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Review

Metabotropic glutamate receptors promote neuronal and vascular plasticity through novel intracellular pathways

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Summary. During the initial development and maturation of an individual, the metabotropic glutamate receptor (mGluR) system becomes a necessary component for the critical integration of cellular function and plasticity. In addition to the maintenance of cellular physiology, the mGluR system plays a critical role during acute and chronic degenerative disorders of the central nervous system. By coupling to guanosine-nucleotide-binding proteins (G-proteins), the mGluR system employs a broad range of signal transduction systems to regulate cell survival and injury. More commonly, it is the activation of specific mGluR subtypes that can prevent programmed cell death (PCD) consisting of two distinct pathways of genomic DNA degradation and membrane phosphatidylserine (PS) residue exposure. To offer this cellular protection, mGluRs modulate a series of down-stream cellular pathways that include protein kinases, mitochondrial membrane potential, cysteine proteases, intracellular pH, endonucleases, and mitogen activated protein kinases. Prevention of cellular injury by the mGluR system is directly applicable to clinical disability, since immediate and delayed injury paradigms demonstrate the ability of this system to reverse PCD in both neuronal and vascular cell populations. Further understanding of the intricate pathways that determine the protective nature of the mGluR system will provide new therapeutic avenues for the treatment of neurodegenerative disorders.

Key words: Apoptosis, Cysteine proteases, Endonucleases, Mitochondrial membrane potential, Protein kinase

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Discovery of the mGluR system

Glutamate is a principal excitatory neurotransmitter in the mammalian nervous system. As a neurotransmitter, glutamate plays important roles in cellular function through the activation of ionotropic glutamate receptors, such as N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) receptors, and kainate (KA) receptors. In addition to its function in neuronal differentiation, migration, and survival in the developing brain (Hack and Balazs, 1994), glutamate is necessary for learning and memory acquisition (Steele et al., 1995). Alternatively, glutamate also can be a principal mediator of cellular injury. Following ionotropic receptor activation, glutamate can result in excitotoxity that may be a critical component for the development of neurodegenerative disease (Beal, 1992).

The actions of glutamate in the mammalian brain were initially thought to be mediated exclusively through the activation of ionotropic glutamate receptors. Yet, further studies provided evidence for the existence of another family of glutamate receptors which were directly coupled to GTP-binding regulatory proteins. Early work, such as the observation of glutamate induced phospholipase C generation in neurons, indicated that glutamate had more complex roles that could not be accounted for by only NMDA, AMPA, or KA receptor families (Sladeczek et al., 1985). Subsequently, it became evident that a new class of glutamate receptors, termed metabotropic glutamate receptors (mGluRs), was coupled to effector systems through guanosine-nucleotide-binding proteins (G-proteins) (Houamed et al., 1991; Prezeau et al., 1992; Yuzaki and Mikoshiba, 1992). The first mGluR, now generally termed mGluR1a, was cloned in 1991 by a functional expression screening procedure (Houamed et al., 1991; Masu et al., 1991). To date, eight members of the mGluRs family have been identified and are numbered mGluR1 through mGluR8 following the order

in which their cDNAs have been cloned.

The mGluR subfamily of G-protein-coupled receptors

In general, G-proteins function as transducers that transfer extracellular information to intracellular signaling cascades. The family of G-proteins consist of heterotrimeric proteins that contain three subunits termed α,ß, and γ. A total of forty-six G-proteins have been identified with twenty-seven classified as Gα, five classified as Gß, and fourteen classified as Gγ. Thus, a variety of heterotrimeric combinations can be formed that may produce a broad spectrum of G-protein signaling (Albert and Robillard, 2002). Activation of G-protein-coupled receptors (GPCRs) results in the dissociation of the heterotrimer of the G-protein into its α and ßγ subunits, which can then bind to a variety of effector molecules. For example, $G\alpha_s$ can bind to adenylate cyclase and increase the activity of adenylate cyclase while binding of $G\alpha_I$ to adenylate cyclase can inhibit the activity of adenylate cyclase. In addition, a

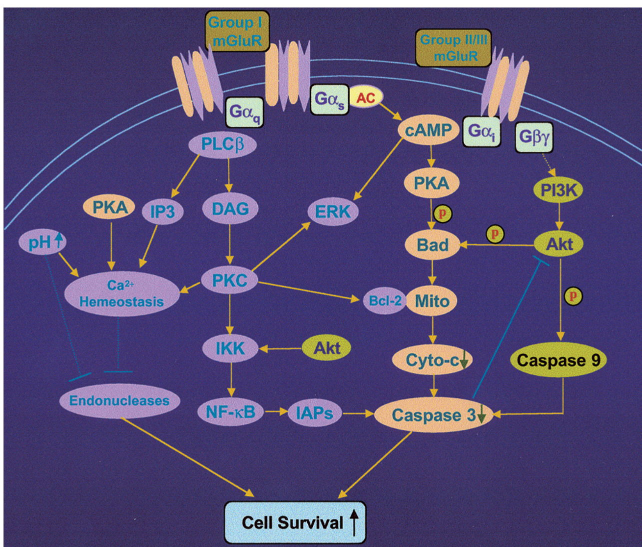

Fig. 1. Metabotropic glutamate receptors (mGluRs) modulate a broad spectrum of signal transduction pathways to offer cellular protection. Group I mGluRs employ the $G\alpha_q$ protein to activate phospholipase ß (PLCß), diacylglycerol (DAG), and inositol triphosphate (IP3). These pathways lead to the activation of protein kinase C (PKC) and the mobilization of intracellular calcium. PKC activation may result in the activation of IκB kinase (IKK) that can precipitate the phosphorylation and degradation of IκB. This is followed by liberation of free NF-κB and activation of inhibitors of apoptosis proteins (IAPs). PKC also can phosphorylate Bcl-2 to block apoptosis. Group I mGluRs also can couple to $G\alpha_s$ to activate adenylate cyclase (AC), cyclic adenosine monophosphate (cAMP), and protein kinase A (PKA). PKA may then inactivate Bad, a pro-apoptotic member of Bcl-2 family resulting in the attenuation of cytochrome c (Cyto-c) release from mitochondria. Group II and group III mGluRs may stimulate the phosphoinositol 3 kinase (PI3K)/Akt pathway to initiate their protective signaling and block caspase 9 activity. In addition, activation of the mGluR system can inhibit caspase 3 activity to prolong the half-life of Akt. Modulation of intracellular pH, PKC, and PKA by mGluRs also may contribute to the cellular homeostasis of intracellular calcium and the prevention of endonuclease activity.

particular G-protein may be responsible for the modulation of a series of signal transduction pathways. The G-protein ßγ has been associated with many effector molecules including adenylate cyclase, phospholipase C-ß (PLC-ß), mitogen-activated protein kinases (MAPKs), and phosphoinositide 3 kinase (PI3K) (Hur and Kim, 2002).

As a family of proteins, GPCRs are considered to comprise one of the largest. All GPCRs share a common structure consisting of a single polypeptide chain. This polypeptide chain forms seven trans-membrane helices that are linked by three alternating extracellular and intracellular loops. The amino terminus is present on the extracellular side and the carboxy terminus is present on the intracellular region (Shacham et al., 2001). Ligands of GPCRs activate these receptors by binding to the extracellular domains (Schwartz, 1994). As a result, the intracellular domains are responsible for the initiation of intracellular signaling (Wess, 1997).

The GPCRs can be divided into three major subfamilies based on nucleotide and amino acid sequence similarity. Family A consists of the rhodopsin/adrenergic receptors, which is by far the largest and one of the better established receptor families for their structure and function. Family A is characterized by the presence of a restricted number of conserved residues (Asp-Arg-Tyr). Family B consists of peptide hormone and neuropeptide receptors that are characterized by a large extracellular NH2 terminus containing six cysteine residues. The metabotropic glutamate receptors (mGluRs) belong to family C of GPCRs, which also includes gamma aminobutyric acid (GABA) receptors and ionotropic calcium receptors. Unlike other GPCR families, mGluRs contain a long NH2 terminal chain and couple to G-proteins through their second intracellular loop rather than the third intracellular loop of the receptor.

Based on sequence homology, G-protein coupling specificity, and agonist selectivity, mGluRs are classified into three major groups. Group I mGluRs (mGluR1 and 5) couple preferentially to G_q to stimulate PLC-ß. Activation of PLC-ß results in the generation of inositol-1, 4, 5-triphosphate (IP3) and diacylglycerol (DG) to mobilize intracellular calcium and activate protein kinase C (PKC) (Fig. 1). Group I mGluRs also can activate adenylate cyclase via coupling to Gs to result in an increase in cyclic adenosine monophosphate (cAMP) (Francesconi and Duvoisin, 1998). In contrast to group I, group II mGluRs (mGluR2 and 3) and group III mGluRs (mGluR4, 6, 7, and 8) are negatively coupled to adenylate cyclase to reduce the amount of intracellular cAMP (Pin and Duvoisin, 1995). In addition, activation of group II/III can modulate activity of extracellular signal-regulated protein kinases (ERK) and PI3K (Ferraguti et al., 1999).

Expression of mGluRs in the central nervous system

Metabotropic glutamate receptors are expressed

throughout the mammalian central nervous system (CNS). In neurons, mGluRs are present in the cerebral cortex (Berger et al., 2001), cerebellum (Berthele et al., 1999), striatum (Tanabe et al., 1993; Mao and Wang, 2001), thalamus (Saugstad et al., 1997), spinal cord (Aronica et al., 2001), and other regions of the brain (Hay et al., 1999) (Table 1). For example, mGluR1 and 5 have been shown to exist in the substantia nigra of primate brains (Hubert et al., 2001). In the hippocampus, a more restricted expression of the receptor subtypes mGluR1b, mGluR2/3, mGluR4a and mGluR5 has been demonstrated (Blumcke et al., 1996). The receptor subtype mGluR4 also has a distinct distribution in the thalamus, hypothalamus, and caudate nucleus (Makoff et al., 1996a). In contrast, mGluR3 is expressed throughout the brain with dense expression in neurons of the cerebral cortex, caudate-putamen, thalamus, and cerebellum (Makoff et al., 1996b).

The mGluR system and its receptors are distributed in specific subcellular regions and subjected to changes during development of the nervous system. Group I mGluRs (mGluR1α and mGluR5) predominantly exist on the post-synaptic membrane of the glutamatergic synapse junctions (Lujan et al., 1997). Yet, in the initial postnatal period, mGluR1α and mGluR5 can be found in proximal dendrites and the somata of the cell. With age, these receptors become densely distributed in the distal part of dendrites to participate in synaptic function (Liu et al., 1998). Group II mGluRs (mGluR2/3) are present primarily in astrocytic processes surrounding the neuronal somata and synapses. A less dense population of group II mGluRs are also located in presynaptic axon terminals. The distribution pattern of mGluR2/3 is believed to be consistently maintained during postnatal development (Liu et al., 1998). Of the group III mGluRs, mGluR6 is initially distributed in both the neuronal soma and dendrites in rat retinal bipolar cells, but later redistributes to postsynaptic sites (Nomura et al., 1994). Presynaptic expression is more common for the mGluR7 subtypes (Shigemoto et al., 1996).

Little is known of the role of the mGluR system in the cerebral vascular system, but recent work is beginning to provide evidence for a vital function for the mGluR system in brain endothelial cells (ECs). Initial work has outlined the expression of mGluRs in cultured rat cerebrovascular ECs (Krizbai et al., 1998) and in cardiac cells (Gill et al., 1999). Further studies have now demonstrated not only the expression of specific group I mGluRs in cerebral ECs, but also the potential for the mGluR system to protect against apoptotic injury (Lin and Maiese, 2001a,b).

Physiology and function of mGluRs

In addition to the anatomical redistribution of mGluRs, functional changes in the mGluR system also may occur during development. The generation of second messengers, such as cAMP, has been reported to

Table 1. Expression of mGluRs in mammalian brain.

mGluR GROUPS	EXPRESSION AND CONTRIBUTORS
Group I	
mGluR 1	Rat cerebral cortex (Berger et al., 2001), striatum (Mao and Wang, 2001; Berger et al., 2001), medulla oblongata (Hay et al., 1999; Aronica et al., 2001), substantia nigra (Hubert et al., 2001), and spinal cord (Aronica et al., 2001) Monkey substantia nigra (Hubert et al., 2001) Human hippocampus (Blumcke et al., 1996), thalamus, and cerebellum (Berthele et al., 1999)
mGluR 5	Rat striatum (caudate nuclei) (Mao and Wang, 2001), medulla oblongata (autonomic nuclei) (Hay et al., 1999), and substantia nigra (Hubert et al., 2001) Monkey substantia nigra (Hubert et al., 2001) Human hippocampus (Blumcke et al., 1996), thalamus, and cerebellum (Makoff et al., 1996), (Berthele et al., 1999)
Group II	
mGluR 2/3	Rat cerebral cortex (Berger et al., 2001), striatum (Tanabe et al., 1993; Berger et al., 2001), medulla oblongata (autonomic nuclei) (Hay et al., 1999), and olfactory bulb Human cerebral cortex, caudate-putamen, thalamus, cerebellum (Makoff et al., 1996; Berthele et al., 1999), and hippocampus (Blumcke et al., 1996)
Group III	
mGluR 4	Rat cerebral cortex (Berger et al., 2001) and striatum (Berger et al., 2001) Human hippocampus (Blumcke et al., 1996), thalamus, hypothalamus, and caudate nucleus (Makoff et al., 1996; Berthele et al., 1999)
mGluR 7	Rat medulla oblongata (Hay et al., 1999) Human cerebellum (Berthele et al., 1999)
mGluR 8	Rat olfactory bulb, pontine gray, thalamus, piriform cortex, cerebral cortex, hippocampus, cerebellum, mammillary body (Saugstad et al., 1997) Human cerebellum (Berthele et al., 1999)

vary under mGluR control during critical periods of ocular dominance and plasticity (Reid et al., 1996). Electrophysiological studies have shown that activation of mGluRs may lead to a range of cellular changes such as the inhibition of calcium and potassium currents, mediation of slow excitatory postsynaptic potential, and inhibition of presynaptic neurotransmitter release (Anwyl, 1999).

Activation of the three groups of mGluRs results in a potent inhibitory action on voltage-gated calcium channels and potassium channels (McCool et al., 1998). The mGluR system can inhibit N-type calcium channels that is modulated through G-protein-dependent pathways (Anwyl, 1999). Potassium channels are also major targets of mGluRs. Activation of group I mGluRs can inhibit slow calcium-dependent potassium currents (Abdul-Ghani et al., 1996), voltage-dependent potassium currents, and slow inactivating potassium currents (Charpak and Gahwiler, 1991). Yet, in some instances, mGluRs also may mediate a weak activation of both calcium and potassium currents (Anwyl, 1999).

Modulation of synaptic transmission is one of the primary functions of mGluRs. In excitatory synapses, mGluRs serve to block synaptic transmission by preventing the release of excitatory transmitters. Activation of group I mGluRs can suppress excitatory transmission through the presynaptic inhibition of glutamate release in hippocampal CA1 cells (Watabe et al., 2002). Similarly, activation of group II mGluRs (mGluR2/3) can depress glutamate release in the dentate gyrus through a mechanism that involves presynaptic voltage gated calcium-entry (Dietrich et al., 2002). Group III mGluRs also have been found to suppress glutamate release from cone presynaptic terminals through the inhibition of excitatory postsynaptic currents (Hirasawa et al., 2002). In addition, mGluR autoreceptors may limit excessive glutamate release through a negative feedback mechanism (Hirasawa et al., 2002).

The mGluR system also can modulate synaptic function at GABA terminals. Agonists of all three groups of mGluRs depress GABA inhibitory postsynaptic currents in rat mesencephalic neurons (Bonci et al., 1997). Group II mGluRs have a significant role in the modulation of GABAergic afferent inhibition in the ventrobasal thalamus that controls functions of sleep, arousal, and sensation (Salt and Turner, 1998). Group III mGluRs support coordination and motor function through the inhibition of GABAergic and glutamatergic transmission in the substantia nigra and pars reticulata (Wittmann et al., 2001).

The ubiquitous distribution of glutamatergic synapses in the brain offers a great potential for mGluRs to modulate global CNS function. Behavioral and physiological studies have demonstrated that mGluRs can regulate fast synaptic transmission and changes in synaptic plasticity (Dietrich et al., 2002). During memory imprinting, group I mGluRs which are juxtaposition to NMDA receptors, can modulate the potentiation of NMDA receptor activity to influence both long-term potential (LTP) and long-term depression (LTD) (Manahan-Vaughan, 1997; Chen et al., 2000). The involvement of mGluRs in synaptic plasticity and in the induction of LTP and LDP is believed to be necessary for the processing of sensory information (Matthies, 1989; Riedel et al., 1996). Activation of mGluRs can also lead to depolarization-induced synapsin I phosphorylation, a process that may be involved in synaptic vesicle exocytosis in visceral sensory neurons (Hay et al., 2000).

Cytoprotection of mGluRs during neuronal and vascular injury

Current interest has focused on the protective role of the mGluR system in the nervous system. Several observations support a prominent role for the mGluR system during neurodegenerative disease. In trisomy 21 disorders, a congenital disorder leading to mental retardation, enhanced expression of the mGluR subtype 5 has been reported (Oka and Takashima, 1999). Some evidence supports the premise that group I mGluRs can regulate the metabolism of amyloid precursor protein (APP) and accelerate the processing of APP into non-amyloidogenic APP (Lee et al., 1996). Modulation of synaptic activity by mGluRs during chronic neuronal disorders, such as in Huntington's disease, has been suggested to alter cellular susceptibility to injury (Calabresi et al., 1999). During models of spinal cord trauma, both group I and group III mGluRs demonstrate increased expression (Mills et al., 2001). The significance of the mGluR system in the CNS has several broad implications and even extends to primary tumors of the brain. In neoplastic disorders of the nervous system, mGluR activation has been considered to represent an important component in the prevention of excitotoxicity during glioblastoma multiforme growth (Ye and Sontheimer, 1999).

In experimental models of injury, activation of mGluR subtypes usually can protect cells against several types of insults. During traumatic brain injury models, activation of group II mGluRs can reduce neuronal loss (Zwienenberg et al., 2001). Neuronal injury during NMDA excitotoxicity also is prevented by the activation of the mGluR system (Pizzi et al., 1996; Wang et al., 1999; Adamchik and Baskys, 2000). Activation of group II receptors during both excitotoxic in vitro and ischemic in vivo conditions can prevent neuronal degeneration and limit infarct size (Cai et al., 1999). Neuronal protection through the activation of the mGluR has been extended to several other models of neuronal injury such as glucose deprivation (Sagara and Schubert, 1998), potassium depletion (Borodezt and D'Mello, 1998), combined oxygen and glucose deprivation (Kalda et al., 2000), nitric oxide (NO) (Maiese et al., 1996, 2000), and anoxia (Maiese et al., 1996; Vincent and Maiese, 2000; Lin and Maiese, 2001b). Studies demonstrating the protective role of the mGluRs system are listed in Table 2.

Further analysis has illustrated that protection by the

mGluR system is mediated through more downstream pathways of cellular injury. For example, mGluR activation prevents, and in some cases, reverses genomic DNA degradation (Vincent et al., 1997), modulates endonuclease activation (Vincent et al., 1999a), and maintains cellular membrane asymmetry (Vincent and Maiese, 2000). Cytoprotection by the mGluR system is believed to act at or below the level of free radical generation and oxidative stress (Maiese et al., 1996; Vincent et al., 1997; Sagara and Schubert, 1998). More recent work has suggested that the mGluR offers similar protective capacity to the vascular system by preventing endothelial cell DNA degradation and inhibiting a thrombotic state through the maintenance of membrane asymmetry (Lin and Maiese, 2001a,b).

Although activation of the mGluR system usually provides a supportive environment for cell survival, both the nature and extent of the cellular injury as well as the state of the cell itself may determine whether activation or inhibition of the mGluR system is ultimately required for cellular protection. As a result, some studies provide evidence that antagonism of mGluRs may be beneficial. For example, inhibition of mGluR activity during the progression of a toxic insult in some experimental models may subsequently improve neuronal survival (Shuaib and Kanthan, 1997; Pellegrini-Giampietro et al., 1999). Furthermore, changes in the cellular environment, such as decreased intracellular calcium release, may allow antagonism of the mGluR system to exert cytoprotection (Maiese et al., 1999).

It is important to realize that the role of the mGluRs in the nervous system is complex in nature and may, at times, have adverse consequences. Down-regulation of the mGluR system has been suggested to lead to the generation of post-injury pain following spinal cord injury during both pharmacological (Abraham et al., 2001) and knockdown studies (Fundytus et al., 2001). In other scenarios, activation of the mGluR system may increase activity of the capsaicin receptor and contribute to hyperalgesia (Tominaga et al., 2001).

Programmed cell death: An end-point for mGluRs

Programmed cell death (PCD), also known as apoptosis, plays a significant role in both neuronal and vascular degeneration. Several general biochemical and physiologic features of PCD have been identified (Kerr et al., 1972). These processes include the loss of plasma membrane asymmetry, nuclear chromatin condensation, and DNA fragmentation. In the neuronal population, PCD is considered to be a significant contributor of cellular injury during a variety of neurologic disorders such as stroke (Johnson et al., 1995), trauma (Satake et al., 2000), and Alzheimer's disease (Shimohama, 2000). Experimental models that employ injury models of free radical exposure (Maiese, 2001), beta-amyloid deposition (Kajkowski et al., 2001), or ultraviolet radiation (Kimura et al., 1998) provide additional support for the role of PCD during neuronal degeneration. Induction of PCD in vascular ECs also can represent a critical step in the development of several disease states such as arteriosclerosis, intravascular coagulation, and ischemic injury (Kurose et al., 1994; Grammas et al., 1997). Loss of vascular ECs can lead to thrombosis, immune dysfunction, and inflammation throughout the vascular system (Staub, 1981; Sage et al., 1982).

Two independent pathways have been demonstrated to lead to cellular PCD. One pathway involves the degradation of genomic DNA through the activity of endogenous neuronal endonucleases. This pathway is considered to be a committed event that results in neuronal demise (Okamoto et al., 1993; Tominaga et al., 1993; Vincent and Maiese, 1999b; Vincent et al., 1999a,b; Love et al., 2000). The second component of neuronal PCD consists of the externalization of membrane phosphatidylserine (PS) residues (Chang et al., 2000; Maiese and Vincent, 2000). Exposure of PS

Table 2. Cytoprotection by the mGluR system in various injury models .

SUBJECTS	MODELS	TYPE OF mGluRs			CONTRIBUTORS
		I	II	III	
In neuronal cultures					
Rat hippocampal neurons	Nitric oxide and anoxia	+	+	+	Maiese et al., 1996
Rat cerebellar granule neurons	Low potassium			+	Borodezt and D'Mello, 1998
Hippocampal HT-22 cells, rat cortical neurons	Oxidative stress	+			Sagara and Schnbert, 1998
Rat cerebellar granule neurons	Oxygen-glucose deprivation	+			Kalda et al., 2000
Rat hippocampal neurons	Membrane asymmetry	+		+	Vincent and Maiese, 2000
In hippocampal slices					
Rat hippocampal slice	NMDA		+		Pizzi et al., 1996
Rat hippocampal slice	Hypoxia/hypoglycemia	+		+	Schroder et al., 1999
Rat hippocampal slice	NMDA	+	+		Adamchik and Baskys, 2000
In animal models					
Rat hippocampus	Hypoxic injury		+		Cai et al., 1999
Rat cortex	Fluid percussion injury		+	+	Zwienenberg et al., 2001
Rat spinal cord	Impact injury		+	+	Mills et al., 2001
Rat striatum	NMDA injury		+	+	Wang et al., 1999

residues is believed to occur early and prior to the appearance of genomic DNA degradation. In neurons, membrane PS exposure serves to identify injured cells for phagocytosis (Vincent and Maiese, 1999a; Chang et al., 2000; Lin et al., 2001) and initiates the activation of coagulation in the vascular cell system. The externalization of PS in platelets or in ECs can promote the formation of a procoagulant surface (Bombeli et al., 1997).

The mGluR system offers an extremely attractive foundation for the provision of cellular protection during both acute and chronic injury paradigms. Recent work has illustrated that mGluRs address cellular survival at each of the independent levels of PCD. Agonism of each group of the mGluR system can prevent the early exposure of membrane PS residues and also inhibit the later stages of genomic DNA destruction (Maiese et al., 2000; Vincent and Maiese, 2000; Lin and Maiese, 2001a,b). Post-treatment studies further support the potential for clinical utility, since administration of mGluR agonists following an injury can prevent the further progression of membrane PS residue exposure. The maintenance of membrane PS asymmetry also provides more long-term protection by inhibiting the destruction of cells through phagocytosis (Savill, 1997) and maintaining a normal anticoagulant state in ECs (Bombeli et al., 1997; Lin and Maiese, 2001b).

Cellular pathways that mediate mGluR protection

Several mechanisms that regulate cytoprotection of the mGluR system in both neuronal and vascular systems have been proposed. Initial work focused on the potential of the mGluR system to modulate the release of intracellular calcium in a variety of animal models (Stefani et al., 1994; Lachica et al., 1995; Yoshino and Kamiya, 1995). Regulation of voltage-gated calcium channels by mGluRs was found to limit excitotoxic injury in neurons (Colwell and Levine, 1999). Other investigations examined the ability of the mGluR system to directly inhibit excitatory transmission (Bonci et al., 1997) and offer protection through the inhibition of NMDA activity (Lafon-Cazal et al., 1999) and free radical generation (Maiese et al., 1996; Vincent and Maiese, 2000). These investigations have matured to identify the ability of the mGluR system to regulate specific cellular and molecular targets that may ultimately determine the fate of a cell. These pathways include the modulation of protein kinase activity, mitochondrial membrane potential, cysteine protease generation, intracellular pH, endonuclease activity, and mitogen activated protein kinase activity.

Protein kinase A and mGluRs

Activation of protein kinase A (PKA) by the mGluR system is one potential pathway that may offer cytoprotection during toxic cellular insults. The mGluR system employs PKA activation for the regulation of memory retrieval (Szapiro et al., 2000) and LTD (Huang et al., 1999). Group I mGluRs can increase adenylate cyclase levels to foster the accumulation of cAMP and the subsequent activation of PKA by coupling to the G-protein $G\alpha_s$ subtype (Hermans and Challiss, 2001). Activation of PKA can regulate cellular calcium homeostasis. Inhibition of PKA activity can lead to a perturbation of calcium homeostasis and subsequent cell death (Zirpel et al., 1998).

During paradigms of cellular injury, activation of PKA can prevent the progression of PCD in a number of cell types, including neurons, neutrophils, and smooth muscle cells (Maiese et al., 1993; Rossi et al., 1995; Orlov et al., 1999). In addition, loss of PKA activity during toxic insults can lead to the progression of PCD (Maiese et al., 1993; Findik et al., 1995; Nishio and Watanabe, 1997). Protection by PKA is believed to reside upstream from the inhibition of caspase 3-like activity (Parvathenani et al., 1998). Furthermore, PKA has been shown to phosphorylate BAD, a member of Bcl-2 protein family, which can prevent the induction of cell injury (Lizcano et al., 2000). In the mGluR system, the subtype mGluR4 requires the activation of PKA to prevent cellular injury following acute neuro-degenerative insults (Maiese et al., 1996).

Protein kinase B and mGluRs

Protein kinase B, also known as Akt, delivers an anti-apoptotic survival signal by several mechanisms. The components of the Akt pathway include Bad (Li et al., 2001), caspase 9 (Cardone et al., 1998), the forkhead transcription factor (FHKRL1) (Brunet et al., 1999), and glycogen synthase kinase-3ß (GSK3ß) (Cross et al., 1995). Each of the cellular systems targeted by Akt is inactivated by phosphorylation resulting in the blockade of PCD. Phosphorylation of Bad precipitates an interaction with the cytosolic 14-3-3 protein resulting in the liberation of the anti-apoptotic protein Bcl-2/Bcl-xL (Li et al., 2001). The phosphorylation of FKHRL1 leads to its association with the cytosolic 14-3-3 protein and the subsequent retention of FKHRL1 in the cytoplasm, rendering it unable to regulate its target genes in the nucleus for the induction of PCD (Brunet et al., 1999). Over expression of GSK-3ß can trigger apoptosis. Yet, direct inhibition of GSK-3ß activity by Akt can block induction of PCD (Yu et al., 2001).

Akt inhibits PCD through its ability to prevent caspase activation that is initiated at either a pre- or a post-mitochondrial level. Recent studies have illustrated that Akt acts to prevent the release of cytochrome c from mitochondria (Kennedy et al., 1999) and functions to inhibit the activation of cysteine proteases following the release of cytochrome c (Rytomaa et al., 2000; Zhou et al., 2000). Yet, feedback systems exist that can modulate the half-life of Akt. Activity of Akt can be eliminated by caspase 3 induction, since caspase 3 has been shown to cleave Akt leading to the inhibition of Akt kinase activity (Bachelder et al., 2001).

Activation of mGluRs may be linked to the Akt pathway by coupling to the G-protein Gßγ (Fig. 1). In some cellular systems, recent work has suggested that glutamate can increase Akt activity through phosphatidylinositol-3-kinase (Perkinton et al., 2002). As a second potential protective mechanism, mGluR inhibition of caspase 3-like activity may serve to prevent caspase 3 mediated cleavage of Akt and foster increased cellular survival through a prolonged half-life of Akt (Maiese and Vincent, 1999; Lin and Maiese, 2001a,b).

Protein kinase C and mGluRs

Protein kinase C (PKC) represents a family of serine-threonine kinases that are physiologically activated by a number of lipid cofactors and are considered to be important transducers in several cell signaling cascades. PKC comprises at least 12 distinct serine/threonine kinase isoenzymes that have important actions in transmembrane signal transduction pathways regulating cell proliferation, differentiation, cytoskeletal functions, gene transcription, PCD, and drug resistance (Musashi et al., 2000). Studies have shown that activation of PKC may be either pro-apoptotic or anti-apoptotic depending on the cell type (Maiese and Boccone, 1995; Maiese et al., 1996; Musashi et al., 2000). Recently, work has begun to define isoform-specific functions of PKC in the apoptotic pathway. For example, PKC isoforms that appear to be anti-apoptotic include PKC-α, PKC-ßII, and PKC-ε and the atypical isoforms PKC-γ and PKC-ζ (Lin et al., 1997).

Several downstream events contribute to the anti-apoptotic actions of PKC. In some cellular environments, PKC activation can depress glutamate release and inhibit excitatory synaptic transmission (Evans et al., 2001). PKC also may synergistically combine with the actions of PKA to regulate intracellular calcium homeostasis that is critical to cell survival (Zirpel et al., 1998). Recent investigations suggest that PKC acts upstream of the IκB kinase (IKK) complex and is associated with activation of the transcription factor nuclear factor κB (NF-κB) (Chen and Lin, 2001). In addition, phosphorylation of Bcl-2 by PKC is required for the suppression of apoptosis by Bcl-2 in some cell systems (Ruvolo et al., 1998) (Fig. 1).

Reliance on the modulation of PKC for cytoprotection by the mGluR system has been supported by several experimental investigations. In the rat cerebellar granule cells subjected to oxygen-glucose deprivation, protection by group I mGluRs against apoptosis required the inhibition of PKC (Kalda et al., 2000). Similar results were obtained in rat hippocampal slices exposed to hypoxic/hypoglycemic injury (Schroder et al., 1999), suggesting that protection against oxygen-glucose deprivation by mGluRs was associated with the modulation of PKC activity. During more specific insult models such as free radical NO exposure and anoxia, activation of mGluRs also has been shown to protect neurons against apoptosis through PKC modulation. Neuroprotection by the mGluR subtypes mGluR1, mGluR2, and mGluR5 appears to be dependent on the direct modulation of PKC activity (Maiese et al., 1996).

Maintenance of mitochondrial integrity by mGluRs

Maintenance of mitochondrial integrity can be a key determinant for normal cellular physiology as well as for cellular recovery from toxic insults. Loss of mitochondria membrane integrity can lead to cellular impairment (Kroemer and Reed, 2000). A decrease in mitochondrial membrane potential can be an important trigger for the release of cytochrome c, a critical determinant of cell survival. Normally residing exclusively in the inner membrane space of mitochondria, cytochrome c, once released into the cytosol, can activate both upstream and downstream caspases such as caspase 8, caspase 9, and caspase 3 through apoptotic protease-activating factor 1 (Apaf-1) (Slee et al., 1999). In addition to cytochrome c release, other mitochondrial proteins, such as endonuclease G (Li et al., 2001), Smac/Diablo (Verhagen et al., 2000), and apoptosis-inducing factor (AIF) (Susin et al., 1999) can also be released in response to injury. Endonuclease G is recognized as a DNase responsible for DNA fragmentation during apoptosis. The caspase co-activator Smac/Diablo competes with caspase-9 for binding to inhibitor of apoptosis proteins (IAPs) to block IAP activity (Srinivasula et al., 2001). AIF translocates from the mitochondria to the nucleus to lead to chromatin condensation and large-scale DNA fragmentation (Susin et al., 1999).

Given the critical function of mitochondria in the release of pro-apoptotic proteins during cell injury, it is conceivable that maintenance of mitochondrial membrane potential by the mGluR system may offer one avenue for cytoprotection. Recent investigations of the mitochondrial membrane potential during cellular injury in cultured rat hippocampal neurons and cerebral microvascular ECs support this premise (Maiese, 2001; Maiese et al., 2001; Chong et al., 2002b). As illustrated in Fig. 2, exposure to the free radical NO or anoxia results in the loss of mitochondrial membrane potential. Pretreatment of ECs one hour prior to nitric oxide exposure with DHPG (750 μM, group I mGluR agonist), but not AIDA (750 μM, group I mGluR antagonist), prevents the loss of membrane potential in mitochondria. The precise mechanisms by which the mGluRs employ to preserve mitochondrial integrity are not clear, but may involve the modulation of intracellular calcium stores and reactive oxygen species. Reduction in mitochondrial intracellular calcium stores and free radical levels has been suggested to promote the maintenance of mitochondrial membrane potential and integrity (Sullivan et al., 1999). Activation of group I mGluRs has been demonstrated to regulate the release of intracellular calcium from both Ins(1,4,5)P3-sensitive and ryanodine-sensitive calcium stores (Maiese et al., 1999). In

addition, group I mGluRs can modulate free radical signal transduction cascades in both neuronal and endothelial cell populations (Maiese et al., 2000; Vincent and Maiese, 2000; Lin and Maiese, 2001b).

Modulation of cysteine protease activity by mGluRs

Cytoprotection through the mGluR system occurs at several levels, but ultimate protection against genomic DNA degradation and membrane PS exposure may be dependent upon the modulation of cysteine protease (caspase) activity. Caspases are a family of cysteine proteases that cleave their substrates after aspartic residues. These proteins are usually synthesized as inactive zymogens that are cleaved into subunits during the initiation of apoptosis. The caspases are then activated by forming heteromeric complexes with accessory molecules such as a death factor receptor. Caspases can be functionally categorized into three groups. Group I is the cytokine-processing caspases which include caspase 1, 4, 5, 11, 12, and 13. Group II caspases consist of caspase 3, 6, and 7 and are termed executioner or effector caspases that cleave crucial cellular protein substrates leading to cell destruction. Group III members include caspase 2, 8, 9, and 10 and are described as initiator caspases that activate downstream executioner caspases resulting in an amplification of caspase activity (Wolf and Green, 1999).

Two pathways are involved in caspase activation leading to apoptosis. As illustrated in Fig. 3, one pathway occurs prior to alterations in mitochondrial membrane potential. This pathway, termed the extrinsic pathway, is initiated by death receptor activation at the cell surface and results in enhanced caspase 8 and 10 activities. As a result, Bid is cleaved by caspase 8 and translocates to mitochondria to release cytochrome c through the Bax subfamily of Bcl-2 proteins. This leads

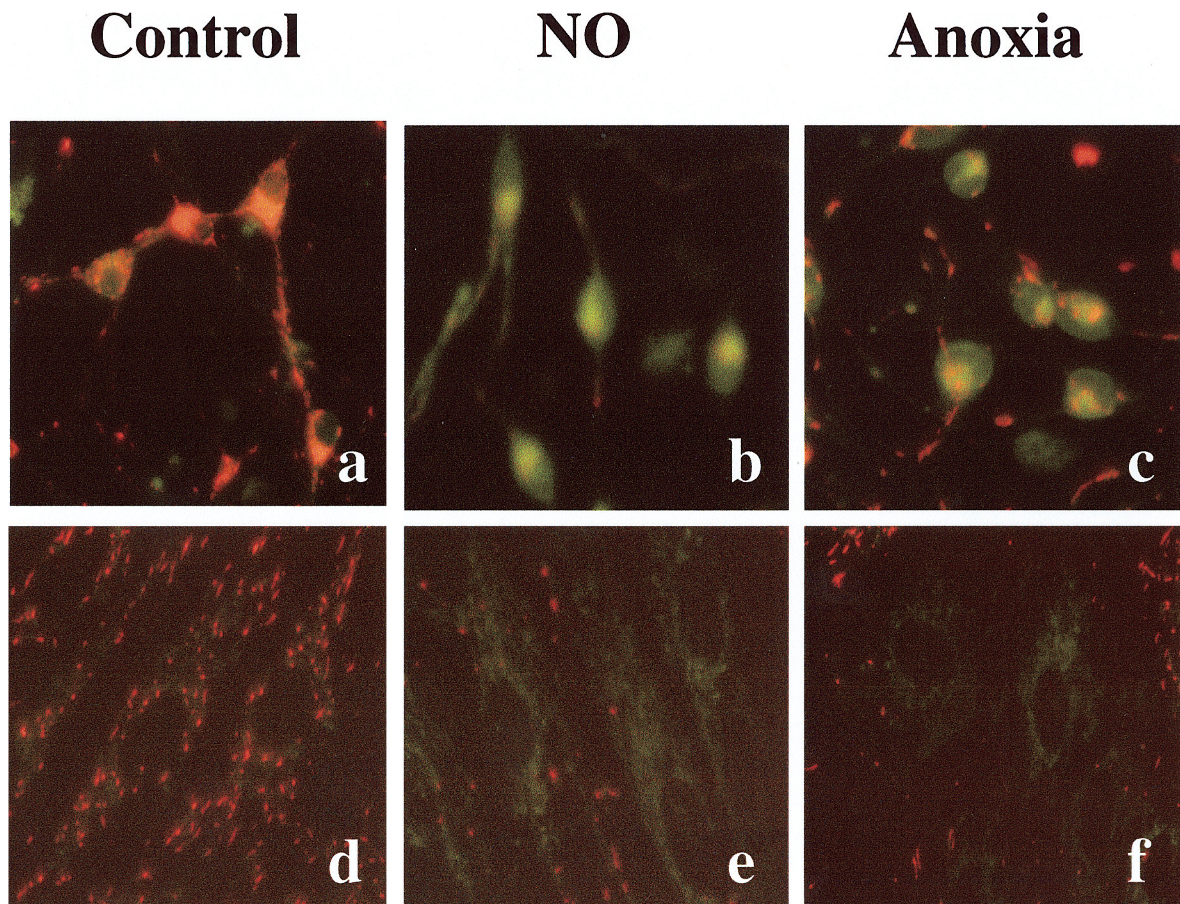

Fig. 2. Mitochondria membrane depolarization occurs during nitric oxide (NO) and anoxia exposure in rat hippocampal neurons and cerebral endothelial cells (ECs). Mitochondrial membrane potential was determined in rat hippocampal neurons **(a, b, and c)** and cerebral ECs **(d, e, and f)** 3 hours following NO exposure (NOC-9, 300 μM in neurons, and 1000 μM in ECs) or anoxia (4 hours) with the fluorescent probe JC-1. Cells were analyzed with a dual emission fluorescence filter (515-545 nm for green fluorescein and emission at 585-615 nm for red fluorescein). Compared with untreated control cells not exposed to NO or anoxia, NO **(b, e)** or anoxia **(c, f)** exposure results in the depolarization of mitochondrial membranes illustrated by green fluorescence staining. x 400

to the subsequent activation of executioner caspases (Green, 2000). The second pathway is mediated by caspase 9 following the release of mitochondrial cytochrome c. Cytochrome c binds to Apaf-1 followed by activation of capase-9 (Cain et al., 2000). The active caspase 9 can then activate executioner caspases 3 and 7 (Fig. 3).

Caspase 1 and caspase 3 function as death proteases to catalyze the specific cleavage of many key cellular proteins (Brown and Borutaite, 1999; Yabuki et al., 2000; Lin and Maiese, 2001b; Maiese, 2001). In particular, caspase 1 - like proteases promote DNA degradation through the activation of other proteins, such as protein kinase C (Emoto et al., 1995) and caspase 3 (Enari et al., 1996). Caspase 3 is a prominent mediator of genomic DNA degradation. Experimental models that use caspase 3 gene deletions illustrate little or no DNA fragmentation following toxic cellular insults (Keramaris et al., 2000). Direct inhibition of caspase 1 and caspase 3-like activities also can protect neuronal cells against injury (Maiese et al., 2001).

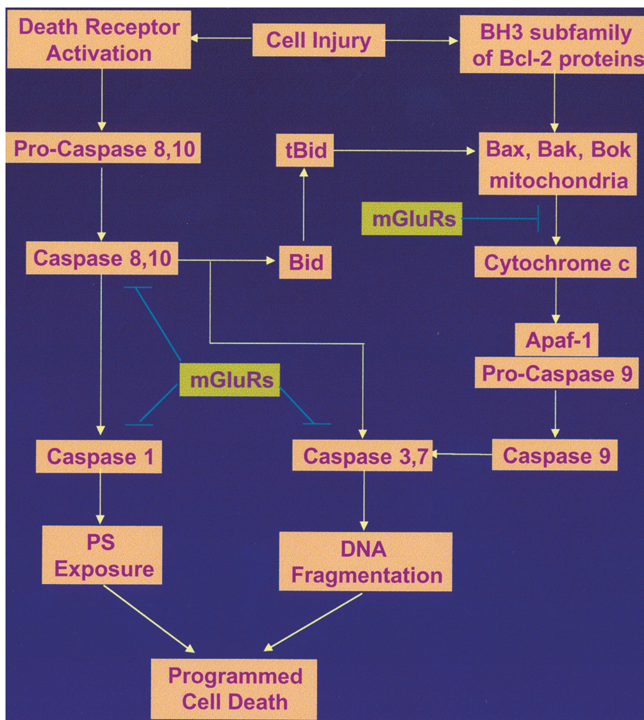

Fig. 3. The mGluR system modulates cysteine protease activity through direct and indirect pathways. The mGluR system modulates two independent pathways that lead to caspase activation. One pathway for the mGluRs to offer cytoprotection involves the direct inhibition of caspase activity, such as caspase 8 and caspase 1. Caspase 8 can activate caspase 3 and caspase 7 or cleave Bid to yield a truncated Bid (tBid) that translocates to the mitochondria to modulate the release of cytochrome c. The second pathway regulated by mGluRs is considered to be indirect in nature and involves the prevention of mitochondrial membrane depolarization. Stress induced apoptotic signals can be transduced to mitochondria by members of the BH3-only family of Bcl-2 proteins that promote activation of the pro-apoptotic subfamily of Bcl-2 proteins (Bax, Bak, Bok). Cytochrome c is subsequently released from mitochondria. Once released, cytochrome c binds to the apoptotic protease activating factor-1 (Apaf-1) followed by activation of caspase 9. These pathways then activate the executioner caspase 3.

The induction of cysteine protease activity is also responsible for the externalization of membrane PS residues (Rimon et al., 1997). During cytokine mediated injury, the externalization of PS residues has been linked to the activity of caspase 1-like proteases through a mechanism that may involve the cleavage of membrane cytoskeletal proteins such as fodrin (Kayalar et al., 1996). In some cellular systems, caspase 3-like proteases have been reported to cleave fodrin and focal adhesion kinase to also induce membrane PS residue exposure during PCD (Levkau et al., 1998). Studies that analyze PS externalization in individual living cells have demonstrated that PS externalization is primarily related to caspase 1-like activity (Maiese and Vincent, 2000; Vincent and Maiese, 2000; Chong et al., 2002a).

Recently, protection by the mGluR system against cellular injury has been attributed to the modulation of caspase activity. Activation of mGluRs attenuates the induction of both caspase 1 and caspase 3 activities (Maiese et al., 1999; Maiese et al., 2000; Vincent and Maiese, 2000,b). Protection by the mGluR system appears to function at two distinct levels. The mGluR system can directly prevent the induction of cysteine protease activity. In addition, mGluRs can indirectly block caspase activity by preserving mitochondrial membrane potential and preventing the release of cytochrome c (Lin and Maiese, 2001b).

Poly(ADP-ribose) polymerase and mGluRs

Poly(ADP-ribose) polymerase (PARP) is a nuclear protein that functions in the regulation of several cellular processes. PARP is responsible for the poly(ADP-ribosyl)ation of nuclear proteins that are involved in DNA repair, chromatin function, and genomic stability (Burkle, 2001). Inhibition or loss of PARP activity can significantly mediate apoptotic DNA degradation and DNA fragmentation (Thies and Autor, 1991; Lin et al., 2000; Lin and Maiese, 2001b). Since NAD^+ is used as the substrate of PARP, the process of poly(ADP-ribosyl)ation or excessive activation of PARP will result in the depletion of intracellular NAD^+ and limit cellular ATP reserves (Wielckens et al., 1982). As a result, a fine modulation of PARP activity must be achieved for efficient utilization of PARP for DNA repair.

PARP cleavage has been identified as a frequent event during apoptosis (Kaufmann et al., 1993; Lin et al., 2000). Caspase 3-like activity leads to the specific cleavage of PARP (Lin et al., 2000; Maiese et al., 2000; Lin and Maiese, 2001b). The significant role of PARP cleavage has been demonstrated by using cytoprotectants such as nicotinamide (Lin et al., 2000; Chong et al., 2002b) or by non-cleavable PARP mutant generations (Oliver et al., 1998). Nicotinamide prevents apoptosis by blocking PARP cleavage through the inhibition of

caspase 3 - like activity in hippocampal neurons (Lin et al., 2000) and cerebral microvascular ECs (Maiese et al., 2001; Chong et al., 2002b). Transfection of cells with non-cleavable PARP results in its resistance to caspase cleavage and prevents apoptosis (Oliver et al., 1998). Recently, it has been demonstrated that the mGluR system appears to prevent PARP degradation and allow for DNA repair through either the maintenance of mitochondrial membrane potential or through the direct inhibition of caspase 3-like activity (Maiese and Vincent, 1999; Vincent and Maiese, 2000; Lin and Maiese, 2001b). Preserving mitochondrial integrity not only inhibits caspase 3 activation through the prevention of cytochrome c release, but also maintains intracellular metabolic homeostasis preventing energy depletion during the DNA repair process.

Intracellular pH, endonuclease activity, and mGluRs

Changes in intracellular pH can significantly influence cellular survival, since cellular proteins such as enzymes, ion channels, and ion transporters are especially sensitive to alterations in intracellular pH (Willoughby et al., 2001). In several experimental injury models of the CNS, including hypercapnia (Ritucci et al., 1997), hypoxia (Roberts and Chih, 1997), and glutamate toxicity (Zhan et al., 1997), cell injury has been linked to disturbances in intracellular pH. During cellular injury, reactive oxygen species have been postulated as a potential mechanism for the induction of acidosis-induced cellular toxicity (Shen et al., 1995). Under some cellular conditions, intracellular acidification has been demonstrated to be both necessary and sufficient for the induction of PCD (Vincent et al., 1999b). Free radical generation results in a biphasic, transient intracellular acidification that can occur within 30 minutes and directly precipitate cellular degeneration (Vincent et al., 1999b; Lin et al., 2000). Prevention of intracellular acidification markedly improves cell survival, suggesting that intracellular acidification is required for free radical induced cellular degeneration.

Cellular injury mediated by intracellular pH also is tied to the activation of acidic-dependent endonucleases (Villalba et al., 1995; Vincent and Maiese, 1999b). Cleavage of chromosomal DNA into oligonucleosomal size fragments is an integral part of cell injury that involves hydrolysis of genomic DNA catalyzed by a number of endonucleases. Endonucleases can be divided into several groups according to their ionic sensitivities to zinc (Torriglia et al., 1997) and magnesium (Sun and Cohen, 1994). Endonucleases associated with PCD induction may be classified as calcium-magnesium dependent endonucleases, magnesium dependent endonucleases, and cation-independent endonucleases. Activation of endonucleases can generate apoptotic chromatin cleavage patterns in a variety of cell types (Hewish and Burgoyne, 1973). DNase I is a secreted digestive enzyme that is a calcium-magnesium dependent endonuclease and consists of a single polypeptide with a molecular weight of 31 kDa (Madaio et al., 1996). DNase II, a lysosomal, acidic pH activated enzyme, also is involved in DNA cleavage during injury (Torriglia et al., 1995). Activation of other endonucleases, such as a magnesium dependent endonuclease and a caspase-activated DNase, can result in the induction of PCD (Pandey et al., 1997).

Within the nervous system, modulation of endonuclease activity directly influences cell survival (Vincent and Maiese, 1999b; Vincent et al., 1999b). Three separate endonuclease activities are present in neurons. They are a constitutive acidic cation independent endonuclease, a constitutive calcium/magnesium-dependent endonuclease, and an inducible magnesium dependent endonuclease (Vincent and Maiese, 1999b). The inducible magnesium dependent endonuclease may be unique for the nervous system (Vincent and Maiese, 1999b). The physiologic characteristics of the magnesium dependent endonuclease, such as a pH range of 7.4-8.0, a dependence on magnesium, and a molecular weight of 95-108 kDa, are consistent with a recently described constitutive 97 kDa endonuclease in non-neuronal tissues, but in contrast the endonuclease in the nervous system is inducible rather than constitutive in nature.

The mGluR system may offer protection against PCD through the regulation of intracellular pH, since activation of the mGluRs directly prevents the induction of intracellular acidification (Vincent et al., 1999a). In addition, activation of mGluRs prevents cellular injury through the modulation of endonuclease activity that is linked to changes in intracellular pH (Vincent et al., 1999a). Prior work has demonstrated that specific mGluRs subtypes can modulate neuronal endonuclease activity during PCD. For example, activation of group III mGluRs inhibits calcium-magnesium dependent endonuclease activity. Yet, it is the activation of group I mGluRs that inhibits magnesium dependent endonuclease activity (Vincent et al., 1999a). Thus, the ability of mGluRs to protect genomic DNA integrity is closely linked to the modulation of both intracellular pH and endonuclease activity by the mGluR system.

Mitogen-activated protein kinases and mGluRs

In addition to classical second messenger systems, other kinases, such as the mitogen-activated protein kinases (MAPKs) also function as second-messengers. MAPKs are serine/threonine kinases that modulate cell differentiation, growth, and death. Phosphorylated MAPKs can translocate to the cell nucleus to regulate both the activation of transcription factors and the subsequent expression of genes. The MAPKs consist of three distinct components. These are the extracellular signal-related kinases (ERKs), the c-Jun-amino terminal kinases (JNKs), and the p38 kinase.

Enhanced activity of the MAPKs p38 and JNK can sometimes lead to cell injury. The MAPKs p38 and JNK have been shown to increase the activity of both caspase

1 and caspase 3 (Ko et al., 2000; MacFarlane et al., 2000). Yet, the function of p38 and JNK during injury paradigms in the neuronal and vascular systems is not entirely evident. The MAPK p38 and possibly JNK are believed to have a role during cellular stress and during neurodegenerative diseases (Hensley et al., 1999). Other work has linked p38 activation to Bax translocation during free radical exposure (Ghatan et al., 2000) and to endothelial injury during cytokine administration (Yue et al., 1999).

In contrast, the activation of ERK is usually associated with inhibition of apoptosis (Xia et al., 1995). During hypoxia, ERK activation can potentially prevent neuronal injury (Vartiainen et al., 2001). Protection by ERK activation also has been observed in many cellular injury models, such as serum deprivation (Desire et al., 2000), hypoxic-ischemia (Han and Holtzman, 2000), and oxidative stress (Wang et al., 1998). The downstream events of ERK that mediate anti-apoptotic signaling appear to be the phosphorylation of the pro-apoptotic protein Bad and induction of pro-survival gene expression via the cAMP response element-binding protein dependent pathway (Bonni et al., 1999). In addition, ERK can prevent caspase activation following cytochrome c release (Erhardt et al., 1999).

Since mGluRs modulate both neuronal and endothelial cell survival during a variety of injury paradigms (Maiese et al., 2000; Vincent and Maiese, 2000; Lin and Maiese, 2001b), it is possible that the protective mechanisms utilized by mGluRs may involve the cellular pathways of the MAPKs. Work has suggested that activation of p38 and JNK may contribute to either neuronal or endothelial degeneration. Within 10 minutes following an ischemic insult, significant activation of p38 and JNK is present in both neurons and ECs (Lin and Maiese, 2001a; Chong et al., 2002b). Yet, this activation of p38 and JNK does not appear to be linked to the protective effect of mGluRs. Activation of mGluRs does not alter the activity of either p38 or JNK, suggesting that protection by mGluRs is independent or below the level of p38 activation (Lin and Maiese, 2001a; Chong et al., 2002b). In contrast, activation of mGluRs can lead to the phosphorylation of ERK (Choe and Wang, 2001), offering support for the hypothesis that ERK activation may be one of the underlying mechanisms that mGluRs used to mediate their protective roles during cellular injury.

Conclusion

The mGluR system can significantly impact the development, maturation, and survival of cells. These G-protein coupled receptors employ a variety of signal transduction pathways to either foster or end cell survival. Usually, it is activation of specific mGluR subtypes that prevent progression of distinct pathways of PCD that involve the degradation of genomic DNA and the exposure of membrane PS residues.

Neuronal and vascular protection by the mGluR system is coupled to various subtypes of G-proteins such as $G\alpha_q$, $G\alpha_s$, and Gßγ that occurs at a series of signal transduction levels (Fig. 1). One level involves the modulation of specific signal transduction pathways that include PKA, PKB, PKC, IKK, and ERK. A second level links the modulation of mitochondrial membrane potential with cytochrome c release, intracellular pH, and intracellular calcium. A third tier oversees the regulation of specific cysteine protease activity that includes caspase 1 and caspase 3-like activities and endonuclease activation. Yet, these cytoprotective mechanisms offered by the mGluR system remain independent from the MAPKs p38 and JNK. Modulation of cellular pathways by the mGluR system during degenerative disease in neuronal or endothelial cell populations is far from complete. Future investigations that identify specific cellular mGluR targets will not only enhance the clinical therapeutic utility of the mGluR system, but also foster greater understanding of the cellular pathways that initially lead to neuronal and vascular disease.

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