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Parkin Promotes Mitophagic Cell Death in Adult Hippocampal Neural Stem Cells Following Insulin Withdrawal

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2020
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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.

11.26.2019

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Parkin Promotes Mitophagic Cell Death in Adult Hippocampal Neural Stem Cells Following Insulin Withdrawal

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Accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Regulated cell death (RCD) plays a fundamental role in human health and disease. Apoptosis is the best-studied mode of RCD, but the importance of other modes has recently been gaining attention. We have previously demonstrated that adult rat hippocampal neural stem (HCN) cells undergo autophagy-dependent cell death (ACD) following insulin withdrawal. Here, I show that Parkin mediates mitophagy and ADCD in insulin-deprived HCN cells. Insulin withdrawal increased the amount of depolarized mitochondria and their colocalization with autophagosomes. Insulin withdrawal also upregulated both mRNA and protein levels of Parkin, gene knockout of which prevented mitophagy and ADCD. c-Jun is a transcriptional repressor of Parkin and is degraded by the proteasome following insulin withdrawal. In insulin-deprived HCN cells, Parkin is required for Ca\textsuperscript{2+} accumulation and depolarization of mitochondria at the early stages of mitophagy as well as for recognition and removal of depolarized mitochondria at later stages. In contrast to the pro-death role of Parkin during mitophagy, Parkin deletion rendered HCN cells susceptible to apoptosis, revealing distinct roles of Parkin depending on different modes of RCD. Taken together, these results indicate that Parkin is required for the induction of ADCD accompanying mitochondrial dysfunction in HCN cells following insulin withdrawal. Since impaired insulin signaling is implicated in hippocampal deficits in various neurodegenerative diseases and psychological disorders, these findings may help to understand the mechanisms underlying the death of neural stem cells and develop novel therapeutic strategies aiming to improve neurogenesis and survival of neural stem cells.

Keywords: autophagy-dependent cell death, c-Jun, hippocampal neural stem cells, mitophagy, Parkin
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### List of Abbreviations

| Abbreviation | Description                              |
|--------------|------------------------------------------|
| Actb         | β-actin                                  |
| AD           | Alzheimer's disease                      |
| ADCD         | Autophagy-dependent cell death           |
| AMPK         | AMP-activated protein kinase              |
| Atg          | Autophagy-related                        |
| Aβ           | Amyloid β                                |
| Baf.A1       | Bafilomycin A1                           |
| C.Casp3      | Cleaved caspase 3                        |
| CMA          | Chaperone-mediated autophagy             |
| CNS          | Central nervous system                   |
| DFCP1        | FYVE domain-containing protein 1         |
| DG           | Dentate gyrus                            |
| DISC         | Death-inducing signaling complex         |
| ER           | Endoplasmic reticulum                    |
| ETC          | Electron transport chain                 |
| Abbreviation | Full Form |
|--------------|-----------|
| ETO          | Etoposide |
| FACS         | Fluorescence-activated cell sorting |
| fAD          | familial Alzheimer's disease |
| GSK3-3β      | Glycogen synthase kinase-3β |
| HCN          | Hippocampal neural stem |
| HD           | Huntington's disease |
| HOIL-1/Rbck1 | Heme-oxidized IRP2 ubiquitin ligase-1 |
| HSPA8/HSC70  | heat shock 70 kDa protein 8 |
| IGF          | Insulin/insulin-like growth factor |
| IMM          | Inner mitochondrial membrane |
| IPCs         | Intermediate progenitor cells |
| JNK          | c-Jun NH2 terminal kinase |
| KD           | Knockdown |
| KO           | Knockout |
| Lacta        | Lactacystin |
| LAMP2A       | Lysosomal-associated membrane protein 2A |
LC3   Microtubule-associated proteins 1A/1B light chain 3B
MOM   Mitochondrial outer membrane
MOMP  Mitochondrial outer membrane
mRFP-GFP-LC3 Monomeric RFP-GFP tandem fluorescent LC3
mtDNA Mitochondrial DNA
mTOR  Mammalian target of rapamycin
NSCs  Neural stem cells
OMM   Outer mitochondrial membrane
OXPHOS Oxidative phosphorylation
PBS   Phosphate-buffered saline
PD    Parkinson's disease
PI    Propidium iodide
PI3P  Phosphatidylinositol 3-phosphate
PINK1 Phosphatase and tensin homologue (PTEN)-induced putative kinase 1
qRT-PCR Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction
RCD   Regulated cell death
RIP  Receptor-interacting protein
RIPA  Radioimmunoprecipitation assay
Rnf19a/Dofrin  Ring finger protein 19A
ROS  Reactive oxygen species
RyR3  Type 3 ryanodine receptor
SgRNA  Single guide RNA
SGZ  Subgranular zone
SiRNAs  Small interfering RNAs
STS  Staurosporine
SVZ  Subventricular zone
TBST  Tris-buffered saline with 0.1% Tween 20
TFAM  Mitochondrial transcription factor A
TNFα  Tumor necrosis factor α
TRAIL  TNF-related apoptosis-inducing ligand
TUNEL  Terminal Deoxynucleotidyl Transferase (TdT) dUTP Nick End Labeling
Ub  Ubiquitin
WT        Wild-type
I. Introduction

1.1 Neural stem cells (NSCs) and neurogenesis

NSCs are multipotent stem cells present in neurogenic niches in the brain. NSCs not only possess self-renewal ability, but also can proliferate and differentiate into neural lineage cell types, mainly neurons, astrocytes, and oligodendrocytes (Temple, 2001; Doetsch, 2003). Neurogenesis, which refers to the production of neurons from NSCs, has been known to occur during embryonic development in the mammalian central nervous system. In the 1960s, however, Altman and Das observed the generation of new neurons in the adult rat brain, which revealed adult neurogenesis exists (Altman and Das, 1965).

Adult neurogenesis occurs in specific brain regions of the mammalian central nervous system (CNS) called neurogenic regions (Alvarez-Buylla and Lim, 2004). Adult NSCs are present in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (Figure 1; Gage, 2000; Ming and Song, 2005). NSCs in adult SVZ differentiate to immature neurons and then migrate into the olfactory bulb. NSCs in adult SGZ proliferate and differentiate into immature granule neurons. These newborn neurons from SGZ are incorporated in the hippocampus. Olfactory neurogenesis is implicated in the regulation of sensory experience (Lledo and Saghatelyan, 2005), whereas adult hippocampal neurogenesis is implicated in hippocampal learning and memory, and is impaired in the aged or injured brain (Shors et al., 2001; Rodriguez et al., 2008). Given their highly dynamic nature and differentiation potential, NSCs residing in the neurogenic niches must be under tight control in terms of metabolism, mitochondrial homeostasis, and autophagy level. Of relevance to this notion, a recent report on the characteristics of mt-Keima mice, an in vivo model of mitophagy, suggested a high basal level of mitophagy in the DG areas of the adult hippocampus (Sun et al., 2015).
FIGURE 1 | Adult neurogenesis in spontaneous neurogenic regions and non-neurogenic regions. Neurogenic regions possessing active neural stem cells (NSCs): In the adult brain, neurogenesis in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricle actively supplies newly generated cells. Modified from (Ryu et al., 2016).
1.2 NSC and mitochondria

In the stem cell field, there has been little interest in the role and importance of mitochondria in stem cells. This is because most stem cell populations undergo glycolytic metabolism with little dependence on mitochondrial oxidative phosphorylation (OXPHOS) for ATP production (Figure 2; St. John et al., 2005; Kondoh et al., 2007; Prigione et al., 2010; Folmes et al., 2011). However, recent studies have reported that stem cell abnormalities are induced by abnormal mitochondrial function, increasing the opinion that mitochondria is important for the normal functioning of stem cells (Norddahl et al., 2011; Fox et al., 2012; Berger et al., 2016). In other words, mitochondria are important regulators that regulate stem cell fate by participating in stem cell signaling and epigenetic modulation as well as ATP production in stem cells (Figure 3). Recently, experimental results supporting this suggestion have been reported.

For normal mitochondrial activation, the stability of mitochondrial DNA (mtDNA) should be maintained without mutations, and mitochondrial dynamics such as fusion and fission should occur normally. Mutation in mtDNA can lead to dysfunction of electron transport chain (ETC) comprising complex I–IV and finally change in ROS production. Stem cells become committed cells through the commitment stage, and then differentiated cells through the differentiation process. In this process, stem cells generally maintain low levels of reactive oxygen species (ROS), while ROS levels increase as they enter differentiation stages (Figure 2; Le Belle et al., 2011; Lyublinskaya et al., 2015). The regulation of stem cell commitment and differentiation by ROS provided evidence that mitochondria are key organelles in determining stem cell fate.

1.2.1 Mitochondria in adult neurogenesis

Metabolic processes of mitochondria are important for regulating self-renewal and
FIGURE 2 | Metabolic and redox changes during stem cell commitment. Stem cell populations undergo glycolytic metabolism with little dependence on mitochondrial oxidative phosphorylation (OXPHOS) for ATP production. Stem cells generally maintain low levels of reactive oxygen species (ROS), while ROS levels increase as they enter differentiation stages.
FIGURE 3 | Mitochondrial metabolism and downstream metabolite signaling in stem cells. Mitochondrial metabolites generated by the tricarboxylic acid (TCA) cycle and the electron transport chain (ETC), including ATP, reactive oxygen species (ROS), and TCA metabolites, serve as signaling molecules to regulate several aspects of stem cell function.

**ROS**
- Stem cell commitment
- Differentiation

**ATP**
- Stem cell commitment
- Survival and maturation

**Acetyl CoA**
- Stem cell maintenance

**NAD⁺:NADH ratio**
- Stem cell maintenance
- Stem cell maintenance
differentiation of NSCs during development. Recently, it has been reported that mitochondria play an important role in regulating adult neurogenesis as well as development (Almeida and Vieira, 2017; Beckervordersandforth, 2017; Khacho and Slack, 2018). Dysfunction of mitochondria (such as accumulation of mutation in mtDNA) alters mitochondrial respiration, mt ROS and eventually affects adult neurogenesis. Studies on the effects of mitochondria on NSC and adult neurogenesis in adult mice have been reported using a model of conditional deletion of genes related to mitochondrial respiration. Deletion of mitochondrial transcription factor A (TFAM) induced abnormalities in ETC and OXPHOS functions, resulting in a significant decrease in proliferation and survival of intermediate progenitor cells (IPCs) (Beckervordersandforth et al., 2017). On the other hand, when the ETC complex expression was restored, the proliferation of activated IPCs increased, and hippocampal neurogenesis was also rescued in aged mice (Beckervordersandforth et al., 2017).

The problem of mitochondrial dynamics also affects NSC fate decisions. The deletion of mitochondrial fusion proteins MFN1 and/or MFN2 caused impairment of NSC self-renewal in the adult hippocampus, which finally led to deficits in spatial learning memory (Khacho et al., 2016).

1.2.2 Adult neurogenesis and neurodegeneration.

Since the discovery of adult neurogenesis (Alvarez-Buylla and Garcia-Verdugo, 2002; Gage, 2002), NSC research has recently come into the spotlight because of its therapeutic potential to cure neurodegenerative diseases. Neurodegenerative diseases, including Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease (HD), induce specific neuronal loss depending on the disease, and similar pre-disease symptoms are observed in the early stage of neurodegenerative diseases. These pre-disease symptoms, such as olfactory dysfunction, depression, and cognitive dysfunction, are related to olfactory or hip-
pocampus function (Simuni and Sethi, 2008; Stout et al., 2011; Hinnell et al., 2012), the places where adult neurogenesis occurs. Therefore, alterations in adult neurogenesis are hallmark of various neurodegenerative diseases. Early cognitive impairment has been observed due to reduced adult neurogenesis, particularly in PD (Marxreiter et al., 2013) and AD (Hollands et al., 2016).

PD is characterized mainly by an array of motor impairments associated with the progressive death of dopaminergic neurons in the substantia nigra pars compacta (Dauer and Przedborski, 2003). PD also affects several neuronal systems and causes various nonmotor symptoms including neuropsychiatric manifestations and cognitive deficits such as early premotor dysfunction (Meissner et al., 2011). The relationship between nonmotor symptoms and adult neurogenesis can be confirmed by the PD mouse model, which has been reported to reduce neural precursor proliferation in SGZ and SVZ in 3-week-old mouse (Brandt et al., 2017). Another characteristic of PD is the accumulation of Lewy body inclusions formed by aggregation of α-synuclein. While adult neurogenesis was increased in α-synuclein-deficient mice, overexpression of α-synuclein not only reduced the survival of newborn hippocampal neurons but also decreased adult neurogenesis (Cebollero et al., 2012).

AD is the most common cause of dementia; it is characterized by the progressive accumulation of amyloid plaques and neurofibrillary tangles. In some familial AD (fAD) mouse models, impairment of neurogenesis was reported in the early stages of AD before the accumulation of amyloid β (Aβ) plaques, and progressive cognitive impairment was induced as aging progressed (Hamilton et al., 2015; Hollands et al., 2016; Hollands et al., 2017). Another characteristic of AD is mitochondrial dysfunction, such as changes in mitochondrial dynamics, biogenesis, and disruption of electron transport (Onyango et al., 2016). In some AD mouse models, it has been reported that normal restoration of mitochondria function increases adult neurogenesis and thus improves cognitive impairment. This has led to further support
for the pre-symptom of AD by dysregulation of adult neurogenesis due to mitochondria dysfunction. Activation of the Wnt pathway in the AD mouse model enhanced cognitive function by increasing proliferation and differentiation of NSCs (Zheng et al., 2017), and also Wnt signaling regulated mitochondrial dynamics in rat hippocampal neurons (Godoy et al., 2014).

1.3 RCD

Regulated cell death (RCD) is an evolutionarily conserved process and is tightly controlled by various intracellular signals and extracellular cues (Danial and Korsmeyer, 2004; Galluzzi et al., 2018). RCD is essential for normal development and maintenance of tissue homeostasis (Ellis et al., 1991; Coucouvanis and Martin, 1995). Therefore, dysregulation of RCD underlies a variety of human diseases, such as cancer, neurodegeneration, and autoimmunity. RCD is currently categorized into 12 distinct cell death subroutines, with intrinsic and extrinsic apoptosis, necroptosis, and autophagy-dependent cell death (ADCD) as main subroutines based on morphological and biochemical criteria (Figure 4; Galluzzi et al., 2018).

1.3.1 Apoptosis

Type I cell death, apoptosis, is distinguished from other cell death types by the following morphological features: cell shrinkage, plasma membrane blebbing, chromatin condensation, nuclear DNA fragmentation, and formation of apoptotic bodies (Galluzzi et al., 2007). Apoptosis is strictly regulated by an evolutionarily conserved family of cysteine proteases called caspases (Figure 4). Apoptosis is classified into two types, intrinsic and extrinsic apoptosis.

Intrinsic apoptosis is induced by various microenvironmental changes such as DNA
FIGURE 4 | The key players in regulated cell death (RCD). RCD is currently categorized into 12 distinct cell death subroutines, with apoptosis, necrosis, and autophagy-dependent cell death (ADCD) as main subroutines based on morphological and biochemical criteria. Modified from (Ryu et al., 2016).
damage, endoplasmic reticulum (ER) stress, growth factor withdrawal (Nunez et al., 1990; Czabotar et al., 2014; Roos et al., 2016; Pihán et al., 2017; Vitale et al., 2017). After these signals, pro-apoptotic proteins (Bax and Bak) are activated at the mitochondrial outer membrane (MOM) and form protein-permeable pores for mitochondrial outer membrane permeabilization (MOMP) (Tait and Green, 2010; Moldoveanu et al., 2014; Delbridge et al., 2016). Apoptogenic proteins, including cytochrome c, Smac/Diablo, and AIF, are released into the cytosol through these pores. Released cytochrome c forms apoptosome complex with APAF-1 and pro-caspase 9, and then caspase 9 activations were occurred by processing of pro-caspase 9 — subsequently, downstream caspase (caspase 3, caspase 6, and caspase 7) cascade activation proceeds (Zou et al., 1999). Finally, cells expressing phosphatidylserine at the membrane are eliminated by phagocytic cells.

Extrinsic apoptosis is induced by ligands, including tumor necrosis factor α (TNFα), Fas/CD95, and TNF-related apoptosis-inducing ligand (TRAIL) at death receptors (Aggarwal et al., 2012; Fleten et al., 2016). Death-inducing signaling complex (DISC) is formed at the intracellular tail of the death receptor, which serves as platforms for activation of caspase 8 (Boldin et al., 1996; Muzio et al., 1996; Dickens et al., 2012). Truncated Bid, which is cleaved by activated caspase 8 is translocated to mitochondria, followed by releasing cytochrome c.

### 1.3.2 Necrosis

Although death receptors are activated by death ligands, cells die of necrosis in conditions where apoptosis does not occur. Inhibition of caspase activity or low intracellular ATP concentration, which are insufficient to promote apoptosis, induce necrosis. Receptor-interacting protein (RIP) kinases are regulators of necrosis (Degterev et al., 2008). In particular, RIP1 and RIP3 are activated to form a necrosome complex, resulting in mitochondrial...
permeability transition pore opening (Figure 4). This causes abnormality in mitochondrial respiratory function, which induces ATP depletion in cells. As a consequence, a rapid collapse of the plasma membrane and release of cytoplasmic components are observed (Rello et al., 2005). Recent progress in the understanding of the regulatory mechanisms of necrosis suggests its regulatable nature and has led to the coining of the term “necroptosis” (Galluzzi and Kroemer, 2008; Moriwaki et al., 2016).

1.3.3 Autophagy

Autophagy (“self-eating” in Greek) is a lysosome-dependent catabolic process characterized morphologically by an increased formation of autophagic vesicles (autophagosomes and autolysosomes) (Yang and Klionsky, 2010). Autophagy is essential for the removal of cytoplasmic materials, including long-lived proteins, dysfunctional organelles, and toxic intracellular components (Shintani and Klionsky, 2004). After degradation processes, cellular constituents such as nucleotides, amino acids, fatty acids, and sugars are released into the cytosol to reuse in metabolic pathways. Thereby, autophagy generally has protective and adaptive functions in response to cellular stress.

1.3.3.1 Three types of autophagy

In mammalian cells, there are three major types of autophagy: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy (Figure 5). Microautophagy refers to a process by which cytosolic substances directly enter the lysosome through an invagination of the lysosomal membrane (Kunz et al., 2004). CMA is a type of autophagy that transports a protein or molecule directly into the lysosome and then degrades it through chaperone proteins on the lysosomal membrane. CMA is highly specific because all CMA substrates have a pentapeptide KFERQ consensus motif (Dice, 1990). The heat shock 70 kDa
FIGURE 5 | Three types of autophagy. This figure shows three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy.
protein 8 (HSPA8/HSC70) complex recognizes proteins containing the KFERQ consensus motif and transfers the target protein to the lysosomal membrane. Translocated target protein binds to CMA substrate receptor called lysosomal-associated membrane protein 2A (LAMP2A) at the lysosomal membrane and then moves into the lysosome for degradation (Agarraberes and Dice, 2001).

Macroautophagy is classically referred to as autophagy. Autophagy is mediated by autophagy-related (Atg) genes (Figure 6; Klionsky et al., 2011). It begins by phagophore formation and elongation to generate double-membraned autophagosomes. For this process, microtubule-associated proteins 1A/1B light chain 3B (LC3, the mammalian homologue of the yeast Atg8 protein) is cleaved by Atg4 to produce LC3-I, which is a cytosolic form of LC3 (Satoo et al., 2009). When autophagosome formation is triggered, LC3-I is conjugated to phosphatidylethanolamine to form LC3-II, which is then recruited to the expanding phagophore membranes to facilitate autophagosome formation (Ichimura et al., 2000). Autophagosomes then mature into autolysosomes through fusion with the lysosomes for degradation and recycling of the autophagy cargos. An increase in the amount of LC3-II or the number of LC3 puncta in autophagic vesicles can serve as an indicator of increased autophagy flux (Klionsky et al., 2016). p62 (sequestosome 1/SQSTM1) is a ubiquitin-binding autophagy adaptor protein, which becomes incorporated into the autophagosomes together with the delivered cargos for autophagic degradation (Pankiv et al., 2007). Thus, the degradation of p62 is regarded as another index of autophagy flux (Ichimura et al., 2008).

1.3.4 Mitophagy

Autophagy can be classified into non-selective and selective autophagy (Figure 7). Unlike non-selective mechanisms that break down entire vesicle contents, selective autophagy can only degrade specific cargoes. In other words, autophagosomes can form around a
**FIGURE 6 | Autophagy machinery and process.** Autophagy begins by phagophore formation and elongation to generate double-membraned autophagosomes. Autophagosomes then mature into autolysosomes through fusion with the lysosomes for degradation and recycling of the autophagy cargos.
Non-selective autophagy degrades a range of cytosolic contents, including proteins and many types of organelles. By contrast, mitophagy occurs to eliminate damaged or defective mitochondria. Mitochondria are selectively recruited into the isolation membrane, and then fuse with the lysosome.
particular cargo such as mitochondria, peroxisomes, and other specific organelles, excluding the rest of the cytoplasm.

Mitophagy is selective autophagy that degrades damaged or defective mitochondria (Figure 7). Molecular pathways of mitophagy are classified as ubiquitin-dependent or -independent (Khaminets et al., 2016). First, phosphatase and tensin homologue (PTEN)-induced putative kinase 1 (PINK1)-Parkin-dependent mitophagy is the most representative of the ubiquitin-dependent pathway (Pickles et al., 2018). In healthy mitochondria, PINK1 is transferred to inner mitochondrial membrane (IMM) and then it is cleaved by several proteases to form truncated forms (Figure 8; Harper et al., 2018). This truncated PINK1 is degraded by ubiquitin-proteasome system. In contrast, in damaged or depolarized mitochondria, PINK1 is located in outer mitochondrial membrane (OMM), not translocated to IMM (Figure 8; Harper et al., 2018; Sekine and Youle, 2018). PINK1, activated by autophosphorylation in OMM, phosphorylates ubiquitin (Ub) and poly-Ub chains and recruits cytosolic Parkin to mitochondria. Parkin is phosphorylated by PINK1, resulting in conformational change of it, which is activated and has E3 ligase activity (Aguirre et al., 2017). Activated Parkin continues to form poly-Ub chains, resulting in Parkin-mediated signal amplification (Ordureau et al., 2014). Because poly-Ub chains are phosphorylated by PINK1, autophagy adaptor proteins such as p62 recognize and bind them. After this, LC3 binds to adaptor proteins, and autophagosome formation is initiated. Second, receptor-mediated mitophagy is an example of ubiquitin-independent mitophagy. Autophagy receptors such as FUNDC1, NIX, BNIP3 can also progress mitophagy by directly binding to LC3 at OMM (Sandoval et al., 2008; Liu et al., 2012; Palikaras et al., 2016).
FIGURE 8 | PINK1/Parkin-dependent mitophagy. In healthy mitochondria, PINK1 is transferred to inner mitochondrial membrane (IMM), and then it is cleaved by several proteases to form truncated forms. By contrast, in damaged mitochondria, PINK1 is stabilized at outer mitochondrial membrane (OMM) and activates Parkin. Activated Parkin continues to form poly-Ub chains, resulting in Parkin-mediated signal amplification.
1.4 ADCD

Contrary to the general notion of autophagy as a survival mechanism, there are reports that excessive or prolonged autophagy can induce cell death (Tsujimoto and Shimizu, 2005). Together with the frequent observation of autophagy in cells undergoing RCD, these findings led to the concept of ADCD. ADCD, previously also known as autophagic cell death, was first proposed based on widespread morphological association of autophagy with cell death (Shen and Codogno, 2011), but without implication of the causative role of autophagy in cell death (Tsujimoto and Shimizu, 2005). Therefore, it is still contentious whether autophagy plays a causative role in cell death, especially in mammals. Taking this controversy into account, ADCD is suggested as a type of RCD that requires autophagic machinery without participation of other cell death pathways (Galluzzi et al., 2018).

1.4.1 Insulin withdrawal model in hippocampal neural stem cells

Insulin/insulin-like growth factor (IGF) family proteins are vital extracellular cues for the differentiation and survival of NSCs in the hippocampus (Åberg et al., 2000; Lichtenwalner et al., 2001). As such, adult hippocampal NSCs, abbreviated as HCN cells following the original description of their isolation, depend on insulin/IGF for survival in in vitro culture (Palmer et al., 1997). Interestingly, we found that insulin-deprived HCN cells undergo ADCD rather than apoptosis despite their intact apoptotic capability (Figure 9; Yu et al., 2008; Baek et al., 2009). Further study revealed that glycogen synthase kinase-3β (GSK3-3β) mediates ADCD in HCN cells (Yu et al., 2008; Baek et al., 2009; Ha et al., 2015). Pharmacological or genetic inactivation of GSK-3β decreased ADCD, while over-expression of the wild-type (WT) or constitutively active form of GSK-3β facilitated ADCD without apoptosis induction (Ha et al., 2015). Because a rise in the intracellular Ca^{2+} level is known to trigger autophagy (Høyer-Hansen et al., 2007), we next focused on the regulation of ADCD
FIGURE 9 | Insulin withdrawal drives the mode of cell death towards ADCD in adult hippocampal neural stem cells. Modified from (Hong et al., 2016).
by Ca\(^{2+}\). In insulin-deprived HCN cells, intracellular Ca\(^{2+}\) level increases, mainly owing to its release from the endoplasmic reticulum (ER) mediated by the type 3 ryanodine receptor (RyR3) (Chung et al., 2016). RyR3-mediated increase in cytosolic Ca\(^{2+}\) activates AMP-activated protein kinase (AMPK), which leads to novel phosphorylation of p62 and promotes mitophagy (Ha et al., 2017). Further study is needed to understand how mitophagy is regulated in insulin-deprived HCN cells.

1.5 Mitophagic cell death

Properly controlled mitophagy is beneficial for cells, but excessive mitophagy can lead to cell death, which is called mitophagic cell death. During mitophagic cell death, colocalization between mitochondria and autophagy markers increases, and the mitochondrial compartment reduced. Early findings supporting the potential for cell death through mitophagy include the smARF molecule. Overexpression of smARF, an alternative translation product of p19ARF (p14ARF in humans, also known as CDKN2A), induced mitochondrial depolarization and caspase-independent cell death, which did not occur when the autophagy genes were knocked down (Reef et al., 2006). Subsequently, several reports have reported that deregulated smARF expression induces PINK1-Parkin-dependent mitophagy (Budina-Kolomets et al., 2013; Grenier et al., 2014), and consequently summarizes that excessive mitophagy can induce cell death.

1.6 Parkin

Parkin is an E3 ubiquitin ligase, and more than 100 mutations in the Parkin-encoding Park2 gene are known to cause an autosomal recessive form of PD (Dawson and Dawson, 2010). An emerging role of Parkin is the regulation of mitophagy (Narendra et al., 2008). Since mitochondrial dysfunction is implicated in the pathogenesis of PD, the role of Parkin-
mediated mitophagy in the regulation of mitochondrial function and dynamics has gained great attention. However, the relevance of Parkin in these cognitive symptoms is not well understood.

Previous reports indicate that mutant Parkin proteins could induce PD through its dysfunctional role in mitophagy. We are interested in Parkin to dissect the molecular mechanisms underlying ADCD in HCN cells as Parkin is intimately associated with RCD and mitophagy. Thus, Parkin is a highly likely candidate to modulate the insulin withdrawal-induced ADCD in HCN cells – specifically through the regulation of mitochondrial dynamics and mitophagy.

The purpose of our research is to identify the mitochondria-associated modulation of autophagy and the subsequent cell death in HCN cells following insulin withdrawal. In particular, we investigated the Parkin regulation of mitochondrial dynamics and autophagy in relation to ADCD using the insulin withdrawal model of ADCD in HCN cells. Here we propose that Parkin promotes the mitophagic cell death of HCN cells upon insulin deprivation. These findings will help understanding the mechanisms of neuronal cell death and develop novel therapeutic strategies for neurodegenerative diseases.
II. Materials and Methods

2.1 Reagents and Antibodies

Antibodies against Parkin (4211), cleaved caspase 3 (9664), poly(ADP-ribose) polymerase (PARP) (9542), c-Jun (9165), and voltage-dependent anion channel (VDAC) (4866), phospho-SAPK/JNK (Thr183/Tyr185) (9251), horseradish peroxidase (HRP)-linked antimouse IgG (7076) were purchased from Cell Signaling Technology (Danvers, MA, United States). Antibodies against p62 (P0067, Sigma-Aldrich, Saint Louis, MO, United States), LC3B (100-2220, Novus Biologicals, City of Centennial, CO, United States), HRP-conjugated b-actin (47778, Santa Cruz Biotechnology, Dallas, TX, United States) and goat anti-rabbit IgG (HCL) secondary antibody (31460, Thermo Fisher Scientific, Carlsbad, CA, United States), PTEN-induced putative kinase 1 (PINK1) (BC100-494, Novus Biologicals) and ubiquitin phosphorylated at S65 residue (p-Ub-S65) (ABS1513-I, EMD Millipore, Burlington, MA, United States) were purchased at the indicated companies. Bafilomycin A1 (Baf.A1, Sigma-Aldrich), carbonyl cyanide 3-chlorophenylhydrazone (CCCP, Sigma-Aldrich), staurosporine (STS, Cell Signaling Technology), and necrostatin-1 (Invitrogen, Carlsbad, CA, United States) were purchased from the indicated companies.

2.2 Cell Culture

HCN cells were cultured as we previously reported (Chung et al., 2015; Chung et al., 2016). In brief, cells were grown in chemically composed serum-free medium containing Dulbecco’s modified Eagle’s medium/F-12 (12400-024, Thermo Fisher Scientific) supplemented with our own-made N2 components, which we made by mixing individual components including 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). The medium was adjusted to pH 7.2 after adding 1.27
g/l sodium bicarbonate (Sigma-Aldrich). Insulin was omitted to prepare I(+) medium. In this paper, I(+) and I(−) denote insulin-containing and insulin-deprived media, respectively.

2.3 Cell Death Assay

HCN cells were seeded in a 96-well plate at a density of $1.0 \times 10^5$ cells/ml. To assess cell death, cells were stained with Hoechst 33342 (Invitrogen) and propidium iodide (PI) (Sigma-Aldrich) and were imaged under a fluorescence microscope (Axiovert 40 CFL, Carl Zeiss, Oberkochen, Germany). We used DAPI filter for Hoechst stained cells and Cy3 filter for PI-positive cells. Images were obtained by AxioVision 4 module Multichannel software (Carl Zeiss) and were analyzed by Pixcavator student edition analysis software (Intelligent Perception Co.). The contrast value of the software was adjusted according to the brightness of the image, and total 7,000–8,000 cells were counted per condition, and more than three experiments were performed. The cell death rates were calculated as follows: Cell death (%) = (PI-positive cell number/Hoechst positive cell number) × 100.

2.4 Caspase 3 Activity Assay

HCN cells were seeded onto 96-well white plates, and caspase 3 activity was measured using a Caspase 3 activity assay kit (Promega, Madison, WI, United States) according to the manufacturer’s instructions. A freshly prepared Caspase 3 Glo reagent solution was added to cultured HCN cells, and luminescence was measured in a luminometer (SpectraMax L, Molecular Devices, San Jose, CA, United States) and was analyzed by SoftMax Pro Software (Molecular Devices). Luminescence values were normalized to protein concentration. We calculated the average value by analyzing duplet samples per condition and performed a total three repeat experiments.
2.5 Terminal Deoxynucleotidyl Transferase (TdT) dUTP Nick End Labeling (TUNEL) Assay

Cells were fixed in 4% paraformaldehyde for 25 min at 4°C, washed twice in phosphate-buffered saline (PBS), 5 min each time. After permeabilization with 0.2% Triton X-100 in PBS for 5 min, the assay was performed using the TUNEL System kit (G3250, Promega) according to the manufacturer’s instruction. In brief, 100 ml of equilibration buffer was added to the cells for 5–10 min at room temperature. After that, 50 ml TdT reaction mix was added for 60 min at 37°C. Cells were immersed in 2 × SSC buffer to stop reaction. Hoechst was added to stain nucleus, and the cells were mounted and analyzed by under a confocal microscope (LSM 700, Carl Zeiss).

2.6 Western Blotting

HCN cells were harvested and lysed on ice in radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific) with 1 × Halt Protease and Phosphatase inhibitor cocktail (Thermo Fisher Scientific), 1 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol. Lysates were centrifuged 12,000g for 10 min, and protein concentration was measured using a Pierce BCA protein assay kit (Thermo Fisher Scientific). Usually, 10–20 mg total protein was loaded per well. After electrophoresis, proteins were transferred to polyvinylidene fluoride membranes in a semidy electrophoretic transfer cell (Bio-Rad, Richmond, CA, United States). The membranes were blocked in blocking buffer consisting of 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature and incubated overnight with diluted primary antibodies. Each primary antibody was diluted in 5% bovine serum Albumin (Thermo Fisher Scientific) or 5% skim milk in TBST. After three washes with TBST, membranes were incubated with suitable HRP-conjugated secondary antibodies (10,000 dilutions) diluted in blocking buffer for 1 h. After 3 washes with TBST, proteins
were detected using Super Signal West Pico PLUS Chemiluminescent Substrate (34580, Thermo Fisher Scientific). SRX 201A (Konica Minolta Medical Imaging, Wayne, NJ, United States) was used to develop films. Obtained images were analyzed using Image J software, and each protein level was finally quantified after normalization via $\beta$-actin.

2.7 Mitochondrial and Cytosolic Fractionation

Subcellular fractionation was performed according to the published protocol (Dimauro et al., 2012) with a slight modification. HCN cells were homogenized by vortexing for 15 min in lysis buffer (250 mM sucrose, 50 mM Tris-HCl, pH 7.4, 5 mM MgCl$_2$, 1 $\times$ Halt protease, and phosphatase inhibitor cocktail) and kept on ice for 30 min. The homogenate was centrifuged at 600 $\times g$ for 15 min, and the supernatant ($S_0$) was further centrifuged at 11,000 $\times g$ for 10 min to separate the supernatant ($S_1$) and pellet ($P_1$). An equal volume of cold 100% acetone was added to $S_1$, and the samples were incubated overnight at $-20^\circ$C to precipitate the proteins. After centrifugation at 12,000 $\times g$ for 5 min, the pellet was resuspended in lysis buffer and used as the cytosolic fraction. $P_1$, which contained the mitochondrial fraction, was resuspended in lysis buffer and centrifuged at 11,000 $\times g$ for 10 min; the pellet ($P_2$) was resuspended in extraction buffer (50 mM Tris-HCl pH 6.8, 1 mM EDTA, 0.5% Triton-X 100, and protease and phosphatase inhibitor cocktails) and sonicated with a Bioruptor KRB-01 (CosmoBio, Japan) on ice 3 times for 10 s with 30 s intervals. We used this fraction as the mitochondrial fraction.

2.8 Plasmids and Transfection

Plasmids encoding RFP-LC3, EGFP-LC3, monomeric RFP-GFP tandem fluorescent LC3 (mRFP-GFP-LC3), and GFP-double FYVE domain-containing protein 1 (DFCP1) were purchased from Addgene (Cambridge, MA, United States).DsRed2-Mito was purchased
from Takara Bio United States (Mountain View, CA, United States) and Case12-mito from Evrogen (Moscow, Russia). Cells were seeded in a 6-well plate 24 h before transfection. Transfection was conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The Plasmids list is shown in Table 1.

| Plasmid name | Plasmid numbers | Characteristics | References |
|--------------|-----------------|-----------------|------------|
| pmRFP-LC3    | 21075           | Encoding a fusion of rat LC3 and mRFP | (Kimura et al., 2007) |
| pEGFP-LC3    | 21073           | Encoding a fusion of rat LC3 and EGFP | (Kabeya et al., 2000) |
| ptfLC3       | 21074           | Encoding a fusion of rat LC3 and mRFP and EGFP | (Kimura et al., 2007) |
| pMXs-puro GFP-DFCP1 | 38269 | Encoding a fusion of M. musculus DFCP1 and EGFP | (Itakura and Mizushima, 2010) |
| pDsRed2-Mito | 632421          | Encoding a fusion of Discosoma sp. red fluorescent protein (DsRed2) and a mitochondrial targeting sequence of human cytochrome c oxidase subunit VIII (Mito). | |
| pCase12-mito | FP992           | Encoding mitochondria-targeted fluorescent Ca^{2+} sensor Case12 | (Gorman, 1985) |

### 2.9 siRNA mediated knockdown by nucleofection

The seeding density of HCN cells was $1.0 \times 10^5$ cells per mL. Parkin was knocked down using on target plus SMART pool small interfering RNAs (siRNAs) against rat Park2 (Dharmacon; L-0907 09-02) and control non-targeting siRNAs (Dharmacon; D-001810-01-20). 100-200 nM of siRNAs were transfected to HCN cells using a Nucleofector Kit (Lonza; V4XP-4024) according to the manufacturer’s instructions. Transfected HCN cells were cultured in insulin-positive media without penicillin/streptomycin for 3-4 hours. After that, transfection media were replaced by normal culture medium.
2.10 Park2 and Pink1 Knockout

Rat Park2 single guide RNA (sgRNA), rat Pink1 sgRNA, and Cas9 were designed by and purchased from Toolgen (Republic of Korea). The target sequence for Park2 was 5’-ATCACTCGCAGCTGGTCAGCTGG-3’, and the target sequence for Pink1 was 5’-CCTGACACCGGCCCGCTTGG-3’. HCN cells were transfected with Park2 sgRNA or Pink1 sgRNA and Cas9 for 24 h and selected by hygromycin B (InvivoGen, San Diego, CA, United States).

2.11 Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

RNA was isolated from HCN cells using QIAzol lysis reagent (Qiagen, Germantown, MD, United States) and used to synthesize cDNA with an ImProm-II Reverse Transcriptase kit (Promega). A CFX96 Real-Time PCR detection system (Bio-Rad) and TOPreal qPCR 2X premix (Enzynomics, Republic of Korea) were used for real-time PCR with the following primers: rat Park2 forward (5’-ATG ATA GTG TTT GTC AGG TT -3’) and reverse (5’-AGA CAA AAA AGC TGT GGT AG-3’); rat ring finger protein 19A (Rnf19a/Dofrin) forward (5’-ATC TCC AAT CGT CTG CTT CGT CTG -3’) and reverse (5’-CGT TCA GTG CATTCT GGA CAA CTG-3’); rat heme-oxidized IRP2 ubiquitin ligase-1 (HOIL-1/Rbck1) forward (5’-ATG GAC GAG AAGACC AAG AAA GCA-3’) and reverse (5’-GTT GAG TGA TGT GTT GCG GGC T-3’); β-actin (Actb) forward (5’-AGC CAT GTA CGT AGC CAT CC-3’) and reverse (5’-CTC TCA GCT GTG GTG GTG AA-3’).

2.12 Flow Cytometry Analysis

Before harvesting, HCN cells were incubated with 100 nM MitoTracker Deep Red and 50 nM MitoTracker Green (Thermo Fisher Scientific) for 15 min. Harvested cells were
washed in cold PBS twice and analyzed using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, United States). MitoTracker Green and MitoTracker Deep Red-stained cells were detected by FL1 and FL3 channels, respectively. Total 30,000 cells were counted in each condition. For negative control, non-stained cells and single stained cells were counted. Data were analyzed by BD Accuri C6 software.

2.13 Immunocytochemistry and Quantification of Relative Fluorescence Intensity or Colocalization Coefficient

Cells were fixed in 4% paraformaldehyde or methanol for 5–10 min, washed in PBS twice and mounted on a glass slide with mount solution (Dako, Carpinteria, CA, United States). Samples were examined under a confocal microscope (LSM 700, Carl Zeiss). Relative fluorescence intensities of TMRE, Case12-mito, p-Ub-S65 were analyzed using Image J software. The cytosol region excluding nucleus area was drawn with drawing tools, and the integrated density of this region was measured. The relative fluorescence intensity in each experimental condition was quantified based on the mean value of the I(+) condition. The colocalization coefficient of mitochondria with LC3 or DFCP1 was analyzed using “colocalization” tool from Zen software (Carl Zeiss) and then quantified using colocalization coefficient following the guideline of the software.

2.14 Promoter Activity Assay

pGL3 and pRL-TK vectors (Promega) contain modified coding regions for firefly luciferase and Renilla luciferase, respectively. We used firefly luciferase as a reporter for the transcriptional activity of Park2 promoter. Renilla luciferase, which was expressed constitutively, served as a control for normalization. Cells were seeded in a 12-well plate 24 h before transfection, and pGL3-basic vectors carrying a series of truncated forms of the rat Park2
promoter region were co-transfected with pRLTK using Lipofectamine 2000. After transfection for 12 h, the cells were collected in I(−) medium and re-seeded in a 24-well plate. Luciferase assay was conducted using a Dual-Glo Luciferase Assay System (Promega) according to the manufacturer’s instructions. Freshly prepared Dual-Glo luciferase reagent solution was added to the cells and luciferase activity was measured in a white 96-well plate in a SpectraMax L luminometer. We measured and analyzed the data using “photon counting mode” of SoftMax Pro Software. We calculated the relative values of each result based on I(+).

2.15 Statistics

Data from at least three independent experiments were expressed as the mean ± standard error (SEM). “n = ” represents a number of cells, unless otherwise stated. Statistical significance was determined using the paired t-test for two-group experiments. For experiments with three or more groups, comparisons were made using one-way analysis of variance and Tukey’s test. GraphPad Prism 5 (GraphPad Software, San Diego, CA, United States) was used to analyze the data. Differences were considered statistically significant for p-values < 0.05.
III. RESULTS

3.1 Insulin Withdrawal Induces ADCD With Mitochondrial Alterations in HCN Cells

HCN cells depend on insulin for their survival and proliferation (Palmer et al., 1997). Insulin withdrawal significantly increased cell death (Figure 10A). HCN cells have about 10% basal cell death levels in I(+) media. However, in the condition where insulin was removed for 24 or 48 hours, the cells died by more than 20% and more than 40%, respectively. Consistent with our previous reports (Yu et al., 2008; Baek et al., 2009) on the non-apoptotic nature of insulin withdrawal-induced death of HCN cells, caspase 3 activity (Figure 10B) and the number of activated, cleaved caspase 3 (C.Casp3)-positive cells remained very low despite an increase in cell death, indicating no involvement of apoptosis (Figures 10C, D). On the other hand, a robust induction of caspase 3 activation by the well-known prototypical apoptosis inducer STS demonstrates normal apoptotic capability of HCN cells (Figures 10B–D). We also confirmed that DNA fragmentation, a marker of apoptosis, did not occur in insulin-deprived HCN cells (Figure 10E). Because TdT, an enzyme that catalyzes attachment of deoxynucleotides, labels blunt ends of double-stranded DNA breaks, we performed TUNEL assay to determine whether insulin-deprived HCN cells death is apoptosis. 63% of total cells were TUNEL-positive by STS-treatments, but TUNEL-positive cells were not observed in either I(+) or I(−) condition. Also, ineffectiveness of pan-caspase inhibitor Z-VAD against insulin withdrawal-induced death of HCN cells has been reported in our previous studies (Chung et al., 2015; Ha et al., 2015). These results support non-apoptotic nature of insulin withdrawal-induced HCN cell death. Besides, insulin-deprived HCN cells did not undergo necrosis. Necrostatin-1, an inhibitor of necroptosis, blocked cell death induced by \( \text{H}_2\text{O}_2 \) but did not prevent cell death induced by insulin withdrawal (Figure 10F). We also reported that suppression of autophagy by knockdown of Atg7 prevented HCN cell death following insulin withdrawal, indicating the causative role of autophagy in cell death (Yu et al., 2008). Charac-
terization of cell death mode excluded apoptosis and necroptosis. Therefore, we presumed that PI/Hoechst-based cell death assay approximately measures total cell death under insulin withdrawal condition.

To measure autophagic flux, immunofluorescence assay using mRFP-GFP-LC3 was performed. The GFP, but not RFP signal is readily quenched in acidic conditions, such as within the autolysosomes (Kimura et al., 2007). As a result, autophagosomes and autolysosomes are observed as yellow (RFP\(^{+}\)GFP\(^{+}\)) and red (RFP\(^{+}\)GFP\(^{-}\)) puncta, respectively (Kimura et al., 2007). In insulin-deprived HCN cells, the number of both the yellow and red LC3 puncta increased, indicating an increase in autophagy flux in comparison with I(+) HCN cells (Figures 11A, B). In addition, when autophagosome maturation was blocked by treatment with Baf.A1, which inhibits fusion between autophagosomes and lysosomes, accumulation of yellow LC3 puncta in I(−) HCN cells was much higher than without Baf.A1 treatment, confirming a robust biogenesis of autophagic vesicles in I(−) HCN cells (Figures 11A, B).

Taken together, insulin withdrawal-induced cell death meets the following criteria: (i) increased autophagic flux is observed (this study; Yu et al., 2008; Baek et al., 2009); (ii) suppression of autophagy prevents cell death (Yu et al., 2008); and (iii) alternative cell death pathways such as apoptosis and necrosis are not involved (this study; Yu et al., 2008; Chung et al., 2015; Ha et al., 2015). Thus, from the current and our previous data, we concluded that insulin-deprived HCN cells undergo ADCD.

Our previous observation suggests that insulin withdrawal induces an increase in cytosolic Ca\(^{2+}\) that originates from the ER (Chung et al., 2016). We hypothesized that this increase might induce mitochondrial Ca\(^{2+}\) accumulation. To measure mitochondrial Ca\(^{2+}\) level, we co-transfected HCN cells with RFPLC3 and Case12-mito, which is an indicator of mitochondrial Ca\(^{2+}\) level (Takeuchi et al., 2013). Co-transfection with RFPLC3 and Case12-mito showed an increase in mitochondrial Ca\(^{2+}\) levels in autophagy-induced cells following insulin
FIGURE 10 | Non-apoptotic and non-necrosis nature of insulin withdrawal-induced HCN cell death. (A) Cell death rates in insulin-deprived HCN cells after insulin withdrawal for 24 and 48 h (n = 3). (B) Caspase 3 activity after insulin withdrawal for 24 h. HCN cells were treated with staurosporine (STS; 0.5 mM) for 3 h as a positive control of apoptosis (n = 4). (C) Immunocytochemical detection of cleaved caspase 3 (C.Casp3) in HCN cells after insulin withdrawal for 24 h. Cells were treated with STS (0.5 mM, 8 h) (n = 47 cells for I(+), 50 cells for I(−), 56 cells for I(+)/STS from 3 independent experiments). Arrows indicate fragmented nuclei in C.Casp3-positive cells. Scale bars, 10 mm. (D) Quantification of C.Casp3-positive cells from (C) (n = 47 cells for I(+), 50 cells for I(−), 56 cells for I(+)/STS from 3 independent experiments). (E) TUNEL assay in HCN cells after insulin withdrawal for 24 h. Cells were treated with STS (0.5 mM, 8 h) (n = 258 cells for I(+), n = 310 cells for I(−), n = 340 cells for I(+)/STS from 3 independent experiments). Scale bar, 10 mm. (F) Cell death rates in insulin-deprived HCN cells treated with necrostatin-1 after insulin withdrawal for 24 h (n = 3). ***$p < 0.001$; ns, not significant.
FIGURE 11 | Insulin withdrawal induces autophagy-dependent cell death (ADCD) in adult hippocampal neural stem (HCN) cells. (A) Assessment of autophagy flux by mRFP-GFP-LC3 puncta assay after insulin withdrawal for 24 h. Cells were treated with Baf.A1 (10 nM) for 1 h before harvesting. Scale bar, 10 mm. (B) Quantification of red and yellow LC3 puncta from (A) (n = 47 cells for I(+), 40 cells for I(+)/Baf.A1, 52 cells for I(−), 50 for I(−)/Baf.A1 from 3 independent experiments). *p < 0.05, ***p < 0.001 for the red puncta; ##p < 0.01, ###p < 0.001 for the yellow puncta; ns, not significant.
withdrawal (Figures 12A,B). Since elevated mitochondrial Ca\(^{2+}\) could cause mitochondrial depolarization (Baumgartner et al., 2009), we next examined mitochondrial depolarization by staining HCN cells with MitoTracker Deep Red or TMRE, which are sequestered in healthy, polarized mitochondria, but are excluded from depolarized mitochondria (Scaduto Jr and Grotyohann, 1999). Fluorescence-activated cell sorting (FACS) analysis of HCN cells co-stained with MitoTracker Green (to ensure the equal number of total mitochondria) and MitoTracker Deep Red (for measurement of mitochondria membrane potential) revealed a reduced number of MitoTracker Deep Red–positive cells, indicating an increase in mitochondrial depolarization following insulin withdrawal (Figure 12C). This result was confirmed by fluorescence microscopy, which showed lower staining intensity of TMRE in I(−) HCN cells than in I(+) HCN cells (Figures 12D,E).

3.2 Insulin-Deprived HCN Cells Undergo Excessive Mitophagy

Because we observed mitochondrial Ca\(^{2+}\) accumulation and depolarization during ADCD, we next examined whether depolarized mitochondria underwent mitophagy. Since LC3 is recruited around mitochondria to mitophagy, colocalization of LC3 and mitochondria was confirmed. The co-transfection of HCN cells with GFP-LC3 and DsRed-Mito showed that their overlap was increased by insulin withdrawal (Figures 13A,B,E). Phosphatidylinositol 3-phosphate (PI3P) is a key signaling lipid for recruitment of autophagy effector proteins to initiate autophagosome generation (Burman and Ktistakis, 2010). DFCP1 contains PI3P-recognizing FYVE domains and binds to PI3P, and thus can serve as a marker of autophagosome nucleation (Axe et al., 2008). Therefore, we assessed translocation of DFCP1 to the vicinity of damaged mitochondria as another measure of mitophagy (Lazarou et al., 2015). I(−) HCN cells showed not only more DFCP1 puncta but also increased colocalization of DFCP1 and mitochondria in comparison with I(+) HCN cells, confirming the occurrence of mitopha-
gy (Figures 13C,D,F). As an alternative approach to demonstrate the localization of LC3 in mitochondria, we used subcellular fractionation. Successful separation of mitochondrial and cytosolic fractions was verified by Western blotting analysis using VDAC and β-tubulin as respective markers for these fractions. We observed more LC3-II in the mitochondrial fraction of I(−) HCN cells than in that of I(+) cells (Figures 13G,H). These results suggest that mitophagy is induced in HCN cells following insulin withdrawal.

Mitochondria are dynamic organelles that regulate their shape and size through fusion and fission according to various cellular signals and conditions in the cell. For mitophagy to occur, mitochondria need to be fragmented and reduced in size before they can be engulfed into autophagosomes. For this reason, mitochondrial fragmentation is observed during mitophagy (Arnoult et al., 2005; Twig et al., 2008). We transfected DsRed-Mito to compare mitochondrial morphology under I(+) and I(−) conditions. As expected, mitochondria showed elongated form in normal conditions, but fragmented mitochondria were observed in insulin-removed media (Figure 14A). When Mdivi-1, mitophagy inhibitor, is treated in I(−) HCN cells, mitochondria did not fragment and maintained the elongation form (Figure 14A). The level of autophagy marker LC3-II was also changed by Mdivi-1 treatment. LC3-II did not increase as much as non-treated I(−) HCN cells (Figure 14B–E). In addition, p62 is an autophagy receptor protein that is degraded during autophagy, but it accumulates without degradation by Mdivi-1 treated I(−) HCN cells (Figure 14B–E). In conclusion, ADCD can also be reduced by inhibition of mitophagy in I(−) HCN cells.

3.3 Parkin Is Upregulated Through Inhibition of Its Transcriptional Repressor c-Jun in Insulin-Deprived HCN Cells

Parkin-mediated mitophagy has been actively studied (Narendra et al., 2008). However, it remains ill-defined whether Parkin-dependent mitophagy can contribute to cell death.
**FIGURE 12 | Changes in mitochondria of HCN cells after insulin withdrawal.** (A) Assessment of mitochondrial Ca\(^{2+}\) levels using Case12-mito after insulin withdrawal for 24 h. Scale bar, 10 mm. (B) Quantification of fluorescence intensity of Case12-mito in HCN cells (n = 33 cells for I(+) , 25 cells for I(−), 36 cells for I(+) /CCCP from 3 independent experiments). (C) FACS analysis of HCN cells stained with MitoTracker Green to determine the total number of mitochondria and MitoTracker Deep Red to determine the number of depolarized mitochondria after insulin withdrawal for 24 h. 30,000 cells were analyzed in each experiment. (D) Fluorescence imaging analysis of TMRE-stained HCN cells. Scale bar, 10 mm. (E) Quantification of fluorescence intensity of TMRE in HCN cells (n = 12 cells from 2 independent experiments). Relative TMRE intensity of fluorescence microscope image was measured by image J. *p < 0.05, **p < 0.01.
FIGURE 13 | Insulin-deprived HCN cells undergo excessive mitophagy. (A,B) Colocalization of GFP-LC3 and DsRed-Mito (arrowheads) after insulin withdrawal for 24 h. Scale bars, 5 mm for (A), 2 mm for (B). (C,D) Immunofluorescence of GFP-DFCP1 and DsRed-Mito after insulin withdrawal for 24 h. Arrowheads indicate colocalization of GFP-DFCP1 and DsRed-Mito. Scale bars, 5 mm for (C), 2 mm for (D). (E) Quantification of colocalization of LC3 and mitochondria (n = 31 cells for I(+), 42 cells for I(−) from 3 independent experiments). (F) Quantification of colocalization of DFCP1 and mitochondria (n = 30 cells for I(+), 33 cells for I(−) from 3 independent experiments). (G) Western blotting analysis of mitochondrial translocation of LC3 after insulin withdrawal for 24 h. Mito, mitochondrial fraction; cytosol, cytosolic fraction. The blots shown are representative of four experiments with similar results. (H) Quantification of LC3-II levels (n = 4) from (G). *p < 0.05, **p < 0.01, ***p < 0.001.
FIGURE 14 | ADCD can be reduced by inhibition of mitophagy in I(−) HCN cells (A) Mitochondrial morphology in I(−) HCN cells. (B) A decrease in autophagy level in Mdivi-1 (100nM) treated HCN cells after insulin withdrawal. (C) Quantification of LC3-II levels from (B). (D) Autophagic flux in insulin-deprived HCN cells. Cells were treated with Baf.A1 (10 nM). (E) Quantification of LC3-II levels from (D). *p < 0.05, **p < 0.01, ***p < 0.001.
Since we observed mitophagy in insulin-deprived HCN cells undergoing ADCD, we wondered if Parkin is involved in ADCD through the regulation of mitophagy. To that end, we first checked Parkin levels in HCN cells following insulin withdrawal. Parkin protein levels were significantly increased 24 and 48 h after insulin withdrawal (Figures 15 A,B). We compared the time course of Parkin upregulation with that of autophagy flux induction following insulin withdrawal. An increase in Parkin protein amount was first observed as early as 3 h after insulin withdrawal, which coincided well with an increase in autophagy flux (Figures 15 C,D). Related to its protein levels, its transcript levels also increased significantly following insulin withdrawal (Figure 15E).

Interestingly, Parkin protein level was decreased when apoptosis was induced by STS, suggesting the potential ADCD-specific role of Parkin in HCN cells (Figures 15 A,B). Like Parkin, Dorfin, and HOIL-1 are also RING-IBR-RING-finger domain E3 ligases present in the brain (Niwa et al., 2002; Tanaka et al., 2004). Interestingly, their mRNA levels were not altered in response to insulin withdrawal (Figure 15F).

Because the upregulation of the Parkin mRNA level by insulin withdrawal seemed to play an important role in ADCD of HCN cells, we investigated the regulation of Parkin gene transcription in insulin-deprived HCN cells. First, to delineate the Park2 promoter region critical for transcription, we cloned a 2.5-kilobase rat Park2 promoter fragment in the pGL3-luc vector in front of a sequence encoding luciferase and measured Park2 promoter activity by luciferase assay. Luciferase expression driven by the cloned Park2 promoter fragment led to substantial luciferase activity in the basal condition (presence of insulin) in comparison with the empty vector (Figure 16A). The activity was further increased by insulin withdrawal (Figure 16A). Next, we cloned a series of truncated versions of the Park2 promoter to find the sequences critical for Park2 transcription and found that 500 bp upstream of the Park2 gene is the core promoter region (Figure 16B).
FIGURE 15 | Parkin expression is upregulated following insulin withdrawal. (A) Western blotting analysis of Parkin protein level in HCN cells after insulin withdrawal for 24 and 48 h. (B) Quantification of Parkin protein level (n = 3 or 4). (C) Time course analysis of Parkin protein levels following insulin withdrawal. (D) Quantification of Parkin protein levels (n = 5) from (C). (E) Time course analysis of mRNA levels of Parkin following insulin withdrawal (n = 4). (F) Changes in the mRNA levels of Parkin, Dorfin, and HOIL-1 (n = 3–7).

*p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant.
FIGURE 16 | Upregulation of the Parkin mRNA level by insulin withdrawal. (A) Luciferase activity assay to analyze Park2 promoter activity after insulin withdrawal for 24 h (n = 3). (B) Activities of various truncated fragments of the Park2 promoter in I(+) cells. Promoter activity was measured using a luciferase assay (n = 3–7). *p < 0.05, ***p < 0.001
Next, we used the PROMO program (Meseguer et al., 2002; Farré et al., 2003) to predict the candidate transcription factors that may participate in both Parkin transcription and autophagy. Binding element analyses predicted several candidate transcription factors, including c-Jun, because there were three potential c-Jun-binding sites in the Park2 promoter region (Figure 17A). c-Jun is a transcription factor phosphorylated and activated mainly by c-Jun NH2 terminal kinase (JNK) (Minden et al., 1994). Based on prior reports on c-Jun functions in autophagy inhibition (Yogev and Shaulian, 2010) and Park2 transcriptional repression (Bouman et al., 2011), we hypothesized that c-Jun decreases Parkin level. To test this hypothesis, we used an ATP-competitive JNK inhibitor, SP60015, to block phosphorylation of c-Jun by JNK. SP600125 treatment increased Parkin mRNA and protein levels in the presence of insulin, suggesting that Park2 is under transcriptional repression by c-Jun in the I(+) condition when Parkin level is low (Figures 17B–E). However, Parkin expression levels were not further increased by SP600125 treatment in insulin-deprived HCN cells (Figures 17D,E), probably due to already high Parkin protein level in I(−) HCN cells. Inactivation of the mammalian target of rapamycin (mTOR)-Akt pathway in I(−) HCN cells was observed previously (Yu et al., 2008). To test whether the mTOR-Akt cascade is involved in Parkin expression, we checked the Parkin expression levels in I(+) condition with rapamycin, which is an mTOR inhibitor. We observed increased Parkin expression in rapamycin-treated I(+) HCN cells, suggesting the involvement of mTOR-Akt pathway in regulation of Parkin following insulin withdrawal in HCN cells (Figure 17F). If c-Jun is a transcriptional repressor of the Park2 gene, then its level should decrease following insulin withdrawal to allow the upregulation of the Parkin mRNA level. To validate this notion, we assessed changes in c-Jun protein in the absence of insulin. c-Jun protein amount was reduced in a time-dependent manner with kinetics similar to that of Parkin increase (Figure 17G). This gradual decrease in c-Jun level was due to its degradation via the ubiquitin–proteasome system, since the pro-
teasome inhibitor lactacystin prevented c-Jun degradation (Figures 17G,H). Lactacystin-mediated blockage of c-Jun degradation decreased the Parkin protein level under insulin withdrawal conditions, confirming the inhibitory role of c-Jun in Parkin expression (Figures 17G,H). Taken together, these data reveal that c-Jun is a transcriptional repressor of the Park2 gene, and proteasome-dependent degradation of c-Jun leads to upregulation of Parkin in insulin-deprived HCN cells.

3.4 Parkin Knockdown/Knockout Prevents ADCD in HCN Cells Following Insulin Withdrawal

To investigate the role of Parkin in ADCD of insulin-deprived HCN cells, we over-expressed Parkin. However, there was no significant change in the ADCD rate (data not shown). We speculated that Parkin level was already high in insulin-deprived HCN cells and therefore over-expression of Parkin did not induce additional effects. Therefore, Parkin knockdown (KD) experiments are performed. Decreased LC3-II level which is an autophagy marker is observed in Parkin transcriptional silencing cells (Figures 18A,B).

Moreover, Parkin expression levels have influence on autophagy flux. LC3-II accumulated in I(−) HCN cells when late phase of autophagy was inhibited by Baf.A1. The degree of accumulated LC3-II is different between normal and Parkin knockdown HCN cells. Autophagy flux in Park2 siRNA transfected HCN cells is slower than control HCN cells (Figures 18C). Ultimately, Parkin affects ADCD level as well as autophagy flux. Increased ADCD level in insulin removed condition is rescued by inhibition of Parkin expression (Figures 18D). All of these things taken together, Parkin has an important role in ADCD.

Next, we ablated the Park2 gene in HCN cells by using the CRISPR/Cas9 gene-editing technique and designated the knockout (KO) cells as sgParkin and control cells as sgCon. Parkin KO attenuated an increase in LC3-II levels following insulin withdrawal.
FIGURE 17 | Parkin is upregulated in HCN cells by degradation of c-Jun, a transcriptional repressor, following insulin withdrawal. (A) Prediction of c-Jun-binding sites in the Park2 promoter region. (B) Parkin mRNA level in HCN cells treated with SP600125, an ATP-dependent JNK inhibitor, (10 mM) for 8 h in comparison with that in I(−) HCN cells after insulin withdrawal for 24 h (n = 3). (C) Western blotting analysis of p-JNK levels in HCN cells after insulin withdrawal for 2 h. SP600125 (10 mM) was treated 10 mM for 2 h. The blots shown are representative of three experiments with similar results. (D) Western blotting analysis of Parkin protein levels in SP600125 (10 mM, 8 h) -treated HCN cells. The blots shown are representative of five experiments with similar results. (E) Quantification of Parkin levels (n = 5) from (D). (F) Increased Parkin protein levels in HCN cells treated with Rapamycin (20 nM, 2 h), a mTOR inhibitor. (G) Time course analysis of c-Jun protein levels following insulin withdrawal in comparison with lactacystin treatment (Lacta, 1 mM). (H) Quantification of c-Jun and Parkin protein levels in I(−) HCN cells treated with or without Lacta (1 mM) for 6 h (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant.
FIGURE 18 | ADCD in I(-) HCN cells is reduced by knockdown of Parkin. (A) As a result of Parkin knockdown, levels of autophagy marker proteins such as p62 and LC3-II are changed. (B) Quantification of LC3-II expression levels by normalized to β-actin. Decreased LC3-II signifies decline of autophagy level. Totally, rate of autophagy decreases when Parkin is knocked down. (C) Autophagy flux in control and Parkin knockdown HCN cells. Levels of accumulated LC3-II by treatment of Baf.A1 much smaller in Parkin knockdown cells than normal cells. (D) Cell death rate of HCN cells. Parkin expression levels are important to regulate autophagic cell death rate. Repressed Parkin expression induces decreased autophagic cell death. *p < 0.05, ***p < 0.001.
FIGURE 19 | Parkin knockout (KO) prevents ADCD in HCN cells following insulin withdrawal. (A) A decrease in autophagy level in sgParkin cells after insulin withdrawal for 24 h. (B) Quantification of LC3-II levels (n = 6). (C) Autophagic flux in sgParkin cells after insulin withdrawal. Cells were treated with Baf.A1 (10 nM) before harvesting. The blots shown are representative of four experiments with similar results. (D) Quantification of LC3-II levels (n = 4) from (C). (E) Cell death rates in sgCon and sgParkin cells after insulin withdrawal for 24 h (n = 11). *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant.
Autophagy flux was also decreased in sgParkin cells, as revealed by a decrease in accumulation of LC3-II induced by Baf.A1 (Figures 19C,D). Also, ablation of the Park2 gene reduced cell death in I(−) HCN cells (Figure 19E). However, Parkin KO did not prevent, but rather promoted apoptosis, since the two prototypical apoptotic inducers STS and etoposide (ETO) increased cell death rate to a greater extent and led to more robust activation of caspase 3 and PARP in sgParkin than in sgCon cells (Figures 20A–C). These results suggest that Parkin has opposite pro-death or anti-death roles depending on HCN cell death mode. Parkin is essential for insulin withdrawal-triggered mitophagy and plays a pro-death role in ADCD. However, Parkin has anti-apoptotic activity against well-known apoptotic inducers, and its deletion renders HCN cells more susceptible to apoptosis.

In the Parkin-dependent mitophagy model, PINK1 is essential for the action of Parkin (Narendra et al., 2010; Youle and Narendra, 2011). PINK1 constitutively shuttles between the cytosol and mitochondria in normal cells but accumulates on the outer membrane of depolarized mitochondria (Narendra et al., 2010). Therefore, we examined whether PINK1 is recruited to mitochondria in I(−) HCN cells. We transfected with mitochondria marker DsRed-Mito in HCN cells. Fluorescence imaging analyses showed an increased accumulation of PINK1 on the mitochondria in I(−) HCN cells (Figures 21A–C). Also, it was confirmed that colocalization of PINK1 and mitochondria increased when mitophagy was induced using CCCP as a positive control (Figures 21A–C). During mitophagy, PINK1, which is stably located in the mitochondria without degradation, recruits cytosolic Parkin. Therefore, we confirmed the sublocalization of Parkin under I(+) and I(−) conditions by transfection of GFP-Parkin and DsRed-Mito. As expected, colocalization of Parkin and mitochondria increased in insulin-deprived HCN cells (Figures 22A). Consequently, it was confirmed that Parkin, which located in cytosolic region in I(+) condition, was translocated to mitochondria in insulin removed condition.
FIGURE 20 | Parkin KO did not prevent, but rather promoted apoptosis (A) Western blotting analysis of C.Casp3, PARP after STS (0.5 mM) or etoposide (ETO) (20 mM) treatment for 8 h. (B) Quantification of C.Casp3 level after normalization to β-actin (n = 5–7). (C) Cell death rates of sgCon and sgParkin cells after STS or ETO treatment for 8 h (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001.
To further examine the involvement of PINK1 in mitophagy, we used an anti-p-Ub-S65 antibody. PINK1 is a ubiquitin kinase. Once stabilized on the outer membrane the depolarized mitochondria, PINK1 phosphorylates ubiquitin at S65, which further stimulates the E3 ligase activity of Parkin (Kane et al., 2014). As expected, in accordance with PINK1 accumulation, the signal intensity p-Ub-S65 was substantially increased in I(−) HCN cells (Figures 22B,C). In this experiment, the potent depolarizing agent CCCP was used as a positive control to chemically induce mitophagy (Figures 22B,C). In addition, ADCD was reduced by PINK1 KO (Figure 22D). In conclusion, PINK1 acts upstream of Parkin in insulin-deprived HCN cells, and HCN cells undergo ADCD through PINK1/Parkin-dependent mitophagy.

### 3.5 Parkin KO Rescues Mitochondrial Alterations and Prevents Initiation of Mitophagy in Insulin-Deprived HCN Cells

Next, we examined whether Parkin deletion affects events upstream of mitophagy. To measure mitochondrial Ca$^{2+}$ levels, we transfected case12-mito into sgCon and sgParkin cells and measured their relative intensities, respectively. In sgCon cells, mitochondrial Ca$^{2+}$ levels were increased in insulin-removed media, but this phenomenon was not observed in Parkin KO cells. Interestingly, mitochondrial Ca$^{2+}$ level was reduced in sgParkin cells (Figures 23A, B). Unexpectedly, we found that the ratio of depolarized mitochondria was lower in sgParkin than sgCon cells following insulin withdrawal (Figure 23C). These results suggest that Parkin is required for efficient ER-to-mitochondria Ca$^{2+}$ transfer and ensuing depolarization. In accordance with decreases in the mitochondrial Ca$^{2+}$ accumulation and depolarization, subsequent mitophagy-related events, such as mitochondrial translocation of LC3 (Figures 24A,B,E) and DFCP1 (Figures 24C,D,F), were all attenuated in I(−) sgParkin cells, suggesting that Parkin KO interfered with the initial steps of mitophagy. Our results suggest
FIGURE 21 | HCN cells undergo PINK1-dependent mitophagy following insulin withdrawal. (A,B) An increase in colocalization of PINK1 and mitochondria after insulin withdrawal for 24 h. Arrowheads indicate DsRed-Mito and PINK1 colocalization. Scale bars, 5 mm. (C) Intensity profile graphs of PINK1 and DsRed-Mito. Scale bar, 5 mm.
FIGURE 22 | HCN cells undergo PINK1/Parkin-dependent mitophagy following insulin withdrawal. (A) Different sublocalization of Parkin is observed in I(+) and I(-) HCN cells. Colocalization of GFP-Parkin and dsRed-Mito higher in I(-) HCN cells compare to I(+) HCN cells. (B) An increase in the level of p-Ub-S65 after insulin withdrawal for 24 h. Cells were treated with CCCP (10 mM) for 0.5 h as a positive control of mitophagy. Blue staining indicates Hoechst, and red staining indicates p-Ub-S65. Intensity is indicated in arbitrary units. Scale bar, 10 mm. (C) The mean intensity of p-Ub-S65 was quantified using ImageJ software (n = 33 cells for I(+), 38 cells for I(-), 46 cells for I(+)/CCCP from 3 independent experiments). (D) Cell death rates of sgCon and sgPINK1 cells (n = 4). *p < 0.05, ***p < 0.001.
FIGURE 23 | Parkin KO Rescues Mitochondrial Alterations

(A) Fluorescence images of Case12-mito in sgParkin cells after insulin withdrawal for 24 h. Scale bar, 5 mm. (B) Quantification of fluorescence intensity of Case12-mito (n = 39 cells for sgCon(+), 28 cells for sgCon(-), 36 cells for sgCon(+)/CCCP, 80 cells for sgParkin(+), 73 cells for sgParkin(-), 37 cells for sgParkin(+)/CCCP from 3 independent experiments). (C) Double staining with MitoTracker Green and MitoTracker Deep Red to measure the ratio of depolarized mitochondria relative to total mitochondria after insulin withdrawal for 24 h. **p < 0.01, ***p < 0.001; ns, not significant.
FIGURE 24 | Parkin KO prevents initiation of mitophagy following insulin withdrawal.

(A,B) Colocalization of LC3 and mitochondria in sgParkin cells after co-transfection with GFP-LC3 and DsRed-Mito. Colocalization was analyzed after insulin withdrawal for 24 h. Arrowheads indicate colocalization of GFP-LC3 and DsRed-Mito. Scale bars, 5 mm for (A), 10 mm for (B). (C,D) Colocalization of DFCP1 and mitochondria (arrowheads) in sgParkin cells after insulin withdrawal for 24 h. Scale bars, 5 mm for (C), 10 mm for (D). (E) Quantification of colocalization of LC3 and mitochondria (n = 26–52 cells from 3 independent experiments). (F) Quantitative analysis of colocalization of DFCP1 and mitochondria (n = 23–36 cells from 3 independent experiments). ***p < 0.001; ns, not significant.
that Parkin deletion not only impairs the recognition of the depolarized mitochondria and their removal but also blocks early changes in mitochondrial physiology and prevents initiation of mitophagy. Therefore, mechanistically, Parkin is intimately involved in mitophagy from its very early steps, including ER-to-mitochondria Ca$^{2+}$ transfer and recruitment of the autophagy-initiating/elongating molecules to mitochondria.
DISCUSSION

Parkin can suppress or promote apoptosis depending on cell type and stressor. Our results are more in line with the anti-apoptotic role of Parkin in HCN cells challenged with well-known apoptotic stimuli. In contrast to this pro-survival role, we also found that Parkin promotes ADCD following the removal of insulin, the key survival neurotrophic factor for HCN cells. Currently, it is not known how these opposite roles of Parkin in the control of distinct modes of cell death are regulated. Overall, deregulated Parkin activity and mishandling of depolarized mitochondria will be detrimental to the cells and lead to neurodegeneration through various pathways depending on cell type and degeneration cue.

Although PD is mainly a movement disorder with motor symptoms, non-motor symptoms such as sleep abnormalities, autonomic failure, and a range of neuropsychiatric symptoms including depression, anxiety, cognitive impairment, dementia, and impulse control disorders are increasingly being recognized as features of PD pathology (Meissner et al., 2011). So far, most therapeutic efforts have been focused on motor control, and alleviation of non-motor symptoms has received less attention. Therefore, there is an unmet need to alleviate non-motor symptoms for better therapeutic design and management of PD. However, currently there is no good animal or cellular model to help address the neuropsychiatric symptoms of PD. In that regard, our present study on Parkin-mediated mitophagy in HCN cells could contribute to understanding the various roles of Parkin in brain areas other than substantia nigra.

What is the interconnection between mitophagy and conventional autophagy? Although it may not be easy to distinguish the effects of mitophagy from those of conventional bulk autophagy in the same cell, it is interesting that the ablation of Parkin attenuates not only mitophagy but also conventional autophagy. Parkin may play additional roles in facilitation of conventional autophagy in insulin-deprived HCN cells. Another possibility is that elimina-
tion of mitochondria has a greater effect on autophagy than elimination of other intracellular constituents in HCN cells. Since interference with mitophagy by using Mdivi-1 reduced autophagy level in HCN cells, the latter possibility seems plausible. During bulk autophagy, as in nutrient starvation conditions, autophagy can be selective for certain intracellular organelles, such as ribosomes and peroxisomes (Hara-Kuge and Fujiki, 2008; Cebollero et al., 2012). In certain cell types, including HCN cells, mitochondria could be the main cargo for autophagic degradation.

In this study, we also identified a new mode of action of Parkin in the regulation of mitophagy. Our data suggest that ER-to-mitochondria Ca\textsuperscript{2+} mobilization triggers Parkin/PINK1-dependent mitophagy, and at the same time, Parkin is required for this Ca\textsuperscript{2+} mobilization. In line with this idea, a previous study reported that Parkin upregulation increases ER?Mitochondria contact to regulate Ca\textsuperscript{2+} transfer (Cali et al., 2013). Upon insulin withdrawal, Parkin mediates Ca\textsuperscript{2+} accumulation in mitochondria and thereby instigates mitochondrial depolarization at the early stage of mitophagy. This may allow recruitment of PINK1 and Parkin and progress of mitophagy. Then, recruited Parkin recognizes and removes dysfunctional mitochondria. According to our model, Parkin is intimately involved from the beginning of mitophagy, and liaises with PINK1 in a mutually cooperative way for the progress of mitophagy. Parkin’s role is more than recognition and removal of depolarized mitochondria; it is an initiator and actuator of mitophagy in the context of ADCD. A schematic diagram shown in Figure 25 summarizes the regulation of Parkin expression and its roles during ADCD.

It is also puzzling how Parkin-mediated selective elimination of dysfunctional mitochondria leads to cell death in insulin-deprived HCN cells, whereas Parkin-dependent mitophagy promotes and cellular homeostasis according to other studies. Currently, the link between mitophagy and cell death remains under-studied. What mechanisms determine the out-
come of Parkin-driven mitophagy, i.e., whether it ensures mitochondrial quality control or facilitates mitophagic cell death? Further studies are warranted to resolve this issue.
FIGURE 25 | A schematic diagram of Parkin-dependent mitophagy and ADCD. Parkin expression is increased by the degradation of c-Jun, a transcriptional repressor of Parkin in insulin-deprived HCN cells. Insulin withdrawal induced mitochondrial depolarization and recruitment of Parkin and PINK1 to mitochondria, where Parkin is also required for ER-to-mitochondria Ca\textsuperscript{2+} transfer. PINK1/Parkin-dependent mitophagy occurs in HCN cells following insulin withdrawal causing excessive mitophagy and ADCD.
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Parkin에 의한 성체해마줄기세포에서의 mitophagic cell death 촉진
조절된 세포 사멸은(Regulated cell death) 인간 건강 및 질병에서 근본적인 역할을 한다. 우리는 이전에 렛의 성체해마줄기세포가 (rat hippocampal neural stem cells; HCN cells) 인슐린을 제거한 배지에서 오토파지 종속 세포 죽음 (Autophagy dependent cell death; ADCD)을 겪고 있음을 확인했다. 이번 연구에서 파킨(Parkin)은 인슐린이 결핍된 HCN 세포에서 mitophagy 와 ADCD를 촉진한다는 것을 보여준다. 인슐린 제거로 인해 탈분극된 미토콘드리아의 수가 증가하였으며 또한 autophagosome 과의 공동 국소화(colocalization)를 증가시켰다. 인슐린 결핍은 또한 Parkin의 mRNA 및 단백질 수준들 다를 상향 조절하였고, 이의 유전자 녹아웃은 mitophagy 및 ADCD를 방지하였다. c-Jun은 Parkin의 전사 억제 인자이며 인슐린 제거 후 프로 프로테아좀 (proteasome)에 의해 분해된다. 인슐린이 제거된 HCN 세포에서, 파킨은 mitophagy의 초기 단계에서 미토콘드리아의 Ca²⁺ 축적 및 탈분극뿐만 아니라 후기 단계에서 탈분극된 미토콘드리아의 인식 및 제거를 위해 필요하다. Mitophagy 동안 Parkin의 pro-death 역할과 대조적으로, Parkin 결식은 HCN 세포가 apoptosis 에 취약하게 하는 등 RCD의 세포사멸의 다른 모드에 따라 Parkin의 독특한 역할을 나타냈다. 종합하면, 이들 결과는 Parkin이 인슐린 제거된 HCN 세포에서 미토콘드리아 기능 장애를 수반하는 ADCD의 유도에 필요하다는 것을 나타낸다. 인슐린 신호전달 장애는 다양한 신경 퇴행성 질환과 관련이 있기 때문에, 이러한 발견은 신경세포줄기 세포의 사멸의 기전을 이해하고 신경 발생 및 생존을 개선시키는 것을 목표로 하는 새로운 치료 전략을 개발하는 데 도움이 될 수 있다.