Published in final edited form as:

Prog Neurobiol. 2018 ; 165-167: 103–116. doi:10.1016/j.pneurobio.2018.03.002.

Notch signaling and neuronal death in stroke

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Abstract

Ischemic stroke is a leading cause of morbidity and death, with the outcome largely determined by the amount of hypoxia-related neuronal death in the affected brain regions. Cerebral ischemia and hypoxia activate the Notch1 signaling pathway and four prominent interacting pathways (NF-κB, p53, HIF-1α and Pin1) that converge on a conserved DNA-associated nuclear multi-protein complex, which controls the expression of genes that can determine the fate of neurons. When neurons experience a moderate level of ischemic insult, the nuclear multiprotein complex up-regulates adaptive stress response genes encoding proteins that promote neuronal survival, but when ischemia is more severe the nuclear multi-protein complex induces genes encoding proteins that trigger and execute a neuronal death program. We propose that the nuclear multi-protein transcriptional complex is a molecular mediator of neuronal hormesis and a target for therapeutic intervention in stroke.

Keywords

HIF-1α; Hypoxia; Ischemic stroke; Neuronal cell death; NF-κB; Notch; p-53; Pin-1

1. Introduction

The most common proximate pathogenic event in stroke is the formation of a clot in a cerebral artery, resulting in severely reduced perfusion of brain tissue supplied by the

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Competing financial interests
The authors declare no competing financial interests.

affected vessel. The clot often resolves within several minutes to hours and blood flow resumes. In human patients and animal stroke models, neurons which are supplied with blood solely by the affected artery die rapidly by necrosis (the ischemic core), whereas neurons peripheral to the core which are also perfused to some extent by other arteries (the ischemic penumbra) undergo delayed apoptosis (Broughton et al., 2009; Fann et al., 2013). The pathophysiology of ischemic stroke-induced brain damage is complex. Ischemia and reperfusion can cause in bioenergetic failure, loss of cellular ion homeostasis, excitotoxicity, impaired mitochondrial function, generation of reactive oxygen species (ROS) and activation of caspases in neuronal cells. It can also lead to inflammatory responses in the brain by promoting generation of arachidonic acid products, production of cytokines, activation of complement pathways, inflammasomes, and various membrane receptors by damage associated molecular patterns (DAMPs), disruption of the blood-brain barrier, and infiltration of immune cells (Arumugam et al., 2005; Broughton et al., 2009; Brait et al., 2012; Fann et al., 2013) (Fig. 1). The triggering of membrane receptors such as toll-like receptors (TLRs), C-type lectin Mincle and the receptor for advanced glycation end products (RAGE) by DAMPs results in the stimulation of numerous signaling cascades and transcription factors, primarily in inflammatory glial cells but also in neurons (Tang et al., 2013a, b; Fann et al., 2013; Arumugam et al., 2017; Wang et al., 2017) (Fig. 1).

Preclinical studies have shown that many neurons in the ischemic penumbra can be rescued and their functional outcomes can be improved, by interventions that target one or more of the above-mentioned neurodegenerative processes. Such neuroprotective approaches include agents that inhibit glutamate receptors, ROS, leukocyte infiltration and immune responses, and interventions that bolster mitochondrial function (Broughton et al., 2009; Lai et al., 2014). In addition, insight into the factors that determine inter-individual differences in stroke outcomes has come from studies showing that antecedent exercise, intermittent fasting, and pharmacological bioenergetic challenges can improve neurological deficit and reduce development of infarcted tissue in animal models of focal ischemic stroke (Liu et al., 2002; Endres et al., 2003; Mattiasson et al., 2003; Arumugam et al., 2007, 2010; Moskowitz et al., 2010; Fann et al., 2014). Despite evidence that numerous pharmacological agents can target this signaling cascade that promotes ischemic neuronal death and consequently improve outcome in various animal models of stroke, such protective effects have not been substantiated in human clinical trials. Recombinant tissue plasminogen activator (rtPA) is the only approved treatment that has proven to be effective in the clinical setting. Indeed, intravenous administration of rtPA results in a significant overall improvement in outcome in patients by dissolving blot the clots that caused the ischemic episode (Nogueira et al., 2016). However, the use of rtPA is limited to 4.5 h after stroke onset and is not as robust and consistently beneficial as was originally predicted.

The mechanism by which such bioenergetic challenges (i.e. exercise, fasting, etc.) increase resistance to neuronal stress is an example of ‘hormesis’, a fundamental property of biological systems in which transient/intermittent exposure to low doses of a stressor results in augmented resistance to more severe stress (Mattson, 2014). Indeed, even as neurons succumb in the ischemic penumbra, others that experience lower levels and/or durations of the same cellular stressors survive and retain functionality. Hypoxic/ischemic stress engages multiple evolutionarily conserved signaling pathways that bolster neuronal stress resistance.

Here we consider five prominent signaling pathways that mediate the hormesis-like response to hypoxia, namely, those involving Notch1, nuclear factor kappa B (NF-κB), p53, hypoxia inducible factor 1 alpha (HIF-1α), and peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1) (Fig. 2). These pathways converge on a conserved DNA-associated nuclear multi-protein complex, which controls the expression of genes that can regulate the fate of brain cells in stroke and other neurodegenerative conditions.

2. Notch in ischemic stroke

Notch (Notch1) is a membrane receptor that regulates cell proliferation, differentiation, and developmental fate switching in a range of tissues. In the developing brain, Notch signaling is involved in the preservation of neural progenitors in an undifferentiated state, in part by inhibition of neurogenesis (Lathia et al., 2008). Notch signaling also influences synaptic plasticity and learning and memory in the adult brain (Lathia et al., 2008; Alberi et al., 2013; Sargin et al., 2013). Notch is an important membrane receptor with a large extracellular N-terminal epidermal growth factor-like repeat domain followed by a cysteine-rich LIN-12 Notch repeat (LNR) region and a juxtamembrane heterodimerization (HD) domain (Fig. 2). On the cytoplasmic side of the membrane, Notch contains recombination binding protein-J-associated molecule (RAM), trans-activating (TAD) domains, ankyrin (ANK) and a C-terminal region rich in proline, glutamic acid, serine and threonine (PEST domain). Notch is cleaved by an extracellular metalloproteinase (tumor-necrosis-factor-α-converting enzyme; TACE) at a site (S2) adjacent to the plasma membrane following ligand binding (Fig. 2). Immediately after its cleavage by TACE, γ-secretase cleaves Notch at the transmembrane domain (S3) and generates the Notch intracellular domain (NICD) (Figs. 2 and 3). NICD is the active intracellular portion of Notch that translocates to the nucleus and cooperates with the co-activator protein Mastermind transcription factor and the recombination signal binding protein Jκ (RBP-Jκ). This results in transcriptional activation of RBP-Jκ and leads to target gene expression (Lathia et al., 2008)

The earliest evidence of Notch-mediated apoptosis was obtained in a study showing that active Notch suppresses B cell growth, interrupts the cell cycle, and induces apoptosis (Morimura et al., 2000). Notch was also shown to regulate apoptosis in early neural progenitor cells (Yang et al., 2004). Evidence for an apoptotic role for Notch was further supported by findings showing that mice transgenic for Notch1 anti-sense (NAS) are protected from ischemic stroke-induced infarct development and neurological deficits. We first determined that γ-secretase-mediated Notch activation endangers neurons in ischemic stroke by modulating pathways that increase their vulnerability to apoptosis, pro-inflammatory leukocyte infiltration, and microglial activation (Arumugam et al., 2006). Specifically, mice transgenic for NAS showed reduced damage to brain cells and improved functional outcome compared with WT mice following focal ischemic stroke. Consistently, WT mice treated with inhibitors of the Notch-activating enzyme γ-secretase also exhibit reduced brain damage and improved functional outcome after stroke via down-regulating Notch signaling (Arumugam et al., 2006). The levels of both γ-secretase and NICD are elevated in primary neurons as well as in ipsilateral tissue following ischemia (Li et al., 2012; Arumugam et al., 2010). Besides enhancing apoptotic cascades in neurons, Notch may have an important role in microglial activation and lymphocyte infiltration in stroke (Wei et

al., 2011; Marumo et al., 2013). Moreover, an apoptotic role for Notch2 was also documented in ischemic stroke (Alberi et al., 2010). Notch2 expression was observed shortly after neonatal ischemia in hippocampal areas with increased cell death. Simulated ischemic conditions in cultured hippocampal neurons results in a similar increase in Notch2 in apoptotic cells and treatment with an active form of Notch2 is neurotoxic (Alberi et al., 2010).

While we and others have established the role of Notch in neuronal death during the early hours following ischemic stroke, Notch signaling may also promote the survival of neuronal stem cells during the recovery and remodeling phases (Androutsellis-Theotokis et al., 2006; Guo et al., 2012). Androutsellis-Theotokis et al. have shown that the transient administration of Notch ligands during 45 days of the post-stroke survival period increases neurogenesis and improves motor skills following ischemic injury (Androutsellis-Theotokis et al., 2006). Furthermore, Notch1 signaling modulates neurogenesis in the sub-ventricular zone (SVZ) following ischemia and may promote cell survival in aged animals (Sun et al., 2013). These results are in accordance with previous studies showing that Notch can promote neuronal stem cell survival (Oishi et al., 2004).

3. Interaction of Notch with NF-κB, p53, HIF-1α, and Pin1 in ischemic stroke

Cerebral ischemia activates prominent signaling pathways in neurons, including those involving Notch1, NF-κB, p53, HIF-1α and Pin1. We propose that these signaling pathways converge on a conserved DNA-associated nuclear multi-protein transcriptional complex, which controls the expression of genes that can ultimately determine the fate of neurons. This section discusses the putative components of this multi-protein transcriptional complex and the evidence for its formation following ischemic stroke.

A role for Notch in the modulation of NF-κB-mediated cell death and inflammatory signaling pathways has been suggested in several studies (Arumugam et al., 2011; Wei et al., 2011). The transcription factor NF-κB is a p65/p50 dimer located in the cytoplasm and associated with an inhibitory protein called IκBα (Fig. 2). Following activation by upstream kinases, IκBα is degraded and the p65/p50 dimer translocates into the nucleus where it binds NF-κB-responsive genes (Mattson and Camandola, 2001). Roles for NF-κB in ischemic stroke are complex because neuronal NF-κB may promote cell survival following mild ischemic stress, but neuronal death following severe ischemia. Roles for NF-κB in determining the fate of neurons following severe ischemic/hypoxic conditions are complex and not fully understood (Mattson and Meffert, 2006; Schwaninger et al., 2006). There is abundant experimental evidence that NF-κB activation is increased in brain cells following ischemia. Early studies showed that activation of NF-κB in hippocampal neurons by tumor necrosis factor (TNF) is involved in an anti-apoptotic function of TNF and could be simulated by administration with I-κB antisense oligonucleotides (Barger et al., 1995). Many animal studies have reported NF-κB to be activated in neurons (Schneider et al., 1999), neurovascular endothelial cells, microglia and astrocytes following stroke (Howard et al., 1998; Zhang et al., 2005; Kaushal and Schlichter, 2008). Furthermore, the proposal that

activation of NF-κB occurs following experimental stroke is supported by a study analyzing brain samples from human stroke patients in which nuclear translocation of NF-κB-p65 was detected in the penumbra region (Nurmi et al., 2004).

A detrimental role of NF-κB in stroke was suggested in a study of p50 knockout mice, in which ischemic damage was drastically reduced compared to WT control animals (Schneider et al., 1999). This finding was further supported by a number of others indicating a detrimental role of NF-κB in ischemic stroke. Transgenic mice that express the 1-κBα super-repressor in different types of brain cells were used to demonstrate that neuronal, but not astrocytic, expression of I-κBα reduced brain damage after permanent cerebral ischemia (Zhang et al., 2005). NF-κB has also been shown to contribute to glial cell activation following ischemic injury by regulating pro-inflammatory pathways (Dvoriantchikova et al., 2009). The NF-κB inhibitor peptide, IKK-NBD, reduces inflammation and apoptosis and protects against ischemic brain injury in adults and neonates (Nijboer et al., 2008; Desai et al., 2010). In addition, pharmacological blockade of NF-κB signaling or knockout of NF-κB-p50 markedly eliminates the injurious effects of ischemic stroke and promotes functional recovery (Venna et al., 2012). These studies confirm that NF-κB can indeed promote cell death following stroke. However, because its effects vary depending on the cell type, degrees of ischemic severity, stages of infarct development, recovery or remodelling, the activation of NF-κB can in fact either promote neuronal death or survival following stroke. We have also shown that NF-κB activation may protect neurons against glucose deprivation- or excitotoxicity-induced death (Mattson et al., 1997; Furukawa and Mattson, 1998).

We have shown that a number of γ-secretase inhibitors reduce levels of NICD and protect against ischemic stroke-induced brain injury by targeting NF-κB-mediated pro-death BH3-only protein, Bcl-2-inter-acting mediator of cell death (Bim) induction (Arumugam et al., 2011). The interaction of Notch and NF-κB signaling pathways in other brain cells such as microglia was also demonstrated. NAS transgenic mice exhibit fewer proinflammatory microglia in the ischemic cortex than non-transgenic control mice (Wei et al., 2011). In addition, blocking Notch signaling using γ-secretase inhibitors attenuates microglial activation following ischemia-like conditions (Wei et al., 2011). Nuclear translocation of NICD, together with RBP-Jκ, was observed in activated microglia following ischemia, and furthermore the ischemia-induced increase in NF-κB in microglia can be prevented by γ-secretase inhibition (Yao et al., 2013). These studies confirm that interactions between Notch and NF-κB pathways in neurons and microglia following cerebral ischemia.

Another protein shown to interact with Notch in neurons and promote cell death following ischemic stroke is hypoxia inducible factor 1 (HIF-1). HIF-1 is considered the master controller of an hypoxic environment and responsible for facilitating adaptive responses to reduced oxygen (Semenza, 2014). HIF is a heterodimeric protein constituting α and β subunits. There are three oxygen-regulated HIF-α isoforms and a constitutively expressed HIF-1β isoform. Hypoxia rapidly upregulates expression of HIF-1α, and under normoxic conditions HIF-1α is rapidly degraded (Semenza, 2014). HIF-1α possesses several domains that mediate either protein–protein or protein-DNA interactions. At its N-terminus are regions that mediate protein dimerization and DNA-binding including a basic helix loop helix (bHLH) domain and a per-ARNT-SIM (PAS) domain (Fig. 2). The C-terminal half of

HIF-1α includes an oxygen-dependent degradation domain (ODD; which includes a TAD domain), an inhibitory domain (IH) and a C-terminal activation domain (Fig. 2). In normoxic conditions, the von Hippel-Lindau protein (pVHL) is bound to HIF-1α and recruits a ubiquitin ligase. This leads to proteasomal degradation of HIF-1α. HIF-1α becomes stable and translocates from the cytoplasm following hypoxic conditions. In the nucleus, HIF-1α dimerizes with HIF-1β and binds to hypoxia response elements (HREs) to begin transcription (Kaelin and Ratcliffe, 2008). Following ischemic stroke, HIF-1 responds to low tissue levels of oxygen, and the nuclear content of HIF-1α increases in the ischemic brain areas (Stroka et al., 2001).

Similar to NF-κB, HIF-1 has also been shown to exert both neuroprotective and harmful effects in ischemic stroke (Helton et al., 2005; Liu et al., 2005; Baranova, 2007). Neuronal specific knockdown of HIF-1α increases infarct volume and mortality following cerebral ischemia (Baranova, 2007). In contrast, knockdown or pharmacological inhibition of HIF-1α results in protection against global ischemic brain damage, indicating a detrimental effect (Helton et al., 2005; Chen et al., 2009). A role for HIF-1α in hypoxic preconditioning-mediated neuroprotection has also been demonstrated (Liu et al., 2005). These findings could suggest that HIF-1α is a mediator of a hormesis-based process in which low levels of HIF-1α activation are neuroprotective, whereas higher levels of activation can induce cell death.

We have established that expression of HIF-1α increases in neurons following ischemic stroke and that it can bind directly with NICD and NF-κB (Cheng et al., 2014a,b). Inhibition of either the Notch pathway using a γ-secretase inhibitor in combination with HIF-1α antagonist or inhibition of HIF-1α alone reduced NF-κB, phospho-p65, and pro-apoptotic cleaved caspase-3 under hypoxic conditions (Cheng et al., 2014a,b). Compared to inhibition of either one alone, inhibition of both γ-secretase/Notch and HIF-1α further reduced apoptotic cell death. These findings are further strengthened by the observation that expression of NF-κB is increased following overexpression of NICD, HIF-1α, or both, in normal or hypoxic conditions (Cheng et al., 2014a,b). Furthermore, inhibition of HIF-1α blocks the abilities of NICD and HIF-1α to increase p-p65 levels (Cheng et al., 2014a,b). Collectively, these findings suggest that NICD, HIF-1α and NF-κB interact functionally to determine the fate of neurons following ischemic stroke.

Another important protein that collaborates with Notch to promote neuronal death following ischemic stroke is p53. p53-mediated apoptosis is a common mechanism of cell death that can be triggered by multiple cellular insults, including oxidative stress and DNA damage (Amundson et al., 1998). p53 is a transcription factor that regulates the cell cycle, and is widely known as a tumor suppressor protein. p53 transcriptionally regulates genes that mediate auto-regulatory feedback loop control of the cell cycle, as well as genes involved in angiogenesis, cellular stress responses, DNA repair and apoptosis (Amundson et al., 1998; Tokino and Nakamura, 2000; Rahman-Roblick et al., 2007). The structure of p53 includes conserved regions with specialized functions such as the N-terminal domain, DNA binding domain, tetramerization domain and a C-terminal domain. The N-terminal domain is involved in transcriptional transactivation and C-terminal domain cooperates with single-stranded DNA to facilitate transcription (Balagurumoorthy et al., 1995; Joerger and Fersht,

2010; Saha et al., 2015) (Fig. 2). p53 is activated by several stress signals, including hypoxia, DNA damage, radiation and chemotherapeutic drugs (Norbury and Zhivotovsky, 2004). Multiple post-translational modifications of p53 regulate its functions. For example, phosphorylation of N-terminal residues, and poly ADP-ribosylation or acetylation of residues in the C-terminal region promote stability of p53 protein and enhance its DNA-binding affinity (Malanga et al., 1998; Coultas and Strasser, 2000; Appella and Anderson, 2001; Brooks and Gu, 2003).

Hypoxia down-regulates the E3 ubiquitin-protein ligase, MDM2, a negative regulator of p53, resulting in the accumulation of phosphorylated p53 (Yang et al., 1997; Mielke and Herdegen, 2000; Zhu et al., 2002). HIF-1α interacts with MDM2 during cellular stress and may indirectly influence activation of p53 by inhibiting its negative regulation by MDM2. Moreover, de-phosphorylated HIF-1α stabilizes p53 via to promote cell death during hypoxia (An et al., 1998). Also, the interaction between p53 and HIF-1α is enhanced by de-phosphorylation of HIF-1α executed during cellular stress and facilitates the apoptosis pathway. Further, p53 and HIF-1α share the same co-activator, p300, and by competing with HIF-1α, p53 attracts co-activators to shift towards an apoptotic pathway (Halterman and Federoff, 1999; Chen et al., 2003; Schmid et al., 2004a,b,c).

The Notch – p53 association in apoptosis was first reported in a study of early neural progenitor cells by Yang et al. (2004), who showed that expression of an active form of Notch1 (NICD) selectively induces extensive apoptosis by elevating levels of nuclear p53 and up-regulating transcription of pro-apoptotic genes such as those encoding Bax and Noxa (Yang et al., 2004). We recently established a novel attribute of Notch in endangering neurons by promoting p53-mediated apoptosis. The interaction of Notch with p53 is a critical molecular step in apoptotic signaling in neurons after ischemic stroke (Balaganapathy et al., 2017). The functional role of the NICD-p53 interaction in modifying disease outcome following stroke involves the stabilization of p53 and the transcriptional regulation of p53 and NICD target genes which can lead to cell death. In addition, p53 inhibition studies have revealed that p53 can increase NICD levels by γ-secretase-mediated cleavage of Notch (Balaganapathy et al., 2017). Importantly, the latter study demonstrated that NICD stabilizes p53 and rescues it from MDM2-mediated ubiquitination and degradation.

Interestingly, Pin1, a peptidyl-prolyl isomerase (PPIase) that regulates p53 transactivation under stress, is implicated in the pathogenesis of ischemic stroke by a mechanism involving Notch signaling. Pin1 has an established role in the stabilization of post-translationally modified proteins by catalyzing protein isomerization when phosphorylated at the peptide bond between proline and a phosphorylated serine or threonine (Figs. 2 and 3), and it is known to play a role in pathogenesis of Alzheimer’s disease (Butterfield et al., 2006; Blair et al., 2015). We recently established that Pin1 also plays a critical role in cerebral ischemia-induced neuronal cell death and neurological deficit (Baik et al., 2015). Specifically, we showed that the Pin1-FBW7-Notch1 axis confers neuronal vulnerability in ischemic stroke, and that neuronal injury can be ameliorated by either a Pin1 inhibitor or Pin1 deficiency (Baik et al., 2015) (Fig. 3). Pin1 interacts with NICD in the brain to increase its stability after ischemic stroke by inhibiting FBW7-mediated polyubiquitination of NICD, resulting in

facilitation of NICD-induced neuronal death (Baik et al., 2015). Notably, NICD mutants with reduced stability due to loss of the Pin1 interaction protect neurons against death. Thus, the coordinated interactions between Pin1, FBW7 and NICD may promote neuronal death in ischemic stroke through the stabilization of NICD (Baik et al., 2015).

It is also important to note that phosphorylated p53 interacts with Pin1 to form a complex and undergoes a conformational modification that induces apoptotic gene expression and mitochondria-mediated cell death effector cascades (Buschmann et al., 2001; Zacchi et al., 2002; Zheng et al., 2002; Sorrentino et al., 2013). In addition to the finding that Pin1 interacts with NICD, we identified that Pin1 also interacts with p53 following ischemic stroke. Thus, Pin1-mediated NICD stabilization and/or p53 phosphorylation play vital roles in the pro-apoptotic function of the p53-NICD complex (Baik et al., 2015). Furthermore, the Pin1 interaction is lost upon deletion of several NICD domains, with the ANK domain of NICD being particularly critical for the interaction (Baik et al., 2015). Similarly, the interaction of p53 with NICD occurs via the NICD ANK domain under simulated ischemic conditions (Balaganapathy et al., 2017). This finding is noteworthy as ANK-domain mutant cells exhibit profound resistance to ischemic conditions compared to NICD mutants involving RAM, TAD, OPA and PEST domains (Baik et al., 2015).

Another transcription factor that is known to partner with Notch is activator protein-1 (AP-1) (Chu et al., 2002; Benhra et al., 2011; Forghany et al., 2018). AP-1 comprises dimers that include members of the Jun and Fos families (Raivich and Behrens, 2006). An early study by Chu and Bresnick (2004) established that NICD binding to RBPJ is compulsory for AP-1 suppression. It was also shown that AP-1 acts as a negative regulator of the Notch pathway by controlling Notch activator Sanpodo recycling (Benhra et al., 2011). A role for AP-1 in ischemic stroke-induced neuronal cell death and post-stroke inflammation is well established. It was shown that while AP-1 may promote survival in an ischemia-resistant area of hippocampus, it might promote delayed neuronal apoptosis with a simultaneous upsurge of c-Jun in the AP-1 dimer (Domańska-Janik et al., 1999). Our group established that activation of TLR in neurons in response to ischemia promotes death by activating the JNK/AP-1 pathway (Tang et al., 2007). This was supported by reports that inhibition of the JNK/AP-1 pathway protects against glutamate excitotoxicity in primary cortical neuronal cultures (Meade et al., 2010) and against neuronal cell death after hypoxic brain injury (Nijboer et al., 2010). It is also important to note that AP-1 has been shown to play a pivotal role in axonal regeneration by regulation of CD44, galanin and integrin (Raivich et al., 2004). While there are studies showing AP-1 to be activated in response to ischemia, and that AP-1 and Notch may interact to regulate cell differentiation during development, no studies have investigated AP-1/Notch interactions in neurons in ischemia models.

Collectively, the evidence described above suggests that NICD can interact with NF-κB, HIF-1α, p53 and Pin1 signaling pathways and it may determine cell fate following ischemic stroke. The remainder of this article considers the emerging evidence that these five hypoxia/ischemia-responsive pathways converge on a nuclear multi-protein transcriptional complex. This protein complex consists of NF-κB, HIF-1α, p53, Pin1 and NICD, and may contain several interacting proteins including p300, CSL, MAML, RBP-Jκ, CBP, CREB, ARNT, p16, AP-1 and c-JUN and regulates the expression of adaptive stress response genes

encoding proteins that may promote cell survival during neural circuit remodeling or mild stress conditions. However, under severe hypoxic or ischemic conditions, this transcriptional complex induces the expression of genes that initiate cell death.

4. Mechanisms of cell death by Notch in ischemic stroke

Unlike many other signaling pathways that regulate gene transcription, the Notch signaling pathway to the nucleus is not amplified by cytoplasmic second messengers and kinases. Upon ligand (Jagged or Delta) binding to Notch, the NICD translocates to the nucleus and interacts with its binding partners such as CSL transcription factor complex (Schroeter et al., 1998; Brou et al., 2000; Miele, 2006; Lubman et al., 2007). NICD binding to CSL recruits the adaptor protein Mastermind-like (MAML), and MAML recruits other proteins including HAT p300 which are essential components of the Notch pathway transcription machinery (Schroeter et al., 1998; Brou et al., 2000; Miele, 2006; Kopan and Ilagan, 2009). While molecular level data on how these proteins interact is lacking, at least some interactions are likely to involve the MAML cofactor, an integral part of Notch transcriptional activities. NICD-mediated transcription induces expression of genes such as Hairy-Enhancer of Split (HES) and HES-related protein (HERP) genes (Iso et al., 2001; Iso et al., 2003; Borggrefe and Oswald, 2009). As pointed out above, Notch-mediated neuronal cell fate determination or other events could be influenced not only by HES but also by the interaction of NICD with other targets, such as NF-κB (Cheng et al., 2001; Arumugam et al., 2011), HIF-1α (Cheng et al., 2014b), p53 (Balaganapathy et al., 2017), Pin1 (Baik et al., 2015) and c-Jun (Cheng et al., 2014a).

Roles for Notch in survival and apoptosis are evolutionarily conserved. In the developing nerve cord of *Drosophila*, active Notch signaling dictates death of the dying lineages, while inactive Notch promotes death of hemilineages (Lundell et al., 2003; Lee et al., 2013). Notch has also been reported to induce apoptosis in other *Drosophila* tissues (Orgogozo et al., 2002; Yu et al., 2002). One possible mechanism for Notch-mediated apoptosis is that Notch may enhance the expression of NF-κB-regulated apoptotic genes (Fig. 4a). Although Notch and NF-κB are well known to have pro-survival and anti-apoptotic functions in diverse cell types, they play a detrimental role in prolonged cerebral ischemia. One NF-κB-regulated neuronal gene that may mediate neuronal death following a stroke is the pro-death BH3-only protein Bim (Inta et al., 2006; Arumugam et al., 2011). We showed that inhibition of γ-secretase protects against neuronal cell death following ischemic/hypoxic conditions by targeting Bim (Arumugam et al., 2011). Furthermore, treatment of mice with a γ-secretase inhibitor reduces levels of NICD, p-p65 and Bim together with the severity of ischemic injury (Arumugam et al., 2011). Similar to Bim, Noxa is another Bcl-2 family pro-apoptotic protein that may play a role in NICD-NF-κB-regulated cell death following ischemic stroke (Ridder and Schwaninger, 2009). Notch signaling can also potentiate the NF-κB-mediated inflammatory processes in microglia which, in turn, release neurotoxic factors (reactive oxygen species and cytokines). Activation of the Notch pathway was shown to be required for the maximal level of NF-κB activation and production of IFN-γ, suggesting that the crosstalk between these signaling pathways participates in inflammatory processes (Shin et al., 2006; Cao et al., 2011).

A Notch-NF-κB interaction occurs in microglial cells following ischemic stroke and promotes post-stroke inflammation. One potential consequence of microglial activation following stroke is the production cytokines such as interleukin (IL)-1β and TNF, which are the major NF-κB-dependent molecules that promote secondary/delayed neuronal death associated with brain ischemia (Wei et al., 2011) (Fig. 4a). Translocation of NF-κB/p65 to the nucleus is weakened in NAS microglial cultures under ischemic conditions, implying that Notch plays a role in NF-κB activity, to stimulate the production of IL-1β and TNF in microglia following stroke. Consistent with this are findings that both Notch and NF-κB pathways function synergistically to produce pro-inflammatory cytokines and nitric oxide (NO) in activated microglia during stroke (Cao et al., 2011; Yao et al., 2013). Furthermore, an elegant study by Shin et al. (2006) demonstrated that Notch signaling prolongs NF-κB activity in leukocytes (Shin et al., 2006). Notch transcriptionally promotes the expression of the NF-κB signaling complex and enhances NF-κB activation (Oswald et al., 1998). Notch also promotes neurogenesis, vascularization, astrocyte proliferation and post-ischemic inflammation during remodeling phase following stroke (Fig. 4b).

Under persistent or severe hypoxic conditions, such as ischemic stroke, HIF-1α modulates a number of gene targets that contribute to apoptotic cell death. Pro-apoptotic Nip3 is effectively activated by the overexpression of HIF-1α and hypoxia (Bruick, 2000), and another HIF-1α-dependent gene target, RTP801, also contributes to cell death following stroke (Shoshani et al., 2002). The promoter for the BH3-only Bcl-2 family protein, Noxa, responds directly to hypoxia via HIF-1α (Kim et al., 2004). Noxa is also a candidate molecule for the mediation of p53-induced apoptosis. p53 stabilization in hypoxia occurs parallel with HIF-1α accumulation and is dependent on the presence of HIF-1α (Schmid et al., 2004a,b,c; Zhou et al., 2015). Moreover, a direct association between p53 and HIF-1α was detected in hypoxic cells (An et al., 1998; Chen et al., 2003). Stabilization of p53 in hypoxia by HIF-1α increases the expression of p53 target genes, such as p21, and results in the initiation of apoptosis. While the gene targets of the NICD/HIF-1α/NF-κB/p53 interaction are largely unexplored, we found that this interaction indeed promotes neuronal death following exposure to ischemic stroke conditions (Arumugam et al., 2011; Cheng et al., 2014b; Baik et al., 2015; Balaganapathy et al., 2017).

Neuronal apoptosis is potentiated by the overexpression of both NICD and HIF-1α as compared with either protein alone, and HIF-1α inhibitor reduces cell death caused by expression of either HIF-1α alone or expression together with NICD (Cheng et al., 2014b). The latter evidence from studies using cell lines exposed to simulated ischemic conditions supports the collaborative neuronal death-promoting roles of Notch and HIF-1α. In vivo models of ischemic stroke further support these findings. Early inhibition of HIF-1α reduces brain damage and neurological deficits, and additional protection is achieved when animals are treated with both γ-secretase and HIF-1α inhibitors. Immunoprecipitation analysis of ischemic brain tissues, as well as primary cortical neurons following simulated ischemic conditions, revealed that NICD directly interacts with HIF-1α (Cheng et al., 2014b). Moreover, the idea of a multi-protein transcriptional complex that promotes cell death following ischemic/hypoxic conditions is further strengthened by the findings that NICD and HIF-1α overexpression in neuronal cells upturns the expression of phosphorylated NF-κB-p65, and inhibition of HIF-1α results in diminished levels of phosphorylated NF-κB-p65,

suggesting that both Notch and HIF-1α may determine NF-κB expression and activation during ischemia (Cheng et al., 2014b) (Fig. 5).

Other possible factors involved in the formation of such a multiprotein transcriptional complex, and the regulation of cell survival- and cell death-promoting genes, are JNK/c-Jun/AP1 and p53. JNK and cleaved caspase-3 levels were reduced by inhibition of Notch, either alone or in combination with HIF-1α inhibition, following simulated ischemia (Cheng et al., 2014a). Notch signaling may endanger neurons under ischemic conditions by controlling the JNK/c-Jun pathways (Cheng et al., 2014a). We have shown that inhibition of JNK can salvage the NICD-overexpressing cells under ischemic conditions which may indicate that apoptotic pathways are activated by the NICD/JNK-c-Jun pathway in association with NF-κB and HIF-1α (Fig. 5). However, it was also shown that NICD inhibits the activation of JNK by interacting with the JNK binding domain of JNK-interacting protein 1 (JIP) (Kim et al., 2005). Therefore, the NICD-JNK interaction and cell death under ischemic conditions need further investigation to clarify these relationships. Nevertheless, interplay between HIF-1α and AP-1 was shown to increase gene transcription (Michiels et al., 2001).

In addition to Notch, the β-amyloid precursor protein (APP) is a major substrate for γ-secretase that has received intensive investigation in the field of Alzheimer's disease because cleavage of APP by γ-secretase produces amyloid β-peptide, the key molecule of the disease-defining extracellular amyloid plaques (Mattson, 2004). However, similar to Notch, cleavage of APP by γ-secretase generates an APP intracellular domain (AICD), which translocates to the nucleus (Kimberly et al., 2001). Data from studies of cultured neural cells suggest that AICD can trigger apoptosis (Ozaki et al., 2006; Kögel et al., 2012). It is therefore possible that, similar to the NICD, AICD is a component of a multi-protein transcriptional complex. Further research will be required to determine whether AICD interacts with Notch and its partners and to elucidate the relative contributions of AICD versus NICD generation plays in the neuroprotective effects of γ-secretase inhibitors in stroke.

Notch interacts with p53 in neuronal cells to promote cell death by inducing expression of apoptotic proteins such as Puma, Noxa and Bad following ischemic stroke (Yang et al., 2004; Yonekura et al., 2006; Balaganapathy et al., 2017). After the onset of ischemia, p53 transactivation is instigated to regulate target genes including those involved in cellular stress responses, DNA repair and apoptosis (Gomez-Lazaro et al., 2008; Menendez et al., 2009; Hong et al., 2010; Filichia et al., 2015; Li et al., 2015). The proteins encoded by p53-inducible genes include Bcl-2 family members that contain various BH domains, such as BH1, BH2, BH3 or BH4. Regulated expression of pro-apoptotic proteins includes the multidomain Bcl-2 family member Bax, BH3-only members such as p53-upregulated modulator of apoptosis (PUMA), Noxa, and Bid, which play key roles in specific cases of neuronal apoptosis (Cregan et al., 2004; Kiryu-Seo et al., 2005; Hong et al., 2010; Akhter et al., 2014). The proapoptotic protein Bax is up-regulated by p53 in response to DNA strand breaks that occur during ischemia and mediates p53-induced neuronal apoptosis. Glutamate-induced calcium influx and oxidative stress, both of which increase in neurons subjected to ischemia, can trigger Bax translocation to the mitochondrial membrane, a pivotal event in

apoptosis (Xiang et al., 1998; Uo et al., 2009; Kotipatruni et al., 2011; Wan et al., 2013). The pro-apoptotic BH3-only proteins, Puma and Noxa, are also target genes of p53 and act by inhibiting the anti-apoptotic Bcl-2 family proteins located at the mitochondria, thus allowing the pro-apoptotic Bax to trigger membrane permeability pore formation and cytochrome c release (Vousden and Lu, 2002; Jeffers et al., 2003; Reimertz et al., 2003).

Other target genes of p53 involved in the cellular death pathway, including insulin-like growth factor binding protein-3 (IGF-BP3), p53-activated gene 608 (PAG608), DR5/KILLER, FAS1 and FAS ligand (FASL/CD95L), have also been found to play a role in ischemic conditions. IGF-BP3 and PAG608 are upregulated during ischemia in humans, however their signaling mechanism has not been elucidated (Tomasevic et al., 1999; Hermann et al., 2001; Johnsen et al., 2005). Genes encoding DR5, FAS1 and FASL are involved in an extrinsic (mitochondria-independent) apoptotic pathway. DR5 and FAS are well-characterized receptors that are activated in ischemic stroke, and FASL controls activation of the FAS receptor (Owen-Schaub et al., 1995; Geske et al., 2001; Reimertz et al., 2003; Culmsee and Mattson, 2005; Webster et al., 2006). Other proteins expressed by p53 include p53-regulated apoptosis inducing protein-1 (p53AIP1) and p53-inducible gene (PIG), which are related to mitochondrial dysfunction leading to ROS accumulation, along with SIVA, which has an indispensable role in ischemia establishing direct interaction with TNFR family proteins as well as anti-apoptotic Bcl-2 family proteins (Prasad et al., 1997; Oda et al., 2000; Matsuda et al., 2002; Xue et al., 2002; Fortin et al., 2004). Critically, the factors that determine whether the interplay of Notch with p53 switches to become detrimental for neurons following ischemic stroke need further investigation. Further Notch-p53 interaction studies will be required to determine in detail the intricate mechanism (s) underlying the contribution of this complex of proteins to the apoptotic pathway. As explained above, since p53 is also involved in transcription-independent mechanisms of apoptosis, the role of the established NICD/p53 complex on this functional aspect of p53 remains to be investigated.

It is possible that many other apoptosis-promoting transcription factors may contribute to the formation of this multi-protein transcriptional complex following stroke. These factors may include p300, MAML, AICD and forkhead box O3 (FOXO3). While evidence for the interaction of Notch with NF-κB, HIF-1α, JNK/c-Jun, Pin1 and p53 in stroke exists, its interaction with other transcription factors in stroke models has not been established.

5. Notch/NF-κB/p53/HIF-1α axis in neuronal plasticity and resilience

While physiological roles for the Notch/NF-κB/p53/HIF-1α axis remain to be established, the responsivity of its various components to cellular stress, suggest that this multi-protein transcriptional complex may also mediate adaptive responses of neurons to bioenergetic challenges and promote survival of neurons during recovery and remodeling phases following ischemic stroke. Based on the published evidence, we propose that Notch/NF-κB/p53/HIF-1α axis may influence responses of neurons to increased excitatory synaptic activity and increased energy demand. In this view, when neurons experience relatively moderate bioenergetic stress, Notch up-regulates adaptive stress response genes encoding proteins that promote neuronal survival and plasticity (Figs. 4b and 6).

Notch, NF-κB and HIF-1 are all involved in developmental and adult neuroplasticity. During embryonic stages of development of the central nervous system, Notch signaling promotes neural progenitor cell (NPC) proliferation, and a reduction in Notch signaling can trigger the differentiation of NPCs into neurons (Corbin et al., 2008). Notch knockout mice die during embryonic development, and exhibit severe cortical dysplasia involving premature neuronal differentiation and depletion of the NPC pool, a phenotype indistinguishable from that of mice lacking presenilin 1 (γ-secretase) (Hartmann et al., 1999; Handler et al., 2000). Notch signaling also plays an important role in synaptic plasticity (Wang et al., 2004; Alberi et al., 2011; Liu et al., 2015). Long-term potentiation (LTP) at hippocampal CA1 synapses is impaired in mice with reduced levels of Notch, and application of the Notch ligand Jagged1 enhances LTP (Wang et al., 2004). The latter study further demonstrated that reduction in Notch expression suppresses basal and stimulated NF-κB activity in hippocampal cells. NF-κB plays a vital role in regulating LTP (Albensi and Mattson, 2000) suggesting that cross-talk/convergence of Notch and NF-κB pathways is involved in hippocampal synaptic plasticity. Notch mutant mice exhibit learning and memory deficits consistent with impaired hippocampal synaptic plasticity (Costa et al., 2005). Jagged1 may be the major ligand that activates the Notch signaling pathway during learning and memory (Sargin et al., 2013), while the immediate early gene Arc has also been implicated as an upstream effector of Notch signaling in synaptic plasticity and learning and memory (Alberi et al., 2011). Emerging findings indicate that NF-κB plays a vital role in neuroplasticity (Engelmann and Haenold, 2016). Synaptic activation of glutamate receptors induces NF-κB activity in cerebellar neurons in culture (Guerrini et al., 1995) and in the hippocampus (Meberg et al., 1996). Mice lacking p65 exhibit a spatial learning deficit (Meffert et al., 2003), and c-Rel-deficient mice also exhibit impaired hippocampal synaptic plasticity and memory deficits (Ahn et al., 2008). It has been suggested that CREB plays an important role in the regulation of synaptic plasticity and memory by NF-κB (Kaltschmidt et al., 2006). The formation of new synapses during brain development and in response to environmental inputs may also require NF-κB activation (Boersma et al., 2011).

HIF-1α mediates the survival of NPCs in hypoxic niches in the subgranular zone of the adult hippocampus (Chatzi et al., 2015), which may occur by modulation of the Wnt/β-catenin signaling pathway (Mazumdar et al., 2010). NPCs isolated from neonatal mice subjected to intermittent hypoxia exhibit increased proliferative potential when propagated as neurospheres, an effect of hypoxia mediated by HIF-1α (Ross et al., 2012). In contrast to Notch and NF-κB, it is unclear whether HIF-1 plays an important role in synaptic plasticity, although it was reported that inhibition or genetic knockdown of HIF-1α blocks the ability IGF-1 to enhance synaptic transmission in cultured hippocampal neurons (Huang et al., 2010).

A collaborative role for Notch and NF-κB in cellular differentiation was first shown in hemopoietic progenitor cells (HPCs), wherein the ability of NF-κB to bind DNA and trigger transcription was strongly decreased in cells from NAS mice (Cheng et al., 2001). Differentiation of dendritic cells was significantly affected in NAS mice and restored by transduction of activated Notch into HPCs. Moreover, increases in p65-NF-κB protein levels augment the Hes1 promoter, and other promoters such as serum response element (SRE), and may induce cell death mechanisms under hypoxic conditions (Espinosa et al., 2002).

Collaboration between NF-κB and Notch occurs in primary cortical neurons and contributes to the regulation of neurite outgrowth, while impaired neurite outgrowth in p50 NF-κB-deficient cortical neurons is mediated by hyperactivation of the Notch pathway (Bonini et al., 2011). Furthermore, NAS transgenic mice exhibit reduced NF-κB activity and impaired LTP at hippocampal CA1 synapses, suggesting a fundamental role for Notch-NF-κB signaling in synaptic plasticity (Wang et al., 2004). However, except for a few such studies, investigations of interactive roles for neural signaling pathways that converge on the Notch/NF-κB/p53/HIF-1α axis are lacking. It will therefore be of considerable interest to interrogate the status of this multi-protein transcriptional complex in various experimental models of neuroplasticity at the neuronal network and behavioral levels.

6. When and how does Notch signaling and its interacting pathways switch from cell survival to cell death?

The dual role of the Notch/NF-κB/p53/HIF-1α axis to either promote neuroplasticity and cellular stress resistance when activated at physiological levels, or trigger cell death when over-activated, can be attributed to its protein components. Accumulating evidence has demonstrated that many putative constituents of this complex, including Notch, NF-κB, HIF-1α, p-53 and Pin1, can exert completely opposite functions in the modulation of cell death in a context-dependent manner (Fig. 6).

Studies have shown that mild or intermittent hypoxia stimulates hippocampal neurogenesis *in vivo* (Tsai et al., 2013; Zhang et al., 2014; Chatzi et al., 2015). *In vitro* studies also confirm that hypoxia can promote survival, proliferation and differentiation of central nervous system stem cells (Studer et al., 2000; Ara and De Montpellier, 2013). On a consistent basis, ischemic stroke temporarily activates the Notch pathway in neural progenitor cells in the adult rat, and Notch activation coincides with an increase in the proliferation of NPCs (Wang et al., 2009a,b). Administration of Notch-activating antibodies enhances ischemic stroke-induced neurogenesis in the SVZ, reduces infarct volume and improves motor deficits in aged ischemic rats (Sun et al., 2013). A recent study showed that intermittent hypoxia enhances neurogenesis and dendritic spine formation in neurons, and these adaptive responses to mild hypoxia of NPCs and neurons are attenuated in Notch heterozygous (*Notch*+/−) mice (Zhang et al., 2014).

As discussed in Section 4, under conditions of severe stress, the collaboration of p53 with other proteins such as NF-κB and HIF-1α may play a major role in detrimental outcomes. However, p53 also plays a role in the cellular response to hypoxia and is maintained at a low level by MDM2-mediated ubiquitination and proteasomal degradation in normal conditions, while it can be stabilized by various cellular stresses. The severity and duration of hypoxia can very differently influence the levels and activation state of p53 (Hubert et al., 2006; Sano et al., 2007). HIF-1α-mediated stabilization and activation of p53 may result from HIF-1α binding to MDM2, which leads to an accumulation of p53 (An et al., 1998; Chen et al., 2003). Additionally, a moderate level of p53 suppresses transcriptional activity of HIF-1α by competing for the limited amount of shared p300, whereas a high level of p53 leads to degradation of HIF-1α (Schmid et al., 2004a, b, c). Other reports propose that HIF-1α

mediated p53 stabilization promotes hypoxia-induced p53-dependent apoptosis. During severe hypoxia, induction of p53 is HIF-1α dependent (An et al., 1998) because p53 stabilization is mediated by its binding with HIF-1α (Suzuki et al., 2001). Similarly, as mentioned, our recent findings suggest that the interplay between NICD and p53 contributes to neuronal injury and brain infarct development following cerebral ischemia (Balaganapathy et al., 2017). These findings suggest that p53 in the transcriptional complex may play a pivotal role in determining pro-apoptotic signaling after ischemic stroke.

Pin1 may also have a dual function in the pathogenesis of certain diseases. We found that the Pin1-FBW7-Notch axis had a compromising function on neuronal vulnerability after ischemic stroke, and that neuronal injury can be ameliorated by treatment with a Pin1 inhibitor or Pin1 deficiency (Baik et al., 2015). Pin1 interacts with NICD and increases its stability in the brain following ischemia by inhibiting FBW7-mediated polyubiquitination, resulting in facilitation of NICD-induced neuronal death. Therefore, the coordinated interaction between Pin1, FBW7 and NICD, and the well-established function of Notch in brain damage following ischemic stroke, provide evidence that Pin1 regulates neuronal death by interacting with Notch.

When considered in the light of our general knowledge of signaling cascades and transcriptional regulation, the emerging evidence for a Notch/NF-κB/p53/HIF-1α axis suggests several potential molecular mechanisms for a transcriptional switch that regulates neuronal cell fate following a stroke. One level of outcome determination is the timing and intensity of activation of different upstream pathways that converge on the Notch/NF-κB/p53/HIF-1α axis. For example, early activation of HIF-1α and/or NF-κB prior to Notch and p53 may bias this transcriptional complex towards induction of genes that promote cell survival (e.g., antioxidant enzymes and anti-apoptotic Bcl-2 family members). Similarly, a pro-survival response may occur with relatively greater levels of Notch/NF-κB/HIF-1α axis that favor pro-survival gene expression. On the other hand, early and intense up-regulation of Pin1 and p53 would bias neuronal fate towards apoptosis. A second level of outcome determination would be protein-protein interactions, which are regulated by protein stoichiometries and posttranslational modifications. Kinases and phosphatases responsive to Ca^{2+} influx likely play important roles in this regard (Mattson, 2000; Gilman and Mattson, 2002), but little is known concerning how the phosphorylation state of components of the proposed transcriptional complex influences the expression of pro-survival and pro-apoptotic target genes. Additional levels of regulation of this protein complex may include protein acetylation (acetylase and deacetylase activities) and chromatin conformation. Clearly, there is much to be learned regarding how the transcriptional complex is regulated, and how it controls neuronal fate.

7. Concluding remarks and future perspectives

Neurons, which demand high oxygen and energy, are exceptionally vulnerable when subjected to even very short periods of hypoxia. Therefore, the brain has evolved adaptive mechanisms to cope with hypoxic and bioenergetic stress (Fig. 6). The endogenous neuronal adaptive responses activated by hypoxic brain cells may determine whether they survive or die under conditions of high demands for oxygen and energy. The mechanisms triggering

multi-protein transcriptional complex assembly (activation) or disassembly including the different kinetics of inflammatory, deleterious, protective, and/or reparative functions need to be investigated in depth. Emerging evidence not only supports the existence of this transcriptional complex, but also suggests that this protein complex plays a crucial role in adaptive responses of the brain to ischemic stroke. In stroke, the major components of this transcriptional complex are involved in neurodegeneration by exerting multiple roles in the regulation of neuronal death, survival and inflammation. However, these contrasting functions may be due to differences in animal strain, target cells, strength and duration of a stimulus or spatial and temporal context. The factors that define whether activation of the Notch/NF-κB/p53/HIF-1α/Pin1 complex is beneficial or disadvantageous for brain cells in the context of ischemic stroke are poorly understood; however, they are likely to involve the composition of the complex formation, which will determine whether the expression of inflammatory and pro-apoptotic genes or anti-apoptotic genes should be increased.

Acknowledgements

This work was supported by the Singapore Ministry of Education Tier 1 grant [T1-BSRG-2015-01], Singapore National Medical Research Council Research Grants (NMRC-CBRG-0102/2016 and NMRC-OFIRG-0036/2017), an Australian National Health and Medical Research Council grant (NHMRC APP1008048), National Research Foundation (NRF) grant (2015R1A2A1A01003530;2017M3C7A1048268), Republic of Korea, the Ministry of Education, Science, Technology, and a grant of the Korean Health 21 R&D Project (HI14C2539), Ministry of Health & Welfare, Republic of Korea and the Intramural Research Program of the National Institute on Aging, National Institute of Health, USA. We thank Dr. Yi-Lin Cheng and Dr. Mathias Gelderblom for helpful discussion.

Abbreviations:

AP-1	activator protein-1
APP	amyloid precursor protein
AICD	APP intracellular domain
DAMPs	damage associated molecular patterns
FOX03	forkhead box O3
HES	hairy-enhancer of split
HPCs	hematopoietic progenitor cells
HDAC	histone deacetylase
MAML	mastermind/Lag3
HIF-1α	hypoxia inducible factor 1 alpha
IGF-BP3	insulin-like growth factor binding protein-3
JIP	JNK-interacting protein 1
LTP	long-term potentiation
MCAO	middle cerebral artery occlusion

NICD	Notch intra-cellular domain
NF-κB	nuclear factor kappa B
PAG608	p53-activated gene 608
PIG	p53-inducible gene
NPC	neural progenitor cell
p53AIP1	p53-regulated apoptosis inducing protein-1
Pin1	peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
ROS	reactive oxygen species
RAGE	receptor for advanced glycation end products
SRE	serum response element
SMRT	silencing mediator of retinoid and thyroid hormone receptor
TLRs	toll-like receptors
TNF	tumor necrosis factor

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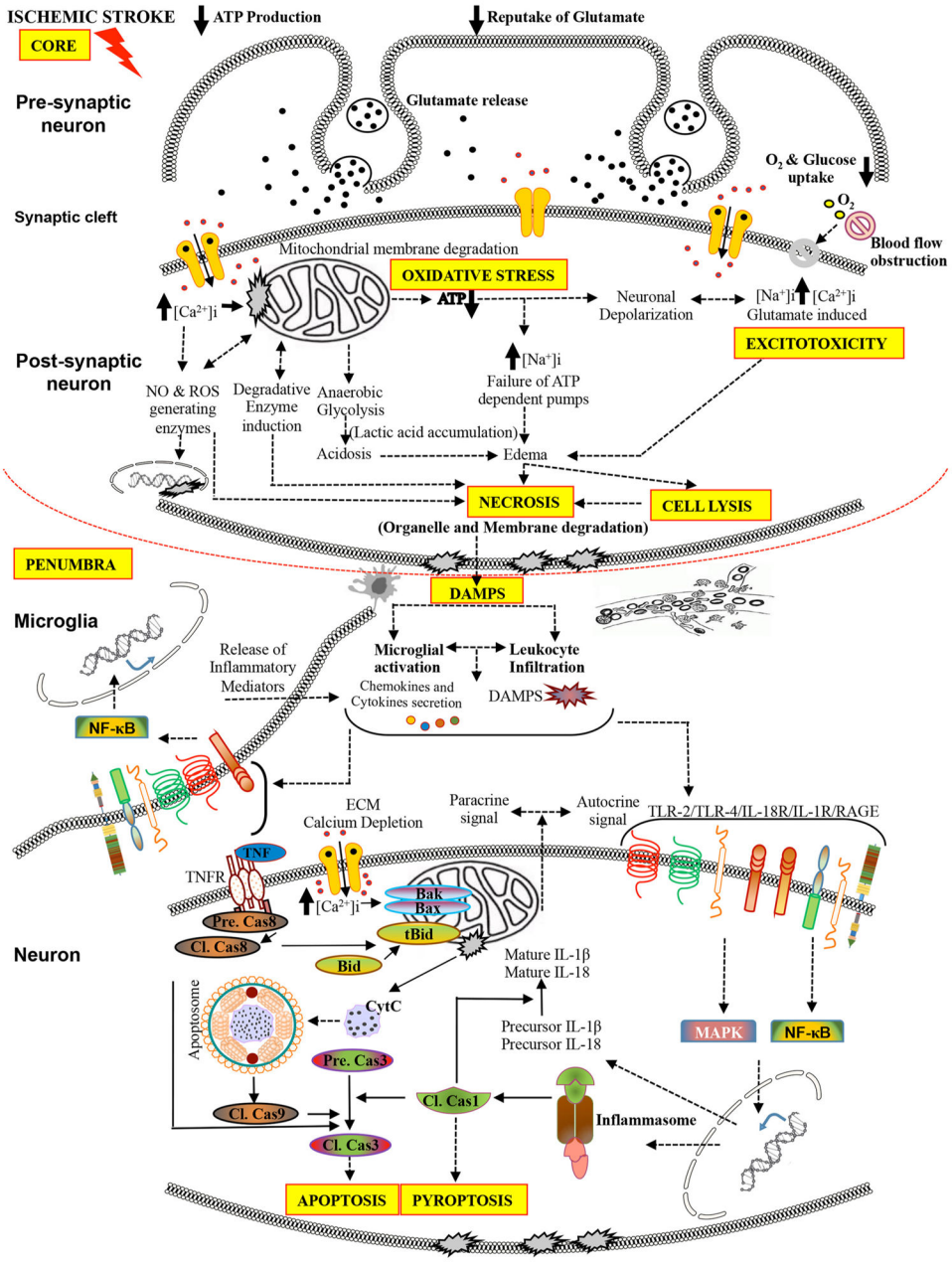

Fig. 1.

Ischemic stroke initiates cell death mechanisms in brain cells following an acute depletion of blood flow, resulting in the formation of an infarct core. ATP production is decreased due to impaired glycolysis and oxidative phosphorylation. The reduced production of ATP results in Na^+ influx due to failure of ATP-dependent Na^+ pumps. At neuronal pre-synaptic terminals, the failure of ATP-pumps causes anoxic depolarization and opening of voltage-gated Ca^{2+} channels which allows influx of Ca^{2+}, inducing uncontrolled glutamate release. Function of the ATP-dependent glutamate uptake transporter is also impaired, causing accumulation of glutamate at the synaptic cleft, which continues to activate Ca^{2+} channels on post-synaptic neurons and results in excitotoxicity. This acute increase in intracellular

Ca^{2+}, along with acidotic toxicity and increased Na^{+} influx, results in cell swelling a characteristic of the osmotic movement of water, which contributes to necrosis. High levels of intracellular Ca^{2+} triggers degradative enzymes and production of reactive oxygen species (ROS), which degrade key membrane and cytoskeletal proteins, lipids and nucleic acids. This also contributes to necrosis as well as the release of DAMPS (damage associated molecular patterns) molecules from the infarct core region. In the region of the penumbra, where limited blood is supplied collaterally, the DAMPS released from the infarct core region may activate surface receptors on glial cells and neurons, initiating signaling cascades such as MAPK and NF-κB, resulting in a positive feedback cycle that further accumulates chemokines and cytokines produced through the inflammasome pathway. The inflammasome complex cleaves pro-caspase-1, making it the executioner of pyroptosis and converting interleukins 1β and 18 to their mature forms, which are then secreted to trigger paracrine and autocrine signaling. The increase in intracellular Ca^{2+} recruits the apoptotic proteins Bax and Bak to the mitochondrial outer membrane, thus compromising the mitochondrial membrane potential and mediating release of cytochrome-C (Cyt-C). Cyt-C forms an apoptosome complex in conjunction with other pro-apoptotic proteins to execute apoptosis by cleaving pro-caspase-9, which in turn cleaves pro-caspase-3 to cleaved caspase-3. Alternatively, activation of the tumor necrosis factor receptor (TNFR) by TNF activates pro-caspase-8, impacting mitochondrial integrity by converting the pro-apoptotic protein Bid to its active truncated form. Then, in combination with cleaved caspase-1 from the inflammasome pathway, it initiates apoptosis via production of cleaved caspase-3, an executioner caspase of apoptosis.

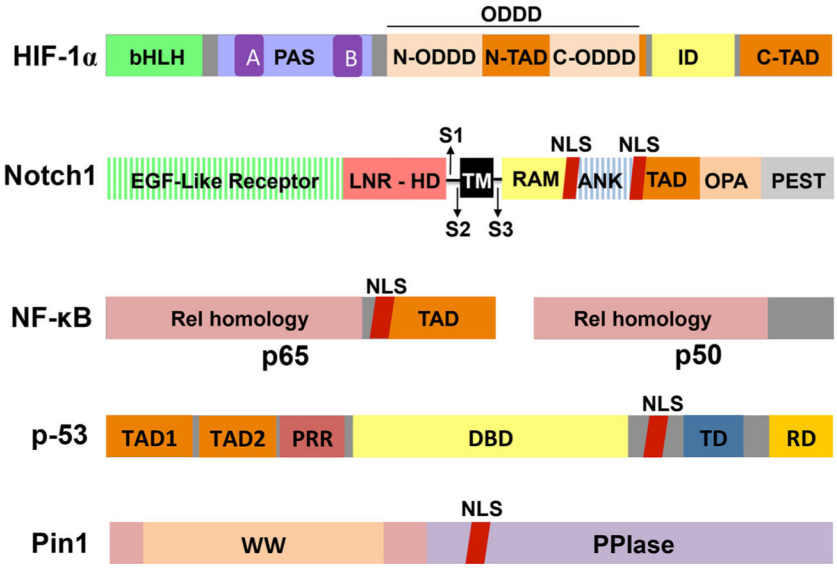

Fig. 2.

Schematic diagrams show the domain structures of a multiprotein transcriptional complex including Notch, HIF-1α, NF-κB, p53 and Pin1. The receptor Notch1 (Notch) contains an extracellular ligand binding region and an intracellular domain (NICD) that arbitrates transcriptional activation of target genes. EGF-like repeats (EGF-LR) of Notch are vital for ligand binding. LIN12-Notch repeats (LNR) and heterodimerization domains (HD) form the negative regulatory region (NRR) which prevents ligand-independent Notch activation. The NICD contains the RBPJ association molecule (RAM) domain, ankyrin repeats (ANK), nuclear localization signal (NLS), a transactivation domain (TAD) and a domain enriched in proline, glutamate, serine and threonine (PEST) residues important for degradation of Notch. Functionally active HIFs consist of HIF-α and HIF-1β (also called ARNT) subunits. The HIF-1α protein is comprised of many domains that are involved in DNA binding. Five proteins, p50, p65/RelA, c-Rel, p52, and RelB constitute the entire family of NF-κB subunits coded by the human genome. p53 contains amino-terminal TADs, followed by a proline-rich region (PRR). The DNA-binding (DBD) is found in the middle of p53, and tetramerization domain (TD) and regulatory region (RD) are located at the C-terminus. Pin1 consists of an N-terminal WW domain and a C-terminal peptidyl-prolyl *cis-trans* isomerase (PPIase) domain. WW domain involves in binding to pSer/Thr-Pro motifs, and PPIase domain catalyses isomerization of pSer/Thr-Pro motifs of the substrates.

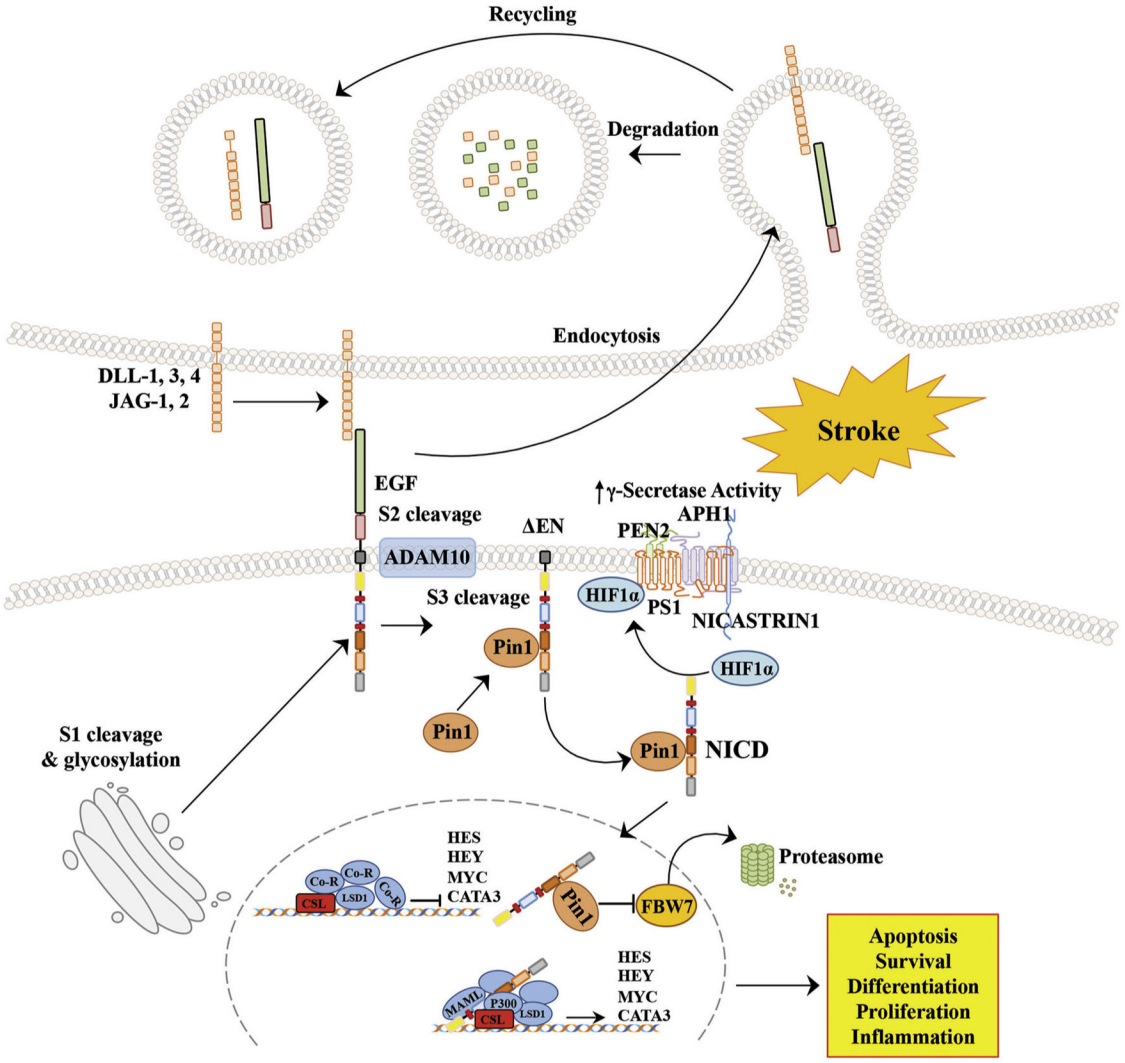

Fig. 3.

The evolutionarily conserved Notch signaling pathway is a fundamental system for communication with other components of the transcriptional complex. Activation of the canonical Notch signaling pathway requires a sequential enzymatic cleavage cascade. Notch is cleaved by Furin (S1 cleavage) at the trans-Golgi and glycosylation occurs, followed by its insertion into the plasma membrane. The extracellular domain of Notch, EGF repeats, interacts with several ligands such as Jagged-1, -3, and -4 and Delta 1 and 2 on neighboring cells or γ-secretase-dependent soluble ligands. Interaction between the ligands and Notch leads to cleavage of the Notch extracellular domain (S2 cleavage), which is then recycled or degraded through endocytosis by a neighboring cell. The remaining trans-membrane anchored Notch domain. ΔEN is subsequently cleaved by the γ-secretase complex (S3 cleavage). Soluble Notch ligands and same-cell ligands can antagonize Notch signaling by direct interaction. γ-secretase activity is increased by HIF-1α in hypoxic conditions. The Pin1-induced conformational change in Notch enhances the γ-secretase-induced cleavage of Notch and increases the stability of NICD (the Notch intracellular domain) via inhibition of

the E3 ligase FBW7. NICD translocates to the nucleus where it interacts with transcriptional cofactors such as p300, CSL, LSD1, and MAML1 to promote target gene expression. Unlike the canonical pathway, TCR activates Notch signaling in a ligand-independent manner by its interaction with Notch. The non-canonical Notch pathway interacts with proteins such as IκB, CARMA1, and β-catenin in the cytosol.

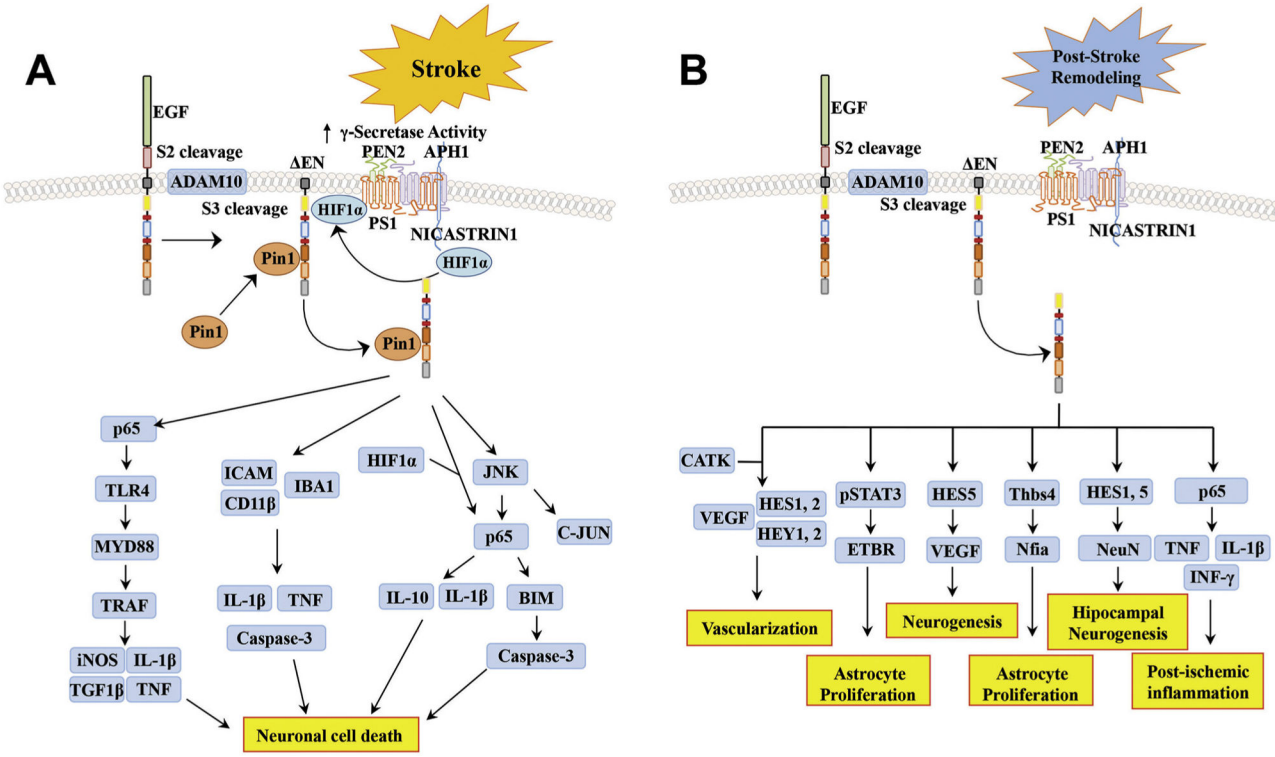

Fig. 4.

Notch can play multiple roles in brain injury during stroke. (**A**) Activated Notch increases apoptotic and inflammatory response proteins, but also interacts with other proteins such as p65, HIF-1α and Pin1. (**B**) During post-stroke remodeling, Notch1 signaling may be involved in repair mechanisms by initiating or interacting with numerous pathways. Notch1 increases neo-vascularization and neurogenesis by up-regulating canonical target genes such as HES1, 2, and 5, and HEY1 and 5. In addition, Notch1 can increase expression of ET-BR and Nfia via activation of pSTAT3 and Thbs4, respectively, both of which can promote astrocyte proliferation.

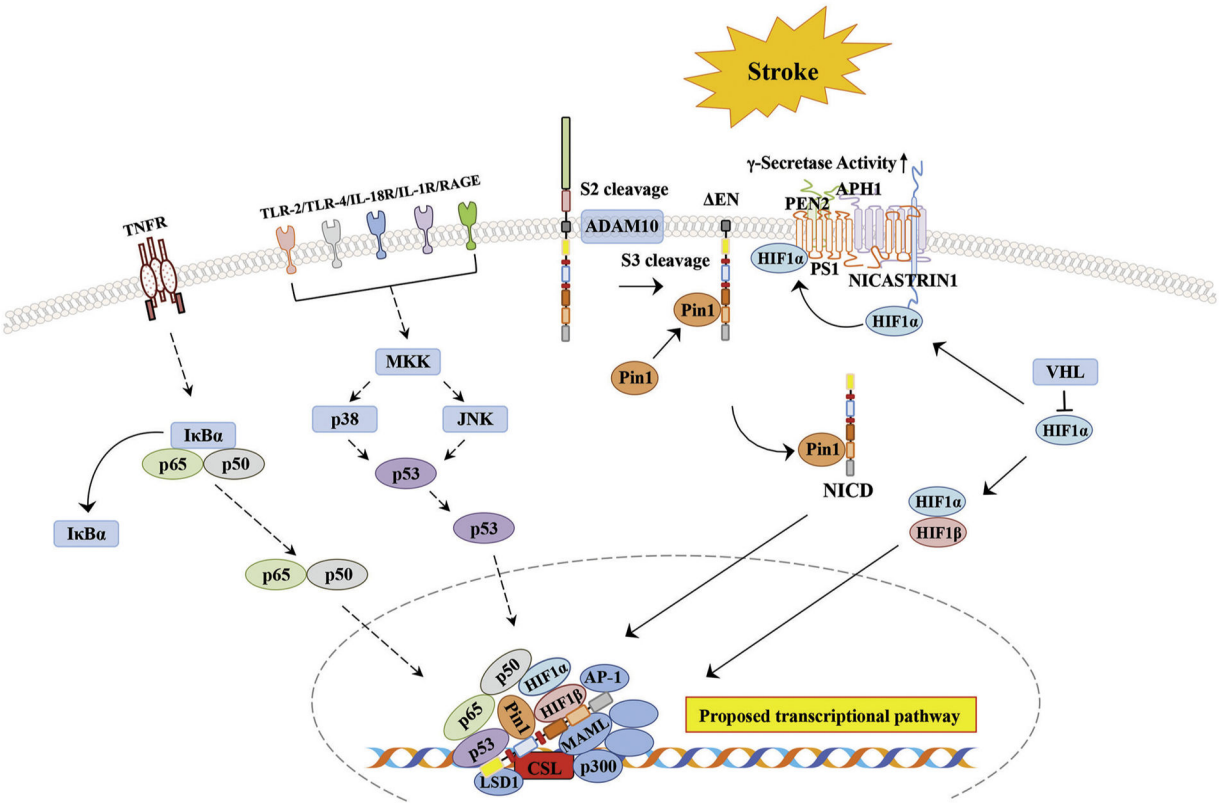

Fig. 5.

Following stroke, the stability of NICD is increased by its interaction with Pin1, and the NICD-Pin1 complex then translocates to the nucleus. Simultaneously, the reduced oxygen level leads to activation of HIF-1α, which then translocates to the nucleus where it functions as a transcription factor. Cell surface receptors elicit phosphorylation of IκBα, the Inhibitor protein of the NF-complex (p65 and p50), making it ubiquitinated and resulting in activation of the NF-κB pathway where the complex also functions as a transcription factor after nuclear translocation. The MAPK pathway is also elicited during activation of cell surface receptors and downstream kinases of the MAPK pathway, p38 and JNK, which then phosphorylate key residues of p53 to facilitate its nuclear translocation once stabilized by its interaction with Pin1. The nuclear proteins, HIF-1α, p65/p50, p53/Pin1 and NICD/Pin1. along with the NICD-binding protein RBP-J/MAML, and co-activator proteins p300, CREB and AP-1, form the ‘multi-protein complex’, a proposed transcriptional complex assembled during ischemic stroke.

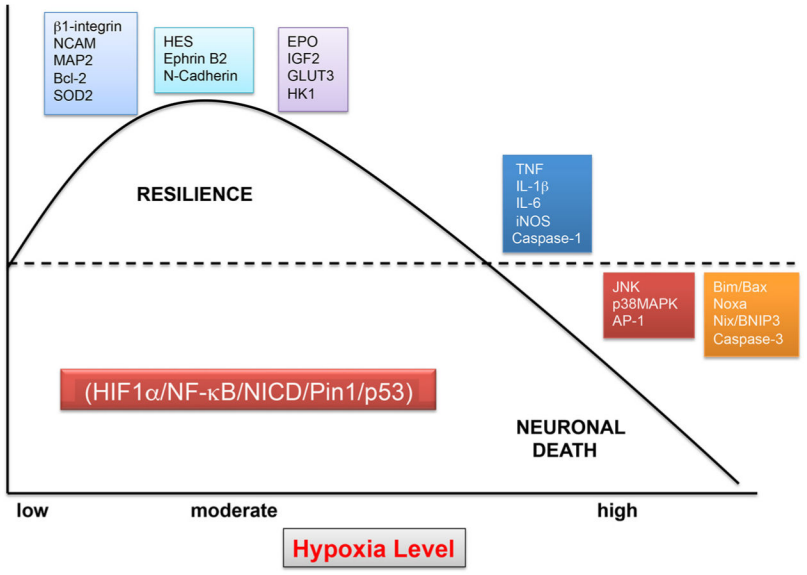

Fig. 6.

The Notch/NF-κB/p53/HIF-1α/Pin1 axis may confer neuronal resilience in response to physiological levels of hypoxic stress. The multiprotein transcriptional complex up-regulates adaptive stress response genes encoding proteins that promote neuronal survival under modest hypoxia (eg. Bcl-2, SOD2, HES, EPO, ect.). When hypoxia is more severe, the multi-protein transcriptional complex induces the expression of genes that trigger (eg. TNF, iNOS, IL-6, JNK, AP-1 ect.) and execute (Bim, Bax, Noxa, Caspase-3, ect.) a neuronal cell death program. The mechanisms by which the Notch/NF-κB/p53/HIF-1α/Pin1 axis activation regulates cellular fate are thought to be controlled by downstream genes and the magnitude and timing of their up-regulation. Various configurations of this multi-protein complex are determined by the expression level of each member of the transcriptional complex in the target cell. Different combinations of protein levels and posttranslational modifications of the multi-protein transcriptional complex components results in different promoter accessibilities, thus resulting in differential expression levels of inflammatory, and anti-apoptotic or apoptotic genes which determine cell fate outcomes under hypoxic stress.