2
Molecular Biology of Brain Injury

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Neurotransmitters and Their Receptors

The concept that electrical activity between neurons is transmitted via chemical messengers was first demonstrated in 1921 by an Austrian physiologist, Otto Loewi. Using two frog hearts, he placed the first heart (still connected to its vagus nerve) into a saline-filled chamber. This chamber was connected to a second saline-filled chamber into which he placed the second heart. Electrical stimulation of the vagus nerve caused the first heart to slow. After a short delay, he noticed the second heart also slowed. From this experiment, he hypothesized that the electrical stimulation of the vagus nerve released a chemical into the first chamber that flowed into the second and caused the second heart to slow just as the first. He referred to the chemical as *Vagusstoff*. We now know this chemical to be acetylcholine, by far the best-studied neurotransmitter [1].

Neurotransmitters: Definition

Neurotransmitters are the chemical messengers synthesized and utilized by neurons to propagate electrical impulses from one neuron to the next. Neurotransmitters are produced and stored within presynaptic neurons, which when depolarized release neurotransmitters into the synaptic cleft. Neurotransmitters bind and activate specific membrane-bound receptors in the postsynaptic cell, leading to ion fluxes, such as inward sodium, calcium, or chloride currents, and to outward potassium efflux. Following their release, neurotransmitters are rapidly inactivated by reuptake and/or degradation.

Neurotransmitters fall into two main categories: peptide neurotransmitters and small-molecule neurotransmitters, such as acetylcholine, biogenic amines, and amino acids. We focus primarily on the role of several major classes of neurotransmission in the normal brain and on the role of amino acid neurotransmitters in excitotoxicity, the process by which overstimulation of glutamate receptors induces cell death.

Neurotransmitter Receptors

There are over 100 putative neurotransmitters and a vast array of neurotransmitter receptors; the same neurotransmitter may be excitatory or inhibitory, depending on whether binding to a specific receptor results in depolarization versus hyperpolarization, respectively. In general, all neurotransmitter receptors function by opening or closing ion channels in the postsynaptic cell membrane. They can do this directly if the receptor functions as an ion channel or indirectly if the receptor lacking an ion channel activates a second messenger system. The former is referred to as an *ionotropic* or *ligand-gated receptor*, the latter as a *metabotropic receptor*.

Ionotropic receptors are generally composed of five membrane-spanning subunits that together form a central channel. The receptor is a multimer with several extracellular neurotransmitter binding sites, a number of transmembrane domains, and a single central ion channel connecting the extra- and intracellular compartments. In contrast, metabotropic receptors are monomeric, membrane-spanning proteins that stimulate intracellular G proteins that interact with separate membrane-spanning ion channels. When neurotransmitters bind the extracellular sites of metabotropic receptors, G proteins linked to the intracellular domain are activated, dissociate, and interact with ion channels or through intermediary proteins to alter conductance of neighboring ion channels. Metabotropic receptors generally modulate the function of ionotropic receptors and have longer lasting electrical effects as well as effects on gene expression and intracellular signaling important for synaptic plasticity, learning, and memory.

Acetylcholine

The two types of acetylcholine (ACh) receptors are nicotinic and muscarinic, named for synthetic chemicals that activate their respective extracellular binding sites on the ACh receptor. Nicotinic ACh (nACh) receptors are excitatory ligand-gated channels localized at the neuromuscular junction, as well as within the brain and autonomic nervous system. Although the role of ACh at the neuromuscular junction and autonomic ganglia is well understood, its role in the brain is less clear. Nicotinic ACh receptors, found throughout the cortex, induce arousal, euphoria, and relaxation. Nicotine and other nACh receptor agonists improve attention, enhance learning, and shorten reaction time. Muscarinic ACh receptors are metabotropic and are responsible for the majority of

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acetylcholine effects in the brain. These receptors are found in abundance in the striatum and other forebrain regions in addition to postganglionic parasympathetic neurons.

Biogenic Amines

Biogenic amines are a group of small-molecule neurotransmitters and includes the three main catecholamines (dopamine, norepinephrine, and epinephrine) as well as serotonin and histamine. Together, the biogenic amines account for a complex array of brain function and clinical behavior.

Dopamine

Inhibitory dopaminergic neurons project from the substantia nigra to the corpus striatum, where they mediate control of motor activity. Disruption of dopaminergic neurons results in the abnormal shuffling gait and pill-rolling tremor described in patients suffering from Parkinson's disease. In addition, dopaminergic neurons arising from the ventral tegmental area and extending to the nucleus accumbens are believed to be involved in motivation, reward, and addictive behavior. Cocaine blocks norepinephrine, serotonin, and dopamine reuptake into presynaptic terminals by inhibiting the dopamine transporter, leading to an accumulation of dopamine in the synaptic cleft. This results in prolongation of dopaminergic effects in the limbic system, producing intense euphoria. Of note, dopamine receptors are exclusively metabotropic.

Norepinephrine

Norepinephrine is a key neurotransmitter of neurons in the locus coeruleus that project to the cerebral cortex, thalamus, and midbrain reticular activating system. Norepinephrine is an excitatory neurotransmitter that mediates sleep and wakefulness, attention, and feeding behavior. Norepinephrine is normally cleared from the synaptic cleft by the norepinephrine transporter (NET). As with cocaine, amphetamine blocks this reuptake mechanism. In addition, amphetamine inhibits monoamine oxidase (MAO) and catechol O-methyltransferase (COMT), the major enzymes that metabolize norepinephrine in neurons and glia. Disruption of norepinephrine metabolism increases synaptic norepinephrine, leading to insomnia, decreased appetite, and increased alertness.

Histamine

Histamine-containing neurons in the hypothalamus project to most regions of the brain and spinal cord, where they influence attention and arousal. Thus, drowsiness is caused by antihistaminic drugs that cross the blood–brain barrier, such as diphenhydramine. Neurons utilizing histamine as a neurotransmitter are also found in the vestibular system. This may explain why another antihistamine, meclizine, is effective as an antiemetic. All three known histamine receptors are metabotropic.

Serotonin

Serotonin, or 5-hydroxytryptamine (5-HT), is implicated in the pathophysiology of a number of psychiatric diseases, including depression, eating disorders, anxiety disorders, and obsessive-compulsive disorder. Serotonin-containing neurons predominate in the raphe region of the pons and upper brain stem and project into the forebrain. A wide variety of 5-HT receptors have been discovered, most of which are metabotropic. These receptors influence sleep and wakefulness, emotion, motor behaviors, and satiety. Once serotonin has been released into a synaptic cleft, its action is terminated by the serotonin reuptake transporter (SERT). The selective serotonin reuptake inhibitors (SSRIs) interfere with SERT and prolong the action of serotonin in the synaptic cleft.

Amino Acids

Four amino acids have been identified as neurotransmitters, including glutamate, aspartate, gamma-aminobutyric acid (GABA), and glycine. Most excitatory neurons in the brain utilize glutamate as a neurotransmitter and are referred to as *glutamatergic neurons*. The most important inhibitory neurotransmitters are GABA and glycine. Together, amino acid neurotransmitters are responsible for the majority of synaptic neurotransmission in the brain.

Gamma-Aminobutyric Acid and Glycine

The majority of inhibitory synapses in the brain utilize either GABA or glycine as neurotransmitters, which act on ionotropic and metabotropic receptors to decrease excitation by causing hyperpolarization of the postsynaptic membrane. In the normal brain, glucose is metabolized to glutamate via the tricarboxylic acid cycle. Glutamate is then converted to GABA by glutamic acid decarboxylase (GAD). Glutamic acid decarboxylase requires a cofactor, pyridoxal phosphate (derived from vitamin B_6), for normal function. Pyridoxine dependency is an autosomal recessive disorder manifest by intractable infantile seizures responsive to vitamin B_6 administration. The disorder is associated with high levels of glutamate in the cerebrospinal fluid and impaired GAD activity.

There are two types of GABA receptors: GABA-A and GABA-B. The GABA-A receptors are ligand gated and function by enhancing Cl^- conduction through the central pore, inducing hyperpolarization and reducing membrane excitability. Benzodiazepines and barbiturates induce sedation and anxiolysis and increase the seizure threshold by binding GABA-A receptors. The GABA-B receptors are metabotropic and inhibit depolarization via recruitment of a G-protein second messenger that blocks neighboring K^+ and Ca^{2+} channels.

Glutamate

Glutamate is a nonessential amino acid that does not cross the blood–brain barrier and therefore must be produced by neurons within the central nervous system in order to function as a neurotransmitter. Glutamine, the primary precursor to glutamate, is supplied by glial cells to neurons. Once within the presynaptic terminal of the neuron, glutaminase converts glutamine to glutamate. Glutamate is stored in vesicles until released by neuronal depolarization and is then transported back to glial cells, reconverted to glutamine via glutamine synthetase, and returned to the neuron.

Glutamate receptors are composed of five monomeric subunits that assemble in various combinations to form a variety of glutamate receptors, several of which may respond to glutamate simultaneously in a given postsynaptic neuron. Three ligand-gated (ionotropic) glutamate receptors have been described: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), and kainite receptors. All three are excitatory. There are three specific properties that make NMDA receptors unique. First, their central pore conducts Na^+, K^+, and Ca^{2+}. Ca^{2+} influx serves as a second messenger to initiate intracellular

signaling cascades and novel gene expression. Second, magnesium binds to glutamate receptors within the central pore, which inhibits channel function by maintaining hyperpolarization of the postsynaptic membrane. Magnesium is extruded from the pore during depolarization to allow free flow of other cations. This unique property adds voltage dependence to ionic flow across the pore and has been linked to brain functions such as learning and memory. Finally, a glycine binding site modulates channel opening in response to glutamate binding, and glycine is required for optimal NMDA receptor function.

Activation of NMDA receptors underlies the formation of novel memories by modulating the strength of the effect of a synapse on a postsynaptic cell [2]. For example, frequent and repetitive stimulation of a synapse containing NMDA receptors leads to augmentation of the postsynaptic response during future synaptic stimulation; this electrophysiologic phenomenon is known as *long-term potentiation* (LTP) and is mediated by calcium influx through the NMDA receptor. Conversely, a low frequency of synaptic stimulation, and failure to recruit firing from additional synapses, leads to long-term depression (LTD) and inhibitory effects on the postsynaptic cell. Long-term depression is also mediated by calcium currents in NMDA receptors. Long-term potentiation and LTD depression are ways in which synaptic strength is regulated, both acutely and on a long-term basis, and both are necessary for normal learning and memory. Both LTP and LTD are inhibited by NMDA receptor antagonists that impair memory function in rodents. Thus, NMDA receptors induce memory formation by calcium-dependent mechanisms that include LTP or LTD in hippocampal as well as cortical brain regions.

In addition to the ligand-gated glutamate receptors, three known metabotropic receptor subtypes modulate neurotransmission by altering postsynaptic Ca^{2+} and Na^{+} channels and thereby modulating excitability of the postsynaptic neuron. Group I mGlu receptors coupled to phospholipase C modulate intracellular calcium signaling, whereas group II and group III receptors inhibit adenylyl cyclase. Metabotropic glutamate receptors also play important roles in synaptic plasticity by potentiating the effects of NMDA receptor activity in brain regions involved in learning and memory.

Excitotoxicity

Drs. Lucas and Newhouse first demonstrated the concept of excitotoxicity in 1957 by feeding glutamate to young mice and finding neuronal loss in the retina [3]. The relationship between increased extracellular glutamate concentrations and neuronal cell death was subsequently described in a number of acute brain injury models [4–8]. During acute insults to the brain, such as stroke, infection, trauma, seizures, hypoglycemia, or hemorrhage, glutamate is released by neurons and glia into the brain extracellular space [8]. High concentrations of glutamate overstimulate NMDA and calcium-permeable AMPA receptors and induce transient, massive influx of extracellular calcium. Calcium may also enter the neuron from voltage-gated calcium channels, sodium/calcium transporters, and from intracellular stores. Intracellular calcium activates proteolytic enzymes that cleave substrates essential for cellular survival, such as cytoskeletal proteins, DNA repair enzymes, and other key cellular constituents. In addition, increased intracellular calcium induces mitochondrial electron transport chain dysfunction and subsequent generation of oxygen free radicals that, in concert with activation of proteases and other "death effectors," leads to necrotic or apoptotic cell death [9]. Recent studies have shown that calpains and caspases (two classes of death proteases activated by increased intracellular calcium) contribute to prolonged increases of intracellular calcium following excitotoxic stimuli by cleaving and inactivating membrane calcium pumps [10,11]. Thus, following an initial (sublethal) calcium increase, defective cellular calcium clearance magnifies the initial insult by prolonging the duration of increased intracellular calcium. Cell injury and death that occur as a result of overactivity of glutamatergic neurotransmission is referred to as *excitotoxicity*.

Despite a wealth of preclinical data implicating excitotoxicity in the pathogenesis of central nervous system injury, efforts to interrupt excitotoxicity using glutamate receptor antagonists are only effective if given before or shortly after the time of ischemic or traumatic injury in experimental animals [12–15]. In human trials, administration of NMDA receptor antagonists up to several hours after stroke and traumatic brain injury was not effective and actually increased mortality and morbidity in some patients [6,16–20]. One explanation for these negative results is that, following traumatic brain injury, NMDA receptor deactivation occurs between 15 min and 1 hr in regions of injured cortex and hippocampus; NMDA receptors remain deactivated for at least 7 days; and NMDA receptor deactivation correlates with deficits in a working memory task at 2 weeks after injury [21]. Interestingly, administration of NMDA reversed the cognitive deficits associated with NMDA receptor deactivation after acute traumatic brain injury [21]. Taken together with other studies implicating acute central nervous system inflammation as one cause of NMDA receptor deactivation [22], the data suggest that long-term memory deficits induced by acute central nervous system injury may be initiated by an acute neuroinflammatory response that inhibits NMDA receptor function in cortical and hippocampal brain regions critical for learning and memory. This hypothesis, testable in the laboratory, may elucidate relationships between acute brain injury, the associated inflammatory response, and lasting learning and memory dysfunction in experimental animals and patients with acute brain injury.

Cell Death After Acute Brain Injury

A number of insults to the central nervous system may initiate complex cascades of intracellular biochemical events that lead to delayed neuronal death, as well as death of other vulnerable cell types remote from the injury center [9,23–29]. Because cell death may occur hours to weeks after central nervous system injury, it is hoped that a better mechanistic understanding will result in novel treatments to preserve tissue and neurologic function. The last 30 years has witnessed impressive advances in understanding basic mechanisms of how cells die after acute brain injury. Excitotoxicity, oxidative stress, and programmed cell death are major pathways that are central to the pathogenesis of ischemic and traumatic brain cell death [30]. Understanding how injured brain cells die is difficult because numerous interrelated, complex mechanisms contribute to the execution phases, and little is known about the mechanisms that initiate death programs after acute brain injury [29,31,32]. This section presents an overview of three modes of cell death, the major pro-cell death pathways, and initiating mechanisms involved in acute brain cell death. Figure 2.1 summarizes some of the pathways involved.

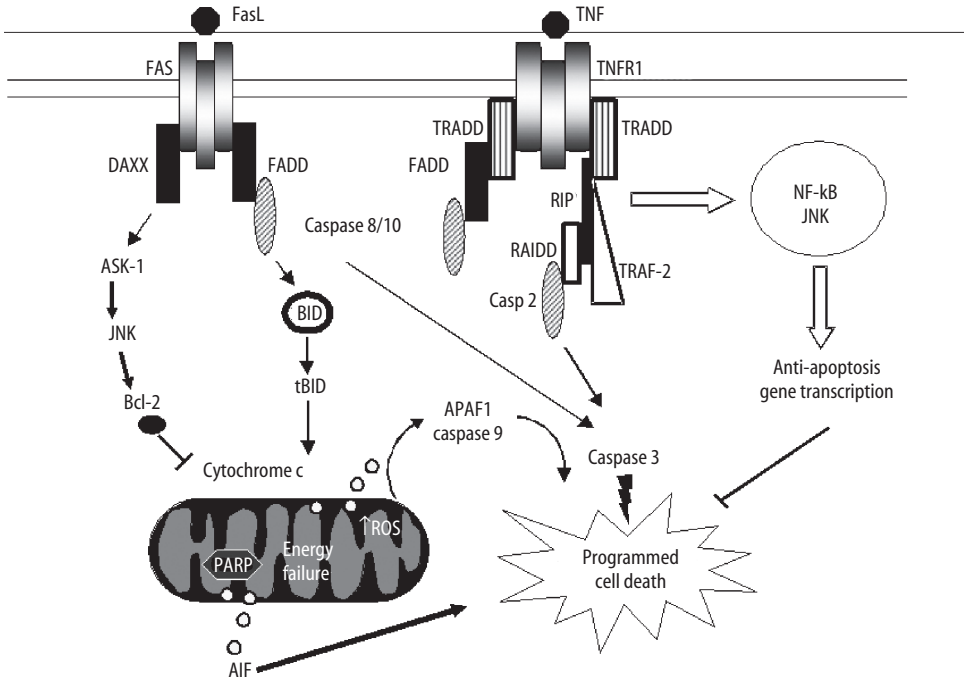

Figure 2.1. Cell death pathways and acute brain injury. Fas and tumor necrosis factor receptor 1 (TNFR1) are prototypical death receptors that signal apoptosis through Fas-associated protein with a death domain (FADD), by activating initiator caspases such as caspases (Casp) 8, 10, and 2. Mitochondria release cytochrome c and other apoptogenic factors (e.g., apoptosis-inducing factor[AIF]), leading to programmed cell death. In addition, TNFR and Bid activation can also induce necrosis through oxidative stress and mechanisms that remain to be clarified. APAF1, apoptotic protease activating factor 1; FasL, Fas ligand; TRADD, TNFR-associated protein with a death domain; RIP, receptor interacting protein; RAIDD, RIP-associated ICH-1 homologous protein with a death domain; TRAF-2, TNF receptor-associated factor-2 ; JNK, jun N–kinase; NFκB, nuclear factor-kappa B; ASK-1, apoptosis signal-regulating kinase 1; Bcl-2, B-cell lymphoma-2; DAXX, death associated protein 6; PARP, poly-ADP(ribose)polymerase; ROS, reactive oxygen species.

Necrosis

Severe ischemic, infectious, epileptogenic, or traumatic insults to the brain induce early cell death that is characterized by cell membrane permeability, organelle swelling, cellular and nuclear shrinkage, metabolic failure and depletion of cellular energy reserves, loss of ion pump function, and cell death that induces a marked local inflammatory response that propagates tissue injury. This mode of neuronal cell death is referred to as *necrosis* [33]. Necrosis is traditionally viewed as resulting from physical cellular disruption, as in severe traumatic brain injury, or from severe ischemic/metabolic insults that induce profound energy failure and cell death, such as severe ischemia or prolonged seizures. The exact biochemical mechanisms that mediate necrosis are relatively unknown but include activation of calpains and other proteolytic enzymes, oxidant injury resulting from generation of toxic oxygen species in the setting of mitochondrial dysfunction, and energy failure resulting from collapse of the mitochondrial transmembrane potential and rapid depletion of intracellular adenosine triphosphate stores [32]. However, recent studies suggest that necrosis can also occur as a form of programmed cell death initiated by activation of tumor necrosis family receptor members [34], and it is likely that necrotic cell death programs will be discovered that contribute to acute brain injury.

Excitotoxic death can be necrotic in the context of extreme insults, whereas milder forms of excitotoxic injury may trigger delayed programmed cell death, commonly referred to as *apoptosis*. Whether necrosis or apoptosis results from excitotoxic insults likely depends on the resulting magnitude and duration of increased intracellular calcium as well as on other factors such as depletion of cellular energy stores. Therapeutic attempts to inhibit necrotic cell death will likely require intervention early after brain injury, as necrosis occurs rapidly after acute central nervous system insults but may also be delayed and progressive.

Programmed Cell Death

Programmed cell death (apoptosis) is an evolutionarily conserved, genetically programmed cell suicide process that is mediated by new protein synthesis and activation of death-inducing proteases [35–37]. Caspase-dependent programmed cell death is mediated by a family of cysteine proteases known as *caspases*, a family of at least 14 known cysteine proteases that promote apoptosis by cleaving substrates at specific tetrapeptide amino acid sequences [33,38]. Caspases exist as proforms that when cleaved at specific aspartate residues form tetrameric active complexes that cleave and inactivate diverse cellular substrates, such as cytoskeletal proteins, inhibitors of DNA endonucleases, and cellular enzymes required for survival. Activity of effector caspases results in classic apoptotic cellular morphology and cell death. Initiator caspases, such as caspases 2, 8, and 9, cleave and activate effector caspases 3, 6, and 7. Other caspases are involved in inflammatory responses and do not directly mediate cell death.

Activated caspases are found in ischemic and traumatic brain tissue and cerebrospinal fluid of humans [29,32]. Genetic or pharmacologic inhibition of caspases reduces tissue damage in experimental stroke and traumatic brain injury but does not always improve functional outcome [39].

Apoptosis is characterized by morphologic and biochemical features distinct from necrosis. Caspase-dependent apoptosis, for example, is characterized by cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation in multiples of 280 bp (known as *DNA laddering* because of the classic pattern produced by DNA gel electrophoresis), formation of nuclear apoptotic bodies, and engulfment of dying cells by phagocytes or neighboring cells in the absence of a local inflammatory response [40]. At the molecular level, activated caspases and proteolytic products of their substrates are detectable, as well as externalization of membrane phosphatidylcholine (which signals phagocytosis) and internucleosomal DNA fragmentation that reacts with terminal transferase in the TUNEL assay, a commonly used in situ marker for apoptosis.

Caspase-independent programmed cell death is mediated by apoptosis-inducing factors released from mitochondria, without concomitant activation of caspases [41–44]. One such factor, named *apoptosis-inducing factor* (AIF), is a phylogenetically ancient flavoprotein encoded by a gene on the X chromosome and expressed in most tissues (45). Apoptosis-inducing factor functions as an electron acceptor/donor and has a second apoptogenic function as well. Precursor AIF is synthesized in the cytosol and imported into the intermembrane space of mitochondria. Following acute cellular injury, AIF translocates through the outer mitochondrial membrane into the cytosol and to the nucleus, where it induces nuclear chromatin condensation and large-scale (approximately 50 kb) DNA fragmentation. This mode of cell death produces margination of nuclear chromatin and cellular morphology distinct from that of caspase-dependent apoptosis. It is likely that other mitochondrial and cytosolic apoptotic proteins also contribute to caspase-independent cell death following acute central nervous system injury.

Intrinsic Pathway of Apoptotic Cell Death

The *intrinsic pathway* is a major apoptotic pathway that involves release of proapoptotic factors from injured mitochondria. Proteins involved in the intrinsic cell death pathway include the Bcl-2 family of proapoptotic proteins (i.e., Bax and Bad), mitochondrial oxidoreductases such as cytochrome c and AIF, some caspases, and DNA fragmentation factors such as caspase-activated DNAse and endonuclease G. Following acute brain injury, apoptosis may be triggered by a number of pathologic mechanisms, including ischemia, trauma, excitotoxicity, oxidative stress, energy failure, and others [24,32]. These pathologic events can lead to depolarization of the mitochondrial inner membrane and release of cytochrome c (or other apoptogenic factors such as AIF in caspase-independent death). Cytochrome c interacts with an adapter protein *apoptotic protease activating factor* (Apaf-1), adenosine triphosphate, and procaspase-9 in the cytosol to form an *apoptosome*, where caspase-9 is autoactivated by self-oligomerization. Caspase-9 cleaves and activates caspase-3, leading to caspase-dependent apoptosis.

Other mechanisms of mitochondrial-mediated cell death include generation of reactive oxygen species in response to excitotoxic and other stimuli and energy failure through overactivation of the DNA repair enzyme poly(ADP)-ribose polymerase (PARP, discussed later). Thus, the mitochondrion not only controls cellular respiration but is a rheostat for cellular damage and a central mediator of cell death after acute central nervous system injury.

The Extrinsic Pathway of Apoptotic Cell Death

Another route to programmed cell death (and even necrosis) involves activation of membrane bound *death receptors* of the tumor necrosis factor receptor (TNFR) superfamily, such as Fas and TNFR1 [46–49]. Ligand binding induces activation of death receptors, which then recruit cytosolic adapter proteins such as Fas-associated protein with a death domain (FADD) and TNFR-associated protein with a death domain (TRADD). Binding between death receptors and their adapter proteins occurs via homotypic interactions between evolutionarily conserved death domain sequences. Activated adapter proteins bind initiator procaspases, such as procaspases 2, 8, and 10, through death effector domain (DED) sequences present in adapter proteins and procaspases. The resulting complex formed by a death receptor, adapter protein(s), and procaspase is a death-inducing signaling complex (DISC). Self-aggregation of initiator procaspases at the DISC induces their autoactivation, and activated initiator caspases process and activate procaspases 3, 6, and 7, which mediate cell death. Alternatively, activated caspase-8 can cleave and activate cytosolic Bid, a proapoptotic Bcl-2 family member that induces release of cytochrome c from mitochondria [50,51]. Of note, mice deficient in Bid have reduced infarct volume and caspase-3 activation after experimental cerebral ischemic injury [52], and Bid can also induce necrotic cell death [53]. Thus, activation of Bid links the extrinsic death pathway, initiated by death receptor activation, to mitochondrial (intrinsic) pathways that may culminate in apoptosis or necrosis.

In addition to cell death pathways, activated TNFRs may induce intracellular signaling pathways and novel gene expression that favor cell survival. For example, activation of nuclear factor-κB (NFκB) is antiapoptotic in the setting of central nervous system injury, whereas activation of jun N–kinase (JNK) by death receptors is proapoptotic in ischemic brain. In neuronal cells, JNK activation is involved in apoptosis in response to stress or withdrawal of survival signals, whereas NFκB protects against TNF- and Fas-induced apoptosis by promoting transcription of antioxidant and antiapoptotic genes, including Bcl-2 family members that inhibit cytochrome c translocation and other apoptotic and necrotic death pathways [54–57]. Thus, TNFR family members may activate multiple intracellular signaling pathways that initiate complex, redundant, and often opposing responses, the net effect of which determines cell survival, death, or even proliferation.

Our group and others have studied death receptor signaling in acute brain injury. In experimental cerebral ischemia, inhibition of TNF-α and Fas ligand together reduces infarction volume by as much as 80% [58]. Following cerebral contusion in mice and humans, a Fas-FADD-procaspase-8 DISC assembles in brain early after trauma and is associated with activation of caspases and ongoing cell death [59]. Because death receptor signaling is highly redundant, it is not surprising that genetic inhibition of Fas alone fails to reduce lesion volume or acute cell death after experimental cerebral contusion [60], although Fas inhibition does reduce cerebral ischemic infarction volume [61] and sequelae of traumatic spinal cord injury [62–64]. We have found that genetic or genetic/pharmacologic inhibition of TNF-α and Fas receptor together reduces post-traumatic brain lesion volume, and, more importantly, seems to improve neurologic function after controlled cortical impact in adult and immature mice [60]. Based on these preliminary findings, we believe that TNFRs, and their downstream adapter proteins, are attractive therapeutic targets to ameliorate tissue damage and functional neurologic deficits after ischemic, traumatic, and other forms of central nervous system injury and degenerative central nervous system diseases.

The Poly(ADP-Ribose) Polymerase Suicide Hypothesis

Poly(ADP-ribose) polymerase-1 (PARP-1) is an abundant nuclear DNA repair enzyme that stabilizes damaged DNA for subsequent repair. Upon activation by severe DNA damage, PARP-1 hydrolyzes NAD(+) to nicotinamide and transfers adenosine diphosphate ribose units to histones and other nuclear proteins, including PARP-1 itself. Adenosine diphosphate ribosylation inhibits protein function and facilitates DNA repair, but overactivation of PARP-1 can deplete cellular stores of NAD(+) and adenosine triphosphate, resulting in energy failure and cell death. DNA damage by oxygen radicals, or excitotoxicity injury, induces PARP-1 activation during acute ischemic and traumatic brain injury. Lesion size after experimental stroke is dramatically reduced in PARP-1 knockout mice. Following traumatic brain injury, PARP-1 knockout mice had similar lesion size but improved neurologic function than wild type [65]. Excessive PARP-1 activation is also implicated in models of Parkinson's disease and traumatic spinal cord injury [66–68]. In addition to necrosis via depleted energy reserves, PARP-1 can also induce release of AIF from mitochondria and induce caspase-independent programmed cell death [69]. Finally, PARP-1 is a transcription factor that modulates expression of genes involved in cell death and survival. Recent studies using specific PARP-1 inhibitors show that partial inhibition of PARP-1 preserves brain NAD(+) stores and improves functional outcome after traumatic brain injury in mice, whereas more complete pharmacologic PARP-1 inhibition impairs spatial learning in naïve as well as injured mice [70]. These studies highlight the multiple roles of PARP-1 in traumatic and ischemic brain injury and underscore the difficulties involved in development of therapies targeting proteins with complex and multiple diverse functions in the brain.

Studies in experimental traumatic brain injury often demonstrate very little correlation between cell death and functional outcome, and interventions that inhibit cell death may or may not influence motor and memory function. Thus, it is not yet clear that inhibiting apoptotic cell death will prove beneficial to patients with head injury [32]. The most effective therapeutic strategies will probably target multiple mechanisms in addition to cell death, such as derangements in cerebral blood flow and energy metabolism, or neurotransmitters and their receptors that are involved in the motor and cognitive functions adversely affected by acute brain injury (discussed below).

The Mitochondrial Permeability Transition Pore

The mitochondrial permeability transition (MPT) pore is a voltage-gated channel that, when open, allows molecules and ions with a mass <1,500 Daltons to pass through the inner mitochondrial membrane to the intermembrane space. Oxidative stress, or rapid and extreme increases in intracellular calcium associated with excitotoxicity, triggers the assembly of an MPT pore, which consists of cyclophilin D binding to an adenine nucleotide translocator [71]. Opening of the MPT pore releases stored calcium into the cytosol, and dissipation of the mitochondrial inner transmembrane potential uncouples the electron transport system from adenosine triphosphate hydrolysis. These events lead to energy failure, enhanced production of reactive oxygen species, a secondary increase in intracellular calcium, release of apoptogenic factors from the mitochondria, and cell death [72]. Compounds that block the MPT pore, such as cyclosporine A and its derivatives, are protective in experimental stroke and traumatic brain injury models, suggesting that the MPT pore is a key regulator of cell death mechanisms, both necrotic and apoptotic [71].

Oxidative Damage in Acute Brain Injury

Under normal conditions, a critical balance exists between the production of oxidant free radicals and the antioxidant defense that protects cells in vivo. Free radicals are defined as molecular species that contain one or more unpaired electrons. During normal metabolism, they are involved in enzymatic reactions, mitochondrial electron transport, signal transduction, activation of nuclear transcription factors, gene expression, and the antimicrobial action of neutrophils and macrophages [73]. The balance between oxidants and antioxidants in injured brain may be disturbed by increased production of free radicals because antioxidant defenses in brain (such as superoxide dismutase, catalase, glutathione, ascorbate, and α-tocopherol) are not adequate to completely neutralize the increase of oxidant species present after trauma or ischemia–reperfusion [74]. The severity of oxidant–antioxidant imbalance determines the magnitude of injury to the cell.

Free radicals can react with almost every molecule found in living cells, including DNA, membrane lipids, proteins, and carbohydrates. A major consequence of oxidative stress is damage to cellular macromolecules. During lipid peroxidation, peroxyl or hydroxyl groups may be added to unsaturated fatty acids, or fatty acid carbon chains may be cleaved during reaction with unpaired electrons to generate aldehydes. Free radical damage to proteins may cause cross-linking, carbonyl formation, and protein denaturation. DNA bases may also be modified by oxidation, resulting in single- and double-strand breaks or mispairing of purine and pyrimidine during DNA replication.

The brain has a number of characteristics that make it especially susceptible to free radical–mediated damage. Brain lipids are highly enriched in polyunsaturated fatty acids, and brain regions such as substantia nigra and striatum have high concentrations of iron, which catalyzes production of free radicals. Both of these factors increase the susceptibility of brain cell membranes to lipid peroxidation. Because the brain is critically dependent on aerobic metabolism, mitochondrial respiratory activity is higher than in many other tissues, increasing the risk of free radical *leak* from mitochondria; conversely, free radical damage to mitochondria in brain may be tolerated relatively poorly because of this dependence on aerobic metabolism.

Free radicals have been implicated in the pathogenesis of central nervous system injury, including traumatic brain injury, spinal cord injury, cerebral ischemia, and neurodegenerative diseases [28,73,75–78]. Reactive oxygen species may modify excitotoxicity by downregulating ion flux through NMDA receptors; however, exposure to oxidative stress can also enhance NMDA receptor-mediated neurotoxicity, particularly when antioxidant defenses are depleted. Free radicals contribute to cell death at several points in the apoptotic cascade, serving as initiators, early signals, and possibly late effectors of apoptotic neuronal death. As previously mentioned, oxidative stress can also contribute to cell death by facilitating mitochondria transition pore formation [74]. Proof that excessive oxygen radical generation is fundamental to the pathogenesis of acute brain injury derives from studies in which superoxide dismutase (SOD) knockout mice had increased damage, and SOD overexpressors had reduced brain damage and improved

functional neurologic outcome after experimental stroke and traumatic brain injury [79–82].

Reactive Oxygen Species

Reactive oxygen species formation during ischemia–reperfusion may originate from several sources (Figure 2.2), including nitric oxide synthase (NOS) activity, mitochondrial electron transport, multiple steps in the metabolism of arachidonic acid, and, in some species (e.g., rodents), xanthine oxidase, which is produced by hydrolysis of xanthine dehydrogenase. Oxygen (O_2) qualifies as a radical because it has two unpaired electrons, each located in a different orbital, both *spinning* in the same direction. This parallel spin is one reason for poor reactivity of O_2 with cellular constituents, despite its potential as an oxidizing agent. Acceptance of a single electron by an O_2 molecule forms the superoxide radical, O_2^-, which has one unpaired electron. Superoxide itself has limited reactivity and is capable of inactivating only a few enzymes directly. The NADH dehydrogenase complex of the mitochondrial electron transport chain is one of the enzymes shown to be a direct target for superoxide attack [83].

Excess superoxide is removed by converting it to H_2O_2, a reaction that is catalyzed by SOD. This reaction is an important defense mechanism in aerobic organisms [83]. Overall, both O_2^- and H_2O_2 have limited chemical reactivity, but they can generate highly reactive hydroxyl radicals ($OH^{\cdot}$) by reacting with transition metals such as iron and copper. After closed head injury in rats, peak hydroxyl radical formation occurred by 40 min, and hydroxyl radicals are increased for several hours after experimental acute subdural hematoma [84–86]. Increased hydroxyl radical production also occurs in brain after focal cerebral ischemic injury in rodents [87]. Superoxide production has been detected after experimental spinal cord injury [88], central nervous system inflammation and ischemia–reperfusion [89], and fluid percussion traumatic brain injury [90]. Superoxide radical is believed to be the principal mediator of microvascular damage after traumatic brain injury, and SOD attenuates brain microvascular damage after traumatic brain injury [91,92].

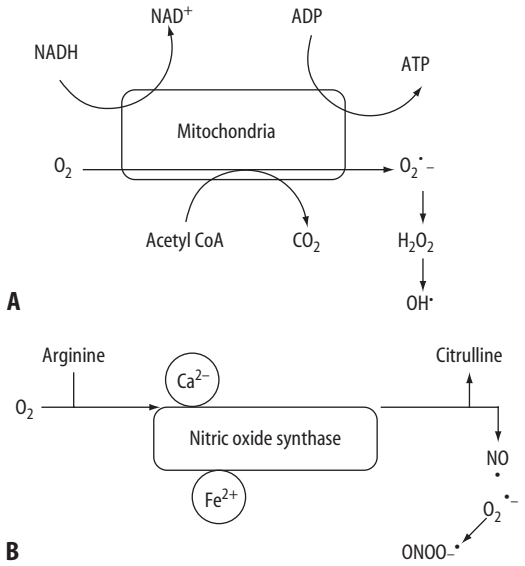

Figure 2.2. Oxidative stress pathways. **(A)** Reactive oxygen species generated by mitochondrial electron transport and **(B)** reactive nitrogen species generated by nitric oxide synthase. Peroxynitrite (ONOO-) is a highly toxic species that signals apoptosis and necrosis after acute brain injury. Other oxidants are generated in damaged mitochondria by the electron transport chain and molecular oxygen. NO, nitric oxide; $O_2^{\cdot -}$, singlet oxygen; H_2O_2, hydrogen peroxide; $OH^{\cdot}$, hydroxyl radical. ADP, adenosine diphosphate; ATP, adenosine triphosphate; NAD^+, oxidized form of nicotinamide adenine dinucleotide; NADH, reduced form of nicotinamide adenine dinucleotide.

Reactive Nitrogen Species

Nitric oxide synthase has been identified as another source of reactive oxygen and reactive nitrogen species with special relevance to pathologic conditions (see Figure 2.2). Nitric oxide synthase is homologous to P-450 cytochrome c reductase; cofactors in the reaction-mediated by NOS are flavin mononucleotide, flavin adenine dinucleotide, tetrahydrobiopterin, and nicotinamide adenine dinucleotide phosphate. Three types of NOS have been identified: Ca^{2+}/calmodulin-activated neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). Nitric oxide synthase normally converts arginine and molecular oxygen to citrulline and nitric oxide (NO), a free radical gas. Nitric oxide is lipid soluble, readily crosses cell membranes, and functions in control of cerebral blood flow (NO mediates vasodilatation), neuronal communication, synaptic plasticity and memory formation, intracellular signal transmission, and release of neurotransmitters [93]. Nitric oxide may exist as nitrosonium (NO^+), $NO^{\cdot}$, and nitroxyl anion (NO^-). NO^+ is thought to contribute to NMDA toxicity, whereas NO^- is thought to be neuroprotective by downregulating the NMDA receptor and inhibiting glutamate release presynaptically, through activation of guanylate cyclase. Nitric oxide, which has limited radical reactivity, can combine readily with O_2 and possibly H_2O_2 to produce peroxynitrite (ONOO-), a highly oxidizing, nonradical compound that oxidizes lipids, proteins, and DNA. Nitric oxide–mediated peroxynitrite contributes to acute brain injury in part by inducing DNA damage and activating PARP, as well as directly by oxidizing key cellular constituents. On the other hand, NO can inhibit excitotoxicity by downregulating NMDA receptor function via S-nitrosylation; NO may inhibit caspase activity in a similar manner. Thus, reactive nitrogen species may have both beneficial and detrimental effects in acute brain injury.

Inhibition of the early peak of NO in brain following traumatic brain injury, which is likely mediated by nNOS, improves neurologic outcome after experimental traumatic brain injury [93]. However, later after injury there is a relative NO deficiency associated with cerebral hypoperfusion; augmentation of NO during this time, by administering L-arginine, improves cerebral blood flow and outcome in several models [93]. A delayed increase in NO after traumatic injury, mediated by iNOS, is also observed in experimental traumatic brain injury; experimental studies suggest both deleterious and protective effects of iNOS in rodent traumatic brain injury models. Formation of peroxynitrite by iNOS early after injury is detrimental, and iNOS inhibition may therefore be protective [94]. In contrast, iNOS knockout mice have impaired long-term spatial memory acquisition after experimental traumatic brain injury, suggesting that iNOS is critical for recovery mechanisms [95]. Recent studies support a beneficial role for iNOS in traumatic brain injury by maintaining endogenous antioxidant reserves [94]. Thus, NO can exert beneficial and detrimental effects in the injured brain, depending on the magnitude of its production, temporal distribution after injury, and other factors.

In the first comprehensive clinical study of oxidative injury in children with traumatic brain injury, Bayir and colleagues found

progressive depletion of antioxidant reserves and evidence for free radical–mediated lipid peroxidation in cerebrospinal fluid samples [96]. These investigators later reported increased S-nitrosothiols (transfer of NO groups to cysteine sulfhydryls on proteins) in cerebrospinal fluid of children with severe traumatic brain injury and increased intracranial pressure and postulated that S-nitrosothiols could be neuroprotective after traumatic brain injury by virtue of nitrosylation and inhibition of NMDA receptors and caspases [97]. In adult patients, lipid peroxidation was noted early after severe traumatic brain injury and was more prominent in males than in females, suggesting that females have less oxidative damage than males during acute brain injury and enhanced neuroprotection mediated by female gonadal hormones [98]. In that study, therapeutic hypothermia tended to decrease lipid peroxidation in males but not females. These data suggest that differences in susceptibility to oxidative injury may explain, at least in part, gender-specific differences in pathophysiology and outcome observed after acute and neurodegenerative brain injury [99].

Oxidative Stress and Neuroinflammation: Mediators of Neurologic Dysfunction After Brain Injury?

Does the brain's endogenous inflammatory response to acute injury influence subsequent neurologic dysfunction observed in patients with traumatic brain injury, meningitis, and other forms of acute central nervous system injury? Our preliminary data suggest that this may indeed be the case, as immature mice lacking TNF-α and Fas receptor had decreased neurologic dysfunction compared with wild-type mice or mice deficient in TNF or Fas alone [60]. Recent studies provide one possible explanation linking neuroinflammation, NMDA receptor deactivation, and motor and cognitive deficits in experimental meningitis and traumatic brain injury [21,22]. In mice subjected to closed head injury, an initial increase in NMDA receptor activation, consistent with acute excitotoxicity, is observed in brain regions proximal to the injury site. From 1 hr to 1 week later, however, pronounced NMDA receptor deactivation is observed in cortex and hippocampal regions involved in learning and memory and is associated with motor and cognitive dysfunction after traumatic brain injury [21]. Downregulation of NMDA receptor function is also observed after injection of lipopolysaccharide into rat brain and is prevented by treatment with an antioxidant [22]. The above observations suggest a link between acute brain injury, neuroinflammation, oxidant stress, and postinjury neurologic dysfunction. Furthermore, desensitization of NMDA receptors after stroke and traumatic brain injury may in part account for the failure of clinical trials using NMDA receptor antagonists to improve outcome in patients with stroke and traumatic brain injury [21].

Extracellular Matrix Proteases

In addition to intracellular proteases, extracellular proteases may also play important roles in brain injury. Data emerging in the past 6 years implicate proteases from the matrix metalloproteinase (MMP) family of genes as well as serine proteases from the plasminogen axis [100]. These proteases play major roles during brain development by altering extracellular matrix and allowing cellular migration and neurite and axonal extension [101]. Dysregulation of MMPs after brain injury leads to aberrant proteolysis of the neurovascular matrix, resulting in blood–brain barrier (BBB) damage and cell death.

In experimental models of cerebral ischemia, many MMPs are significantly increased at the levels of expression and activity [102–105]. Overall data point to a deleterious role for MMPs, at least acutely. Matrix metalloproteinase injection into brain is neurotoxic [106]. Treatment with inhibitors or MMP-neutralizing antibodies reduce edema and infarction in rat and mouse models of cerebral ischemia [103,107–109]. Recently, it was demonstrated that MMP-9 knockout mice had significantly smaller lesion volumes than wild-type mice after focal cerebral ischemia and traumatic brain injury, emphasizing the central role of this protease, at least in murine systems [102,103,110]. A similar finding was obtained after transient global cerebral ischemia, with hippocampal neuron death being significantly ameliorated in MMP-9 knockout mice [111].

After neurovascular injury, MMPs may degrade basal lamina, weaken vessels, and predispose vessels to leakage and rupture. In experimental studies, activation of MMP-9 and degradation of critical protein components of cerebral blood vessels have been correlated with the development of hemorrhage and edema [112,113]. In a recent study, pharmacologic inhibition of MMPs significantly decreased the incidence of hemorrhage in a rabbit model of embolic stroke [102], and matrix degradation and subsequent BBB leakage was reduced after cerebral ischemia in MMP-9 knockout mice [102]. Matrix metalloproteinase activation and BBB disruption is associated with the generation of reactive oxygen radicals [114]; thus interactions between oxidative stress and the proteolytic cascade may ultimately mediate the progression of edema and infarction. Within the context of early neurovascular inflammation, cytokines and vascular adhesion molecules may further amplify MMPs in activated endothelium [115–117]. Cell adhesion molecules themselves may also be substrates for MMPs, thus comprising a complex interactive system of response to brain injury.

In addition to vascular leakage, extracellular matrix proteases may also directly induce cell death. The disruption of homeostatic signals between cells and matrix can initiate specialized modes of apoptosis called *anoikis* [118]. In vivo and in vitro evidence is beginning to accumulate to support the importance of these novel mechanisms in stroke. In a nonhuman primate model of focal cerebral ischemia, areas in which vascular antigens were lost correlated with regions of neuronal injury [119]. Loss of neuron–matrix interactions promotes neurotoxicity by downregulating cell survival pathways associated with integrin signaling [120]. The importance and relevance of these matrix mechanisms has recently been underscored by the finding that fibronectin knockout mice suffered increased neuronal apoptosis and brain infarction after cerebral ischemia [121].

Apart from MMPs, proteases from the plasminogen system are also involved in brain injury. In ischemic stroke, the primary role for tissue plasminogen activator would be beneficial lysis of the offending clot. However, accumulating data now suggest that pleiotropic and deleterious actions of tissue plasminogen activator may also participate in neurovascular pathology. Tsirka, Strickland, Lipton, and colleagues first demonstrated that tissue plasminogen activator knockout mice were protected against excitotoxic hippocampal injury and focal cerebral ischemia [122,123]. Tissue plasminogen activator knockout mice suffered significantly less brain damage after trauma than did wild-type mice [124]. Tissue plasminogen activator may interact with the NR1 subunit of the NMDA receptor complex and amplify damaging calcium currents during

excitotoxicity [125]. Tissue plasminogen activator (and plasmin) may also target nonfibrin substrates in brain extracellular matrix, leading to augmented excitotoxic neuronal death in the hippocampus via degradation of interneuronal laminin and disruption of prosurvival cell-matrix signaling (126). Although the main effect of tissue plasminogen activator administration in stroke certainly occurs within the targeted vessel, these findings suggest that extravascular actions of tissue plasminogen activator may complicate its intended role in clot lysis.

Most brain injury research has been focused on intracellular mechanisms of cell death. However, accumulating data now suggest that extracellular proteases can also play key roles by degrading neurovascular matrix and inducing both BBB disruption and cell death. Hence, targeting both intra- and extracellular proteases may offer more effective approaches for treating stroke and brain trauma in the future.

Cortical Spreading Depression

During acute brain injury such as stroke or trauma, neurons and glia may undergo spontaneous depolarizations that spread in waves to distant regions of uninjured brain. These waves, known as *cortical spreading depression* (CSD), propagate at 2–4 mm/min over cerebral cortex and are associated with marked increases in extracellular K^+ and glutamate and intracellular Na^+ and Ca^{2+} [127]. Although neuronal firing may initially increase, it is subsequently depressed, and the electroencephalographic silence outlasts the period of tissue depolarization by several minutes [127]. Cortical spreading depression causes a large transient cerebral blood flow increase, followed by delayed, prolonged cerebral blood flow decrease [128]. In experimental animal models, NMDA receptor inhibitors (e.g., ketamine) and Ca^{2+} channel antagonists block CSD [129]. The factors that mediate cerebral blood flow changes during CSD remain unknown, although K^+, H^+, prostanoids, nitric oxide (NO), and calcitonin gene-related peptide have been implicated [130–133].

Following experimental cerebral ischemia, repetitive spontaneous waves of spreading depolarizations resembling CSD originate from focal ischemic cortex [134]. Like CSD, these periinfarct spreading depressions cause massive K^+, Na^+, and Ca^{2+} shifts, reduce intracellular adenosine triphosphate, and cause tissue acidosis. Periinfarct spreading depressions decrease cerebral blood flow and worsen hypoperfusion under ischemic conditions and enlarge cerebral infarcts presumably by exacerbating the energy deficit in ischemic neurons [135–137]. More work needs to be done to understand the contribution of CSD to acute brain injury in humans and to determine whether targeting CSD in the postinjury period is a useful therapeutic strategy [138].

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