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REVIEW

The contributions of excitotoxicity, glutathione depletion and DNA repair in chemically induced injury to neurones: exemplified with toxic effects on cerebellar granule cells

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Abstract

Six chemicals, 2-halopropionic acids, thiophene, methylhalides, methylmercury, methylazoxymethanol (MAM) and trichlorfon (Fig. 1), that cause selective necrosis to the cerebellum, in particular to cerebellar granule cells, have been reviewed. The basis for the selective toxicity to these neurones is not fully understood, but mechanisms known to contribute to the neuronal cell death are discussed. All six compounds decrease cerebral glutathione (GSH), due to conjugation with the xenobiotic, thereby reducing cellular antioxidant status and making the cells more vulnerable to reactive oxygen species. 2-Halopropionic acids and methylmercury appear to also act via an excitotoxic mechanism leading to elevated intracellular Ca^{2+}, increased reactive oxygen species and ultimately impaired mitochondrial function. In contrast, the methylhalides, trichlorfon and MAM all methylate DNA and inhibit O^6-guanine-DNA methyltransferase (OGMT), an important DNA repair enzyme. We propose that a combination of reduced antioxidant status plus excitotoxicity or DNA damage is required to cause cerebellar neuronal cell death with these chemicals. The small size of cerebellar granule cells, the unique subunit composition of their *N*-methyl-D-aspartate (NMDA) receptors, their low DNA repair ability, low levels of calcium-binding proteins and vulnerability during postnatal brain development and distribution of glutathione and its conjugating and metabolizing enzymes are all important factors in determining the sensitivity of cerebellar granule cells to toxic compounds.

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Certain foreign compounds (xenobiotics) cause neuronal cell death to granule cells in the cerebellum while sparing granule cells in the hippocampus and in other brain regions. The precise basis for the selective toxicity of foreign compounds to cerebellar granule cells is not well understood. In this review we discuss the possible basis for this selective toxicity. Over the last 10 years there has been an upsurge in interest in mechanisms of cell death in the central nervous system, with particular emphasis on two death processes, excitotoxicity and apoptosis. There is still considerable debate and discussion regarding the morphological and biochemical diagnosis of these two cell death processes and on the contribution of apoptotic-like neuronal cell death in the adult nervous system, and its role in neurological disorders (Nicotera *et al*. 1997, 1999; Sastry and Rao 2000; Olney 2003). In this review we discuss the role of excitotoxicity in the selective toxicity observed with certain foreign compounds to cerebellar neurones and the role of MK-801, a drug that blocks *N*-methyl-D-aspartate (NMDA) glutamate receptors, which can protect against the excitotoxic cell death process. Excitotoxicity refers to a process of neuronal cell death caused by excessive or prolonged

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Abbreviations used: AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; 2-BPA, 2-bromopropionic acid; 2-CPA, L-2-chloropropionic acid; EAAT, excitatory amino acid transporter; GSH, glutathione (γ-L-glutamyl-L-cysteinylglycine); GSSG, glutathione disulphide; MAM, methylazoxymethanol; NMDA, *N*-methyl-D-aspartate; OGMT, O^6-guanine-DNA methyltransferase; oxo^{8}dG, 8-hydroxy-2′-deoxyguanosine; Ogg1, 8-oxoguanine glycosylase.

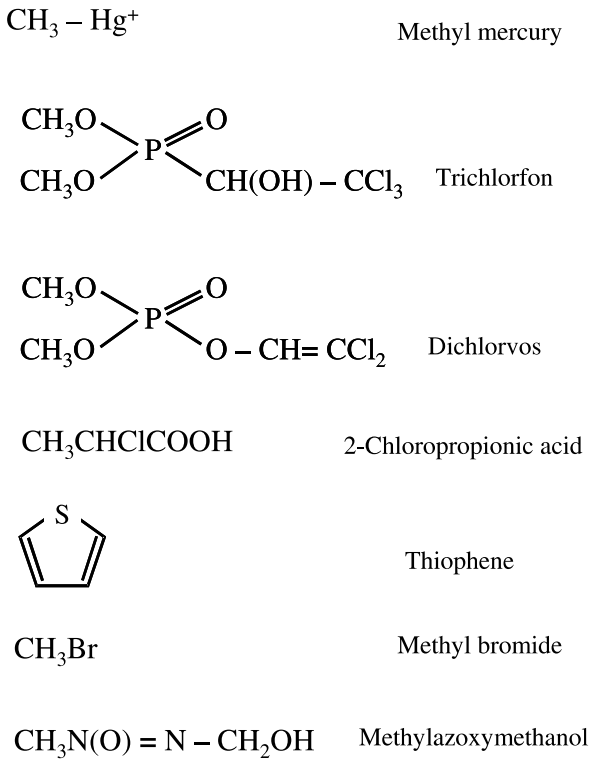

Fig. 1 The chemical formulae of the six xenobiotics and one metabolite dichlorvos discussed in the text.

activation of receptors for the excitatory amino acid neurotransmitter glutamic acid. The principle of excitotoxicity has been well established experimentally in both *in vitro* systems and *in vivo* following administration of excitatory amino acids to the nervous system.

We also discuss the role of the antioxidant glutathione (GSH) in protecting cerebellar neurones against free radical attack and hence mitochondrial damage as a consequence of glutamic acid release. Foreign chemicals may also interact with cerebellar DNA either directly, or indirectly, via free radical generation. Mature neurones do not undergo DNA replication; however, DNA repair is essential for maintaining the nucleotide sequences of genomic DNA over time and hence the viability and function of neurones.

In no case is there evidence of a concentration of the foreign compound in the cerebellum, although it has been pointed out that the vermis is in close contact with the cerebrospinal fluid and the central vein (Cavanagh *et al.* 1997). We suggest that the basis for selective toxicity of foreign compounds to the cerebellum is dependent on a number of factors, such as the unique subunit composition of cerebellar NMDA receptors, which following activation may lead to excessive glutamic acid release; a chemically induced decrease in the intracellular antioxidant GSH, leading to an impaired ability of granule cells to cope with reactive oxygen species; and a low ability of cerebellar granule cells to repair DNA damage and cope with inhibition of DNA repair enzymes. These key factors do not operate in isolation and the sensitivity of these small neurones may be a result of a combination of these events. As in other brain regions it is clear that although cell death is expressed in neurones, underlying this response astroglial cells may be also be impaired, and key functions such as the reuptake of glutamic acid from the extracellular space and maintenance of neuronal antioxidant status (GSH) may also be compromised and hence contribute to the demize of neurones.

The function, structure and development of the cerebellum

The cerebellum is involved in the control of movements, particularly those linked to the voluntary nervous system and movements where timing is of crucial importance. It coordinates different muscle groups so that muscles exert movements fluently and precisely. The cerebellum receives constant feedback information about occurring and intended movements. It should be regarded as a stabilizing control system that receives forewarning of each motor impulse (De Lahunta 1983; Jacobson 1991). Different forms of ataxia and unstable gait usually accompany atrophy of the cerebellum.

The cerebellar cortex is a simple structure consisting of three layers: the molecular layer, the Purkinje cell layer and the granular cell layer. The cerebellum is uniquely organized for the distribution of afferent information. It receives impulses via mossy and climbing fibres from several brain regions involved in motor activity, such as the spinal cord, the vestibular nucleus and the cerebral cortex. Purkinje axons are the only output from the cerebellar cortex. The molecular layer consists of basket cells and stellate cells.

The Purkinje cells are activated by the granular cell axons called parallel fibres and are inhibited by the basket cells and stellate cells in the molecular layer. The granular cells are activated by the mossy fibres, which come from other brain regions except the olivia inferior, which activates Purkinje cells via the climbing fibre.

Neurotransmitters in the cerebellum are well recognized. The granular cell axon terminals, mossy fibres and climbing fibres are predominantly excitatory, using glutamic acid as their transmitter. Purkinje cells, basket cells, stellate cells and Golgi cells are inhibitory and use γ-aminobutyric acid (Fonnum *et al.* 1970; Ottersen 1993). For the subsequent discussion it is important to note that certain neurochemical parameters are special for the cerebellum and that they may contribute to the vulnerability of the cerebellum to injury by foreign compounds.

Different cell types in the cerebellum develop at different times. The Purkinje cells in rats are formed during days 14–15 of gestation; at birth they form a layer that is six cells thick, which by postnatal days 3–4 turns into a single layer. The external germinal layer forms a swamp-like

structure at birth. The granular cell layer is formed by migration from this layer between postnatal days 7–15, with a peak at days 10–11. The basket cells are formed on days 6–7 and the stellate cells on days 10–11 (Altman and Anderson 1972; Bayer and Altman 1995). Agents acting in the perinatal or early postnatal period are thus likely to interfere with the development of the granular cells, and other cerebellar cells may be affected as a result (Altman and Anderson 1972).

Excitotoxicity

Excitotoxic cell death involves prolonged depolarization of neurones, changes in intracellular calcium concentrations and the activation of enzymatic and nuclear mechanisms of cell death. The different aspects of the excitotoxic process have been the subjects of numerous reviews (Rothman and Olney 1987; Meldrum and Garthwaite 1990; Beal 1992; Choi 1992; Lipton and Rosenberg 1994; Fonnum 1997; Doble 1999; Olney 2003). In this review we have only highlighted key aspects of the process and refer readers to the above reviews for additional information.

Glutamic acid uptake

In normal synaptic function the excitatory action of glutamic acid is rapidly terminated due to its efficient removal from the synapse by glutamic acid uptake systems in glial and nerve terminals (Nicholls and Attwell 1990). This function is facilitated by specific transporter proteins that allow the cotransport of glutamic acid with Na^+ ions and concomitant counter transport of K^+ ions, using the electrochemical gradient of Na^+ and K^+ as the driving force. This process is highly efficient and enables glutamic acid to be concentrated in the intracellular compartment up to 10 000-fold, thereby keeping the extracellular level of glutamic acid at around 1 μM. A number of different subtypes of glutamic acid transporters have been cloned and sequenced in human tissue [excitatory amino acid transporters (EAAT) 1–5] and rat tissue and have been reviewed recently (Danbolt 2001; Robinson 2002). Briefly glutamate transporter protein EAAT-1 (GLAST in the rat) is localized on astrocytes and expressed predominantly in the cerebellum. EAAT-2 (GLT-1 in the rat) is localized on perisynaptic astrocytes and expressed widely throughout the central nervous system, but less in the cerebellum. EAAT-3 (EAAC-1 in the rat) is the principal glutamic acid transporter localized on neurones and is thought to be responsible for the reuptake of synaptic glutamic acid into nerve terminals of glutaminergic neurones. EAAT-4 is a transporter that is exclusively localized to Purkinje cells in the cerebellum. EAAT-5 has a very limited localization to the retina. Selective blockade by foreign compounds of glutamic acid transporters such as EAAT-1 or EAAT-4 could lead to selective excitotoxicity to the cerebellum. Indeed selective knockout studies using antisense oligonucleotides to GLAST (EAAT-1) have shown elevation of extracellular glutamic acid and neuronal cell death (Rothstein *et al*. 1996). Similarly, GLAST mutant mice show motor incoordination and increased susceptibility to cerebellar injury (Watase *et al*. 1998), demonstrating the major role of astrocytes in sequestering glutamic acid and preventing excitotoxic cerebellar damage. Cystine is also a substrate for these transporters and any blockade will reduce cystine entry, thereby reducing GSH synthesis (see later) and hence make the cells more susceptible to oxidative stress (McBean 2002). It is now clear that astrocytes play an important role in maintaining extracellular glutamic acid concentrations at very low levels. Glutamic acid is metabolized within astrocytes by glutamine synthase to give glutamine. Glutamine is then released into the extracellular space where it is innocuous, as it cannot activate excitatory amino acid receptors. Nerve terminals then take up extracellular glutamine where it is reconverted into glutamic acid by the enzyme glutaminase. Thus astrocytes are very important in protecting neurones against excitotoxicity.

NMDA receptors

Excitatory amino acid receptors are divided into three main families: NMDA receptors, α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA)/kainate receptors and metabotropic receptors. NMDA and non-NMDA receptors are ligand-gated ion channels and mediate fast synaptic transmission by glutamic acid. NMDA receptors are believed to play an important role in acute excitotoxicity, whereas non-NMDA receptors may be important in certain more chronic forms of excitotoxic stress. Metabotropic receptors, in contrast, are G-protein coupled receptors and are not thought to be primarily involved in fast synaptic transmission or in excitotoxicity. Non-NMDA receptors will not be considered in detail in this review; for further information see Fonnum (1997) and Doble (1999).

NMDA receptors play an important role in excitotoxic mechanisms of neuronal cell death (Rothman and Olney 1987; Meldrum and Garthwaite 1990; Beal 1992; Choi 1992; Lipton and Rosenberg 1994; Fonnum 1997; Doble 1999; Olney 2003) and their expression during the development of the cerebellum has been studied in some detail. The predominant subunits expressed in the cerebellum of the rat, mouse and human are NR1 and NR2C subunits (Akazawa *et al*. 1994; Watanabe *et al*. 1994; Rigby *et al*. 1996; Didier *et al*. 1997). The NR2B subunit is transiently expressed in granule cells during the first 2 weeks after birth, whereas the NR2A and NR2C subunits appear in granule cells during the second postnatal week. The NR2C subunit has a higher affinity towards glycine and a lower affinity towards glutamic acid than the NR2A subunit. The NR2C subunit also has a lower affinity towards Mg^{2+} and antagonists such as MK-801 and displays a unique pharmacology relative to NMDA

receptors in other brain regions (Buller *et al*. 1994; Sucher *et al*. 1996). In contrast, in the adult cerebrum most of the cells express NR1 and NR2A subunits (Thompson *et al*. 2000). Genetically modified mice deficient in NR2A, NR2C or both subunits, show normal cerebellar morphology, although the granule cells showed altered NMDA receptor responses measured electrophysiologically. Mice with both, but not a single subunit, missing showed motor incoordination, demonstrating a role for the NMDA receptor in motor function (Kadotani *et al*. 1996). The subunit composition of NMDA receptors in the cerebellum compared to the rest of the brain may have a bearing on the selective toxicity of foreign compounds for the cerebellum. This will be discussed later with regard to foreign compounds that cause selective injury to the cerebellum.

Mechanisms of excitoxicity

Elevated levels of extracellular glutamic acid cause persistent depolarization of the neurone. Depolarization is initiated primarily by activation of AMPA receptors and subsequently by activation of voltage-dependent Na^+ channels. This leads to Na^+ entry and further depolarization. Chronic depolarization results in release of the Mg^{2+} block on NMDA receptors such that they become available for activation by synaptic glutamic acid. Persistent depolarization also upsets the osmotic balance of the neurone since intracellular sodium concentrations ($[Na^+]_i$) rise. The entry of Na^+ ions is followed by passive entry of Cl^- ion in order to maintain ionic equilibrium. This is followed by entry of water, following the osmotic gradient. The entry of water leads to osmotic swelling and dilution of cytoplasmic contents, leading to disruption of organelles. This can lead to neuronal cell lysis and the release of cell contents (including glutamic acid) into the extracellular space. This osmotic insult to neurones will contribute to cell death, especially if the excitotoxic challenge is sufficient to cause cell lysis. However, it is not an obligatory step, as removal of the extracellular Na^+ and Cl^- will prevent the osmotic swelling but not cell death (Choi 1987).

It is the calcium-dependent part of the cascade of events that appears to be essential for the toxicity. Removal of extracellular Ca^{2+} from the medium of cultured neurones prevents the neurotoxicity of glutamic acid (Choi 1985). Intracellular calcium levels ($[Ca^{2+}]_i$) are usually very low, about 100 nM. However, when neurones are excessively depolarized, $[Ca^{2+}]_i$ rises. The principle source is entry through voltage-dependent calcium channels upon neuronal depolarization and via NMDA receptor channels. However, this Ca^{2+} entry is probably only transient, since both voltage-dependent calcium channels and NMDA receptors desensitize rapidly. Some increase in $[Ca^{2+}]_i$ will arise due to impairment of the Na^+/Ca^{2+} exchanger, whose electrochemical driving force will have been altered by depolarization. In addition, activation of NMDA receptors appears to lead to mobilization of Ca^{2+} from intracellular stores. A marked rise in $[Ca^{2+}]_i$ is probably the irreversible step en route to cell death, since the Ca^{2+} component of excitotoxicity is not reversible on removal of the extracellular depolarizing stimulus. Thus the ability of neurones to withstand excitotoxic damage may well be related to their ability to buffer intracellular Ca^{2+}. In this regard, cerebellar granule cells in the rat and human brain appear to express no or very low levels of the calcium-binding proteins calbindin and parvalbumin, but do express calretinin, and therefore will have a reduced ability to buffer intracellular Ca^{2+}, relative to other neurones (Abe *et al*. 1992; Kadowaki *et al*. 1993; Yew *et al*. 1997). Prolonged elevation of $[Ca^{2+}]_i$ is thought to initiate a complex cascade of interrelated intracellular events, which leads to the destruction of the neurone (Fig. 2). The rise in free $[Ca^{2+}]_i$ will stimulate the activity of numerous enzymes and trigger other Ca^{2+}-dependent protein–protein interactions that are ultimately deleterious to the neurone. Cytosolic proteases activated by calcium, such as calpain, will attack the cytoskeleton (Mills and Kater 1990). Ca^{2+}-dependent cytosolic kinases, such as protein kinase C, will modify the phosphorylation state of cytosolic proteins and thus disrupt cell function (Favaron *et al*. 1990). Lipases, such as phospholipase A_2, activated by $[Ca^{2+}]_i$ will attack the cell membrane, as well as other organelles. Activation of nucleases will disrupt the organization of chromatin in the nucleus and fragment DNA (Orrenius *et al*. 1989; Robertson *et al*. 2000).

Free radicals are produced as a consequence of activation of many of these Ca^{2+}-dependent enzymes, such as phospholipase A_2, nitric oxide synthase and xanthine oxidase, and by oxidative injury to mitochondria. Free radical damage to intracellular organelles is a major contributor to neuronal cell death. Lipid peroxidation results from the attack of free radicals on fatty acids in membranes and this, together with Ca^{2+}-dependent lipases, leads to membrane damage and ultimately cell lysis. Free radical attack on mitochondria compromises energy production within the cell and can also result in mitochondrial and nuclear DNA damage (Gorman *et al*. 2000) (Fig. 2). The importance of free radicals in mediating excitotoxicity is underlined by the ability of antioxidants, free radical scavengers and inhibitors of nitric oxide synthase to attenuate the excitotoxic damage both in cell culture systems and *in vivo*.

During the course of excitotoxicity, the extracellular concentration of glutamic acid will rise as glutamic acid is released by neurones that have undergone lysis, by slowing or reversal of glutamic acid transporters subsequent to depolarization and by Ca^{2+}-dependent exocytosis of synaptic vesicles. This build up of extracellular glutamic acid can then diffuse towards other neurones and depolarize them in their turn, thus amplifying and spreading the neuronal cell injury.

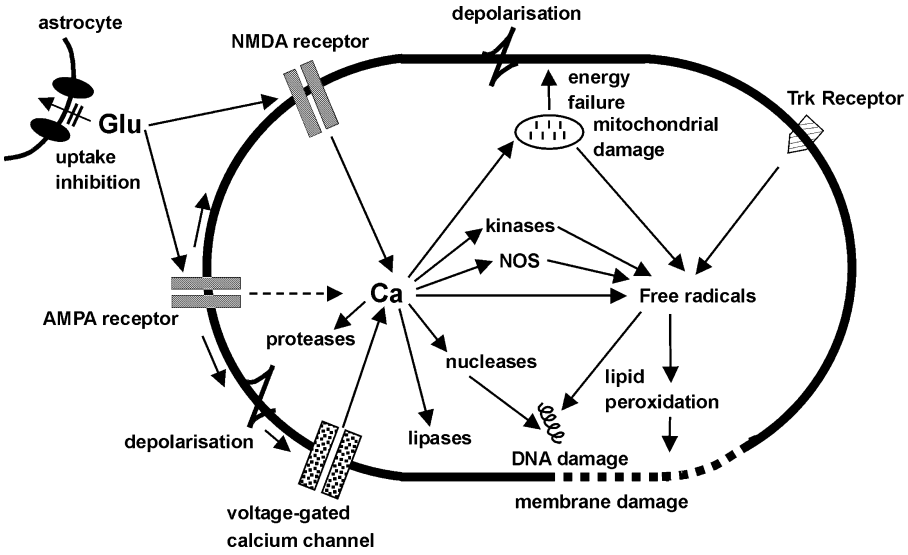

Fig. 2 Intracellular calcium and excitotoxicity. Diagrammatic representation of the various intracelluar processes that can be triggered by a rise in intracellular [Ca^{2+}] in neurones undergoing an excitotoxic insult. The extracellular level of transmitter glutamate will increase if uptake into astrocytes is inhibited. Glutamate will activate AMPA receptor and the subsequent depolarization will open the NMDA receptor for calcium and in turn open voltage-gated calcium channels. The increase in calcium will activate lipases, proteases and nucleases and may increase the production of free radicals in the mitochondria. It can further activate processes [nitric oxide synthetase (NOS), kinases] to increase in free radicals such as superoxide, hydroxyl radical and peroxynitrite. Trk receptor is a receptor coupled to tyrosine kinase or with intrinsic tyrosine kinase activity.

The role of GSH in the brain

The tripeptide GSH is present in the brain at a concentration of 1–3 mM (Cooper 1997) with a concentration in the cerebellum of about 1.8 mM (Wyatt *et al*. 1996b). GSH plays an important role as an antioxidant, is a partner for the detoxification of foreign compounds, a cofactor in isomerization reactions, and a storage and transport form of cysteine (Cooper 1997). It is in this first function that GSH plays an important role in protecting against reactive oxygen species formed during excitotoxicity.

GSH is synthesized in brain cells by the same route as in other tissues. γ-GluCys synthetase uses glutamic acid and cysteine as substrates, forming the dipeptide γ-GluCys, which then combines with glycine in a reaction catalysed by GSH synthetase to generate GSH. The intracellular concentration of GSH is regulated by feedback inhibition of γ-GluCys synthetase by the end product GSH, such that GSH synthesis and consumption are balanced. GSH has been detected in both neurones and astrocytes using histochemical and immunohistochemical techniques (Philbert *et al*. 1991; Hjelle *et al*. 1994). It is now clear that astrocytes are the primary source of GSH in the brain, containing much higher concentrations than neurones (Cooper 1997; Dringen and Hirrlinger 2003). Mitochondria contain a distinct pool of GSH (Griffith and Meister 1985). Mitochondria cannot synthesize GSH and hence rely on importing GSH from the cytosol. Several high affinity transporters for GSH have been identified in mitochondria (Martensson *et al*. 1990)

During detoxification of reactive oxygen species, GSH is involved in two types of reaction: (i) GSH reacts non-enzymatically with radicals such as superoxide anion radical, nitric oxide or hydroxyl radical and (ii) GSH is the electron donor for the reduction of peroxides in the glutathione peroxidase reaction (Fig. 3). The final product of this reaction is glutathione disulphide (GSSG). Within brain

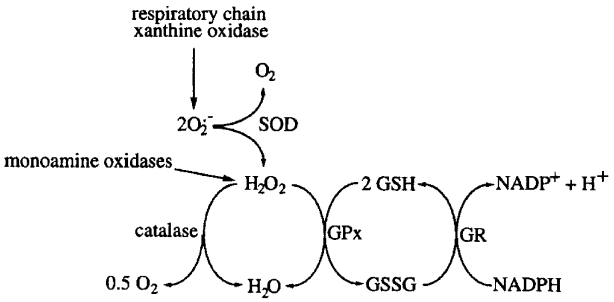

Fig. 3 The role of glutathione in the generation and disposal of reactive oxygen species. Superoxide generated by the respiratory chain or by xanthine oxidase is converted by superoxide dismutase (SOD) to H_2O_2. Monoamine oxidases generate additional H_2O_2. This peroxide is disposed of by catalase and or glutathione peroxidase (GPx). The oxidized glutathione (GSSG) is reconverted back to GSH by glutathione reductase (GR), the process consuming NADPH.

cells GSH is regenerated from GSSG by the reaction catalysed by glutathione reductase (Fig. 3). In contrast, during the generation of GSH-*S*-conjugates by GSH transferases the concentration of cellular GSH is lowered, thus GSH used in this process has to be replaced by re-synthesis from the constituent amino acids. GSH is only part of the brain's defences against reactive oxygen species. Other enzymes such as superoxide dismutase and catalase (Fig. 3), as well as antioxidants such as ascorbic acid and α-tocopherol, are involved in detoxification of reactive oxygen species in neurones and astrocytes (Cooper 1997; Dringen 2000; Dringen and Hirrlinger 2003).

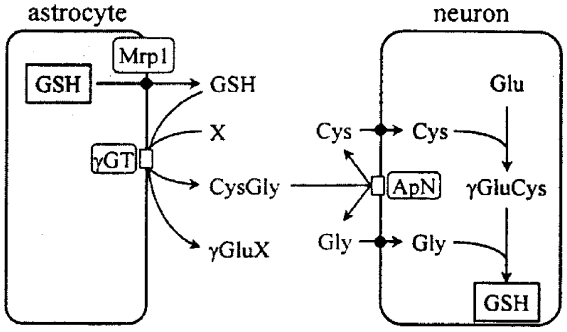

Fig. 4 Metabolic interaction between astrocytes and neurones in glutathione metabolism in the brain. Glutathione (GSH) released from astrocytes, via the multidrug-resistance protein 1 (Mrp1), is a substrate for γ-glutamyltransferase (γGT) located on the surface of astroglial cells. The CysGly generated serves as an extracellular precursor of neuronal GSH. After hydrolysis of the dipeptide by aminopeptidase N (ApN), the release cysteine and glycine are taken up into neurones. X represents an acceptor of the γ-glutamyl moiety transferred by γGT from GSH (from Dringen and Hirrlinger 2003).

GSH synthesis in neurones and astrocytes

GSH synthesis depends on the intracellular availability of glutamic acid, cysteine and glycine. In the brain these amino acids are not present extracellularly in high concentrations, since glutamic acid and glycine are neurotransmitters and cysteine at high concentrations is excitotoxic (Olney *et al*. 1990; Mathisen *et al*. 1996). Neurones rely on the presence of extracellular cysteine for GSH synthesis, as they cannot use the cysteine-oxidation product cystine, as a GSH precursor (Sagara *et al*. 1993; Kranich *et al*. 1996; Dringen *et al*. 1999). Neurones also use glutamine as an extracellular precursor for the glutamic acid moiety of GSH (Kranich *et al*. 1996). In contrast, astroglial cells prefer glutamic acid and cystine as extracellular GSH precursors (Dringen and Hamprecht 1998; Kranich *et al*. 1998). Transport of cystine into astroglial cells is essential for the synthesis of GSH; this can take place via two separate transport systems, the Na^+-dependent glutamic acid transporters EAAT-1 and EAAT-2 as discussed earlier and a cystine-glutamic acid exchanger (McBean 2002). This differential preference for extracellular GSH precursors prevents competition between neurones and astrocytes. Murphy and coworkers have shown that either depriving immature neurones of cystine, or glutamic acid-induced cystine depletion, causes GSH depletion leading to oxidative stress and neuronal cell death (Murphy *et al*. 1989; Ratan *et al*. 1994). Thus, excess extracellular glutamic acid can result in inhibition of cystine uptake, intracellular GSH depletion and cell death. Astrocytes can release GSH under non-stressed conditions (Stone *et al*. 1999; Stewart *et al*. 2002) via the Mrp-1 transporter (Decleves *et al*. 2000). Upon release GSH is a substrate for γ-glutamyltransferase located on the external surface of astrocytes thus liberating CysGly (Fig. 4). The CysGly can then serve as an extracellular precursor of neuronal GSH; following hydrolysis by aminopeptidase N, the amino acids cysteine and glycine are taken up into neurones (Fig. 4). Thus there is metabolic cooperation between astrocytes and neurones in the metabolism and synthesis of GSH (Dringen and Hirrlinger 2003). The turnover of the total GSH pool in rat brain is slow, with an estimated half-life of 70 h (Douglas and Mortensen 1956). More recent studies have indicated that a portion of brain GSH may turnover more rapidly, with a half-life of 30 min that may reflect an astrocyte compartment (Cooper 1997).

GSSG export from astrocytes cells, mediated by Mrp-1, has been reported following oxidative stress (Hirrlinger *et al*. 2001), thus extracellular GSSG can be considered as a marker of oxidative insult. GSSG has been reported to act as an agonist and modulator of glutamic acid NMDA receptors (Sucher and Lipton 1991; Leslie *et al*. 1992; Ogita *et al*. 1995). This may be important in excitotoxicity, since extracellular GSSG down-regulates the stimulation of NMDA receptors by glutamic acid.

GSH conjugation and the mercapturic acid pathway

To reach the central nervous system, drugs or neurotoxic chemicals have to cross either the luminal and antiluminal membranes of endothelial cells, or the basolateral and apical membranes of choroidal cells. The entry and efflux of chemicals or their metabolites will depend on their ability to diffuse through these cells and whether transport proteins can either facilitate or reduce entry of a xenobiotic into the brain compartment. The cerebral capillaries and choroid plexuses contain several enzymes that act as metabolic barriers towards endogenous and foreign chemicals. GSH S-conjugates formed either in the periphery or brain are metabolized and excreted in the urine as mercapturic acids. The first step in this process is removal of the γ-glutamyl residue by the enzyme γ-glutamyltransferase. γ-Glutamyltransferase is present in the brain, as well as many peripheral tissues such as the renal proximal tubules. In the brain it shows regional variability, being strongly expressed in the choroid plexus

and brain capillaries with high activity in endothelial cells and pericytes (Tate *et al*. 1973; Okonkwo *et al*. 1974; Ghandour *et al*. 1980; Frey *et al*. 1991; Ghersi-Egea *et al*. 1994). Blood–brain interfaces can efficiently prevent the blood to brain passage of some xenobiotics by metabolic processes. For example, inhibition of γ-glutamyltransferase by acivicin leads to a large increase in the brain uptake of 5-(glutathione-*S*-yl)-α-methyldopamine, a metabolite of 3,4-(±)-methylenedioxyamphetamine, showing that γ-glutamyltransferase efficiently metabolizes the blood-borne GSH conjugate, thus limiting its entry into the brain (Miller *et al*. 1996). The neuropil can also metabolize xenobiotics that have the capacity to enter the brain parenchyma. GSH transferase isoforms have been detected in the brain and shown regional variability (Abramovitz *et al*. 1988; Cammer *et al*. 1989; Johnson *et al*. 1993; Philbert *et al*. 1995). In general, α-isoforms of GSH *S*-transferases are expressed in nuclei of selected neurones throughout the nervous system. Expression of μ and π-isoforms are confined to astrocytes and oligodendroglial cells, respectively. The elimination of xenobiotic metabolites from the brain, and specifically of conjugates, is complicated by their hydrophilic nature, which limits their passive elimination into the blood through the blood–brain barrier. Transport mechanisms have been shown to play a role in the elimination process, for example the specific uptake of dinitrophenyl-GSH *in vitro* into inside-out membrane vesicles of an endothelial brain cell line has been reported (Homma *et al*. 1999). The efflux of a GSH conjugate that reaches the cerebrospinal fluid can occur via the choroid plexus, where metabolism via γ-glutamyltransferase will generate the cysteinylglycine *S*-conjugate. GSH-derived conjugates synthesized in the brain may then diffuse out of the tissue into the ventricular cerebrospinal fluid, or perhaps drain along the parenchymal and subdural perivascular spaces into the subarachnoid space (Davson and Segal 1996). Here they will be removed from the brain with the bulk flow of cerebrospinal fluid. The exchange processes between the brain parenchyma and cerebrospinal fluid and the role of drug metabolism are complex and require further study. Once the cysteinylglycine *S*-conjugate has entered the bloodstream it can undergo further metabolism via a peptidase to liberate the *S*-conjugate. The final step in mercapturic acid formation involves *N*-acetylation, which is catalysed by the enzyme *N*-acetyltransferase. These latter enzymes are present in the liver and kidney (Dekant *et al*. 1994; Commandeur *et al*. 1995). The extent to which GSH *S*-transferases and the mercapturic acid pathway are involved in detoxification reactions in the brain is unknown and the pathway may actually lead, on occasions, to bioactivation of xenobiotics (Cooper and Kristal 1997; Monks *et al*. 1999).

Thus, GSH conjugation of xenobiotics may occur in the periphery and hence reduce the amount of a xenobiotic entering the brain, while some can also occur in the brain. In general GSH conjugation is a detoxification pathway for xenobiotics. The role of this pathway in cerebellar granule cells and its relevance, if any, to selective toxicity requires investigation.

The above is a brief overview of the key roles of GSH in the brain. For more detailed information on the metabolic compartmentation, synthesis, degradation, entry into the brain and the enzymes involve in protecting the brain against reactive oxygen species see Cooper (1997), Dringen (2000) and Dringen and Hirrlinger (2003).

GSH plays a very important role in protecting neurones from free radical injury following the extracellular release of glutamic acid. This process is applicable to cerebellar granule cells and we will discuss the role of GSH in the selective toxicity produced by foreign compounds to the cerebellum.

DNA damaging agents and neuronal cell death

Neurones must have active DNA repair mechanisms in order to survive. This is consistent with the fact that extensive neurodegeneration is found in people with the rare autosomal recessive photosensitive disorders xeroderma pigmentosum and Cockayne's syndrome, which are associated with a defect in nucleotide excision repair required for the removal of DNA damage induced by UV light and chemical adducts (Kohji *et al*. 1998; Rapin *et al*. 2000). Animals with targeted deletions of genes involved in DNA repair display a similar phenotype to that of humans, including genetic instability and neural degeneration, including abnormal cerebellar development (Gao *et al*. 1998; Murai *et al*. 2001; Sun *et al*. 2001).

Neuronal DNA damage often occurs as a result of oxidative stress and the increased production of reactive oxygen species (Enokido and Hatanaka 1993; Ratan *et al*. 1994; Park *et al*. 1998; Martin *et al*. 2003). 8-Hydroxy-2′-deoxyguanosine (oxo^{8}dG) is one of the most common adducts formed from the reaction of reactive oxygen species with DNA (Dizdaroglu 1991). Oxo^{8}dG is removed from DNA by an active mechanism of base excision repair. Specific *N*-glycosylases identify damaged purines and pyrimidines, and this is followed by apurinic/apyrimidinic endonuclease action that processes strand breaks and sites of base loss. The repair process is then completed by DNA polymerases and DNA ligases. Oxo^{8}dG is removed from DNA by a bifunctional glycolyase/AP lyase enzyme, termed 8-oxoguanine glycosylase (Ogg1). This enzyme is present in the adult cerebellum at low basal levels (Cardozo-Pelaez *et al*. 2000), depletion of GSH with diethylmaleate leads to an increased removal of oxo^{8}dG in the cerebellum, presumably in response to the decreased antioxidant status (Cardozo-Pelaez *et al*. 2002).

Several chemicals that inhibit DNA repair cause neuronal cell death. Camptothecin, which binds to and inhibits DNA topoisomerase I forming a cleavable complex consisting of topo-1 covalently linked to the damaged DNA strand, causes

neuronal cell damage (Hsiang *et al*. 1989). The chain-terminating nucleoside cytosine arabinoside will also kill post-mitotic neurones, probably via inhibition of topoisomerase II-mediated DNA repair or inhibition of DNA ligase (Morris and Geller 1996; Park *et al*. 1998). DNA damage to mammalian cells, including cerebellar granule cells, initiates transient phosphorylation cascades, which modify the activities of proteins regulating both apoptosis and repair. In these cases the production of DNA damage or inhibition of repair commits the damaged neurones to apoptosis (Sastry and Rao 2000; Geller *et al*. 2001; Romero *et al*. 2003). The tumour suppressor gene p53 appears to be involved in the signalling pathway for some agents that induce neuronal cell apoptosis. Cultured cerebellar neurones from p53 null mice, but not from wild-type mice, were protected from cytosine arabinoside toxicity (Enokido *et al*. 1996a). DNA-damaging agents such as etopside and bleomycin also induced apoptosis in wild-type but not p53 null mutant mice cerebellar neurones, indicating p53 is required for apoptotic death due to DNA strand breaks (Enokido *et al*. 1996b). Moreover, p53 is involved in activating nucleotide excision repair (Wani *et al*. 1999), which repairs UV-induced DNA damage, and base excision repair (Zhou *et al*. 2001). The signalling pathways involved in DNA repair both upstream and downstream of p53 are not fully understood in neuronal cells. However, not all chemically induced damage to DNA leads to apoptosis via the p53-dependent pathway; methylazoxymethanol (MAM) causes apoptosis by a p53-independent pathway and without showing DNA fragmentation (Wood and Youle 1995; Johnson *et al*. 1998). It is, however, clear that DNA damage, whether caused by chemically induced DNA alkylation or chemically induced inhibition of DNA repair enzymes, can cause cerebellar neuronal death by an apoptotic pathway (Sastry and Rao 2000). We will discuss the role of DNA damage in the selective toxicity of foreign compounds to granule cells in the cerebellum.

The study of toxic effects of foreign compounds to the cerebellum has been greatly assisted by the use of cerebellar granule cells in culture. These cells can be prepared from the developing cerebellum of rats or mice on days 7–8 and then grown under excitable conditions (Gallo *et al*. 1982; Schousboe *et al*. 1989). The cells may be used between days 3 and 14, but are typically used after 6–8 days in culture. In this review, we have concentrated on compounds that mainly target the granule cell layer of the cerebellum.

Chemically-induced cerebellar granule cell loss

2-Halopropionic acids

Halogen substitution in the 2-position of propionic acid results in chemicals that are toxic to the cerebellar granule cell layer in adult rats, but not in mice, while not affecting granule cells in the forebrain such as those in the hippocampus (O'Donoghue 1985; Simpson *et al*. 1996; Lock *et al*. 2000, 2001a). L-2-Chloropropionic acid (2-CPA) is used as a chemical intermediate in the synthesis of certain agrochemicals. A single large oral dose or daily exposure to lower doses of 2-CPA, 2-bromopropionic acid (2-BPA) and to a lesser extent 2-iodopropionic acid will produce cerebellar granule cell necrosis. Both the D and L isomers of 2-CPA are neurotoxic, whereas only the racemate of the other analogues has been examined. Exposure of 21-day-old rats to 2-CPA does not result in cerebellar injury. Granule cells in the developing rat cerebellum only become sensitive to 2-CPA at about 32 days of age (Lock *et al*. 2001b).

The neuronal injury is preceded by a marked depletion in the GSH status of the liver (Wyatt *et al*. 1996a; Lock *et al*. 2001a). This reflects depletion of the hepatic cytosolic GSH pool with little effect on the mitochondrial compartment. The thiol status of the liver then recovers over time, but decreases following each dose of 2-CPA. This response reflects conjugation of the 2-halopropionic acid with GSH, prior to its excretion in the urine as a mercapturic acid (Wyatt *et al*. 1997). No increase in the content of hepatic oxidized GSH was seen to support the idea of oxidative stress (Wyatt *et al*. 1996a). Conjugation with GSH is enzyme catalysed by the zeta isoform of glutathione *S*-transferase that is found in the liver (Tong *et al*. 1998). In the brain, only a small decrease in non-protein thiol (mainly GSH) depletion is observed 4 h after dosing with either 2-CPA or 2-BPA, but the thiol status is more markedly reduced 12–24 h after exposure, especially following multiple doses (Wyatt *et al*. 1996a; Lock *et al*. 2001a). No regional difference in the extent of thiol depletion was seen in the brain, the depletion being similar in the forebrain (an undamaged brain region) and the cerebellum. Non-protein thiol depletion in the brain was never as extensive as in the liver, being maximally reduced by about 50–60%. It is possible that the depletion of GSH *per se* in the brain is more marked than these numbers indicate, as some of the non-protein thiol component is cysteine. The basis for the delay in GSH depletion in the brain is not understood but may reflect progressive loss from a slowly turning over pool of GSH. Incubation of 2-CPA with GSH in the presence of rat brain cytosolic fraction did not result in GSH conjugate formation analogous to that seen with the liver; however, the amount formed could be small and hence below the limits of detection. Another possible explanation may be related to the finding that the 2-CPA-cysteine conjugate (2-*S*-cysteinylpropanoic acid) is a good non-competitive inhibitor of cystine transport in cerebellar slices with a K_i of 60 μM (Wyatt *et al*. 1996b). Circulating plasma levels of 2-*S*-cysteinylpropanoic acid remain fairly constant at about 100 μM for ~12 h after a neurotoxic dose(s) of 2-CPA (Wyatt *et al*. 1997), and this could be sufficient to modulate cystine transport *in vivo*. Reducing cystine transport into neurones or astrocytes will

reduce GSH synthesis and potential make neurones more sensitive to extracellular glutamic acid, as discussed earlier. Studies are required to establish which cystine/glutamic acid transporters are inhibited by 2-*S*-cysteinylpropanoic acid and hence their cellular localization.

Brain GSH status is thus reduced following exposure to these 2-halopropionic acids, causing a decrease in the antioxidant status of the brain. A decrease in brain GSH content of this magnitude is not sufficient to precipitate neuronal cell death in its own right, but is likely to make neurones more vulnerable to attack by excitotoxic agents. This notion is supported in part by studies showing that prior administration of buthionine sulfoxime, a potent irreversible inhibitor of γ-GluCys synthetase, leading to reduced GSH synthesis (Meister 1991), potentiated the toxicity of 2-CPA. In contrast, supplementing the GSH status by administration of the isopropylester of GSH (Meister 1991) afforded complete protection against the clinical signs of neurotoxicity, neurochemical changes and cerebellar granule cell necrosis produced by 2-CPA (Wyatt *et al*. 1996b). Some caution needs to be attached to these findings for the following reasons.

(i) Buthionine sulfoxime does not readily cross the blood–brain barrier and hence the brain GSH content was not markedly reduced. Depletion of hepatic and renal GSH pools may have made more 2-CPA available in the plasma for entry into the brain, thereby increasing the toxicity.

(ii) The converse is true with the supplementation studies, where more 2-CPA may have been sequestered outside the brain by conjugation with GSH and hence reduced the dose delivered to the brain.

Studies suggest that 2-CPA-induced cerebellar granule cell necrosis may occur via an excitotoxic mechanism, involving the NMDA receptor. Administration of the non-competitive NMDA antagonist, MK-801, to rats 30 min before, or 1 h after 2-CPA, prevented the cerebellar toxicity (Widdowson *et al*. 1996a; Lock *et al*. 1997; Williams *et al*. 2001). Partial protection was also seen with the glutamate antagonist (±)3-(2-carboxy-piperazin-4-yl)-propyl-1-phosphonic acid (Widdowson *et al*. 1996b) and nitric oxide synthase inhibitors (Widdowson *et al*. 1996c). These findings support the view that these 2-halopropionic acids mediate cerebellar toxicity via an excitotoxic mechanism involving glutamic acid. However, electrophysiology studies on single cerebellar granule cells maintained in culture have provided no evidence for a direct agonist or co-agonist action at the NMDA receptor ion channel complex (Widdowson *et al*. 1997a). Metabolic studies in the brains of rats exposed to 2-CPA have indicated activation of the mitochondrial pyruvate dehydrogenase complex in the cerebellum and forebrain soon after dosing (Williams *et al*. 1999, 2001). MK-801 treatment had no effect on activation of the pyruvate dehydrogenase complex but did significantly prevent the increase in cerebellar lactate and reduction in *N*-acetylaspartate, with partial prevention of the fall in energy status (Williams *et al*. 2001). Thus 2-CPA neurotoxicity may only be partially mediated by the NMDA subtype of glutamate receptors.

Release of glutamic acid into the extracellular space is proposed to trigger a cascade of events leading to the generation of cytotoxic free radicals and mitochondrial dysfunction as discussed earlier. Studies with cerebellar granule cells in culture have shown that 2-CPA produced dose- and time-dependent toxicity that could partially be prevented by exposure to MK-801 but not by other glutamate receptor antagonists such as D(−)-2-amino-5-phosphopentanoic acid or (±)3-(2-carboxy-piperazin-4-yl)-propyl-1-phosphonic acid. Exposure of cerebellar granule cells to 2-CPA leads to a concentration-dependent increase in formation of 2,7-dichlorofluorescein, a measure of formation of reactive oxygen species. Addition of the antioxidant vitamin E, the mitochondrial permeability transition pore inhibitor cyclosporin or the spin trap *N*-*tert*-butyl-α-(2-sulfophenyl)-nitrone reduced reactive oxygen species formation and cell death (Sturgess *et al*. 2000; Myhre *et al*. 2001). Interestingly, the ERK-type of MAP kinase inhibitor, U0126, was able to prevent both the formation of reactive oxygen species and neuronal cell death in these *in vitro* experiments (Myhre *et al*. 2001). These findings with cerebellar granule cells *in vitro* support the view that free radicals may play a role in the toxicity of 2-CPA. *In vivo* studies using the aminosteroids U74389G and U83836E, the free radical scavengers 3-methyl-1-phenylpyrazolin-5-one and *N*-*tert*-butylphenylnitrone, or the iron chelator *N*-ethoxy-2-ethyl-3-hydroxypyridin-4-one were all ineffective at reducing 2-CPA neurotoxicity (Widdowson *et al*. 1997b). Whether high enough concentrations of these radical scavengers reached the cerebellum was not determined; adequate delivery and a prolonged retention in the cerebellum is needed to give the best chance of protection *in vivo*. The histopathology studies conducted with 2-CPA supported a necrotic mechanism of cell death (Simpson *et al*. 1996; Jones *et al*. 1997); we found no indication of DNA fragmentation in cerebellar granule cells using the terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) technique (Widdowson *et al*. 1997b).

In summary, the selective toxicity of 2-halopropionic acids to the cerebellum is probably via an excitotoxic mechanism. The specificity is presumably related to the particular NR2C/NR2A subunit composition of the NMDA receptor expressed on cerebellar granule cells and hence unique pharmacology. A combination of increased extracellular glutamic acid and reduced antioxidant status (GSH) is proposed to make the granule cells more vulnerable to free radical attack. The role of astrocytes in the toxicity has not been explored; in particular the inhibition of cystine/glutamate transporter(s) by the cysteine conjugate of 2-CPA requires investigation.

Methylhalides

Inhalation exposure to methyl chloride and methyl bromide have been reported to cause cerebellar granule cell necrosis

in rodents, in addition to producing lesions in other organs such as the liver and olfactory epithelium (Morgan *et al*. 1982; Jiang *et al*. 1985; Hurtt *et al*. 1987; Eustis *et al*. 1988; Reed *et al*. 1995). Poisoning cases in humans with either methyl iodide or methyl bromide have indicated some cerebellar lesions, although other neurotoxic effects were also reported (Hermouet *et al*. 1996; De Haro *et al*. 1997). The methyl halides readily react with GSH, causing depletion in the cerebellum and other organs (Kornbrust and Bus 1984; Chellman *et al*. 1986a; Davenport *et al*. 1992a; Chamberlain *et al*. 1998). This is thought to represent a detoxification pathway of metabolism, but will also lower the antioxidant status in these organs. Methyl bromide and methyl iodide also methylate bases on DNA, with N^3-methyladenine, N^7-methylguanine and O^6-methylguanine having been detected in the liver and lung (Bolt and Gansewendt 1993; Yang *et al*. 1995). Some workers have proposed that further metabolism of the *S*-methylglutathione to *S*-methylcysteine and then methanethiol leads to the ultimate toxic metabolite (Kornbrust and Bus 1983). Depletion of GSH with buthionine sulfoximine in mice reduces the toxicity of methyl chloride (Chellman *et al*. 1986a). In rats, however, buthionine sulfoximine administration increases the toxicity and tissue injury produced by methyl bromide and methyl iodide (Thomas and Morgan 1988; Chamberlain *et al*. 1998).

Studies using neuronal cells, including cerebellar granule cells in culture, have shown a dose and time dependent toxicity of methyl iodide (Bonnefoi *et al*. 1991; Bonnefoi 1992; Davenport *et al*. 1992b; Chamberlain *et al*. 1999). GSH content of the cells was reduced by exposure to methyl iodide (Bonnefoi *et al*. 1991; Chamberlain *et al*. 1999), with some indication that neuronal cell death correlated with depletion of mitochondrial GSH (Bonnefoi 1992). Prior depletion of GSH, by treating cerebellar granule cells with buthionine sulfoximine, significantly enhanced granule cell loss, whereas pre-treatment with the isopropyl ester of GSH afforded protection (Chamberlain *et al*. 1999). One possible consequence of the marked depletion of intracellular GSH following exposure to methyl iodide is that the cells may be more sensitive to free radical attack. 3-Amino-1-[*m*-tri(fluoromethyl)phenyl]-2-pyrazoline (BW755C) (a cyclo-oxygenase/lipoxygenase inhibitor), nordihydroguaiaretic acid (a lipoxygenase inhibitor), *N,N′*-diphenyl-*p*-phenylenediamine, vitamin E, butylated hydroxytoluene and desferrioxamine all protected cerebellar granule cells against methyl iodide exposure (Bonnefoi 1992; Davenport *et al*. 1992b; Chamberlain *et al*. 1999). Thus six different compounds which all have antioxidant activity have been shown to protect neuronal cells against methyl iodide. Furthermore, protection against methyl chloride and methyl bromide toxicity has been observed with BW755C in rats (Chellman *et al*. 1986b; Thomas *et al*. 1989). In contrast, MK-801 had no effect on methyl iodide induced toxicity to neuronal cells in culture, suggesting the NMDA receptor and hence an excitotoxic mechanism is unlikely to be involved (Davenport *et al*. 1992b). The protection elicited by antioxidants may involve other mechanisms in addition to acting as free radical scavengers. One possibility is that these compounds act as trapping agents for the strong methylating potential of methyl iodide and thereby reduce the extent of methylation of critical cellular targets such as DNA. Methyl halides will also methylate O^6-guanine-DNA methyltransferase (OGMT) and thereby reduce or prevent the repair of methylated DNA (Oh *et al*. 1996). DNA methylations are removed by OGMT, which repairs the damage by transferring the methyl group to a cysteine residue on the protein (Karran *et al*. 1979; Olsson and Lindahl 1980). Methylation of the active site of OGMT leads to suicide inactivation of the enzyme, and synthesis of new enzyme is required to maintain DNA integrity. The effects on DNA repair and hence the integrity of DNA may be a contributory factor in the neuronal cell death. The role of apoptosis in this process is unclear. Preliminary studies with isolated cerebellar granule cells in culture, using the Hoechst dye 344, indicated some apoptotic-like cells following methyl iodide (Chamberlain *et al*. 1999). More detailed dose–response studies are required to support this finding.

In summary, the current mechanism of toxicity of the methylhalides involves depletion of tissue GSH thereby reducing the antioxidant status of the cerebellar granule cell, plus their ability to methylate DNA and enzymes such as OGMT and thereby impair repair processes. The basis for the selectivity to the granule cells of the cerebellum awaits further study.

Thiophene

Administration of thiophene to rats produces degeneration of the cerebellar granule cell layer following 5–10 days of subcutaneous injection (Christomanos and Scholz 1933; Upners 1939; Herndon 1968; Albrechtsen and Jensen 1973). Recently Mori *et al*. (2000) administered thiophene by intramuscular injection to rats and reported neuronal cell injury in the cerebral cortex, inferior collicullus and inferior olives, in addition to the predominant lesion in the granular layer of the cerebellum.

Few mechanistic studies have been undertaken with thiophene. Thiophene is metabolized in both rats and rabbits by cytochrome P-450 to form thiophene *S*-oxide that readily reacts with GSH to form a conjugate. Between 30 and 40% of an oral dose of thiophene is excreted in the urine as the mercapturic acid (Bray *et al*. 1971; Dansette *et al*. 1992), indicating that GSH conjugation is a major route of metabolism. Recent studies with cerebellar granule cells in culture indicate that thiophene is not toxic *per se.* However, co-incubation of thiophene with cerebellar granule cells and a drug metabolizing system (liver S9 fraction from Arochlor-treated rats) results in cell death, supporting the formation of a toxic intermediate. Addition of the antioxidant vitamin E, GSH or the spin trap *N-tert*-butyl-α-(2-sulfophenyl)-nitrone

protected the cells against thiophene-induced neuronal cell death, possibly by scavenging the reactive thiophene *S*-oxide outside the cells (Dreiem and Fonnum, submitted). Thus, analogous to the 2-halopropionic acid and methyl halides, thiophene has the potential to cause GSH depletion in tissues including the brain. Studies examining the thiol status of the cerebellum and other brain regions following exposure to thiophene need to be carried out. Prior treatment of rats with MK-801 followed by thiophene may give some indication as to whether glutamic acid and hence an excitotoxic mechanism is involved in this toxicity.

Methylmercury

Methylmercury also targets the cerebellar granule cell and other small neurones in the central nervous system. In adult humans and primates the granule cell layer of the cerebellum and visual cortex are especially susceptible (Hunter and Russell 1954; Shiraki 1979; Harada 1995), whereas in rats peripheral sensory nerve cells are far more severely affected than neurones in the central nervous system (Chang and Hartman 1972; Herman *et al*. 1973; Jacobs *et al*. 1975). The basis for the selectivity to small neurones is not known. Syversen (1977) has suggested that susceptible neurones are those incapable of repairing the initial damage inflicted by methyl mercury, such as inhibition of protein synthesis. Others have questioned whether inhibition of protein synthesis *per se* is sufficient to be a direct cause of cell death (Sarafian and Verity 1991).

The affinity of methylmercury for thiol groups is well known (Webb 1966), thus intracellular methylmercury readily attaches itself to thiol residues in proteins, GSH and cysteine and hence methylmercury will perturb many cellular processes. It has thus proved exceedingly difficult to elucidate which enzymes/proteins are more sensitive to methylmercury and hence the primary trigger of events leading to neuronal cell necrosis. Recent advances have demonstrated that methylmercury cysteine can enter the brain via a carrier-mediated process, mimicking the neutral amino acid methionine for transport across the blood–brain barrier (Kerper *et al*. 1992; Mokrzan *et al*. 1995). Once inside the brain, many targets have been identified. One of the most sensitive is thought to be changes in $[Ca^{2+}]_i$ in cerebellar granule cells, which is accompanied by a rise in inositol phosphate, indicating a role for this signalling pathway following exposure to methylmercury (Sarafian 1993). Studies with isolated cerebellar granule cells have shown that the rise in $[Ca^{2+}]_i$ produced by methylmercury can be reduced by nifedipine and ω-conotoxin-MVIIC (Marty and Atchison 1997, 1998), suggesting a role for L-, N- and/or Q-type Ca^{2+} channels. Subsequent studies have indicated that all types of Ca^{2+} channels may be affected by methylmercury and that these channels may be a means of entry for methylmercury (Sirois and Atchison 2000). Changes in $[Ca^{2+}]_i$ can trigger a cascade of events leading to formation of reactive oxygen species and ultimately interference with mitochondrial energy metabolism. Methylmercury produces an initial phase of release of Ca^{2+} from intracellular stores into the cytosol and a second influx phase of extracellular Ca^{2+} (Marty and Atchison 1997). Recent findings suggest that mitochondria contribute significantly to both the initial phase of $[Ca^{2+}]_i$ increase and subsequent cell death through opening of the mitochondrial permeability transition pore (Limke and Atchison 2002; Limke *et al*. 2003). Cerebellar granule cells express high levels of muscarinic cholinergic receptors (Neustadt *et al*. 1988), and methylmercury has been shown to act as a strong competitive inhibitor of these receptors in ligand-binding studies (Coccini *et al*. 2000). Treatment of cerebellar granule cells in culture with atropine, to block muscarinic receptors, prevents the initial rise in $[Ca^{2+}]_i$ following exposure to methylmercury (Atchison, personal communication), indicating a role for these receptors in the selective toxicity. No evidence for an action of methylmercury at NMDA receptors was found in isolated cerebellar granule cells (Marty and Atchison 1997). Methylmercury at low concentrations, up to 300 nM, will induce apoptosis in cerebellar neurones in culture (Kunimoto 1994) but causes cell death by a non-apoptotic manner at higher concentrations.

It should be noted that methylmercury is preferentially accumulated in astrocytes (Garman *et al*. 1975; Charleston *et al*. 1994). Recent studies have shown that methylmercury can inhibit glutamic acid transporters (EAAT-1 and EAAT-2) located on astrocytes, thereby reducing glutamic acid uptake, and can also stimulate glutamic acid release from astrocytes (Aschner *et al*. 1994, 2000; Allen *et al*. 2001a). This will result in an increase in the extracellular concentration of glutamic acid, thereby sensitizing neurones to excitotoxic injury. Methylmercury also inhibits the transport of cystine into astrocytes, thereby reducing intracellular GSH, and hence the antioxidant status (Allen *et al*. 2001b; Shanker and Aschner 2001). A role for an excitotoxic mechanism is also indicated by reports that NMDA receptor antagonists provide some attenuation of the injury (Park *et al*. 1996; Miyamoto *et al*. 2001). Depletion of the GSH status of neurones is an early response to exposure, leading to a reduced antioxidant status (see Sarafian *et al*. 1996). There is also strong evidence for the involvement of reactive oxygen species in methylmercury toxicity *in vivo* in the cerebellum (Ali *et al*. 1992) and from studies in cerebellar and other neurones in culture (Sarafian *et al*. 1994; Choi *et al*. 1996; Sorg *et al*. 1998; Aschner 2000). Recent studies with astrocytes in culture have demonstrated methylmercury-induced formation of reactive oxygen species and their attenuation by a range of antioxidants and free radical scavengers (Shanker and Aschner 2003). In contrast, prior inhibition of GSH synthesis with buthionine sulfoximine increased reactive oxygen species formation in methylmercury-treated astrocytes (Shanker and Aschner 2003).

In summary, methylmercury is highly reactive with thiol residues, leading to GSH depletion and inactivation of proteins with thiol residues in their active site. In cerebellar granule cells in culture, low concentrations of methylmercury cause a rise in $[Ca^{2+}]_i$, which may trigger a cascade of events leading to mitochondrial impairment and generation of reactive oxygen species. Astroglial cells may also play a role in methylmercury-induced toxicity. Methylmercury inhibits glutamic acid uptake and stimulates its release from astroglial cells, thereby sensitizing neurones to excitotoxic injury. Methylmercury also inhibits cystine transport into astrocytes, thereby reducing the concentration of intracellular GSH. The combination of these events leads to production of reactive oxygen species and mitochondrial dysfunction and hence cerebellar granule cell necrosis. The precise involvement of muscarinic cholinergic receptors, which are abundant on cerebellar granule cells, awaits further study.

The effect of foreign compounds on the cerebellum during development

Methylazoxymethanol

As discussed earlier, the cell types in the cerebellum develop at different times during brain development. Exposure to methylazoxymethanol (MAM) has been used to help determine the time of their appearance. MAM is an aglycone and is prepared from cycasin, a toxic component of the cycad plant (Morgan and Hoffmann 1983). Cleavage of cycasin by β-glucosidase in the brain liberates free MAM, enabling it to exert its toxic effects (Kisby *et al*. 1999). Treatment of pregnant rats with MAM at gestational day 14, during the formation of Purkinje cells, leads to a dramatic loss of these cells in the adult animal. Interestingly, the numbers of granule cells are reduced in the same proportion so that the numbers of granule cells per Purkinje cells remain constant (Chen and Hillman 1989). In contrast, administration of MAM to newborn rats within 24 h of birth is accompanied by a large reduction in cerebellar granule cells, whereas the numbers of Purkinje cells are not reduced. Treatment of rats and mice around birth and during the first neonatal week may lead to other abnormal developments of the cerebellum. This involves the formation of synapses between mossy fibre and Purkinje cells, alteration of Purkinje cell positioning or the appearance of an ectopic granule cell layer (De Barry *et al*. 1987; Garcia-Ladona *et al*. 1991).

MAM is toxic to cerebellar granule cells in culture (Wood and Youle 1995; Johnson *et al*. 1998; Mehl *et al*. 2000) but the effect of antioxidants and NMDA receptor antagonists do not appear to have been examined. The mechanism by which MAM exerts its toxicity involves methylation at the O^6 and N^7 positions on guanine in DNA (Matsumoto and Higa 1966; Sohn *et al*. 1985). In addition, MAM compromises DNA repair by inhibiting OGMT (Esclaire *et al*. 1999; Mehl *et al*. 2000). Methylation of GSH may also occur (although we have found no studies on this topic) and this would be expected to be a detoxification pathway, reducing alkylation of cerebellar DNA and inhibition of OGMT. MAM has been reported to cause cytotoxicity to cerebellar granule cells in culture by apoptosis, via a p53-independent pathway (Wood and Youle 1995; Johnson *et al*. 1998) without showing DNA fragmentation. The precise mechanism whereby MAM causes selective cerebellar granule cell death and the involvement of inhibition of DNA repair, GSH and glutamic acid requires further studies.

Trichlorfon

Trichlorfon is an organophosphorous compound that is spontaneously converted to dichlorvos, an acetylcholinesterase inhibitor. In veterinarian medicine trichlorfon has been used against ecto- and endoparasites, and in aquaculture against salmon lice. In human medicine trichlorfon has been used for the treatment of *Schistosomiasis* and is undergoing clinical trials for treatment of Alzheimer's disease. There has been a single report of teratogenic effects in humans. Women in a small village in Hungary consumed fish killed by apparently high doses of trichlorfon. The offspring developed lesions, which were assumed to be due to trisomy resulting from an error of meiosis in oogenesis (Czeizel *et al*. 1993).

When trichlorfon is given to pregnant sows and guinea pigs, the offspring show cerebellar atrophy (Berge *et al*. 1987; Mehl *et al*. 1994). In guinea pigs the most sensitive period is found to be between days 40–50 of gestation (Hjelde *et al*. 1998). The toxic effects can be seen in other parts of the brain, but the most sensitive region by far is the cerebellum, followed by the medulla spinalis and hypothalamus. The cerebellar lesion is characterized by degeneration of the granule cells in both the external and inner layers. In the guinea pig a single high dose of trichlorfon at day 43 in gestation completely prevents any further growth of the cerebellum (Mehl *et al*. unpublished). Cerebellar atrophy is not seen in the rat, as the metabolite of trichlorfon, dichlorvos does not reach the brain, being readily metabolized.

The effects are not due to inhibition of acetylcholinesterase or neuropathy target esterase (Mehl *et al*. 1994). Trichlorfon and its active metabolite dichlorvos methylate DNA, the major product being N^3-methyl adenine. Recently Badawi (1998) has shown that trichlorfon given to rats forms O^6-methylguanine in the liver and kidney and inhibits the DNA repair enzyme OGMT in the liver. *In vitro* studies in our laboratory have shown that the active metabolite of trichlorfon, dichlorvos inhibited OGMT enzyme from *Escherichia coli*, which is similar to the human enzyme. Further, dichlorvos was lethal to two different *E. coli* mutants deficient in DNA repair, but not to a wild-type *E. coli*. One of the mutants was deficient in OGMT, whereas

the other mutant was deficient in 3-methyladenine DNA glycosylase. These studies indicate that methylation of DNA and inhibition of its repair could be a possible mechanism of toxicity (Mehl *et al*. 2000). Dichlorvos will also methylate GSH and this reaction will sequester some of the methylating potential, thereby protecting OGMT and DNA.

The *in vivo* observations in guinea pig offspring could be reproduced in rat cerebellar granule cells in culture following exposure to dichlorvos and trichlorfon, further supporting the view that trichlorfon exerts its toxic effect mainly through its conversion to dichlorvos. No protection was seen with NMDA receptor antagonists or by the addition of antioxidants (Fonnum, unpublished observation), suggesting an excitotoxic mechanism may not be involved. Cerebellar granule cells in culture were, however, killed by exposure to DNA methylating agents such as MAM, methyl methane sulphonate and by O^6-benzylguanine, a potent inhibitor of OGMT (Mehl *et al*. 2000). Agents with weaker DNA alkylating ability, such as ethyl methane sulphonate, ethyltrichlorfon or dimethoate, were much less toxic to granule cells (Mehl *et al*. 2000). The role of apoptosis in the cell death caused by these agents in cerebellar granule cells has not been reported. These findings support a role for inhibition of DNA repair and DNA methylation in the mechanism of cytotoxicity; the basis for the selectivity to cerebellar granule cells requires further study.

In summary, we have discussed six foreign compounds that, *in vivo*, cause cerebellar granule cell loss in the developing or adult brain. The mechanism of neuronal cell loss in two cases, 2-halopropionic acids and methylmercury, appears in part to involve accumulation of extracellular glutamic acid and hence an excitotoxic mechanism of cell death. This mechanism also involves depletion of the intracellular antioxidant GSH, increased $[Ca^{2+}]_i$ and the generation of reactive oxygen species (Fig. 5). There is a marked co-operativity between neurones and astrocytes with regard to the removal and maintenance of extracellular glutamic acid and in the supply of intermediates for the synthesis and maintenance of GSH. Both compounds are toxic to isolated cerebellar granule cells in culture, and methylmercury is also toxic to astrocytes in culture. Thus, a combination of events in both cell types may lead to cerebellar granule cell death, primarily by necrosis. The mechanism of neuronal cell loss with the methylhalides, methylazoxymethanol and trichlorfon would appear to involve DNA damage, methylation and inhibition of DNA repair plus depletion of the intracellular antioxidant GSH and oxidative stress (Fig. 5). In this situation apoptotic pathways of neuronal cell death may be switched on, detailed studies to assess this have not been conducted.

The pathways involved in cerebellar granule cell death are also present in other neurones and hence the basis for the selectivity of these chemicals for cerebellar granule cells *in vivo* is not clear, but probably involves a number of

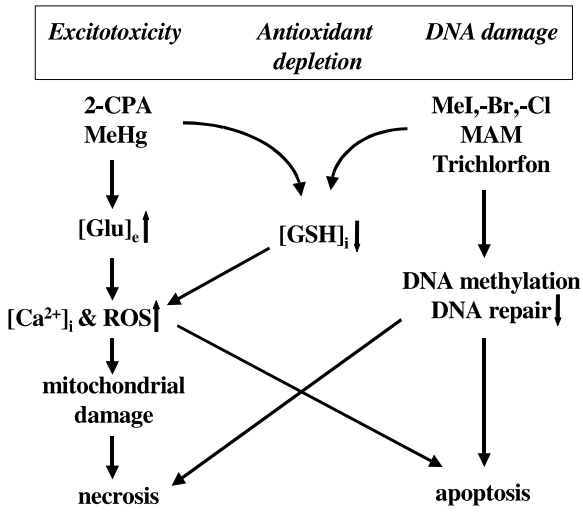

Fig. 5 Model for the differential pathways altered by the six foreign compounds that cause selective death to cerebellar granule cells. Glu, glutamic acid; ROS, reactive oxygen species.

factors. The unique pharmacology associated with the NMDA receptor subunit composition in these neurones, their small size and their vulnerability during postnatal development are all contributory factors to their sensitivity. The absence or low expression of Ca^{2+}-binding proteins will also reduce their ability to sequester and hence buffer against intracellular release of Ca^{2+}. The density of muscarinic cholinergic receptors on cerebellar granule cells may also be important for some chemicals.

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