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BRAIN CHEMOTHERAPY FROM THE BENCH TO THE CLINIC: TARGETING NEURONAL SURVIVAL WITH SMALL MOLECULE INHIBITORS OF APOPTOSIS

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1. ABSTRACT

Increasing evidence implicates aberrant apoptosis as a fundamental cause of neurodegenerative diseases. Thus elucidating the underlying causes of neuronal programmed cell death may foster the development of therapeutic interventions. Research in the last 15 years provided a solid foundation for understanding molecular mechanisms of neuronal apoptosis. This review discusses the major molecules and signaling pathways leading to neuronal survival or apoptosis with emphasis on several small molecule inhibitors that target neuronal survival with the hope of impeding the detrimental effects of neurodegenerative diseases.

2. INTRODUCTION

Thirty years ago, considerable skepticism challenged the idea that massive cell populations of the developing nervous system underwent naturally occurring, programmed cell death (PCD) during terminal steps towards maturation. Although in the late 1940s pioneering studies by future Nobel Prize recipient Rita Levi-Montalcini and Viktor Hamburger quantitatively reported normal and experimentally induced neuronal cell death in chick embryos, the notion that the fate of at least 50% of the neurons of the developing nervous system would be death seemed "counterintuitive" (1). How could it be possible that massive death would accompany naturally occurring, non-pathological development of the nervous system? However, in a relatively short time, the seemingly counterintuitive idea, which holds programmed cell death as vital to normal neurodevelopment, has become an accepted truth. A commonly accepted rationale to explain neuronal programmed cell death during development is that neurons proliferate in excess and compete for limiting amounts of neurotrophic factors such as nerve growth factor (NGF). Neurons that form proper connections continue to receive nourishing factors while neurons that fail to integrate into proper circuitry (during synaptogenesis) die through PCD, or more specifically apoptosis (2). Subsequently, proper establishment of neuronal connections ensures neuronal activity, which independent from neurotrophic factors, can maintain the survival of some populations of neurons (3). Apoptosis is a term coined by Kerr et al. (1972) to describe the morphological changes associated with programmed cell death. It is a biologically conserved and necessary form of active (requiring ATP) cell suicide characterized by cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, and phagocytosis by neighboring cells (4). This form of cell death contrasts with necrotic cell death that usually results from injury and causes inflammation because of cellular swelling and rupture (4).

Mounting evidence has implicated aberrant apoptosis as an important step in the progression of a wide variety of chronic and acute neurodegenerative disorders

including but not limited to: Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), and stroke-induced neuronal loss (2,5). As these disorders are largely age-associated, their frequency will likely increase due to the increasing age of the nation's population. Surprisingly, there are few effective treatments and no cures for any neurodegenerative ailments. Thus, using *in vitro* and *in vivo* model systems to investigate the molecular mechanisms of neurodegeneration may provide valuable insight towards development of potential therapeutic interventions. The focus of this article is: 1) to review the current understanding of neuronal apoptosis by highlighting the basic pathways and molecules involved in apoptosis and survival promotion of neurons, and 2) to examine several small molecule inhibitors of neuronal apoptosis that may provide a foundation for potential chemo-neuroprotection therapies.

The foundations of our understanding of apoptosis stems from the work of Sydney Brenner, John Sulston, and Robert Horvitz, and accordingly, their efforts were awarded the 2002 Nobel Prize in Physiology or Medicine. Using the nematode *Caenorhabditis elegans* as a model system to study cell lineage determination, they discovered that of the 1090 cells that arose during development, PCD was responsible for removing 131 cells; in fact, 105 of the apoptotic cells were neurons (6). Subsequently, biochemical analyses identified the key molecular components of the canonical apoptotic cascade found in *C. elegans*. According to the 'central dogma' of PCD in C. elegans, EGL-1 is induced upon apoptotic stimuli and interacts with a cell death inhibitor CED-9, thus supplanting an adaptor CED-4 which promotes activation of an apoptosis executioner CED-3 (6,7). Thus, in the last fifteen years, research sought to find mammalian counterparts to the *C. elegans* death pathway hoping that an understanding of the molecular mechanisms of cell death will facilitate the creation of therapeutic interventions where the death program has gone awry.

Investigators have used a variety of primary cell culture systems to explore the molecular mechanisms of apoptosis in mammalian neurons. Among the most widely used are cultures of sympathetic neurons (from superior cervical ganglion), cortical neurons, hippocampal neurons, and cerebellar granule neurons (8). This review will highlight, where applicable, the mechanisms of apoptosis and survival observed in one of the most commonly employed systems, cultures of rat cerebellar granule neurons (CGNs). Cultures of CGNs obtained from week-old rat pups can be induced to undergo apoptosis when switched from media containing depolarizing concentrations of potassium (25mM, high potassium, HK) to media containing physiological concentrations of K+ (5mM, referred to as low potassium, LK) (9,10). Treatment with HK is likely to mimic the physiologic stimulation of CGN, hence HK simulates neuronal activity (11). Depolarization with HK leads to activation of voltage-gated calcium channels permitting Ca++ to enter the cell and activate downstream signaling (11). At 8 hours following a switch to LK, neurons begin to exhibit characteristic apoptotic morphological changes including cellular shrinkage, chromatin condensation, and DNA fragmentation. At roughly 24 hours following LK-treatment, approximately 50% of the neurons will have undergone apoptosis. However, the commitment to cell death occurs at around 6 hours of activity deprivation (LK) at which time there is no observable difference in the ability to rescue neurons with HK (12). In the absence of HK, neurons survive in culture in the presence of insulin-like growth factor 1 (IGF-1), which is likely a physiologic neurotrophic factor for CGNs *in vivo* (9). Furthermore, our lab and others have found that cyclic AMP elevating agents and analogs, lithium, and thapsigargin can maintain the survival of granule neuron cultures although not as effectively as HK (9,13-16). These survival factors activate distinct signaling pathways that protect the cell from apoptosis (10,17). In other words, when deprived of survival factors, cultures of rat cerebellar granule neurons will undergo programmed cell death with the characteristic features of apoptosis. Therefore, in order to understand apoptosis in neurons a study of the underlying mechanisms of death progression is imperative.

3. DISCUSSION

3.1. Molecules and Pathways Promoting Neuronal Apoptosis

The following sections describe the signaling cascades that promote neuronal apoptosis. We will start by reviewing caspases and their activation because this usually represents the final phase in the eventual demise of a neuron. Then, We will work backwards to describe the molecular events that initiate this phase.

3.1.1. Caspases and neuronal cell death

Caspases are mammalian homologues of CED-3 and are the executioners of apoptosis (18). The word caspase is a contraction of cysteine-dependent aspartate-specific protease (19); thus, the contraction describes the enzymatic properties of caspases whereby a cysteine sidechain in the active site is responsible for cleaving substrates at aspartic acid residues. Caspases are synthesized as inactive zymogens called procaspases and, in response to specific apoptotic signals, are cleaved to form active heterodimers of two small and two large subunits. To date at least, 15 mammalian caspases, 11 in humans, have been identified and can be divided into initiators and effectors. Initiator caspases, including caspases-2, -8, -9, and -10, are the first to be activated and lead to the activation of effector caspases including -3, -6, and -7 which are responsible for cleaving substrates that orchestrate the eventual demise of the cell (5,20). Moreover, knockout studies have revealed an essential role for caspases in developmental neuronal apoptosis. Mice deficient in either caspase-3 or -9 die prenatally and exhibit deficiencies in developmental neuronal cell death characterized by abnormally large brains (21).

3.1.2. Extrinsic Pathway of Caspase Activation

Caspase activation, in general, occurs in two commonly accepted fashions, the extrinsic and intrinsic pathways (Figure 1) (22,23). The extrinsic pathway of

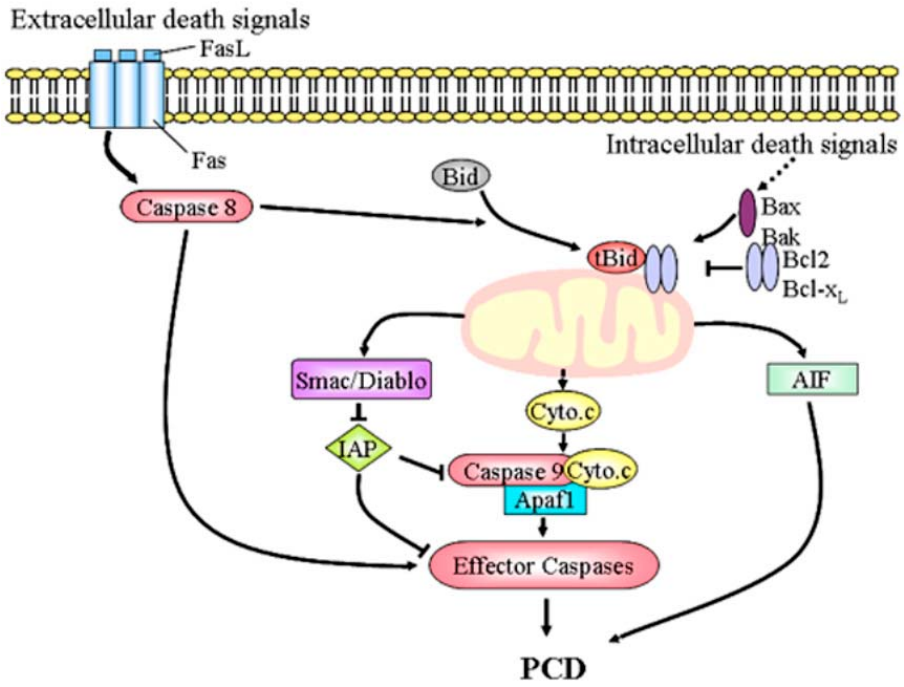

Figure 1. Caspase activation pathway. The Figure delineates the extrinsic and intrinsic pathways of caspase activation. Activation of initiator caspases either by extracellular signals or by internal signals leads to the activation of effector caspases and eventual cell death. AIF, apoptosis-inducing factor; cyto. c, cytochrome *c*; IAP, inhibitor of apoptosis protein; tBid, truncated Bid; PCD, programmed cell death. For details see text.

caspase activation refers to apoptosis induced by death receptors, which include Fas and tumor necrosis factor (TNF) receptor family proteins. In the Fas pathway, Fas activation by Fas ligand (FasL) promotes a conformation change in the cytoplasmic portion of Fas leading to formation of a Death-Inducing Signaling Complex (DISC) at the receptor. In the DISC, Fas interacts, through death domains (DD), with an adaptor protein Fas Associated Death Domain (FADD) that also possesses a death effector domain (DED) promoting its interaction with procaspase-8 (via DEDs) (23,24). Procaspase-8, an initiator caspase, recruited to the DISC in high concentrations results in autoproteolytic activation to caspase-8 and ensuing activation of effector caspases such as caspase-3 and -7 (25). Furthermore, caspase-8 cleaves Bid, a proapoptotic member of the Bcl-2 family of proteins, to a truncated form that translocates to the mitochondria and promotes cytochrome *c* release (26,27). Cleavage of Bid by caspase-8 connects the extrinsic pathway to the intrinsic pathway and underlies the importance of crosstalk and amplification during apoptotic signaling.

3.1.3. Bcl-2 Family Members and the Intrinsic Pathway of Caspase Activation

The mammalian intrinsic pathway, derived from the *C. elegans* model, involves regulation of caspase activity as a function of mitochondrial health in response to apoptotic signaling (22). Readers are reminded that in the C. elegans model, four gene products are responsible for regulating the apoptotic cascade, EGL-1, CED-9, CED-4, and CED-3, of which the mammalian counterparts are Bcl-2 homology (BH) domain 3-only proteins, Bcl-2, Apaf-1, and caspase-3, respectively. Mitochondrial health in response to apoptotic signaling, based on a 'rheostat model,' depends upon the balance between proapoptotic and anti-apoptotic Bcl-2 family proteins that controls the release of cytochrome *c* from the mitochondria (28). Bcl-2 family proteins consist of proapoptotic and anti-apoptotic proteins that contain at least one of four conserved BH domains (23). Proapoptotic proteins come in two forms BH3-only (Bad, Bid, Bim, NOXA, and PUMA) and multi-domain (Bax and Bak) containing BH1-3, while the multi-domain anti-apoptotic proteins (Bcl-2, Bcl-x_L, Mcl-1, A1, and Bcl-W) usually contain BH1-4. Upon an internal death signal, proapoptotic Bcl-2 family proteins translocate to the mitochondria leading to the release of cytochrome *c* by mechanisms that are still under intense investigation (22,23). Once in the cytosol, cytochrome *c* binds to Apaf-1 and in the presence of ATP facilitates activation of caspase-9, thereby promoting activation of caspase-3 (22). In addition to cytochrome *c* release from the mitochondria, other factors exit from the mitochondria and contribute to apoptosis. Release of Smac/Diablo promotes apoptosis by acting as antagonists of inhibitor of apoptosis (IAP) proteins that bind and inhibit caspases (22,29,30). Furthermore, two additional factors, apoptosis-inducing factor (AIF) and endonuclease G, released from the mitochondria are able to promote apoptosis independent of caspases by translocating to the nucleus and regulating chromatin condensation (AIF) and DNA fragmentation (AIF and endonuclease G) (31,32).

Although the mechanisms of caspase-dependent cell death have been elucidated primarily in non-neuronal

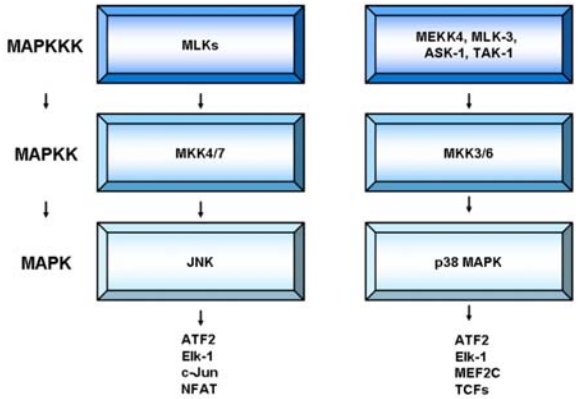

Figure 2. Stress-activated MAP kinase pathways. JNK and p38 MAPK are members of the stress-activated MAP kinase (MAPK) pathways. They are dually phosphorylated on a tripeptide motif (Thr-Xaa-Tyr) by a MAP kinase kinase (MAPKK). The MAPKK are activated by a group of MAP kinase kinase kinases (MAPKKK). ASK-1, apoptosis-signal-regulating kinase-1; ATF2, activating transcription factor 2; Ets-1, ets-like-1; JNK, c-Jun NH_2-terminal kinase; MEF2C, myocyte enhancer factor 2C; MKK3/4/6/7, mitogen-activated protein kinase kinase 3/4/6/7; MLK-3, mixed lineage kinase-3; NFAT, nuclear factor of activated T cells; TAK-1, transforming growth factor-β-activated kinase-1; TCFs, ternary complex factors.

cells, the molecular pathways, generally, are applicable to neurons. The importance of molecules (i.e. Apaf-1 and Bcl-2 family members) in caspase activation and neuronal cell death has been demonstrated by analysis of knockout mice. In addition to caspase-3 and -9, Apaf-1 knockout mice display gross abnormalities in developmental neuronal cell death (33,34). Apaf-1 knockout mice present morphological distortions in many areas of the brain including the diencephalon, midbrain, choroid plexus, and hypothalamus (33). In terms of proapoptotic Bcl-2 family members, CGNs from Bax-deficient mice are resistant to LK-induced apoptosis (35). On the other hand, mice deficient in Bcl-x displayed extensive apoptotic death of neurons in the diencephalon, midbrain, spinal cord, and dorsal root ganglia, while CGNs from Bcl-2-deficient mice are more susceptible to trophic factor withdrawal and LK-induced apoptosis (36,37). However, evidence in granule neurons has elucidated both caspase-dependent and caspase-independent pathways to death for cerebellar granule neurons, in particular the involvement of executioner caspase-3 (35,38-42). For example, the use of general and specific peptide caspase inhibitors yielded contradictory results. While many specific caspase inhibitors (for caspase-1, -2, -3, -6, -7, -8, -9, -10) do not protect CGNs from LK-induced apoptosis, general caspase inhibitors delay apoptosis in CGNs exposed to LK (35,39). Given the irreversible nature of these caspase inhibitors, the eventual loss of viability of CGNs exposed to LK suggests a caspase-independent pathway to death. Correspondingly, work with CGNs isolated from caspase-3 knockout mice demonstrated a requirement for caspase-3 in chromatin condensation and DNA fragmentation but not in death of neurons exposed to LK (42). On the other hand, induction of apoptosis by p53 overexpression in CGNs or by neurotoxins such as 1-methyl-4-phenylpyridinium (MPP+) proceeds in a caspase-3 dependent manner (40,43). Therefore, increasing support implicates caspase activation as a necessary step in the promotion in a variety of neurological diseases including ischemic stroke (caspase-1, -3, -8, -9, -11), Alzheimer's disease (caspase-3, -6, -9), amyotrophic lateral sclerosis (ALS; caspase-1, -3, -9) , Parkinson's disease (caspase-1, -3, -8, -9, -11), and Huntington's disease (caspase-1, -3, -8, -9) (2,20,44). Moreover, disrupting caspase activation in animal models of neurodegeneration can be neuroprotective (2,20,44). Most recently, disruption of Fas signaling (extrinsic pathway) was shown to be neuroprotective in a mouse model of Parkinson's disease induced by 1-methyl-4-phenyl-1,2,3,6-tetrathydropyridine (MPTP) (45).

3.1.4. Apoptosis and the Stress-Activated MAP Kinase Pathways, JNK and p38 MAPK

The mitogen-activated protein (MAP) kinase signaling cascades are highly conserved throughout all eukaryotic organisms and are responsible for relaying external signals toward specific responses including migration, proliferation, differentiation, and death (46). Originally identified from genetic studies in the yeast *Saccharomyces cervisiae,* these cascades are composed of a MAP kinase (MAPK) that is dually phosphorylated on a tripeptide motif (Thr-Xaa-Tyr) by a MAPK kinase (MAPKK) which is in turn phosphorylated by a MAPKK kinase (MAPKKK) (Figure 2) (47). Subsequent studies revealed three MAP kinase cascades in mammals; two of the cascades, c-Jun NH_2-terminal kinase (JNK) and p38, play important roles in environmental stress-activated responses, while the third, ERK cascade, relays extracellular signals directing survival and differentiation.

Of the two stress-activated pathways, the JNK pathway is by far the most extensively studied in neurons. In this pathway, JNK is the MAPK and has ten isoforms in the human adult brain encoded by alternative splicing of three genes: *jnk*1, *jnk*2, and *jnk*3 which generate isoforms with molecular weights of 46 and 55 kD (48-50). Expression of JNK1 and JNK2 is ubiquitous to all tissues, while JNK3 is restricted to the brain, heart, and testis (50). Although individual knockouts show no obvious effects, combined knockout studies of the three genes have yielded interesting roles for JNK1 and JNK2 in region-specific developmental apoptosis of the brain and for JNK3 in neuronal stress response (21,51,52).

In terms of signaling, cytokines like TNF and cellular stresses such as: UV treatment, trophic factor withdrawal, repolarization, cyclohexamide, anisomysin, heat shock, hydrogen peroxide, ceramide, 3-nitropropionic acid (3-NP), and MPTP can activate JNK signaling leading to apoptosis in cell culture and in animal models of disease (53-64). Upon specific external stimuli, activation of JNK pathway specific MAPKKKs initiates. However, the identity of the MAPKKK is dependent upon the stimulus and cell type, and the precise activation mechanisms are presently under intense investigation (50,65). Recently, biochemical (overexpression of active and inactive mutants) and pharmacological evidence has implicated a

family of MAPKKK designated the mixed lineage kinase family (MLK), particularly MLK-3, in JNK activation during neuronal apoptosis (62,66,67). Once activated MLK-3 can phosphorylate and activate MAPKK members in the pathway including mitogen-activated protein kinase kinase 4 and 7 (MKK4 and MKK7). These kinases are dual-specificity kinases that can phosphorylate JNK on both threonine and tyrosine residues (68). Furthermore, kinase assays have demonstrated activation of MKK7 in LK-induced apoptosis, and an inhibitor of MLK-3 can prevent this activation (69). Moreover, JNK pathway activation has an additional level of control mediated by a group of JNK interacting proteins (JIP/IB-1). These proteins are molecular scaffolds that sequester MLKs, MKKs, and JNKs and lead to activation in response to a specific stimulus (50,65). Interestingly, overexpression of JIP either in cell culture or in *in vivo* models of neurodegeneration prevents JNK activation and apoptosis, presumably by separating various components of the JNK pathway into discrete complexes (70,71).

Once activated JNKs are able to phosphorylate a variety of substrates including the transcription factors c-jun, activating transcription factor 2 (ATF2), ets-like-1 (Elk-1), p53, and nuclear factor of activated T cells (NFAT) (65,72). Recently, observations suggest that JNK2/3 mediates c-jun phosphorylation in cerebellar granule cells exposed to LK-inducing apoptosis (73; Chin and D'Mello, unpublished observation). A great deal of research proposes that c-jun is a key component in neuronal apoptosis due to its ability to activate transcription of itself and other proapoptotic genes (74). The proapoptotic targets of c-jun and other JNK-activated transcription factors are the subject of much research. However, two transcriptional targets related to apoptosis include FasL and Bim (59,75-79). In addition, recent work in granule neurons and sympathetic neurons links JNK signaling to the intrinsic death machinery by the ability of JNK to activate Bad and Bim by phosphorylation (79-81).

Another MAPK module implicated in neuronal apoptosis is the p38 MAPK cascade, though, compared to the JNK pathway, research examining this pathway's involvement in neuronal apoptosis is partial at best. Originally identified for contributing to inflammation in response to cytokines, p38 MAPKs consist of five isoforms p38α, p38β, p38β2, p38δ, and p38γ (65,82). Expression of two isoforms p38α and p38β is ubiquitous to all tissues with high expression in brain, while the remaining three seem restricted from the brain (83). Similar to JNK, dual phosphorylation of p38 MAPK in neurons occurs on a tripeptide motif in response to a variety of cellular stresses including UV light, excitotoxins, lipopolysaccharide (LPS), trophic withdrawal, and inflammatory cytokines (83). The direct upstream MAPKKs in p38 MAPK activation are MKK3 and MKK6. Phosphorylation of these MAPKKs follows activation of various MAPKKK including apoptosis-signal-regulating kinase-1 (ASK-1), transforming growth factor-β-activated kinase-1 (TAK-1), and MEKK4 (Figure 2) (83). Efforts primarily in non-neuronal cells have elucidated targets of p38 MAPK to include a mix of transcription factors and protein kinases. Transcription factors that are p38 MAPK targets include ATF2, Elk-1, myocyte enhancer factor 2C (MEF2C), ternary complex factors (TCFs), C/EBP-homologous protein 10 (CHOP10) (72,84). In addition, p38 MAPK can activate various protein kinases such as MAPK-interacting kinase (MNK), mitogen and stress-activated kinase (MSK), and mitogen-activated protein kinase activated protein kinases 2/3/5 (MAPKAP-K2/3/5) (65,72). Furthermore, a few recent articles propose that p38 MAPK is responsible for LK-induced c-jun phosphorylation in cerebellar granule neurons (85-87); however, these results directly contradict reports suggesting that p38 MAPK does not phosphorylate c-jun (73,88). In addition, one recent report demonstrated a pro-survival role for p38 MAPK in activity-dependent (HK) survival of cerebellar granule neurons (89). Nevertheless, increasing evidence points toward a role for p38 MAPK activation in the progression neurodegenerative disorders including Alzheimer's disease, amyotrophic lateral sclerosis (ALS), Parkinson's disease, and multiple sclerosis (83).

3.1.5. CDKs and the Abortive Reentry of Neurons into the Cell Cycle

The cell cycle comprises a series of highly regulated and conserved events that occur in order for one cell to give rise to two equal daughter cells. In normally cycling cells (Figure 3), this process consists of four stages G_1, S, G_2, and M where progression depends on regulation of cyclin-dependent kinases (CDKs) in regards to expression, activation (by cyclins), and inhibition (by CDK inhibitors) (81,90). Post-mitotic terminally differentiated neurons do not undergo mitosis (because they arrest in a quiescent stage G_0). The fact that this observation has been dogma for more than a century does not eliminate the presence of cell cycle related proteins from performing vital functions in neurons. However, one hypothesis is that reactivation of cell cycle proteins in post-mitotic neurons leads to activation of programmed cell death machinery (91). Observations made over ten years ago cited similarities between apoptotic neurons and normal cycling cells to include rounding of cells and chromatin condensation (92). Furthermore, recent evidence implicates elevated activity of cell cycle progression proteins (cyclin D1, cyclin B, cyclin E, CDK4, CDK6, CDK2, CDK1, retinoblastoma protein (Rb), and E2F) and decreased expression of cell cycle inhibitory proteins prior to neuronal apoptosis (81,93). One cell cycle-related protein, CDK5, has high expression in the nervous system. Interestingly, the role of this kinase either proapoptotic or anti-apoptotic evidently stems from the identity of its activating-binding partner either p25 or p35/39, respectively (94,95).

Once elevated, cell cycle proteins can target the cell intrinsic death machinery of neurons. In LK-induced apoptosis (activity deprivation) of CGNs, CDK1 phosphorylates Bad at a residue that prevents its interaction with a Bad-inhibitory protein 14-3-3 thus promoting Bad-mediated apoptosis (96). Furthermore, overexpression of E2F, a transcription factor that regulates expression of cell cycle genes, promotes apoptosis in several neuronal populations, and overexpression of dominant negative

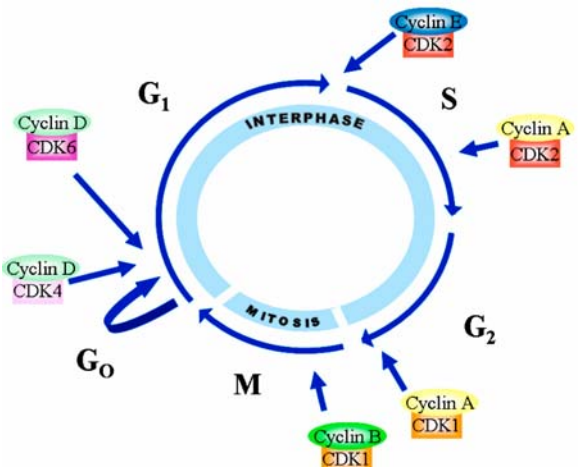

Figure 3. The cell cycle and its regulation by CDKs. In normally cycling cells, the cell cycle is composed of four main stages (G1, S, G2, and M) under the direction of cyclin dependent kinases (CDKs). Increasing evidence supports that abortive reentry into the cell cycle by post-mitotic neurons activates programmed cell death machinery.

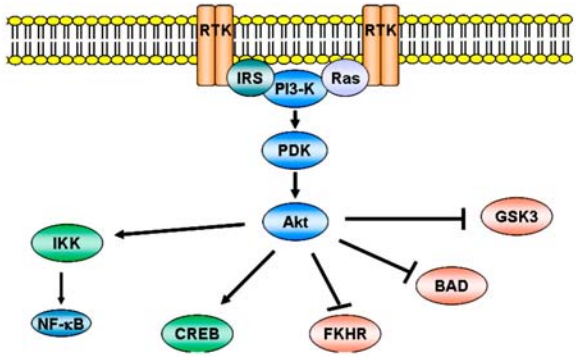

Figure 4. PI3-K/Akt pathway. Growth factors bind to receptor tyrosine kinases and initiate signaling that leads to the activation of Akt. Once activated, Akt can regulate a diverse array of molecules by phosphorylation. Molecules in green are pro-survival molecules that are activated by Akt, and molecules in red are pro-apoptotic molecules that are inactivated by Akt. CREB, cAMP response element-binding protein; FKHR, Forkhead; GSK3, glycogen synthase kinase 3; IKK, inhibitory κB kinase; IRS, insulin receptor substrate; PDK, phosphoinositide-dependent protein kinase; PI3-K, phosphoinositide 3-kinase.

forms of E2F prevents LK-induced apoptosis (81,97,98). Accordingly, aberrant reentry into the cell cycle has been associated with neurological disorders including Alzheimer's disease, amyotrophic lateral sclerosis (ALS), and stroke (81,99,100).

3.2. Molecules and Pathways Promoting Neuronal Survival

One hypothesis is that apoptosis results from a biochemical shift in the balance between life and death. Therefore, if we want to shift the balance in favor of promoting neuronal survival then an examination of key pathways regulating neuronal survival is imperative. Two key survival signaling cascades are the phosphoinositide 3-kinase (PI3-K)/Akt and the Raf-MEK-ERK pathways.

3.2.1. PI3-K/Akt Pathway and Neuronal Survival

PI3-Ks constitute a class of proteins that are responsible for responding to external cues such as growth factors, trophic factors, cytokines, and neurotransmitters to influence fundamental cellular activities (101). Autophosphorylation of activated receptor tyrosine kinases (RTKs) recruits PI3-Ks to the plasma membrane. PI3-Ks interact directly with phosphotyrosine residues or through the adaptor proteins insulin receptor substrates (IRSs) to generate phosphatidylinositol phosphates (PIPs), which serve as second messengers that recruit enzymes to the plasma membrane to relay signaling information (102). In addition, membrane localization of PI3-Ks at the plasma membrane through interaction with Ras can lead to activation of PI3-Ks (102). Akt is the cellular homologue of the transforming retroviral oncogene v-akt and its product bears significant homology to PKA and PKC (103,104). The structural organization of Akt consists of an N-terminal pleckstrin homology (PH) domain, which binds PIPs, and a catalytic domain (103). Akt has diverse functions in the cell including the regulation of glucose metabolism, cell proliferation, transcription, and inhibition of apoptosis (103,104). Growth factors such as IGF-1, PDGF, NGF, and basic FGF, which depend on signaling through Akt, cause the activation of tyrosine kinase receptors resulting in recruitment of PI3-K to the plasma membrane (105,106). PI3-K activation leads to generation and accumulation of phosphoinositide phosphates at the plasma membrane, prompting recruitment of proteins with PH domains to the membrane, including Akt. At the membrane, phosphoinositide-dependent protein kinase-1 (PDK-1), also a PH domain containing protein, phosphorylates Akt at threonine 308, which results in partial activation of Akt (107). Another phosphorylation on Akt occurs at serine 473 by mechanisms still under investigation and leads to full activation of Akt. Mutational analysis has demonstrated that phosphorylation of T308 is sufficient to activate Akt, but phosphorylation of both residues is required for maximal activation (103). Although, activation of Akt through PI3-K is the most common activation mechanism, reports have suggested PI3K-independent mechanisms of Akt activation (108,109). Previous research from our lab demonstrated that treatment with HK, forskolin, IGF-1, or lithium results in the phosphorylation at T308 and/or S473 and activation of Akt; however, Akt is not a requirement for survival directed by HK and forskolin as demonstrated by pharmacological as well as biochemical data (expression of dominant negative Akt) (110,111). Once activated, Akt can phosphorylate and activate anti-apoptotic molecules including inhibitory κB kinase (IKK) and cAMP response element-binding protein (CREB) (Figure 4) (112-114). Furthermore, Akt physically interacts with JIP thereby inhibiting the ability of JIP to activate JNK signaling (115). Akt, also, promotes survival by phosphorylating, thereby inactivating, many proapoptotic molecules including Bad, glycogen synthase kinase 3 (GSK3), Forkhead, and caspase-9 (103,104).

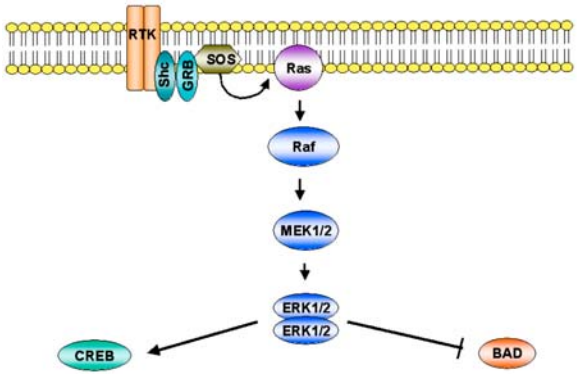

Figure 5. The Raf-MEK-ERK kinase cascade. Activation of receptor tyrosine kinases leads to recruitment of adaptor proteins Shc and GRB. GRB recruits the GTP exchange factor SOS which activates Ras. Ras activation leads to Raf activation in a series of complex changes in phosphorylation on multiple residues, protein-protein interactions, and protein-lipid interactions. Activated Raf, in turn, activates MEK. MEK phosphorylates the MAP kinase ERK on two residues resulting in activation. In certain populations of neurons, activation of ERK leads to activation of the pro-survival molecule CREB and inactivation of the proapoptotic Bcl-2 protein Bad. ERK, extracellular-signal-regulated kinase; Grb2, growth-factor-receptor-binding protein 2; SOS, son of sevenless.

Figure 6. Structures of several small molecule inhibitors. Depicted are the structures of inhibitors designed or discovered to target neuronal survival in cell culture, animal models, and in some cases clinical trials. See text for details.

3.2.2. Raf-MEK-ERK Kinase Cascade and Neuronal Survival

Three members of the proto-oncogenic Raf family of serine/threonine kinases have been identified in mammals, A-Raf, B-Raf, and c-Raf and are critical to the third MAPK signaling cascade which relays extracellular stimuli to the nucleus (Figure 5) (116,117). The Raf proteins share similar structure including an autoinhibitory N-terminal regulatory domain and a C-terminal kinase domain. In vivo, c-Raf activation proceeds in a complex and still not completely understood mechanism. It is known that inactivated c-Raf is present in the cytosol bound to the protein 14-3-3, and this interaction is dependent on a c-Raf inhibitory phosphorylation at serine 259 (118). To be activated, c-Raf is recruited from the cytosol to the plasma membrane by high affinity binding to the GTP bound state of the small G protein Ras (119). Binding of c-Raf to Ras is sufficient to displace 14-3-3 long enough for dephosphorylation of S259 to occur. Next, depending upon the type and duration of the stimulus, a combination of events involving complex changes in phosphorylation of c-Raf on multiple residues, protein-protein interactions, and protein-lipid interactions results in full activation of c-Raf (120). Although A-Raf activation has been less explored, it seems to resemble c-Raf activation in the sense that activation requires multiple steps, whereas B-Raf activation is less complex (121). Activation of B-Raf appears to depend on its binding to the Ras family of proteins alone (122,123). Once activated, Raf proteins regulate the cellular processes of growth, differentiation, and apoptosis (117). The primary substrates of Raf proteins are MAP/ERK kinases 1 and 2 (MEK1/2); however, c-Raf can directly phosphorylate other proteins including Bad, p53, and Rb (124).

Activated MEK1/2 signals to the MAP kinase, ERK, and activation of ERK isoforms ERK1 and ERK2 can lead to neuronal survival of some populations by activation of the transcription factor CREB or inactivation of the proapoptotic, Bcl-2 family member, Bad (125). In addition, a related ERK family member, ERK5, has been implicated in survival of embryonic cortical, dorsal root ganglion, and cerebellar neurons in response to growth factor treatment (126). Furthermore, knockout mice of all three Raf proteins have provided insight into their function. A-Raf knockout mice are born viable with intestinal and neurological defects; however, knockout mice with either c-Raf or B-Raf die during midgestation with evidence of excessive apoptotic death (119). This data points towards essential pro-survival roles for c-Raf and B-Raf. However, recent reports, also, have implicated a proapoptotic role for ERK signaling in neurons. Characterized by biphasic or sustained patterns of activation, ERK signaling has been implicated in neuronal degeneration in response to cerebral ischemia, brain trauma, and neurodegenerative diseases (127,128). Subsequently, pharmacological inhibition of ERK signaling is neuroprotective against animal models of ischemia and a variety of cell culture insults including: trophic factor withdrawal, neuronal activity withdrawal, mechanical trauma, glutathione depletion, MPP+ and 6-hydroxydopamine (128). These studies warrant further investigation into the cellular context of a proapoptotic role of ERK signaling.

3.3. Small Molecule Inhibitors of Neuronal Apoptosis

While symptomatic therapies exist for a number of neurodegenerative diseases, pharmacological drugs directed at reducing or preventing neuronal loss are lacking. Our increasing knowledge of the molecular mechanisms of neuronal apoptosis has facilitated the identification of small molecule inhibitors of these cascades. Subsequently, research has examined their effectiveness in primary cultures of neurons and neuronal cell lines, in *in vivo* paradigms of neurodegeneration in laboratory animals, and, for a few, in human clinical trials. This section of the review will focus on the successes and promise of various small molecule inhibitors in preventing neuronal loss in neuropathological conditions (Figure 6, for inhibitor structures).

3.3.1. Caspases Inhibitors

Caspases seem to be the most obvious targets to prevent unwanted apoptosis, and accordingly, this area has seen the most progress and latitude in the past ten years. For example, one report characterized the inhibitory effects of aged garlic extract on purified caspase-3 activity (129). Furthermore, research in animal models of neurodegeneration has explored overexpression of inactive forms of various caspases, caspase-deficient mice, or peptide ketone caspase inhibitors to test the hypothesis that

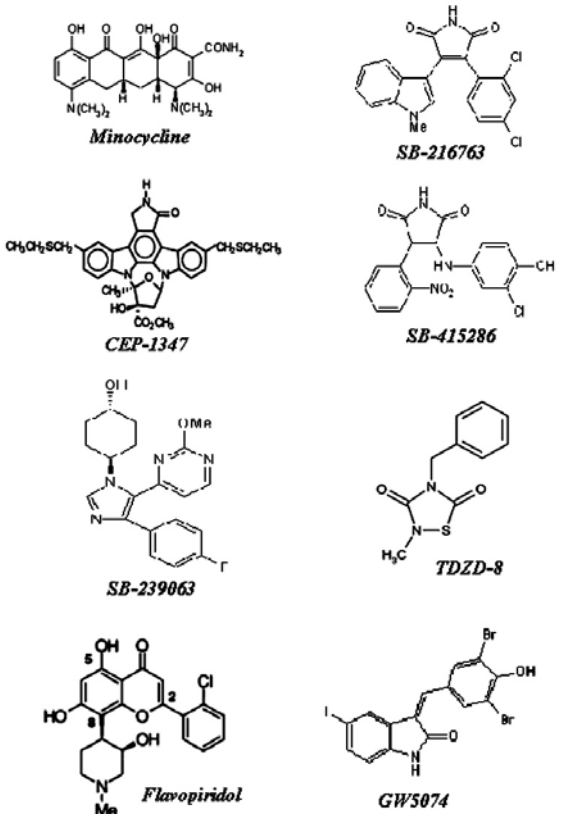

Figure 6. Structures of several small molecule inhibitors. Depicted are the structures of inhibitors designed or discovered to target neuronal survival in cell culture, animal models, and in some cases clinical trials. See text for details.

caspases promote neuronal loss in neurodegenerative disorders (2,20,44,130). Recently, reports of specific and potent small molecule inhibitors of caspases have provided exciting promise towards combating the progression of neurodegenerative disorders. Minocycline is a tetracycline derivative with anti-inflammatory effects and has been a successful antibiotic option for many years (131). Surprisingly, in the past few years, the versatility of this compound has proven invaluable in the charge to prevent neuronal loss. Minocycline prevents neuronal loss in response to global brain ischemia, traumatic brain injury, 6-hydroxydopamine-induced toxicity, MPTP-induced toxicity, R6/2 mouse model of Huntington's (mutant huntingtin gene with expanded CAG repeats), G93A mouse model of ALS, (20,131). One of the key components of neuroprotection is minocycline's ability to thwart activation of caspases including caspase-1 and caspase-3 by preventing cytochrome *c* release from mitochondria (20). An additional measure of protection comes from its anti-inflammatory actions and prevention of p38 MAPK activation (132). The success of minocycline has warranted further investigation, and appropriately minocycline in currently in clinical trials to test its effectiveness against ALS (Phase III, recruiting patients), Parkinson's disease (Phase II; creatine administered concurrently, recruiting completed), and Huntington's disease (Phases I and II, recruiting patients) (www.clinicaltrials.com). Recently, a specific and reversible small molecule inhibitor of caspase-3, named M826, has been reported to prevent neuronal loss in ischemic brain injury and an animal model of Huntington's disease (133,134). Future research will determine if M826 can be as versatile as minocycline in preventing neuronal loss by caspase inhibition.

3.3.2. Inhibitors of MLK-JNK and MLK-p38 MAPK Pathways

Within the last six years, the progression of knowledge about the MLK-3 inhibitor CEP-1347 has increased dramatically. Originally, CEP-1347 demonstrated an ability to block apoptosis of cultured motoneurons (135). Since its first demonstrated neuroprotective properties, this compound, because of its specificity, has been useful in dissecting the importance of the JNK pathway in a variety of paradigms including neuronal and non-neuronal cells (136-143). In terms of the neuroprotective actions, CEP-1347 protects against neuronal loss from MPTP toxicity, arsenite-induced apoptosis in cortical neurons, beta-amyloid-induced neuronal apoptosis, trophic factor withdraw from sympathetic neurons, LK-induced apoptosis in CGN, and grafting of embryonic dopaminergic neuron into the striatum of hemiparkinsonian rats (62,142,144-149). Recently, the Parkinson Study Group reported promising results in a Phase I human clinical trial (30 patients) with CEP-1347 (150). This has subsequently led to the organization of Phase II and III clinical trials (recruitment completed) with CEP-1347 to determine the safety and efficacy in the treatment of Parkinson's disease.

In addition to minocycline's use as an anti-inflammatory agent that can prevent activation of p38 MAPK, SmithKlineBeecham has developed a new second-generation p38 MAPK inhibitor SB-239063 (151). The first generation of this class of inhibitors included SB-203580 and SB-202190 which were useful to dissect the molecular requirements for p38 MAPKs in many processes. However, these two inhibitors received criticism because in addition to inhibiting p38 MAPKs, they inhibit JNKs (73). SB-239063 demonstrates greater *in vitro* kinase selectivity than first generation inhibitors, a 3-fold increase in cellular activity, and a 3- to10-fold increase in *in vivo* activity (152). Thus far, SB239063 has demonstrated an ability to prevent neuronal loss in ischemic and excitotoxic insults (152,153). The greater selectivity of this inhibitor should promote valuable insight and resolve disputes regarding the contribution of p38 MAPK to neuronal apoptosis.

3.3.3. Inhibitors of Cell Cycle Proteins

Small molecule inhibitors of cell cycle proteins have been widely investigated for their potential to inhibit human cancers and only recently have shown potential as therapeutics against neurodegenerative diseases in animal models (154). For example, the general CDK inhibitor flavopiridol (inhibits CDK2, CDK4, CDK1, and CDK5) has been shown to prevent neuronal loss and improve behavioral outcome in response to ischemia and MPTP-induced neurotoxicity (155-157). Smith et al. (2003) demonstrated that the neuroprotective effect of flavopiridol

was due to its inhibition of CDK5 and to some extent CDK2 (157). This offers an interesting opportunity because CDK5 is highly expressed in the nervous system, and more selective inhibitors of CDK5 may be able to counteract the loss of neurons in diseases such as Alzheimer's disease and related tau-associated pathologies where CDK5 and GSK3 have been implicated in disease pathology (154). Thus far, no human clinical trials have commenced to determine the ability of cell cycle inhibitors to slow the progression of neurodegenerative diseases.

3.3.4. Inhibitors of GSK3

Given that pro-survival pathways in neurons inhibit GSK-3 activity, logically, small molecule inhibition of GSK3 may have a neuroprotective outcome. Recently, two small molecule inhibitors of GSK3, SB-216763 and SB-415286, demonstrated neuroprotection against LK-induced apoptosis in CGNs (158). In addition, pertaining to Huntington's disease, GSK3 inhibition by SB-216763 prevented cell death in cell lines overexpressing mutant huntingtin gene with expanded CAG repeats (159). In regards to Parkinson's disease, a non-ATP competitive inhibitor of GSK3 in the thiadiazolidinones (TDZD) family exhibited protection against 6-hydroxydopamine-induced cell death (160). Various reports suggest that GSK3 directly interacts with several proteins that accompany Alzheimer's disease progression (161,162). Counteracting these interactions with small molecule inhibitors of GSK3 is one strategy under intense investigation. Subsequently, three inhibitors of GSK3, SB-216763, SB-415286, and TDZD displayed an ability to reduce tau phosphorylation in cell culture (163,164). Initial attempts at exploring neuroprotection provided by GSK3 inhibition established a strong potential for therapeutic intervention against neurodegenerative conditions; thus, future research should focus on extending these observations *in vivo* in animal models of neurodegenerative disease to establish the therapeutic efficacy.

3.3.5. Inhibitors of c-Raf

Recently, our lab identified GW5074, a small molecule inhibitor of c-Raf, to display neuroprotection against a variety of apoptosis-inducing stimuli (165). GW5074 was protective against activity deprivation (LK), methylmercury, and MPP+ in CGN and homocysteic acid in embryonic cortical neurons. Furthermore, GW5074 prevented neuronal loss and improved behavioral outcome in mice administered 3-NP, a neurotoxin that replicates many of the pathological and pathophysiological hallmarks of Huntington's disease. What is intriguing about this inhibitor is its apparent mechanism of action. Lackey et al. 2000 reported that GW5074 inhibited c-Raf potently and specifically (IC_{50} = 9nM), while exhibiting no effect on the activities of cdk1, cdk2, c-src, p38 MAP kinase, VEGFR2, and c-fms (166). We confirmed GW5074 to be a specific inhibitor of c-Raf while not affecting the kinase activities of JNK1, JNK2, JNK3, MEK1, MKK6, MKK7, CDK1, CDK2, CDK6, CDK5, and GSK3β (165). Previous observations have noted that when treated with a small molecule inhibitor of c-Raf, cells attempt to overcome this inhibition by inducing the activation of c-Raf (167-169). These studies reported a compensatory mechanism where inhibition of c-Raf resulted in activation of c-Raf (when measuring the kinase activity in the absence of the inhibitor). Not surprisingly, treatment of CGNs with the c-Raf inhibitor GW5074 leads to activating modifications on c-Raf as detailed by decrease in an inhibitory phosphorylation and thus, an increase in kinase activity (when assayed in the absence of the compound) (165). Moreover, GW5074 treatment induces the activation B-Raf, an isoform of c-Raf. Whether B-Raf activation occurs simultaneous to the accumulation of activating modifications on c-Raf or to compensate for lack of endogenous c-Raf activity (due to presence of inhibitor), remains to be determined. A variation of the latter possibility has been observed in kinase-impaired mutations of B-Raf that lead to activation of c-Raf activity (170,171). Nevertheless, B-Raf is required for the neuroprotection provided by GW5074, while its neuroprotective properties are independent of the MEK-ERK pathway and Akt-pathway (165; Chin et al., manuscript in preparation). Previous observations have detailed a neuroprotective role for B-Raf. Sensory and motoneurons cultured from B-Raf knockout mice fail to survive in response to neurotrophic factors, but the same neurons survive in response to neurotrophic factors when transfected with a B-Raf expression vector (172). Additionally, the treatment of CGN with GW5074 prevents phosphorylation and expression of c-jun and prevents downregulation of NF-κB DNA-binding activity, two events that occur during activity deprivation-induced (LK) apoptosis (165). Although seemingly counterintuitive, treatment with an inhibitor, GW5074, induces a novel neuroprotective pathway that is dependent on B-Raf and likely involves c-jun and NF-κB. In contrast, to other inhibitors, which impede proapoptotic molecules, GW5074 treatment results in activation of pro-survival molecules. Thus, given the versatility of GW5074 in preventing neurodegeneration in distinct types of cultured neurons, in response to different neurotoxic stimuli, and in an animal model of neurodegeneration, GW5074 could have therapeutic value against neurodegenerative diseases in humans. The potential of GW5074 warrants additional testing in alternative animal models of neurodegeneration and further investigation into the exact mechanism of action for GW5074.

3.4. Perspective

In this review, we have attempted to provide a general understanding of the mechanisms of apoptosis in neurons by reviewing the molecules and pathways that regulate survival and apoptosis. In addition, we have highlighted the success of small molecule inhibitors that have emerged in the quest to enhance neuroprotection in the context of degenerating conditions. One caveat to the use of any inhibitor is the potential undesirable effects that could arise in other non-diseased tissues. In addition, questions remain unanswered regarding whether preventing apoptosis in diseased neurons can lead to an effective rescue and restoration of function. Therefore, continued persistence in identifying neuronal specific isoforms of disease promoting molecules must continue in order to eliminate unwanted side effects of neuroprotective compounds. Conversely, this may not be a problem if we could design ways to target neuroprotective compounds

solely to afflicted areas. The last twenty years of research into the once counterintuitive phenomenon of naturally occurring cell death or programmed cell death in the nervous system has seen enormous strides. Continued research should provide opportunities to combat neurodegenerative diseases with chemo-neuroprotection therapies.

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