H. Y., Chung, K. M., Baek, S. H., Kim, E. K., & Yu, S. W. (2015). Regulation of autophagic cell death by glycogen synthase kinase-3β in adult hippocampal neural stem cells following insulin withdrawal. *Mol Brain*, 8, 30. doi: 10.1186/s13041-015-0119-9

Han, S. I., Kim, Y. S., & Kim, T. H. (2008). Role of apoptotic and necrotic cell death under physiologic conditions. BMB Rep, 41(1), 1-10.

Handler, M., Yang, X. & Shen, J. (2000). Presenilin-1 regulates neuronal differentiation during neurogenesis. *Development*, 127, 2593-606.

Harr, M.W., McColl, K.S., Zhong, F., Molitoris, J.K. & Distelhorst, C.W. (2010). Glucocorticoids downregulate Fyn and inhibit IP(3)-mediated calcium signaling to promote autophagy in T lymphocytes. *Autophagy*, 6, 912-21.

Hong, C. J., Park, H. & Yu, S. W. (2016). Autophagy for the quality control of adult hippocampal neural stem cells. *Brain Research*, doi:10.1016/j.brainres.2016.02.048.

Hou, W., Han, J., Lu, C., Goldstein, L. A. & Rabinowich, H. (2010). Autophagic degradation of active caspase-8: a crosstalk mechanism between autophagy and apoptosis. *Autophagy*, 6(7), 891-900.

Hoyer-Hansen, M., Bastholm, L., Szyniarowski, P., Campanella, M., Szabadkai, G., Farkas, T., Bianchi, K., Fehrenbacher, N., Elling, F., Rizzuto, R., Mathiasen, I.S. & Jaattela, M. (2007). Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2. *Molecular Cell*, 25, 193-205.

Itakura, E. & Mizushima, N. (2010). Characterization of autophagosome formation site by a hierarchical analysis of mammalian Atg proteins. *Autophagy*, 6, 764-76.

Jeong, H. S., Choi, H. Y., Lee, E. R., Kim, J. H., Jeon, K., Lee, H. J. & Cho, S. G. (2011). Involvement of caspase-9 in autophagy-mediated cell survival pathway. *Biochimica et Biophysica Acta*, 1813(1), 80-90.

Kasri, N.N., Kocks, S.L., Verbert, L., Hebert, S.S., Callewaert, G., Parys, J.B., Missiaen, L. & De Smedt, H. (2006). Up-regulation of inositol 1,4,5-trisphosphate receptor type 1 is responsible for a decreased endoplasmic-reticulum Ca^{2+} content in presenilin double knock-out cells. *Cell Calcium*, 40, 41-51.

Kempermann G., Kuhn H. G. & Gage, F.H. (1997). More hippocampal neurons in adult mice living in an enriched environment. *Nature*, 386, 493-95.

Kerr, J. F., Wyllie, A. H. & Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer*, 26, 239-57.

Khachaturian, Z.S. (1987). Hypothesis on the regulation of cytosol calcium concentration and the aging brain. *Neurobiology of Aging*, 8, 345-6.

Khachaturian, Z.S. (1994). Calcium hypothesis of Alzheimer's disease and brain aging. *Annals of the New York Academy of Sciences*, 747, 1-11.

Klionsky, D. J. (2004). Cell biology: regulated self-cannibalism. *Nature*, 431(7004), 31-2. doi: 10.1038/431031a

Kobayashi, S., Bannister, M.L., Gangopadhyay, J.P., Hamada, T., Parness, J. & Ikemoto, N. (2005). Dantrolene stabilizes domain interactions within the ryanodine receptor. *Journal of Biological Chemistry*, 280, 6580-7.

Koike, M., Shibata, M., Tadakoshi, M., Gotoh, K., Komatsu, M., Waguri, S., Kawahara, N., Kuida, K., Nagata, S., Kominami, E., Tanaka K, & Uchiyama, Y. (2008). Inhibition of autophagy prevents hippocampal pyramidal neuron death after hypoxic-ischemic injury. *American Journal of Pathology*, 172(2), 454-69.

Korolchuk, V. I., Menzies, F. M. & Rubinsztein, D. C. (2010). Mechanisms of cross-talk between the ubiquitin-proteasome and autophagy-lysosome systems. FEBS Letter, 584(7), 1393-8.

Kourtis, N. & Tavernarakis, N. (2009). Autophagy and cell death in model organisms. *Cell Death & Differentiation*, 16, 21-30.

Krantic, S., Mechawar, N., Reix, S. & Quirion, R. (2005). Molecular basis of programmed cell death involved in neurodegeneration. *Trends in Neuroscience*, 28, 670-6.

Kreutzberg, G. W. (1996). Microglia: a sensor for pathological events in the CNS. *Trends in Neuroscience*, 9:312–8.

Kroemer, G., & Levine, B. (2008). Autophagic cell death: the story of a misnomer. *Nature Reviews Molecular Cell Biology*, 9(12), 1004-10.

Levy Lahad, E., Wasco, W., Poorkaj, P., Romano, D. M., Oshima, J., Pettingell, W. H., Yu, C. E., Jondro, P. D., Schmidt, S. D. & Wang, K. (1995). Candidate gene for the chromosome 1 familial Alzheimer's disease locus. *Science*, 269, 973-7.

Li, J. & Yuan, J. (2008). Caspases in apoptosis and beyond. Oncogene, 27:6194-6204.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S. & Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*, 91, 479-89.

Lindsten, T., Golden, J.A., Zong, W.X., Minarcik, J., Harris, M.H. & Thompson, C.B. (2003). The proapoptotic activities of Bax and Bak limit the size of the neural stem cell pool. *Journal of Neuroscience*, 23, 11112-9.

Liu, J., Liu, M. C. & Wang, K. K. (2008). Calpain in the CNS: from synaptic function to neurotoxicity. *Science Signaling*, 1(14), 1-7. doi: 10.1126/stke.114re1

Lockshin, R. A. & Williams, C. M. (1965). Programmed Cell Death—I. Cytology of Degeneration in the Intersegmental Muscles of the Pernyi Silkmoth. *Journal of Insect Physiology*, 11, 123-33.

Luo, S. & Rubinsztein, D. C. (2010). Apoptosis blocks Beclin 1-dependent autophagosome synthesis: an effect rescued by Bcl-xL. *Cell Death & Differentiation*, 17(2), 268-77.

Ma, D. K., Kim, W. R., Guo, L. M. & Song, H. (2009). Activity-dependent extrinsic regulation of adult hippocampal and olfactory bulb neurogenesis. *Annals of the New York Academy of Sciences*, 1170, 664-73.

Machado, V. M., Morte, M. I., Carreira, B. P., Azevedo, M. M., Takano, J., Iwata, N., Saido, T. C., Asmussen, H., Horwitz, A. R., Carvalho, C. M. & Araujo, I. M. (2015). Involvement of calpains in adult neurogenesis: implications for stroke. *Frontiers in Cellular Neuroscience*, 9(22), 1-9. doi: 10.3389/fncel.2015.00022

Madden, D. T., Egger, L. & Bredesen, D. E. (2007). A calpain-like protease inhibits autophagic cell death. *Autophagy*, 3(5), 519-522.

Meijer, A. J. & Codogno, P. (2004). Regulation and role of autophagy in mammalian cells. *International Journal of Biochemistry*, 36, 2445-62.

Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M.D., Klionsky, D.J., Ohsumi, M. & Ohsumi, Y. (1998). A protein conjugation system essential for autophagy. *Nature*, 395, 395-8.

Mizushima, N., Yamamoto, A., Matsui, M., Yoshimori, T. & Ohsumi, Y. (2004). In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Molecular Biology of the Cell*. 15, 1101-11.

Mizushima, N. (2009). Methods for monitoring autophagy using GFP-LC3 transgenic mice. *Methods is Enzymology*, 452, 13-23.

Ming, G.L. & Song, H. (2005) Adult neurogenesis in the mammalian central nervous system. *Annual Review of Neuroscience*, 28, 223-50.

de la Monte, S. M. (2009). Insulin resistance and Alzheimer's disease. *BMB Report*, 42(8), 475-481.

Mori, F., Okada, M., Tomiyama, M., Kaneko, S., and Wakabayashi, K. (2005). Effects of ryanodine receptor activation on neurotransmitter release and neuronal cell death following kainic acid-induced status epilepticus. *Epilepsy Research*, 65, 59-70.

Mosser, D. D., Caron, A.W., Bourget, L., Meriin, A. B., Sherman, M. Y., Morimoto, R. I. & Massie B. (2000). The chaperone function of hsp70 is required for protection against stress-induced apoptosis. *Molecular and Cellular Biology*, 20(19):7146-59.

Nixon, R. A. (2013). The role of autophagy in neurodegenerative disease. *Nature Medicine*, 19: 983–97.

Nottebohm, F. (1985). Neuronal replacement in adulthood. *Annals of the New York Academy of Sciences*, 457, 143-61.

Nottebohm, F. (1989). From bird song to neurogenesis. *Scientific American*, 260(2), 74-9.

Ohsumi, Y. (2001). Molecular dissection of autophagy: two ubiquitin-like systems. *Nature Reviews Molecular Cell Biology*, 2, 211-6.

Oppenheim, R.W. (1991). Cell death during development of the nervous system. *Annual Review of Neuroscience*, 14, 453-501.

Oules, B., Del Prete, D., Greco, B., Zhang, X., Lauritzen, I., Sevalle, J., Moreno, S., Paterlini-Brechot, P., Trebak, M., Checler, F., Benfenati, F. & Chami, M. (2012). Ryanodine receptor blockade reduces amyloid-beta load and memory impairments in Tg2576 mouse model of Alzheimer disease. *Journal of Neuroscience*, 32, 11820-34.

Palop, J.J., Jones, B., Kekonius, L., Chin, J., Yu, G.Q., Raber, J., Masliah, E. & Mucke, L. (2003). Neuronal depletion of calcium-dependent proteins in the dentate gyrus is tightly linked to Alzheimer's disease-related cognitive deficits. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 9572-7.

Palmer, T.D., Ray, J. & Gage, F.H. (1995). FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. *Molecular and Cellular Neurosciences*, 6(5), 474-86. doi:10.1006/mcne.1995.1035.

Palmer, T. D., Takahashi, J. & Gage, F. H. (1997). The adult rat hippocampus contains primordial neural stem cells. Molecular and Cellular Neuroscience, 8(6), 389-404.

Pandey, U. B., Batlevi, Y., Baehrecke, E. H. & Taylor, J. P. (2007). HDAC6 at the intersection of autophagy, the ubiquitin-proteasome system and neurodegeneration. *Autophagy*, 3(6), 643-5.

Pickart, C. M. (2001). Mechanisms underlying ubiquitination. *Annual Review of Biochemistry*, 70(1), 503-33.

van Praag, H., Kempermann, G. & Gage, F. H. (1999). Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nature Neuroscience*, 2, 266-70.

de la Rosa, E. J. & de Pablo, F. (2000). Cell death in early neural development: beyond the neurotrophic theory. *Trends in Neuroscience*, 23(10), 454-8.

Rosenberg, S.S. & Spitzer, N.C. (2011). Calcium signaling in neuronal development. *Cold Spring Harbor Perspectives in Biology*, 3, a004259. doi: 10.1101/cshperspect.a004259.

Saito, K., Elce, J. S., Hamos, J. E. & Nixon, R. A. (1993). Widespread activation of calcium-activated neutral proteinase (calpain) in the brain in Alzheimer disease: a potential molecular basis for neuronal degeneration. *Proceedings of the National Academy of Sciences of the United States of America*, 90(7), 2628-32.

Santos, D. M., Xavier, J. M., Morgado, A. L., Sola, S. & Rodrigues, C. M. (2012). Distinct regulatory functions of calpain 1 and 2 during neural stem cell self-renewal and differentiation. *PLoS One*, 7(3), e33468. doi: 10.1371/journal.pone.0033468

Shen, H. M., & Codogno, P. (2011). Autophagic cell death: Loch Ness monster or endangered species? *Autophagy*, 7(5), 457-65.

Sherrington, R., Rogaev, E. I., Liang, Y., Rogaeva, E. A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G. & Holman, K. (1995). Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature*, 375, 754–760

Schin, K. S. & Clever, U. (1965). Lysosomal and free acid phosphatase in salivary glands of chironomus tentans. *Science*, 150(3699), 1053-5.

Shintani, T., & Klionsky, D. J. (2004). Autophagy in health and disease: a double-edged sword. Science, 306(5698), 990-5. doi: 10.1126/science.1099993

Snyder, J. S. , Hong, N. S., McDonald, R. J. & Wojtowicz, J. M. (2005). A role for adult neurogenesis in spatial long-term memory. *Neuroscience*, 130, 843-52.

Stadelmann, C., Deckwerth, T.L., Srinivasan, A., Bancher, C., Bruck, W., Jellinger, K. & Lassmann, H. (1999). Activation of caspase-3 in single neurons and autophagic granules of granulovacuolar degeneration in Alzheimer's disease. Evidence for apoptotic cell death. *American Journal of Pathology*, 155, 1459-66.

Supnet, C., Noonan, C., Richard, K., Bradley, J. & Mayne, M. (2010). Up-regulation of the type 3 ryanodine receptor is neuroprotective in the TgCRND8 mouse model of Alzheimer's disease. *Journal of Neurochemistry*, 112, 356-65.

Talbot, K., Wang, H. Y., Kazi, H., Han, L. Y., Bakshi, K. P., Stucky, A., Fluino, R. L., Kawaguchi, K. R., Samoyedny, A. J., Wilson, R. S., Aryanitakis, Z., Schneider, J. A., Wolf, B. A., Bennett, D. A., Trojanowski, J. Q. & Arnold, S. E. (2012). Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resistance, IRS-1 dysregulation, and cognitive decline. *Journal of Clinical Investigation*, 122(4), 1316-38.

Tsujimoto, Y. & Shimizu, S. (2005). Another way to die: autophagic programmed cell death. *Cell Death & Differentiation*, 12 Suppl 2, 1528-34. doi: 10.1038/sj.cdd.4401777

Tu, H., Nelson, O., Bezprozvanny, A., Wang, Z., Lee, S., Hao, Y., Serneels, L., De Strooper, B., Yu, G. & Bezprozvanny, I. (2006). Presenilins Form ER Ca2+ Leak Channels, a Function Disrupted by Familial Alzheimer's Disease-Linked Mutations. *Cell*, 126(5), 981-93.

Uchiyama, Y., Koike, M., Shibata, M., & Sasaki, M. (2009). Autophagic neuron death. *Methods in Enzymology*, 453, 33-51.

Vila, M. & Przedborski, S. (2003). Targeting programmed cell death in neurodegenerative diseases. *Nature Reviews Neuroscience*, 4, 365-75.

Webb, S.E. & Miller, A.L. (2003). Calcium signalling during embryonic development. *Nature Reviews Molecular Cell Biology*, 4, 539-51.

Willette, A.A., Bendlin, B.B., Starks, E.J., Birdsill, A.C., Johnson, S.C., Christian, B.T., Okonkwo, O.C., La Rue, A., Hermann, B.P., Koscik, R.L., Jonaitis, E.M., Sager, M.A. & Asthana, S. (2015). Association of Insulin Resistance With Cerebral Glucose Uptake in Late Middle-Aged Adults at Risk for Alzheimer Disease. *JAMA Neurology*, 72, 1013-20.

Wu, B., Yamaguchi, H., Lai, F.A. & Shen, J. (2013). Presenilins regulate calcium homeostasis and presynaptic function via ryanodine receptors in hippocampal neurons. *Proceedings of the National Academy of Sciences of the United States of America*, 110, 15091-6.

Yamamoto, A., Tagawa, Y., Yoshimori, T., Moriyama, Y., Masaki, R. & Tashiro, Y. (1998). Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. *Cell Structure and Function*, 23(1), 33-42.

Yamasaki, T.R., Blurton-Jones, M., Morrissette, D.A., Kitazawa, M., Oddo, S. & LaFerla, F.M. (2007). Neural stem cells improve memory in an inducible mouse model of neuronal loss. *Journal of Neuroscience*, 27, 11925-33.

Yang, Z. & Klionsky, D. J. (2010). Mammalian autophagy: core molecular machinery and signaling regulation. *Current Opinion in Cell Biology*, 22, 124-31.

Yano, S., Tokumitsu, H. & Soderling, T.R. (1998). Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. *Nature*, 396, 584-7.

Yeo, B. K., Hong, C. J., Chung, K. M., Woo, H., Kim, K., Jung, S., Kim, E. K. & Yu, S. W. (2016). Valosin-containing protein is a key mediator between autophagic cell death and apoptosis in adult hippocampal neural stem cells following insulin withdrawal. *Molecular Brain*, 9, 31. doi: 10.1186/s13041-016-0212-8.

Young, J.E., Martinez, R.A. & La Spada, A.R. (2009). Nutrient deprivation induces neuronal autophagy and implicates reduced insulin signaling in neuroprotective autophagy activation. *Journal of Biological Chemistry*, 284, 2363-73.

Yousefi, S., Perozzo, R., Schmid, I., Ziemiecki, A., Schaffner, T., Scapozza, L., Brunner, T. & Simon, H. U. (2006). Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. *Nature Cell Biology*, 8(10), 1124-32. doi: 10.1038/ncb1482

Yu, S. W., Baek, S. H., Brennan, R. T., Bradley, C. J., Park, S. K., Lee, Y. S., Jun, E. J., Lookingland, K. J., Kim, E. K. & Kim, S. W. (2008). Autophagic death of adult hippocampal neural stem cells following insulin withdrawal. *Stem Cells*, 26(10), 2602-10.

Zampese, E., Brunello, L., Fasolato, C. & Pizzo, P. (2009). Ca^{2+} dysregulation mediated by presenilins in familial Alzheimer's disease: Causing or modulating factor? *Current Trends in Neurology*, 3, 1–14.

Zampese, E., Fasolato, C., Pozzan, T. & Pizzo, P. (2011). Presenilin-2 modulation of ER-mitochondria interactions: FAD mutations, mechanisms and pathological consequences. *Communicative & Integrative Biology*, 4(3), 357-60.

Zhivotovsky, B. & Orrenius, S. (2011). Calcium and cell death mechanisms: a perspective from the cell death community. *Cell Calcium*, 50, 211-21.

Zhu, C., Qiu, Li., Wang, X., Hallin, U., Cande, C., Kroemer, G., Hagberg, H. & Blomgren, K. (2003). Involvement of apoptosis-inducing factor in neuronal death after hypoxia-ischemia in the neonatal rat brain. *Journal of Neurochemistry*, 86, 306-17.

Zhu, K., Dunner, K., Jr. & McConkey, D. J. (2010). Proteasome inhibitors activate autophagy as a cytoprotective response in human prostate cancer cells. *Oncogene*, 29(3), 451-62.