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NEUROPROTECTIVE EFFECTS OF OVEREXPRESSION OF THE INHIBITOR OF APOPTOSIS PROTEINS IN THE QUINOLINIC ACID MODEL OF EXCITOTOXIC INJURY

by

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Thesis submitted to the Faculty of
Graduate Studies and Research in
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for the degree of M.Sc.

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DEDICATION

I would like to dedicate this thesis to the people who have contributed the most to making me who I am today. Without their love and support, finding direction would have been immeasurably more difficult, thank you.

To my parents, Alison and Brian Lee
and
to Jacquie Cohen

ABSTRACT

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder which results in the selective loss of the medium spiny neurons in the striatum. The neuropathological and behavioural deficits of HD can be modeled in rats by injection of the NMDA receptor agonist quinolinic acid (2,3 pyridine decarboxylate; QA) directly into the striatum [Beal, 1986 #65]. Consistent with the human condition, this animal model displays hallmarks of apoptosis suggesting that amelioration of the HD condition may be achieved through treatments which block programmed cell death.

The first aim of this study was to examine whether intrastriatal QA injections result in neurons dying by apoptosis as assessed by caspase activation. Animals were intrastriatally injected with QA and striatal brain sections processed for several apoptotic markers. It was observed that intrastriatal QA injections results in DNA fragmentation as indicated by in situ nick-end labeling (ISEL), which is a hallmark feature of apoptosis. ISEL labeling was proceeded by a large increase in active caspase-3 located in injured striatal neurons.

Intrastriatal administration of QA resulted in profound neuronal loss as determined by use of the structural Nissl stain [Araujo, 1997 #12]. This pattern of neuronal loss is similar to that observed in HD. Recently, a novel family of inhibitor of apoptosis (IAP) proteins have been identified and subsequently shown to attenuate cell death induced by a variety of apoptotic triggers *in vitro*. The goal of the second study was to determine whether adenovirally-mediated overexpression of the IAPs resulted in protection against QA-induced degeneration. Quantitative assessment of striatal neuron loss was assessed by counting

neurons labeled with the neuron specific marker NeuN. Animals that had been injected with the LacZ vector display a reduction in NeuN positive neurons in the striatum. In contrast, XIAP, and to a lesser degree NAIP, overexpression significantly reduced QA-induced injury.

Intrastriatal injections of QA also result in motor and cognitive deficits that are symptomatic of HD. These behavioural deficits manifest themselves as impairments in spatial learning and motor performance, and can be measured in rats using the Morris water maze test. The goal of the third study was to determine whether neurons saved by overexpression of the IAPs were still functional. In a third study, animals were intrastriatally injected with recombinant adenoviral constructs containing either *xiap*, *naip*, or *lacZ* one week prior to QA injections (120 nmol). QA lesions produced a modest impairment of spatial memory performance that was attenuated by XIAP but not NAIP.

Taken together, these results suggest that treatments capable of elevating XIAP expression may be useful in preventing neuronal damage associated with excitotoxic injury.

ACKNOWLEDGMENT

There are several groups and individuals who have contributed to the successful completion of this body of research.

First, I would like to thank my supervisor, Dr. G.S. Robertson for his guidance and understanding. From initially doing my honour's degree in his lab to the Master's thesis contained within, his sense of professionalism and his sense of humour have made my experience in graduate research productive and rewarding.

I would also like to thank members of Dr. Robertson's lab, who through their assistance and collaboration, I have found friendship and fond memories. Accordingly, I thank Stephen J. Crocker for his help, friendship, and support. I also thank Yanxia Zhu, Daigen Xu, and Charlie S. Thompson for help and assistance along the way.

Since many of these studies were performed using reagents provided by the CHEO research labs and Apoptogen, I would like to thank Drs. Korneluk, Mackenzie, and Liston et al. for their help. Too bad none of them can play hockey!

I would also like to thank Yves Bureau for helping set up and run the Morris water maze experiments.

I would also like to thank Donna, Gillian, Shannon, and Denyse for their help over the last two years and also for putting up with me.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

I. HUNTINGTON'S DISEASE

A. Symptoms of Huntington's Disease

Huntington's disease (HD) is a progressive neurodegenerative disease which affects the medium spiny neurons of the striatum (Reiner *et al*, 1988). HD is transmitted by autosomal dominant inheritance. The average age of onset of symptoms is 40 and the disease is fatal within approximately 20 years of onset of neuropsychological symptoms (Reiner *et al*, 1988). HD starts off with mild symptoms such as depression, mood swings, forgetfulness, and lack of coordination that may go unnoticed. As the disease progresses, the symptoms become progressively worse. The symptoms can be grouped into two main categories: (i) progressively worsening movement deficits resulting in bradykinesia and rigidity; and (ii) neuropsychiatric problems, including cognitive impairment (such as memory function and visuospatial processing), personality changes, psychosis and dementia.

There are several drugs used to treat the symptoms of HD. Antidepressants and neuroleptics may be used to treat emotional and cognitive symptoms. There are however, no known drugs to slow or halt the progress of the disease.

B. Pathology of Huntington's Disease

The progressive degeneration of striatal projection neurons results in the loss of a major striatal output which leads to severe motor and cognitive deficits. The neuronal

degeneration is selective: largely sparing the interneurons and afferent terminals and targeting the medium spiny projection neurons. This leads to a loss of striatal gamma-aminobutyric acid (GABA) concentrations while relatively sparing somatostatin and dopamine concentrations (Beal *et al*, 1986). Recent studies have suggested that the selective vulnerability of this subpopulation of striatal neurons may be due to extensive cortical inputs and expression of huntingtin, a protein from a candidate gene for HD, which may increase susceptibility to excitotoxic death through an apoptotic-like mechanism (Dure *et al*, 1995; Portera-Cailliau *et al*, 1995). The gene which encodes huntingtin, termed *IT15*, is located on chromosome 4. This gene has been found to contain a $(CAG)_n$ trinucleotide repeat that appears to be significantly expanded in patients with HD (Anonymous, 1993). This trinucleotide repeat (polyglutamine expansion) is the genetic alteration responsible for HD (Wellington & Hayden, 1997): In normal subjects, the CAG repeat length is between 9 and 34 triplets, with an average length of 19 on normal chromosomes; the repeat length in persons affected with HD ranges between 38 to over 100.

The mechanism by which the trinucleotide expansion leads to the clinical and pathological features of HD is presently unknown. The HD gene (*IT15*) is widely expressed throughout the body in tissues such as brain, pancreas, liver, testes and muscle (Li *et al*, 1993). Furthermore, *IT15* expression does not appear to be correlated with neuron vulnerability since *IT15* mRNA is expressed equally in all regions of the brain (Landwehrmeyer *et al*, 1995). It has been suggested that the ability of the trinucleotide expansion in the *IT15* gene to produce selective neuronal degeneration in specific areas and specific neuron populations may be related to metabolic or excitotoxic mechanisms

(Wellington & Hayden, 1997). Expression of the huntingtin protein may increase the susceptibility of neurons to excitotoxic damage. The toxic fragment hypothesis posits that the cleavage products of proteins containing long polyglutamine stretches (i.e. huntingtin) may have toxic properties (Wellington & Hayden, 1997). The fragment from huntingtin following caspase-3 cleavage may accumulate and provoke further caspase-3 activation, thus resulting in further huntingtin cleavage and accelerated neuronal apoptosis.

II. EXCITOTOXIC RODENT MODEL OF HD

A. Histological effects of intrastriatal QA injections

The selective pattern of neuronal death and behavioural changes observed in HD are mimicked in the rat by intrastriatal administration of the N-methyl-D-aspartate (NMDA) receptor agonist quinolinic acid (QA). QA selectively kills medium spiny neurons while sparing the large somatostatin, neuropeptide Y, and cholinergic neurons (Beal *et al*, 1986). This pattern of neuronal loss is similar to that observed in HD. QA is an endogenous tryptophan metabolite which has been found in both normal rat and human brains (Sanberg et al., 1989). Since excessive concentrations of QA have been shown to produce excitotoxic injury resembling HD, it has been postulated that intrastriatal injections of QA may be a useful rodent model of the HD pathology and may be a relevant *in vivo* assay for evaluating potential therapeutic strategies for HD.

Intrastriatal administration of QA results in profound neuronal loss as determined by use of the structural Nissl stain (Araujo & Hilt, 1997; Figueredo-Cardenas *et al*, 1994;

Roberts *et al*, 1993). The loss of medium spiny neurons results in a marked shrinkage of the striatum several months following QA injections (Figueredo-Cardenas *et al*, 1994).

B. Behavioural effects of intrastriatal QA injections

Intrastriatal injections of QA also result in motor and cognitive deficits that are symptomatic of HD. These behavioural deficits manifest themselves as impairments in spatial learning and motor performance, and can be measured in rats using the Morris water maze test (Block *et al*, 1993; Furtado & Mazurek, 1996). The Morris Water Maze is a test whereby rats must find a platform in a pool of water (170 cm in diameter) which is submerged just below the surface. Rats which have undergone bilateral striatal QA lesions have been shown to require significantly more time than sham-operated animals to find the platform (Block et al., 1993). Similarly, rats which have been trained to find the platform and then undergone these lesions display an increased swim distance to find the platform, suggesting that there is a loss in the memory for the platform which may relate to the death of higher motor centers in the striatum. Indeed, Furtado and Mazurek (1996) demonstrated that QA-lesioned rats performed normally on tests that examined motor activity, suggesting that impaired performance of these animals in the water maze indicates cognitive dysfunction rather than deficient motivation or inadequate motor skills. Furthermore, cued trials demonstrated that visual, motor, and motivation aspects of QA treated animals were normal. Similar results have been obtained after striatal lesions using the excitotoxin ibotenic acid (Whishaw *et al*, 1985). It is interesting to note that deficits in visuospatial processing and memory, the same deficits observed in QA lesioned rats, are among the first cognitive

impairments to appear in HD.

Previous studies have shown that several neurotrophic factors such as nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF), and glial cell line-derived neurotrophic factor (GDNF) rescue striatal neurons from QA-induced damage, and result in a decrease in cell loss and a reduction in lesion size (Anderson *et al*, 1996; Araujo & Hilt, 1997; Davies & Beardsall, 1992). Araujo and Hilt (1997) also demonstrated that i.c.v. administration of GDNF 30 minutes prior to unilateral intrastriatal QA injections leads to a reduction in amphetamine-induced rotational behaviour.

C. QA-induced seizure activity

It has been shown that intrastriatal injection of QA results in clonic-tonic movements of the contralateral limbs and episodic barrel rotations which begin approximately 30 minutes after the injection and last for several hours (Marrannes & Wauquier, 1988). Intrahippocampal injection of QA, a model of temporal lobe epilepsy, also results in repetitive seizure episodes which are associated with changes in cortical and hippocampal EEGs approximately 30 minutes following injection (corresponding with the appearance of seizure-like activity) (Vezzani *et al*, 1986). Barrel rotations, clonic-tonic movements of the limbs, and excessive chewing are all characteristic of QA-induced seizures. It is not clearly understood whether intrastriatal administration of QA results in actual seizures or simply seizure-like activity. However, it is known that administration of antiepileptic drugs attenuate intrastriatal QA-induced seizure-like activity (Marrannes & Wauquier, 1988).

III. CELL DEATH

A. Apoptosis and necrosis

There are two major types of cell death (i.e. apoptosis and necrosis). Apoptosis, or cell suicide, is the death of a cell in a systematic and orderly manner. Cells that die rapidly by osmotic mechanisms involving cation influx and autophagocytosis in response to an acute injury often do so by an uncontrolled process termed necrosis. Apoptosis and necrosis are differentiated on the basis of morphological abnormalities of cells at the ultrastructural level, dependance on protein synthesis, and in vitro or in vivo evidence of systematic DNA cleavage.

Apoptosis is a fundamental biological process that is required to maintain the integrity and homeostasis of multicellular organisms; it is involved in normal cell turnover, elimination of autoreactive T-cells from the immune system, embryonic development, and chemical-induced cell death (Jacobson *et al*, 1997; Steller, 1995; Vaux & Strasser, 1996). During development, apoptosis is involved in eliminating redundant or unnecessary cells. Apoptosis plays a developmental role by: i) sculpting structures; ii) deleting unneeded structures; iii) controlling cell numbers; iv) eliminating abnormal, misplaced, non-functional, or harmful cells; and v) producing differentiated cells without organelles (Jacobson *et al*, 1997).

The morphology of an apoptotic cell is characterized by membrane blebbing, perinuclear chromatin condensation, compaction of cytoplasmic organelles, swelling of the endoplasmic reticulum, and internucleosomal DNA fragmentation into a ladder pattern which

can be visualized by agarose gel electrophoresis (a biochemical hallmark of apoptosis) (Vaux & Strasser, 1996). The DNA of cells undergoing apoptosis is cleaved into multiples of 180 base pairs, corresponding to internucleosomal spacing (Wyllie, 1980). Changes in the cell membrane are responsible for apoptotic cells being recognized and phagocytosed. Apoptotic cells shrink and are rapidly engulfed by neighbouring cells before there is any leakage of their contents resulting in an inflammatory response.

Apoptotic cell death can be divided into phases. The earliest phase is the stimulus that provokes the apoptotic response - this may occur extracellularly or from within the cell. The second phase includes detection of this signal or metabolic state and transduction of the signal. In the third, or effector phase, proteases and their positive and negative regulators are activated. Finally, in the post-mortem phase, the chromatin condenses and the cell's DNA is degraded. The dying cells are then recognized and engulfed by neighbouring cells (Vaux & Strasser, 1996).

Necrosis occurs as a result of overwhelming cellular injury. It may be caused by an upset in cellular ionic homeostasis, which may lead to dilation of the endoplasmic reticulum, alteration of the mitochondria, swelling of the cell, and rupture of the plasma membrane (Johnson & Deckwerth, 1993). Necrotic cells swell and burst, consequently spilling their contents and eliciting a damaging inflammatory response (Raff, 1998). Necrotic cells show random DNA fragmentation resulting in smearing of DNA on agarose gels.

B. Neuronal apoptosis

Apoptosis is a form of programmed cell death whereby stimuli induce specific

cellular processes resulting in the systematic metabolic, structural, and genomic dismantling of the cell. Apoptosis plays an important role in the development of the central nervous system. In nervous system development, neurons are generated in excess, and up to half are eliminated by apoptosis in order to match projections to target cells which form networks necessary for adult function (Oppenheim, 1991). Although programmed cell death is necessary for maintaining homeostasis, apoptotic dysregulation is believed to result in many pathologies. For example, apoptosis has been shown to play a role in numerous neurodegenerative disorders including Parkinson's disease, Alzheimer's disease, stroke, traumatic brain injury, and Huntington's disease.

C. The role of caspases in apoptosis

The initiation and execution of apoptosis involves the action of specific proteases termed caspases. Horvitz et al. (1986) first implicated caspases in apoptosis using the nematode *Caenorhabditis elegans*. During normal nematode development, 131 cells of the 1090 cells generated die by apoptosis (Ellis *et al*, 1991). *Ced-3* and *ced-4* are two genes which are required for cell death in the *C. elegans*. Mutation or deletion of *ced-3* results in the prevention of the death of the 131 cells which normally die by apoptosis during development (Ellis & Horvitz, 1986; Ellis *et al*, 1991). The *ced-9* gene prevents cell death by antagonizing the function of *ced-3* and *ced-4* (Hengartner *et al*, 1992). The CED-9 protein shows sequence homology with the mammalian Bcl-2 protein, which prevents cell death in mammals. No mammalian homologue of CED-4 has been identified, however the CED-3 protein shows sequence homology with the mammalian interleukin-1β-converting

enzyme (ICE), also known as caspase-1. This sequence homology taken together with the finding that overexpression of ICE induces apoptosis, suggests that ICE may play a key role in apoptosis (Yuan *et al*, 1993). Since the suggestion that ICE may be intimately involved in the induction of apoptosis, a further nine ICE-like proteases have been identified. This family of proteases has now been termed the caspases, the 'c' denoting a cysteine protease and the 'aspase' referring to the tendency of these enzymes to cleave after an aspartic acid residue (Alnemri *et al*, 1996).

There is now significant evidence demonstrating that caspases are involved in apoptosis. Firstly, specific members of the caspase family have been implicated in the proteolysis of key proteins that are selectively cleaved at the onset of apoptosis (Nicholson *et al*, 1995). Secondly, caspase inhibitors have been shown to block apoptosis *in vitro* and *in vivo* (Du *et al*, 1997; Fink *et al*, 1998). Furthermore, activation of several caspases has been shown to be necessary for the apoptotic phenotype to appear (Nicholson *et al*, 1995; Nicholson & Thornberry, 1997). Finally, mice lacking the caspase-3 gene have a severe defect in neuronal apoptosis resulting in neuronal expansion and possible structure duplication that results in death at PD7-14 days (Kuida *et al*, 1996).

There are at least 10 human members of the caspase family. They can be divided into three distinct groups based on the tetrapeptide sequence of their target substrates. Caspases-1, -4, and, -5 comprise group I and all prefer, and cleave following, the tetrapeptide sequence WEHD. The group II caspases, caspases-2, -3, and -7, and the nematode CED-3, prefer the sequence DExD. Caspases-6, -8, and -9 (group III) prefer the sequence (I/L/V)ExD (Cohen, 1997). All caspases have small and large subunits which heterodimerize

and through processing by autocatalysis generate the active enzyme. Generation of the final large subunits requires a further processing of an N terminal region (Nicholson, 1995 #120). Caspases are synthesized as a catalytically dormant proenzyme containing an amino-terminal prodomain followed by the large and small subunits of the active enzyme (Cohen, 1997). The catalytically active caspase consists of a tetramer (with two active sites) containing two small and two large subunits which are generated following autocatalytic cleavage at specific aspartate cleavage sites. Caspases target substrates with a Asp-x-x-Asp amino acid sequence and cleave between the Asp-x. They require Asp in the P1 position of substrates.

Caspases carry out specific functions in apoptosis including the disassembly of cell structures, the breakdown of cytoskeletal proteins, and inactivation of DNA repair enzymes. Caspases target proteins which comprise the cytoskeleton such as gelsolin, actin, and fodrin (Nicholson *et al*, 1995). Caspases cause the fragmentation of DNA by cleaving ICAD, an inhibitor of the nuclease which is responsible for DNA fragmentation, CAD (caspase-activated deoxyribonuclease). In non-apoptotic cells, ICAD forms an inactive complex with CAD. During apoptosis, ICAD is cleaved by caspases, releasing the active CAD (Enari *et al*, 1998). Lastly, caspases inhibit enzymes which function in the repair of damaged DNA such as poly(ADP-ribose) polymerase (PARP).

Caspases may function as either initiators or effectors of apoptosis. It is now believed that their activation occurs in a cascade fashion. A proapoptotic signal results in the activation of initiator caspases which, in turn, activate effector caspases resulting in cellular disassembly. Caspases-8 and -9 are initiator caspases which may be activated through binding of a ligand (i.e. Fas, TNF-α, etc) to a specific receptor or release from mitochondria

of cytochrome c, which then associates with APAF-1 to activate caspase-9 (Li *et al*, 1997).

Recent studies have highlighted the importance of one specific caspase, caspase-3, in neuronal death (Kuida *et al*, 1996) Mice that lack the caspase-3 gene show profound nervous system abnormalities; their brains growing to twice the normal volume. Caspase-3 knock-outs display protrusions of the brain tissue with associated skull defects as a result of abnormal apoptosis. Furthermore, ectopic cell masses were found in the cerebral cortex, hippocampus, and striatum -- presumably cells which failed to die during neuronal development. In contrast, there were no discernible histological abnormalities observed in the heart, lung, liver, kidney, spleen and testes, despite expression of caspase-3 in these tissues in normal animals (Kuida et al., 1996). Fas induced death in the immune system was also normal. This suggests that although there may be redundancies in caspase function in other tissues, caspase-3 is the primary caspase involved in the execution of apoptosis in the brain.

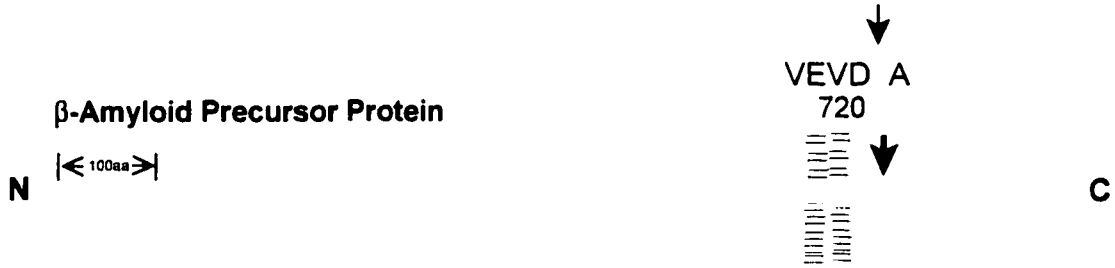
Caspase-3 peptide inhibitors have also been shown to block neurodegeneration (Fink *et al*, 1998). Fink et al. (1998) demonstrated that intracerebroventricular (i.c.v.) injection of the caspase-3 inhibitor N-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethyl-ketone (zDEVD-fmk) up to 9 hours following middle cerebral artery (MCA) occlusion, resulted in a significant reduction in infarct volume. The 9 hour time point corresponds to the appearance of the 20-kDa caspase-3 fragment (p20) following MCA occlusion (Fink *et al*, 1998).

Caspase-3 has several downstream targets which are cleaved in response to a

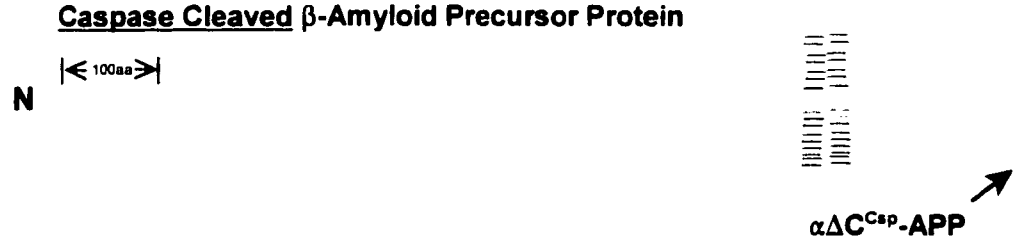
proapoptotic signal, one of which is the amyloid-β precursor protein (APP). APP is cleaved by caspase-3 during apoptosis and results in elevated amyloid-β (Aβ) peptide formation (Gervais *et al*, 1999). Recently Gervais et al. (1999) demonstrated that caspase-3 is the predominant caspase involved in APP cleavage which is markedly elevated in dying neurons of Alzheimer's disease brains. It has previously been demonstrated that treatment of cultured neurons with the Aβ fragment results in apoptosis (Loo *et al*, 1993), and that neurons undergoing apoptosis generate increased Aβ peptide levels (LeBlanc, 1995). Gervais et al. also demonstrated, using an antibody directed against the caspase-3 cleaved form of APP (designated $\alpha\Delta C^{Csp}$-APP), that elevated levels of $\alpha\Delta C^{Csp}$-APP are found in senile plaques as well as apoptotic hippocampal neurons following excitotoxic injury with kainic acid and following ischemia (Fig. 1).

Fig. 1. Caspase-3 has several downstream targets which are cleaved in response to a proapoptotic signal, one of which is the amyloid-β precursor protein (APP). APP is cleaved by caspase-3 during apoptosis and results in elevated amyloid-β (Aβ) peptide formation. An antibody was generated, designated $\alpha\Delta C^{Csp}$-APP (Gervais et al., 1999), which is highly specific for the caspase-generated neoepitope in APP.

APP processing by caspase-3



Generation of the antibody directed against the caspase-3 cleaved form of APP



IV. APOPTOSIS AND HUNTINGTON'S DISEASE

Recent evidence suggests that the neurons which die in HD do so by apoptosis (Portera-Cailliau *et al*, 1995; Thomas *et al*, 1995). Portera-Calliliau *et al.* (1995) demonstrated DNA fragmentation by terminal transferase-mediated (TdT) deoxyuridine triphosphate (d-UTP)-biotin nick end labeling (TUNEL) of neurons in human HD patients post-mortem and in animals which had received intrastriatal injections of QA. The TUNEL reaction labels the 3'-OH ends of DNA which has been cleaved during apoptosis. Gel electrophoresis of DNA extracted from QA-lesioned striata also revealed the DNA laddering typical of apoptosis. Ultrastructural analysis of QA-lesioned animals 6 hours post-lesion showed neurons containing large clumps of chromatin within nuclei as well as numerous vacuoles and swollen organelles in the cytoplasm -- all of these hallmark features of apoptosis.

In addition, there have been reports that the huntingtin protein is specifically cleaved by caspase-3 (Goldberg *et al*, 1996). Furthermore, the longer the polyglutamine tract (in the range associated with the clinical presentation of HD), the more susceptible huntingtin is to caspase-3 cleavage (*ibid.*). The mechanism by which huntingtin is involved in the selective loss of striatal neurons is still unclear. However, recent evidence suggests that the release of proapoptotic proteins resulting from the expansion of CAG repeats may contribute to neuronal apoptosis in HD [Butterworth, 1998 #105].

V. THE INHIBITOR OF APOPTOSIS PROTEINS

A. Discovery of the IAP family

Inhibitor of apoptosis proteins (IAPs) were first characterized as proteins encoded by insect viruses which propagate by inhibiting defensive apoptosis within the host. The first IAPs to be discovered were found in the baculoviruses *Cydia pomonella* granulosis virus (CpGV) and *Orgyia pseudotsygata* nuclear polyhedrosis virus (OpMNPV) (Birnbaum *et al.* 1994; Crook *et al*, 1993). The first mammalian IAP to be isolated was neuronal apoptotic inhibitory protein (NAIP), which is thought to be involved in spinal muscular atrophy (SMA), a disease resulting from the degeneration of motor neurons (Roy *et al.* 1995). The primary abnormality found in SMA is a loss of motor neurons which leads to weakness and wasting of the voluntary muscles. Children afflicted with this disorder rarely survive past the first few years of life due to respiratory muscle weakness (Roy *et al.* 1995). Roy et al. (1995) discovered deletions or mutations in the *naip* locus in patients with type 1 SMA, the most severe form of the disease. Consistent with a role for NAIP in SMA, Xu et al. (1997) found a correlation between neurons that display NAIP immunoreactivity and those neuronal populations that have been reported to suffer pathological alterations in severe forms of SMA.

B. Structure and tissue distribution of the mammalian IAPs

Several members of the mammalian IAP family include NAIP, X-linked IAP (XIAP), the human IAPs - HIAP-1 and HIAP-2, survivin, and BRUCE (Hauser *et al*, 1998). The

gene that encodes the XIAP protein is located on the X chromosome and contains three BIR (baculoviral inhibitor of apoptosis repeat) domains as well as a RING zinc-finger motif. The BIR domain is required to prevent apoptosis and is characteristic of all members of the IAP family (Takahashi *et al*, 1998; Deveraux, 1999 #126; LaCasse, 1998 #127). HIAP-1 and HIAP-2 also contain three BIR domains, a RING zinc-finger motif, and a caspase-recruitment (CARD) domain. NAIP contains the three BIR domains similar to its other family members, however, it lacks the RING zinc-finger motif and has a large N-terminal fragment (Liston *et al*, 1996) (Fig. 2). The most recent addition to the IAP family is survivin which contains a single BIR domain (Ambrosini *et al*, 1997). Survivin is present during fetal development and is undetectable in differentiated adult tissues. However, survivin has been found in all of the most common cancers including lung, colon, pancreas, prostate, and breast (Ambrosini *et al*, 1997).

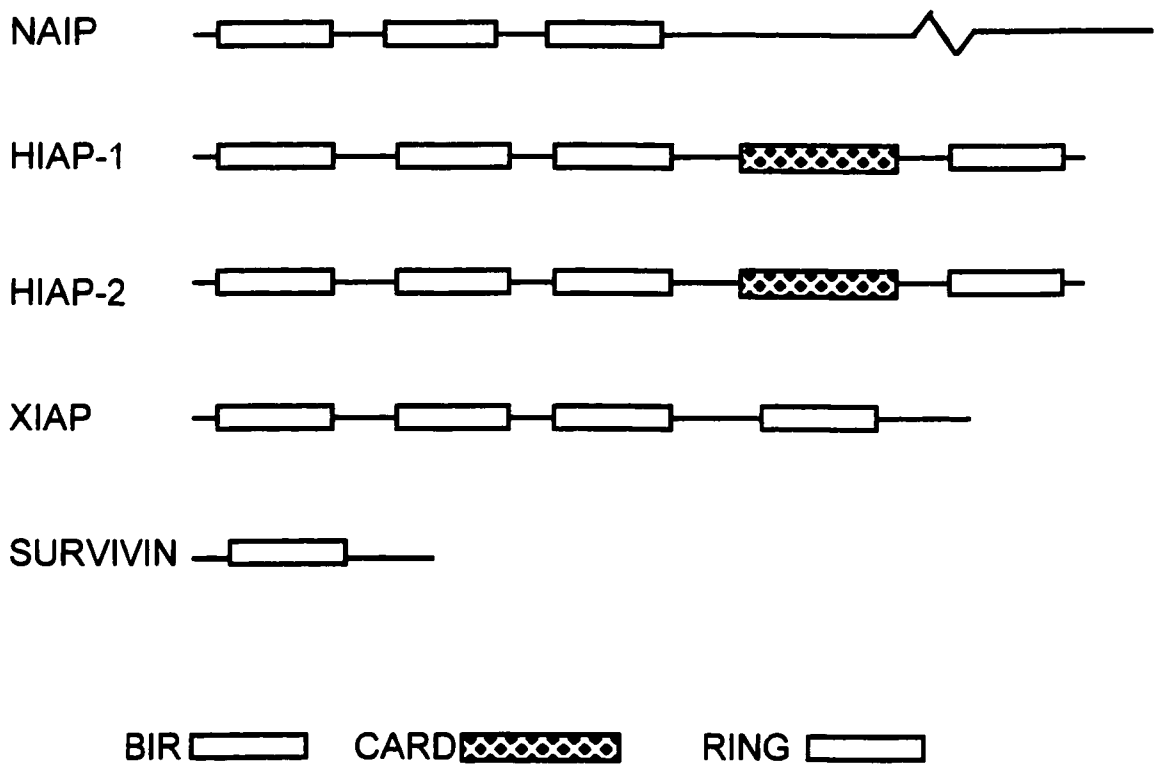
The mammalian IAPs are widely distributed in most tissues. XIAP mRNA is expressed in all fetal and adult tissues examined to date, with the exception of peripheral blood leukocytes (Liston *et al*, 1996). *hiap*-1 mRNA is uniquely distributed in fetal tissues, being present in the lungs and kidneys and absent from the brain and liver. In the adult, *hiap-1* mRNA is highly expressed in lymphoid tissues such as the spleen, thymus and peripheral blood lymphocytes, suggesting a role for HIAP-1 in lymphocyte development (Liston *et al*, 1996). *hiap-2* mRNA is found in most fetal and adult tissues, and is highly expressed in adult skeletal muscle and pancreas and is absent from the brain and peripheral blood leukocytes.

While XIAP, HIAP-1 and HIAP-2 are widely expressed in most tissues, NAIP is

restricted to neurons, liver and macrophages (Roy *et al*, 1995). Within the brain NAIP is found in the cholinergic neurons of the striatum, pyramidal neurons of the hippocampus, the substantia nigra pars compacta, cortical pyramidal neurons of layers V and VI, the thalamus, most of the cranial nerve nuclei in the brainstem, and the motor neurons of the spinal cord (Xu *et al*, 1997b).

Survivin is expressed fetally and not in adult differentiated tissue (Ambrosini *et al*, 1998). Furthermore, survivin is expressed in many cancers including lung, colon, breast, prostate, pancreas, neuroblastomas (Ambrosini *et al*, 1998).

Fig 2. Structure of the mammalian IAPs; neuronal apoptosis inhibitory protein (NAIP), human inhibitor of apoptosis protein 1 (HIAP1), human inhibitor of apoptosis protein 2 (HIAP2), X-linked inhibitor of apoptosis protein (XIAP), and survivin. These figures highlight the locations of the baculoviral IAP repeat (BIR; grey), caspase-recruitment domain (CARD; checkered), and RING (hatched) domains.


NAIP
HIAP-1
HIAP-2
XIAP
SURVIVIN
BIR
CARD
RING

C. Function of the mammalian IAPs

Recent evidence indicates that some members of the IAP family block apoptosis by binding to and inhibiting members of the caspase family. XIAP, HIAP-1 and HIAP-2 have all been shown to potently bind to and inhibit caspases 3 and 7 (Deveraux *et al*, 1997; Roy *et al*, 1997). XIAP, HIAP-1 and HIAP-2 were shown to bind to active caspase-3 and -7 in a cell-free system in which caspases were activated in cytosolic extracts by addition of cytochrome c. Further deletion studies indicate that the BIR2 domain of XIAP is sufficient and necessary for caspase-3 and -7 inhibition (Takahashi *et al*, 1998).

D. Inhibition of apoptosis by overexpression of the IAPs

It has been shown that overexpression of IAPs may confer protection against a variety of apoptotic triggers both *in vitro* and *in vivo* (Liston *et al*, 1996; Xu *et al*, 1997a). Overexpression of the IAPs has been shown to block apoptosis in a variety of cell lines produced by a number of apoptotic triggers. Overexpression of the IAPs by transfection or adenovirus protects cells against apoptosis triggered by serum deprivation, menadione (a potent inducer of free radicals), TNF-α, and cycloheximide (Liston *et al*, 1996). Adenovirally-mediated overexpression of NAIP and XIAP has also been shown to attenuate apoptotic cell death of hippocampal CA1 neurons five days following transient cerebral ischemia (Xu *et al*, 1999; Xu *et al*, 1997a).

Furthermore, overexpression of XIAP prevented the activation of caspase-3 and DNA fragmentation in hippocampal CA1 neurons following transient cerebral ischemia (Xu *et al*,

1999). In addition to preventing neuronal apoptosis, Xu et al. (1999) demonstrated that, following ischemia, hippocampal neurons that express XIAP were still functional. Following an episode of transient forebrain ischemia, rats pretreated with XIAP performed better in the Morris Water Maze task than animals which were pretreated with the control LacZ virus, indicating that not only were the neurons still alive, but they also operated properly. XIAP overexpressing CA1 neurons which survived the ischemic insult also displayed considerably higher levels of the neuronal activity marker NGFI-A than animals injected with the lacZ construct, a finding which is particularly interesting given that NGFI-A is normally depressed in CA1 neurons following an episode of global ischemia (Dragunow *et al*, 1994). Since NGFI-A expression is driven by NMDA receptor activation and elevated intracellular calcium levels, XIAP overexpression may maintain calcium homeostasis in the neuron following excitotoxic/ischemic injury (Xu *et al*, 1999).

VI. RESEARCH OBJECTIVES

A. To determine the effects of adenovirally-mediated IAP overexpression on neuronal loss following intrastriatal QA lesions

Striatal QA lesions in the rat mimic the pathology of HD by selectively targeting the medium spiny neurons of the striatum. QA lesions have also been shown to cause apoptotic cell death as evidenced by TUNEL labeling, DNA laddering on gel electrophoresis and ultrastructural laddering (Portera-Cailliau *et al*, 1995; Thomas *et al*, 1995). Previous studies have demonstrated the anti-apoptotic effects of adenovirally-mediated overexpression of the

IAPs, both *in vitro* (Liston *et al*, 1996)) and *in vivo* (Xu *et al*, 1999; Xu *et al*, 1997a). Thus, the purpose of the present study was to investigate the potential neuroprotective effect of adenovirally-mediated overexpression of NAIP and XIAP against QA-induced neuronal loss in the intrastriatal QA model of HD.

B. To determine the effects of intrastriatal QA administration on caspase-3 activation

Administration of QA and other excitotoxins directly into brain structures has been shown to result in apoptosis (Portera-Cailliau *et al*, 1995; Thomas *et al*, 1995). Furthermore, the IAPs are thought to act as anti-apoptotic proteins by inhibiting caspase-3 and -7 (Deveraux *et al*, 1997; Roy *et al*, 1997). Since caspase-3 has been shown to play a central role in neuronal apoptosis (Kuida *et al*, 1996), one of the goals of the present study was to determine whether QA-induced neuronal apoptosis is a caspase-dependent event.

C. To determine the effects of adenovirally-mediated IAP overexpression on QA-induced deficits in spatial learning

Research has shown that intrastriatal QA lesions in rats result in behavioural deficits similar to those observed in humans with HD (Block *et al*, 1993; Furtado & Mazurek, 1996). Rats which are subjected to intrastriatal injections of the excitotoxin QA take longer to find the platform and have an increased swim distance in the Morris Water Maze task (*ibid.*). Previous studies have demonstrated that IAP overexpression in the hippocampus prevents deficits in spatial learning following an episode of transient forebrain ischemia (Xu *et al*, 1999). Accordingly this study aimed to investigate whether overexpression of the IAPs prior

to an excitotoxic insult will protect against QA-induced deficits in spatial navigation.

MATERIALS AND METHODS

I. ANIMALS

Adult male Wistar rats (200-250 g; Charles River, Montreal) were housed two per cage in a temperature-controlled environment with a 12 hr light/dark cycle and free access to standard laboratory chow and water. All animals were treated in strict accordance with procedures outlined in the Guide for the Care and Use of Experimental Animals endorsed by the Medical Research Council of Canada.

II. EFFECT OF INTRASTRIATAL QUINOLINIC ACID ADMINISTRATION ON NEURONAL SURVIVAL

A. Experimental protocol

Two groups, composed of 2 rats each, were stereotaxically injected with either vehicle (0.01M phosphate-buffered saline) or QA [120 nmol, dissolved in 0.01M PBS; pH 7.4; QA (Sigma, St. Louis, MO; P6, 320-4)] into the striatum (AP = +1.2, ML.= -2.6, DV = -4.5 mm from bregma; according to Paxinos and Watson, 1997 #136). Two weeks following QA lesions, animals were deeply anaesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital (100 mg/kg; MTC Pharmaceuticals, Cambridge, ON). Rats were then transcardially perfused with saline (0.9%; 200ml) followed by an equivalent volume of paraformaldehyde (PFA)(4.0%) in 0.1 M phosphate buffer (PB). Perfused tissues were postfixed in 4% PFA overnight and then cryoprotected in 10% sucrose in 0.1 M PB for 48

hrs. Brains were frozen using gaseous carbon dioxide. Finally, sections (12 μm thick) were cut throughout the striatum using a cryostat (plates 12 to 17 of Paxinos and Watson, 1986).

B. Immunohistochemistry

NeuN immunoreactivity was detected using the monoclonal antibody A60. A60 (or NeuN) was generated against brain cell nuclei and recognizes the neuron-specific protein NeuN (neuronal nuclei). The antibody labels nuclei and perikarya of most neuronal cell types found in the nervous system of adult mice; it crossreacts immunohistochemically with neural tissue from rats, chicks, humans, and salamanders (Mullen *et al*, 1992; Wolf *et al*, 1996).

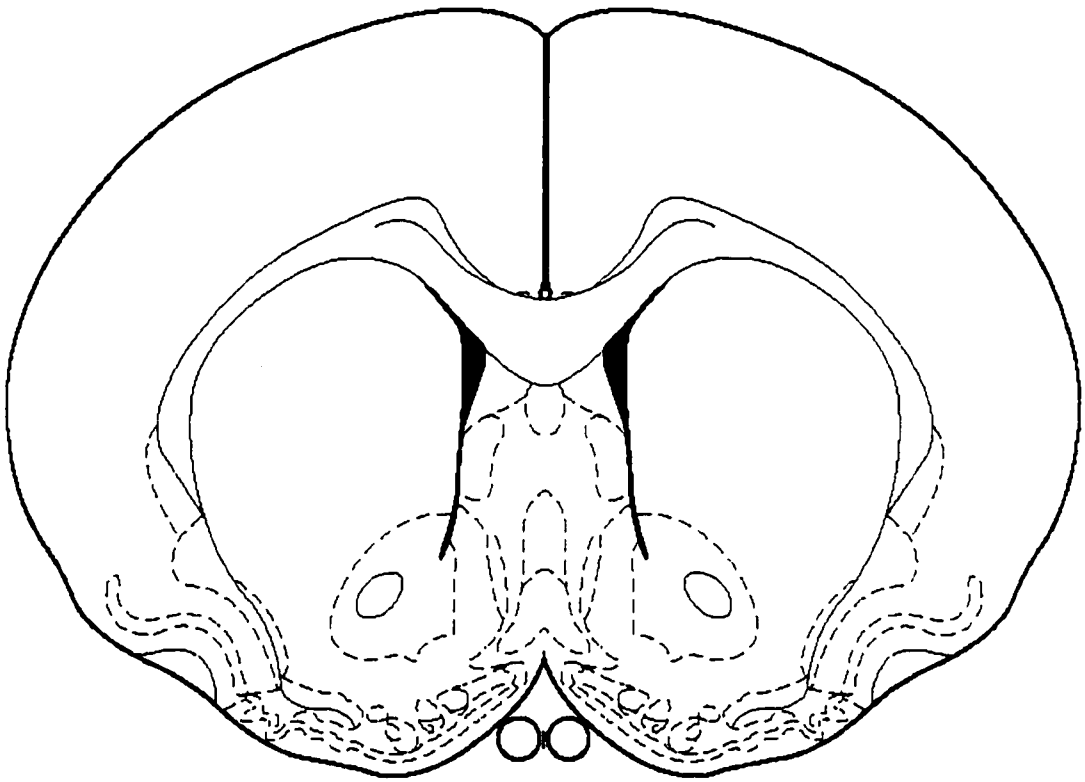
Immunohistochemistry was performed using a standard procedure described by Xu et al. (1997) (Xu *et al*, 1997b). Free floating sections were washed 3 times (10 minutes per wash) with 0.01 M phosphate-buffered saline (PBS) and then incubated for 10 min in PBS containing 0.3% hydrogen peroxide to block endogenous peroxidase activity. Sections were then washed in PBS an additional three times, and incubated for 48 hr at 4°C in NeuN primary antisera [containing PBS with 0.3% Triton-X, 0.02% azide (1:200; provided by Dr. R.J. Mullen)]. Sections were then washed three times in PBS and incubated in biotin-labelled donkey anti-mouse secondary antibody (1:200; Jackson laboratories, Mississauga, ON) for 12 h at 4°C. Once again, sections were washed three times in PBS, followed by incubation for 3 hours at room temperature in PBS containing 0.3% Triton X-100 and streptavidin-horseradish peroxidase (1:200; Amersham Life Science Inc, Oakville, ON). After three more washes, the sections were rinsed in 0.1M acetate buffer (pH 6.0) and the

reaction was visualized using a glucose oxidase-diaminobenzidine (DAB)-nickel method (Shu *et al*, 1988). The reaction was terminated by washing in acetate buffer (0.1M, pH 6.0), and sections were mounted on gelatin-coated slides, dehydrated through a graded series of alcohols and xylene, and coverslipped for microscopic observation.

C. Quantification of NeuN immunoreactivity

NeuN immunoreactivity in the striatum was quantified using an image analysis system equipped with Northern Eclipse 5.0 software (Nigel Banner, EMPIX Imaging). Digitization of sampled areas (600 x 450 μm) was performed using a 10x objective (100x magnification) using a Sony 3CCD colour video camera linked to a microscope. Thresholding was performed on the digitized image in order to eliminate small positive profiles, such as fragments of nuclei and weakly stained cells from the final analysis. To ensure consistency between measurements, the appropriate threshold value was chosen on the basis of the nuclear size. Thresholding was performed on the digitized image until the average diameter of the stained nuclei was 7-8 μm thick. A total of two measurements per section, and a minimum of two sections per animal, were taken for each animal (Fig. 3).

Fig. 3. Schematic diagram of a representative section used for the counting of NeuN-positive neurons in the medial and lateral striatum. Digitization of sampled areas (600 x 450 μm) was performed 100 X magnification. A total of two measurements were performed per section, and a minimum of two sections were sampled for each animal. Measurements were performed at the same coordinates used for injection of the QA and/or adenoviral vectors.



III. UPREGULATION OF IAP PROTEINS FOLLOWING ADENOVIRAL INJECTIONS

A. Detection of upregulation of IAPs by Western blot analysis

Cytoplasmic proteins were extracted from tissue which, one week prior, had been injected with the adenoviral constructs containing either the *xiap* or *lacZ* genes. Animals were decapitated and the ipsilateral striata removed for protein analysis. The contralateral striatum was used as a control for each animal. Cytosolic protein extraction was performed as described by Doucet et al. (1990) using a 0.3 M sucrose buffer (1mM EGTA, 25 mM NaCl, 15 mM Tris-HCl, pH 6.8 (1mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, 2 μg/ml leupeptin). Tissue was placed in sucrose buffer and homogenized using a hand-held dounce. Samples were centrifuged and the supernatant collected and stored at -20°C. The concentration of protein was determined using the Bio-Rad protein colorimetric assay (Hercules, CA, 500-0006), which is based on the Bradford dye-binding procedure whereby the colour change of Coomassie blue in response to various concentrations of proteins is read at 595nm.

Equivalent amounts of cytoplasmic protein were subjected to electrophoresis in a 10% gel SDS-PAGE system as described by Doucet et al. (1990). The proteins were then wet transferred electrophoretically to a polyvinylidene difluoride membrane (NEN, Boston, MA) as described previously (Towbin et al., 1979) at 80 volts for one hour. Membranes were subsequently incubated with the primary antibody, XIAP (1:1000) or α-actin (1:1000; Amersham Life Science Inc, Oakville, ON) or β-Galactosidase (Promega, 1:1000) in a 5%

skim milk solution with Tris-buffered saline with tween-20 (TBST) over-night at 4°C. Membranes were then washed three times in TBST for 10 min at room temperature, and incubated in horseradish peroxidase-linked secondary antibodies, anti-rabbit (XIAP or β-Galactosidase) and anti-mouse (α-actin)(1:2000 and 1:5000, respectively; Amersham Life Science Inc) in a 5% skim milk solution with TBST for one hour at room temperature. The blots were then developed using enhanced chemiluminescence (ECL, Amersham Life Science Inc).

B. Quantification of IAP protein upregulation

The upregulation of striatal IAP proteins by Western blot was quantified using the Image 1.47 software (Wayne Rasband, NIMH). Autoradiographs were scanned using a flatbed scanner (300 DPI), and the bands compared using the actin loading control for calibration.

IV. EFFECTS OF ADENOVIRALLY-MEDIATED OVEREXPRESSION OF THE IAPs ON QA-INDUCED NEURONAL DEATH

A. Experimental Protocol

The neuroprotective potential of NAIP and XIAP in the intrastriatal QA model of HD was assessed by adenovirally-mediated overexpression of *xiap* and *naip* one week prior to QA injections. Three groups of male Wistar rats (n=8; 200-250g) received bilateral stereotaxic injections of a 3 μl suspension containing 2.5×10^7 viral particles encoding either

lacZ, *naip* or *xiap* into the dorsal striata. One week later, all animals received a single deposit of QA (120 nmol in 2 µl, Sigma) into the striatum at the same coordinates as the adenovirus injection (AP = +1.2, ML.= ±2.6, DV = -4.5 mm from bregma). A sham group received bilateral *Ad.lacZ* injections, followed one week later by bilateral saline injections at the same coordinates. One week following QA injections, QA-induced deficits in spatial navigation were assessed using the Morris Water Maze test. One day following completion of the behaviour assessments, animals were deeply anaesthetized and transcardially perfused as described above.

B. Behavioural assessment using the Morris Water Maze

A consequence of bilateral intrastriatal QA lesions is the development of spatial learning and motor performance deficits (Furtado & Mazurek, 1996; Block *et al*, 1993). The Morris Water Maze is used to assess these behavioural changes.

The water maze is a pool 170 cm in diameter and 90 cm deep. A clear plastic platform (approximately 18 cm in diameter) is placed 50 cm from the wall of the pool and 3 cm below the surface of the water so that it is hidden from the view of an animal swimming in the pool. Thus, animals learn to find the platform by relying on external visual cues. The time required for an animal to reach the submerged platform, its escape latency, is used as an indication of the animal's spatial navigation abilities. During pretraining, animals were released into the pool. If they failed to find the platform within 120 seconds they were manually guided to it. Animals were then given a 60 second rest on the platform before commencing another trial. Each animal was trained for four days at four trials per

day.

The water maze was used to determine whether adenovirally-mediated XIAP and NAIP overexpression in QA-lesioned rats, pretrained in the water maze, attenuated deficits in motor performance, and spatial learning capacity. Male Wistar rats (200-250g) received bilateral striatal injections of the adenoviral constructs (3 µl containing 2.5 x 10^7 viral particles) encoding either *lacZ*, *naip*, or *xiap* (n=8 for each group). Seven days later, animals in the treatment groups received bilateral QA injections (120 nmol) at the same coordinates as the adenoviral injections (AP = +1.2, ML.= ±2.6, DV = -4.5 mm from bregma). Animals injected with *lacZ*, followed one week later by saline, served as sham operated controls (n=8).

One week following QA/saline injections, each rat's performance in the water maze was assessed. After three days of measuring the rat's ability to find the submerged platform, the platform was raised 2 cm above the surface of the water and was wrapped in black plastic to make it more visible. This final day of testing served to control for possible deficits in spatial processing, and thus ascertain whether the treatment groups differed from the sham-operated controls in locomotor abilities. The following day, all animals were transcardially perfused with saline (0.9%; 200ml) followed by an equivalent volume of paraformaldehyde (4.0%) in phosphate-buffered saline (PBS). Perfused tissues were postfixed, cryoprotected, and assessed for differences in neuronal survival by immunohistochemical staining with the NeuN antibody.

V. MECHANISM OF CELL DEATH FOLLOWING INTRASTRIATAL QA INJECTIONS

A. Experimental protocol

Various biochemical markers were used to confirm that QA lesions induced neurons to die via apoptosis. Male Wistar rats (n=2 per time point; 200-250 g) were given unilateral intrastriatal injections of QA (120 nmol) as described above. Animals were decapitated 1, 3, 6, 12, 24, and 72 hrs following QA administration and their brains were removed and frozen in isopentane cooled on dry ice (-65°C). Brain sections (12 μm thick) were cut through the striatum using a cryostat.

B. Immunohistochemical detection of DNA fragmentation using *In Situ* End Labeling

QA-induced DNA fragmentation was assessed using *in situ* end labeling as described by Xu et al. (1997a). Fresh frozen cryostat sections were thawed and fixed with 1% glutaraldehyde for 15 minutes at room temperature. Sections were washed three times for 5 minutes in 0.01 M PBS followed by permeabilization in methanol/acetone (1:1) for 10 minutes, and another three washes in PBS. Sections were then incubated with 20 μg/ml proteinase K in 25 mM Tris-HCl (Boehringer Mannheim, Laval, PQ), pH 6.6 for 15 minutes at room temperature. The tissue was then washed twice in double distilled water (ddH_2O) for 15 minutes and stained, in the dark, with Hoescht 33258 (0.5 μg/ml; Sigma)for 30 minutes at room temperature. Sections were once again washed three times (5 minutes per wash) with PBS and incubated in ISEL reaction mixture containing 2 mmol/l $CoCl_2$

(Boehringer Mannheim, Laval, PQ), 10μmol/l biotin-16-dUTP (Boehringer Mannheim), Terminal Transferase buffer (Boehringer Mannheim), ddH_20, and 25 U terminal transferase (Boehringer Mannheim) for 1 hour at 37°C in a humid chamber.

The reaction was terminated by washing the sections three times (1 minute per wash) with PBS and incubating them with 250μl/slide of staining solution containing 2.5 μg/ml avidin-FITC (Sigma), 4 X saline-sodium citrate buffer, 0.1% triton-X and 0.5% powdered milk for 30 minutes at room temperature in the dark. The sections then underwent a final three washes with PBS (1 minute per wash) and were coverslipped with antifade solution containing 1 mg/ml *p*-phenylenediamine in 90% glycerol in PBS. Positive controls were prepared by incubation with 10 U/ml DNAse I for 20 minutes at 37°C before treatment with terminal transferase.

C. Immunohistochemical detection of active caspase-3 and caspase-3-cleaved amyloid-β precursor protein ($\alpha\Delta C^{Csp}$-APP)

Immunohistochemical detection of active caspase-3 was performed using an antibody which specifically recognizes the catalytically active caspase-3 tetramer $(p17/p12)_2$ (Rasper et al., 1998). Fresh frozen cryostat sections (12μm) were cut and processed for immunohistochemical detection. Slide mounted sections were washed three times with 0.01 M phosphate-buffered saline (PBS) and then incubated for 48 hr at 4°C with the antibody that recognizes active caspase-3 (provided by Dr. D.W. Nicholson) in 0.01M PBS containing 0.3% Triton X-100. Sections were washed another three times in 0.01 M PBS and incubated, in the dark, for 3 hours at room temperature in PBS containing 0.3% Triton X-100 and CY3-

labelled donkey anti-rabbit IgG (1:200, Amersham Life Science Inc, Oakville, ON). Sections were washed a further three times in 0.01 M PBS in the dark and cover slipped using antifade, 1 mg/ml *p*-phenylenediamine in 90% glycerol in PBS.

Immunohistochemical detection of $\alpha\Delta C^{Csp}$-APP was used to confirm caspase-3 activation. A similar protocol to the caspase-3 immunofluorescence was used to detect the caspase-3 cleaved APP (denoted $\alpha\Delta C^{Csp}$-APP). Tissue sections were washed three times in 0.01 M PBS and then incubated with the $\alpha\Delta C^{Csp}$-APP primary antibody (1:1000; provided by Dr. D.W. Nicholson) for 48 hrs at 4°C. Sections were washed another three times and incubated, in the dark, for 3 hours at room temperature in PBS containing 0.3% Triton X-100 and CY3-labelled donkey anti-rabbit IgG (1:200, Amersham Life Science Inc.). Sections were washed a final three times in 0.01 M PBS in the dark and cover slipped using antifade, 1 mg/ml *p*-phenylenediamine in 90% glycerol in PBS.

RESULTS

I. EFFECT OF INTRASTRIATAL QUINOLINIC ACID ADMINISTRATION ON NEURONAL SURVIVAL

Intrastriatal injection of QA resulted in significant neuronal destruction, as indicated by NeuN immunohistochemistry, relative to saline-injected controls (Fig. 4). Average cells were determined by counting the number of labelled cells in an area 450 x 600 μm using computer assisted image analysis. The average neuron count was 38 ± 21/area of measurement (450 x 600 μm) for the QA-lesioned animals (n=2), and 331 ± 58 for the saline-injected animals (n=2)(Fig.5).

Fig. 4. Representative sections from animals injected with saline (A) or QA (B). Note the loss of neurons in the dorsal striatum 1 week after injection of QA (120 nmol).

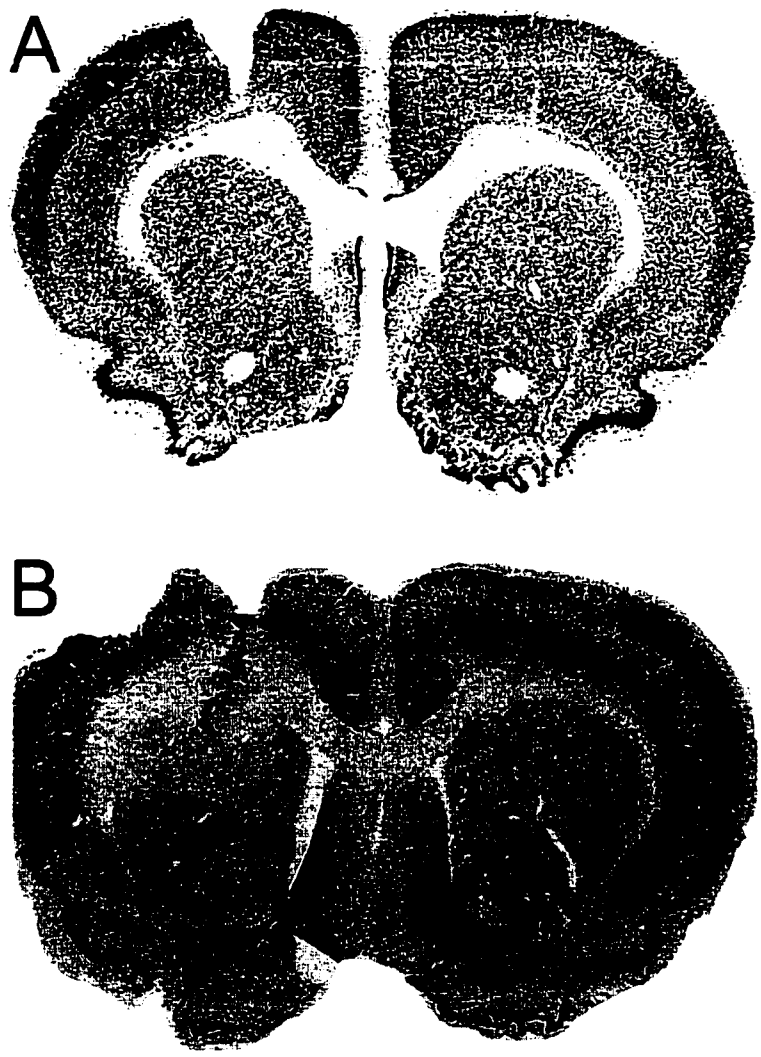
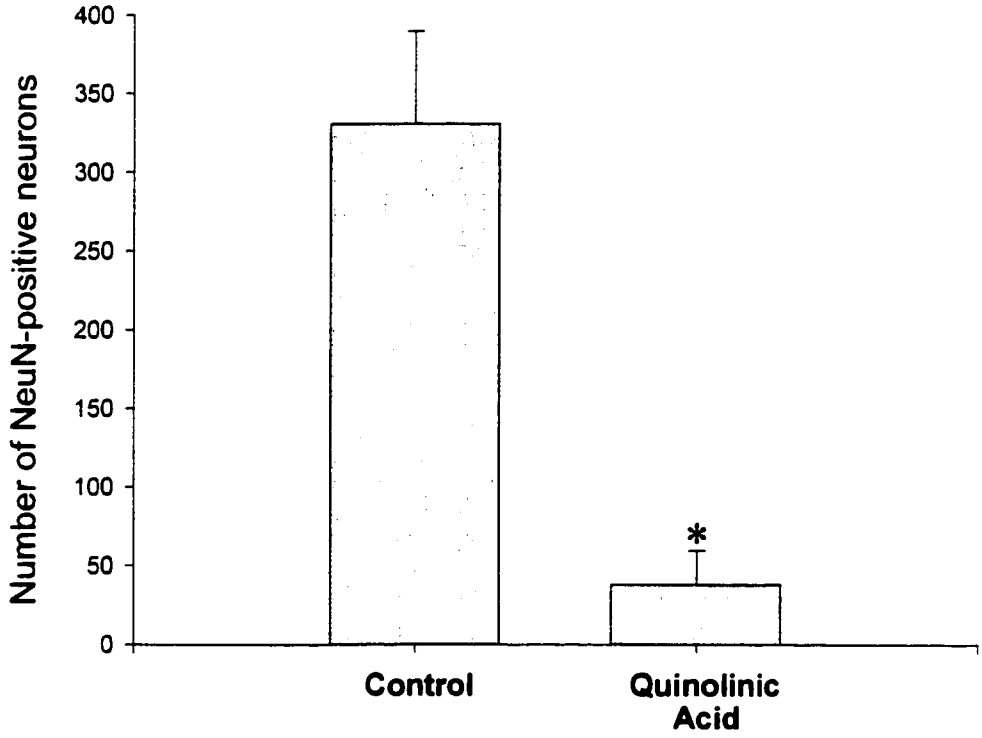

A
B

Fig. 5. Intrastriatal injection of QA results in the destruction of striatal neurons as detected by NeuN immunoreactivity. Cell counts of NeuN-immunoreactive neurons revealed that the average neuronal density for the saline and QA injected groups was 331 ± 58 and 38 ± 21, respectively (n=2). Statistical analysis using an unpaired t-test revealed that significantly more labeled neurons were present in the saline that the QA treated group ($p < 0.01$).


Number of NeuN-positive neurons
400
350
300
250
200
150
100
50
0
*
Control
Quinolinic Acid

II. UPREGULATION OF IAP PROTEINS BY USE OF ADENOVIRAL CONSTRUCTS CONTAINING IAP GENES

A. Intrastriatal injection of the adenovirus containing *xiap* leads to overexpression of XIAP by Western blot analysis

As shown by Western blot analysis (Fig. 6), intrastriatal injection of the adenovirus encoding human XIAP resulted in a ten-fold increase in levels of human XIAP protein in the ipsilateral striatum, relative to the contralateral side which received no injection.

B. Intrastriatal injection of the adenovirus containing the *xiap* gene leads to overexpression of the XIAP protein by immunohistochemistry

Adenovirally-mediated overexpression of XIAP protein in the striatum was also shown using immunohistochemistry (Fig. 7). Intrastriatal injection of the adenovirus containing *xiap* resulted in an upregulation of human XIAP staining in the ipsilateral striatum. Maximal XIAP staining was observed near the cannula tract. No human XIAP staining was observed in the contralateral striatum. Cell counts revealed an average of 60 ± 27.6 immunoreactive neurons/area of measurement (450 x 600 μm) staining for XIAP on the ipsilateral side and no staining observed on the contralateral side (Fig. 8).

Similar results were observed with the adenoviral construct containing *lacZ*. Intrastriatal injection of the LacZ adenoviral vector resulted in similar infectivity and upregulation of the β-Galactosidase protein (Fig. 7). Cell counts for β-Galactosidase showed an average of 48 ± 16 immunoreactive neurons /area of measurement (450 x 600 μm) on the

ipsilateral side and no staining observed on the contralateral side (Fig. 8).

Fig. 6. Western blot analysis indicates that injection of the adenoviral construct containing XIAP leads to an upregulation in XIAP protein one week following injection of 2.5×10^7 pfu. XIAP immunoreactive proteins in cytosolic extractions from the intact and contralateral striatum. Immunoreactivity was detected using a human XIAP antibody. A single band was observed at 57 kDa. Ten micrograms of protein sample was loaded in each well and actin was used as a loading control.

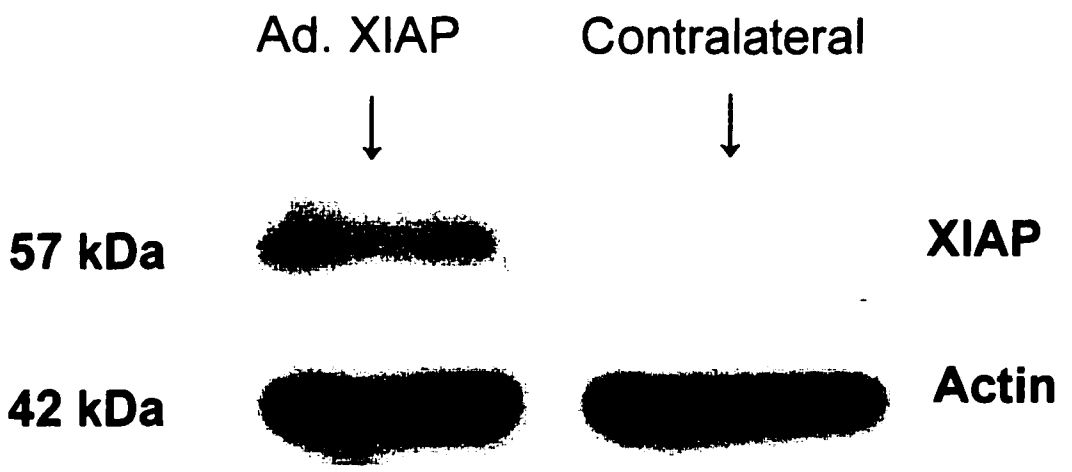
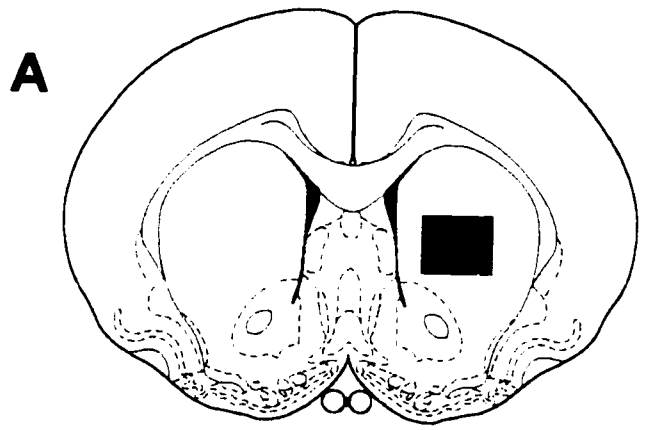

Ad. XIAP
Contralateral
57 kDa
XIAP
42 kDa
Actin

Fig. 7. Schematic diagram representing the area of interest in B and C (A). Virally mediated overexpression of XIAP and lacZ was confirmed by immunohistochemical detection of human XIAP and β-galactosidase in the ipsilateral striata, respectively (B, D). Scale bar = 200 μm. Insets are higher power images. Scale bar = 40 μm. Immunohistochemistry for human XIAP and β-galactosidase in the contralateral striata can be seen in C and E, respectively. Scale bar = 200 μm.


A

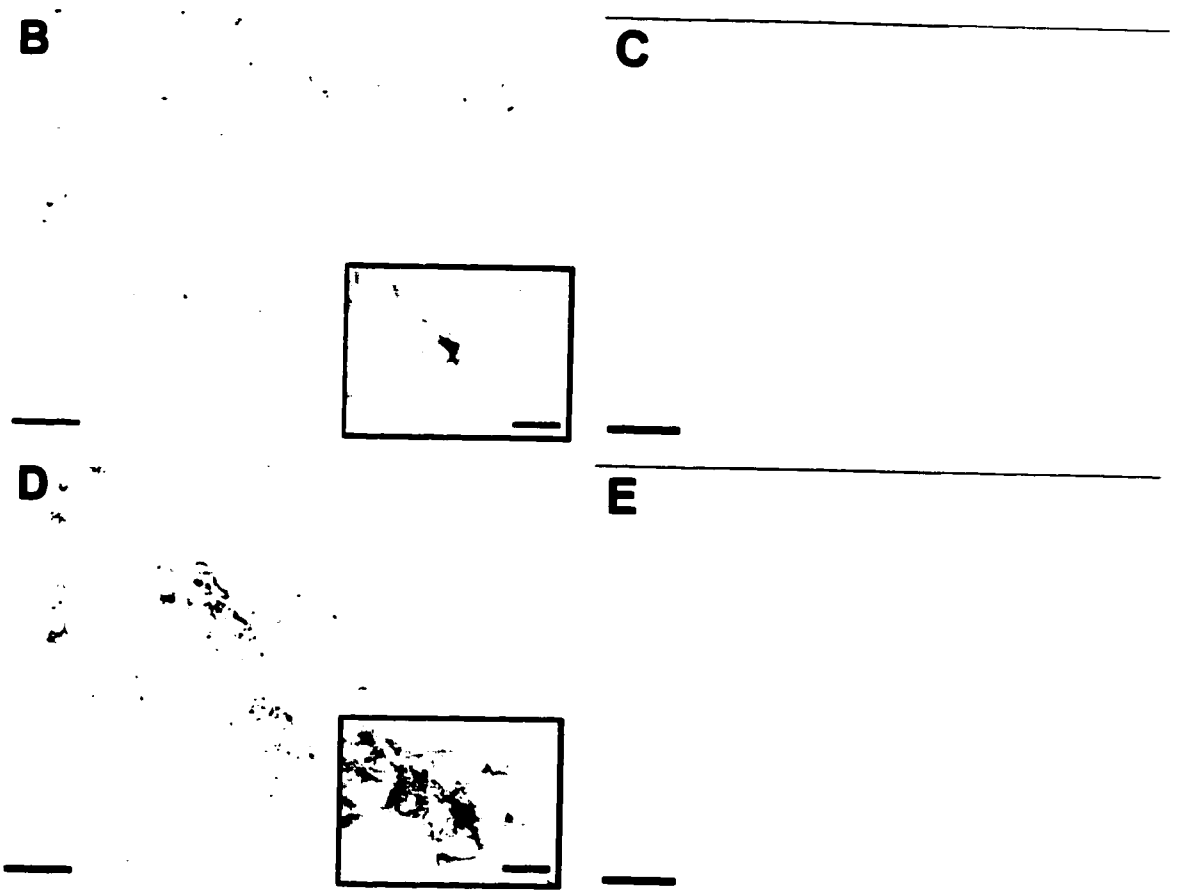
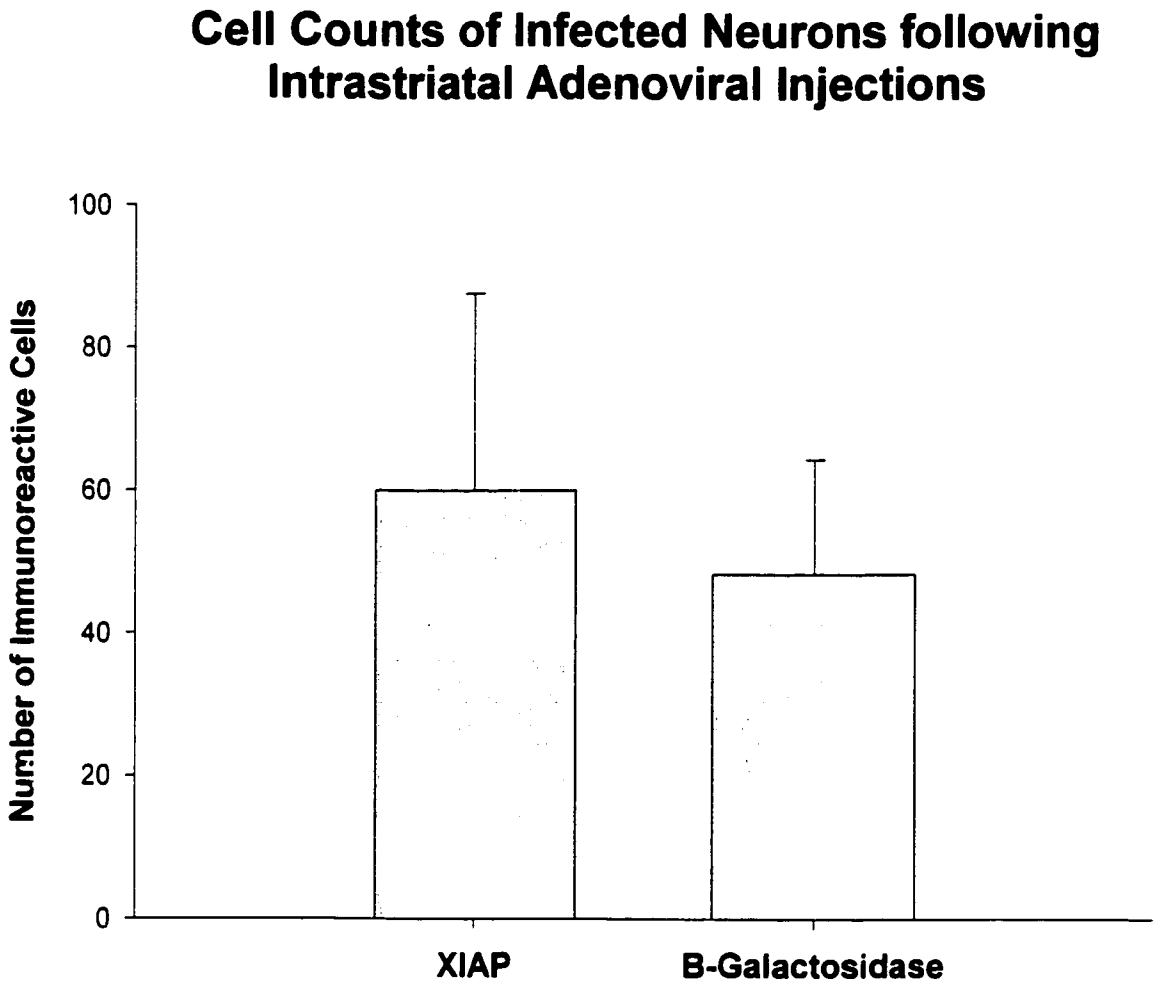

B
C
D
E

Fig. 8. Cell counts of infected neurons following intrastriatal adenoviral injections. Virally mediated overexpression of XIAP and lacZ was confirmed by immunohistochemical detection of human XIAP and β-galactosidase in the ipsilateral striata. Cell counts of XIAP and β-galactosidase immunoreactive neurons in the striatum showed counts of 60 ± 27 and 48 ± 16 /area of measurement (450 x 600 μm), respectively.


Cell Counts of Infected Neurons following Intrastriatal Adenoviral Injections
Number of Immunoreactive Cells
100
80
60
40
20
0
XIAP
B-Galactosidase

III. EFFECTS OF ADENOVIRALLY-MEDIATED OVEREXPRESSION OF THE *iap* GENES ON QA-INDUCED EXCITOTOXIC LESIONS

This study examined whether overexpression of the IAPs conferred protection against striatal QA lesions. This was determined both histologically, using the neuron specific antibody NeuN to assess neuron survival, and behaviourally, using the Morris Water Maze test to determine deficits in spatial learning.

A. Adenovirally-mediated XIAP overexpression reduces neuronal loss following intrastriatal QA administration

The effect of upregulation of adenoviral constructs containing XIAP, NAIP, or LacZ on QA-induced neuronal loss is shown in Fig. 9. Cell counts of NeuN-immunoreactive neurons revealed that sham treated animals, which were injected with the LacZ construct followed, one week later, by an injection of saline, had an average neuronal count of 419 ± 23 (n=8) (Fig. 10). Animals which received injections of the LacZ adenoviral construct, followed one week later, by a QA injection, had an average neuronal count of 80 ± 17 (n=7). Animals injected with XIAP, followed one week later by QA had an average cell count of 362 ± 25 (n=7). Animals injected with NAIP, followed one week later by QA had an average neuronal count of 190 ± 33 (n=8).

Fig. 9. IAP overexpression leads to a reduction in neuronal loss following bilateral intrastriatal QA administration. Immunohistochemical detection of neurons with NeuN antibody of a sham operated animal which received bilateral injections of saline (A). A reduction in NeuN-positive neurons was observed in animals which were injected bilaterally with *lacZ* one week prior to QA injection (B). An attenuation of neuronal loss was observed in animals that received bilateral injections of the adenovirus containing XIAP one week prior to QA lesions (C). A reduction in lesion size was also observed in animals which received bilateral NAIP injections one week prior to QA lesions (D).

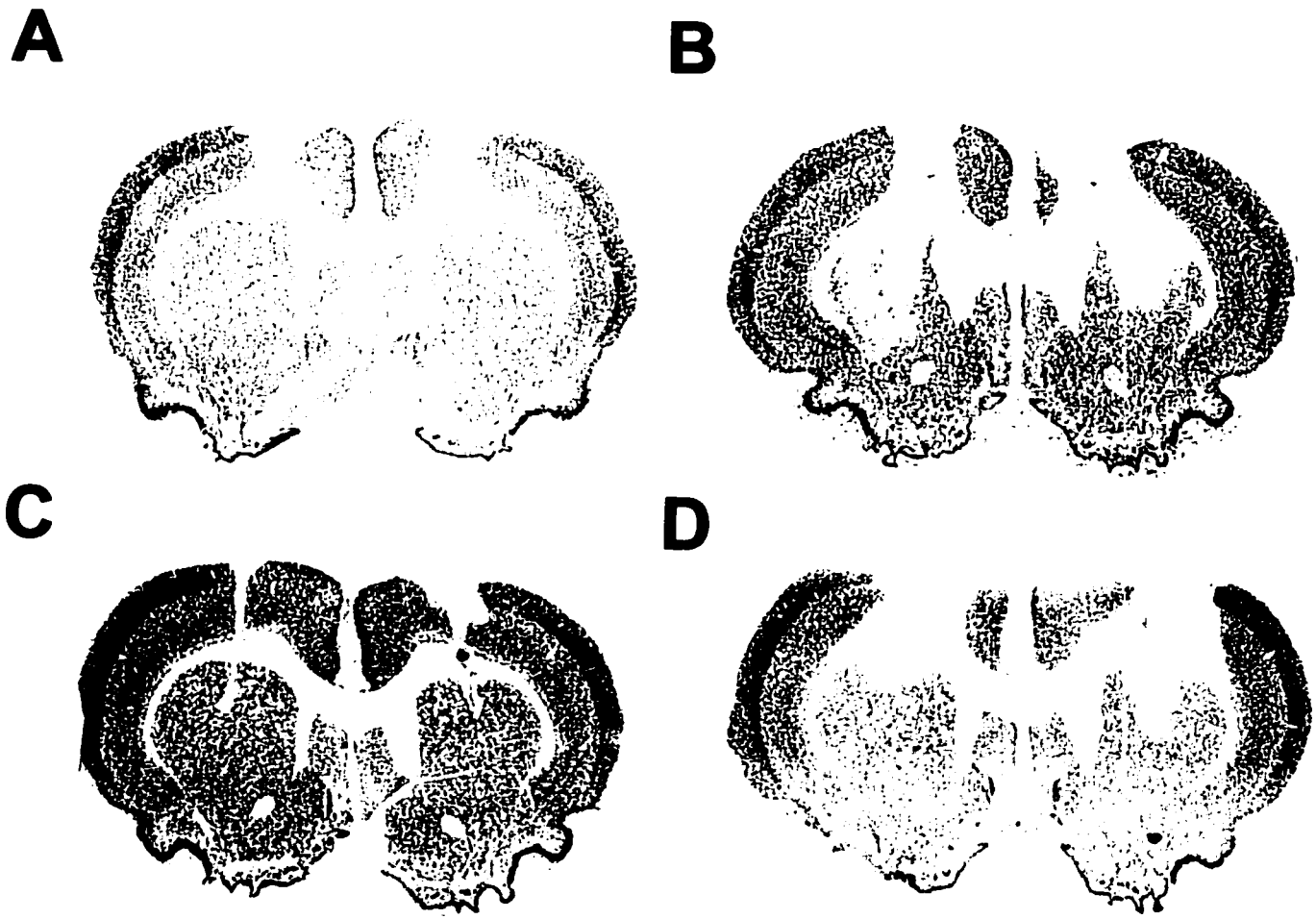
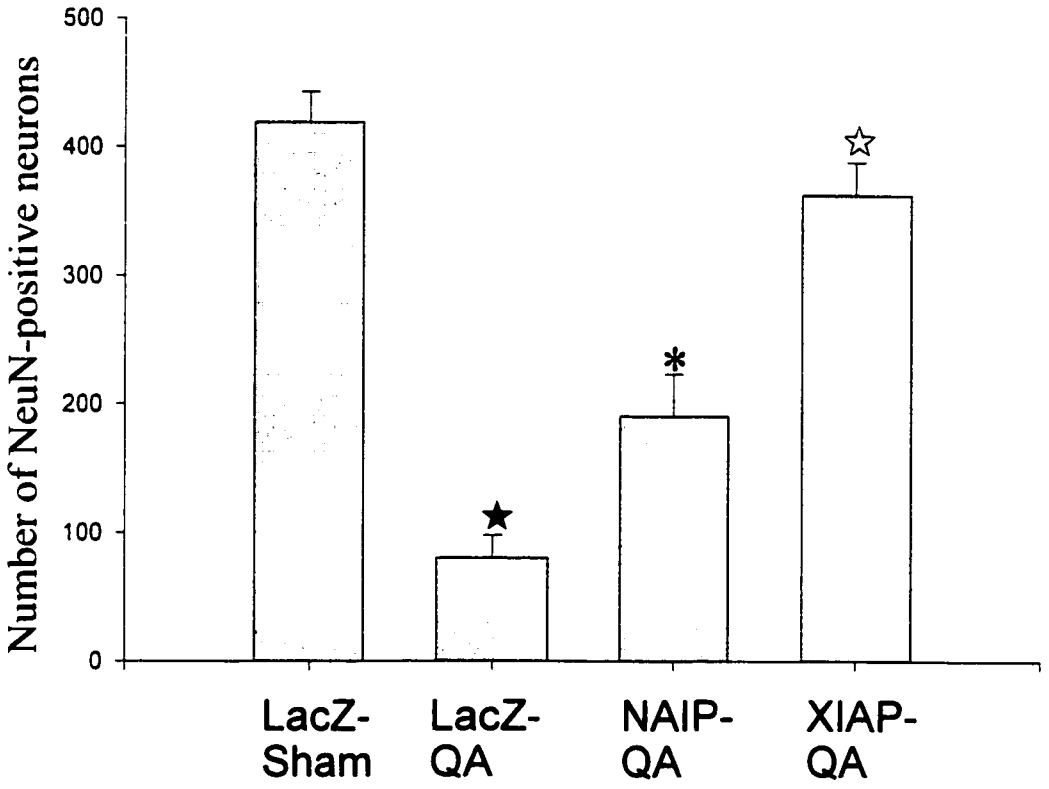

A
B
C
D

Fig. 10. IAP overexpression leads to a reduction in neuronal loss following intrastriatal QA administration. Cell counts of NeuN-immunoreactive neurons revealed that the average neuronal density for the lacZ-Sham, lacZ-QA, NAIP-QA, and XIAP-QA groups was 419 ± 23, 80 ± 17, 190 ± 33, and 362 ± 25, respectively (n=8). Statistical analysis revealed that significantly more labeled neurons were present in the XIAP-QA than the lacZ-QA group and NAIP-QA group ($p < 0.05$), and significantly more labeled neurons were present in the NAIP-QA than the lacZ-QA group ($p < 0.05$). ★ $p < 0.05$ relative to lacZ-Sham, NAIP-QA, and XIAP-QA. * $p < 0.05$ relative to lacZ-Sham, lacZ-QA, and XIAP-QA. ☆ $p < 0.05$ relative to lacZ-QA and NAIP-QA.


Number of NeuN-positive neurons
500
400
300
200
100
0
LacZ-Sham
LacZ-QA
NAIP-QA
XIAP-QA

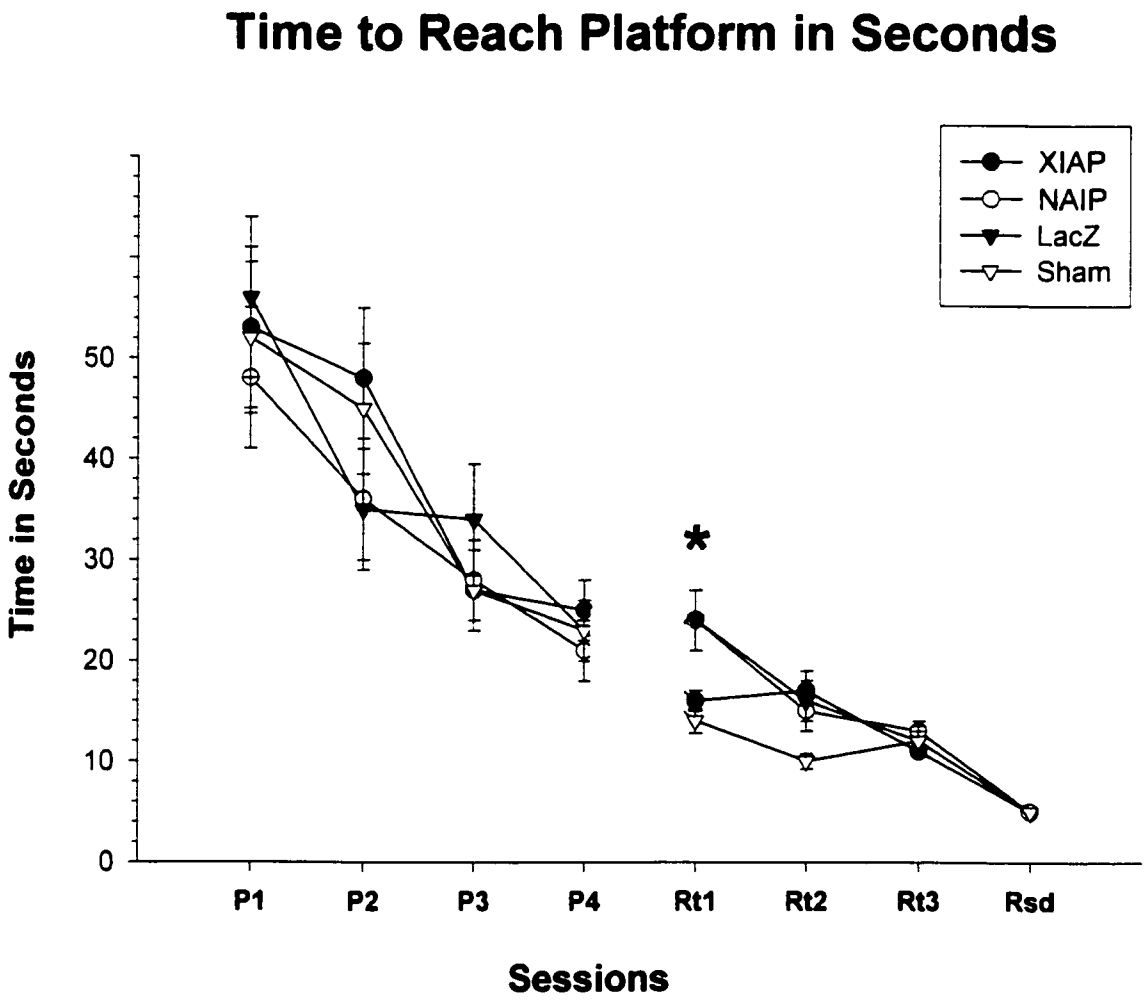
B. Adenovirally-mediated IAP overexpression shows no effect on escape latency following QA lesions using the Morris Water Maze

Animals which have sustained intrastriatal QA lesions, causes deficits in visuospatial memory, take significantly longer to find the platform in the Morris Water Maze task than sham treated controls (Furtado & Mazurek, 1996; Block *et al*, 1993). Since IAP overexpression significantly reduced neuronal death following QA lesions, animals injected with the XIAP adenovirus were expected to perform better that LacZ treated animals.

On the first day of testing (i.e. retention day 1), the sham-treated animals had the shortest escape latency, followed by XIAP, NAIP, and LacZ treated animals. On the first retention day, XIAP animals performed better than LacZ and NAIP animals. Unfortunately this trend did not continue as NAIP and LacZ injected animals rapidly learned to find the platform by the second retention day (Fig. 11).

On the raised platform trial day, the average escape latency was similar for each group indicating that locomotor deficits were not affecting response times.

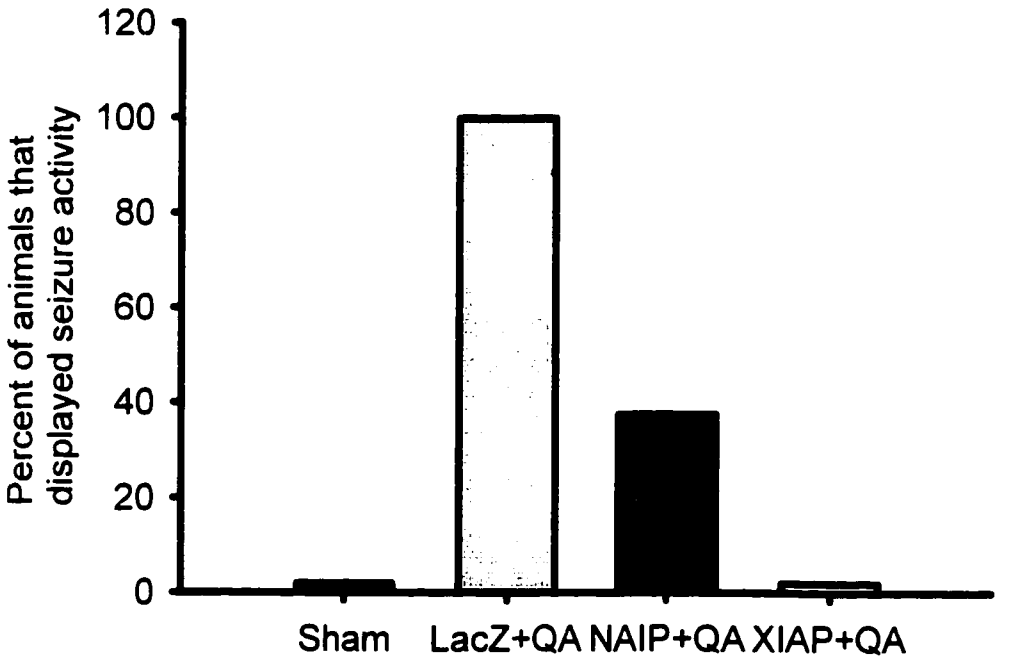
Fig. 11. Animals received bilateral striatal injections of the viral constructs (3 μl containing 2.5 x 10^7 viral particles) encoding either lacZ, NAIP, or XIAP in the dorsal striatum (n=8 for each group). Animals injected with saline served as sham operated controls (n=8). Seven days later, all animals received bilateral QA injections (120 nmol) at the same coordinates as the adenoviral injections. All rats were pretrained in the water maze before injection of the adenoviruses and QA (P1-P4). One week following QA lesions, the performance of all rats was assessed in the water maze using the same protocol employed for the pretraining sessions (Rt1-Rt3). On the last day a cued trial was performed in which the animals were required to find a platform raised 2 cm above the surface of the water and wrapped in black plastic (Rsd).


Time to Reach Platform in Seconds
XIAP
NAIP
LacZ
Sham
Time in Seconds
0
10
20
30
40
50
*
P1
P2
P3
P4
Rt1
Rt2
Rt3
Rsd
Sessions

C. Adenovirally-mediated XIAP overexpression attenuates QA-induced seizure-like activity

Intrastriatal QA administration results in seizure-like activity which begins approximately 30 minutes following the injection and lasts for several hours (Marrannes & Wauquier, 1988). Seizure-like activity was defined as excessive head waving and chewing, rearing, clonic-tonic movements of the contralateral limbs, and barrel rotations. Animals were observed for 5 minutes every 30 minutes for three hours following intrastriatal QA injections. Between episodes of barrel rotations and other seizure-like activities, animals sat quietly in their cages. All animals injected with the LacZ adenovirus followed by QA displayed some form of seizure activity (n=7) (Fig.12). Three of the 8 animals which received NAIP injections followed by QA lesions demonstrated seizure activity. Animals which were injected with XIAP followed by QA showed no seizure activity(n=7). None of the saline treated control animals showed any form of seizure activity (n=8).

Fig. 12. Animals which received prior treatment with XIAP exhibited no QA-induced seizure activity. Animals were intrastriatally injected with QA (120 nmol) and allowed to recover for 30 minutes. Following the onset of seizure activity, animals were observed for a period of 5 minutes every 30 minutes for 3 hours. Seizure activity was defined as excessive chewing, head waving, clonic tonic forepaw extensions, and barrel rotations. The sham group was bilaterally injected with lacZ into the striatum one week before saline injections at the same coordinates. The LacZ + QA group was bilaterally injected with lacZ into the striatum one week before QA injections (120 nmol) at the same coordinates. The NAIP + QA and XIAP + QA groups were bilaterally injected with NAIP and XIAP, respectively one week before QA injections (120 nmol) (n=8).


Percent of animals that displayed seizure activity
120
100
80
60
40
20
0
Sham
LacZ+QA
NAIP+QA
XIAP+QA

IV. CASPASE-3 MEDIATED NEURONAL DEATH FOLLOWING INTRASTRIATAL QA INJECTIONS

Intrastriatal QA administration leads to neuronal death via apoptosis (Portera-Cailliau *et al*, 1995; Thomas *et al*, 1995). The present study examined whether this apoptotic cell death occurred via a caspase-3-dependent mechanism.

A. Effects of intrastriatal QA administration on DNA fragmentation in the striatum

In agreement with previous studies, intrastriatal QA lesions resulted in DNA fragmentation in the ipsilateral striatum (Fig. 13). DNA fragmentation was detected using the *In Situ* End-Labeling (ISEL) technique. No ISEL labeling was observed at 1, 3 or 6 hrs following QA administration (n=2/time-point). ISEL labeling was observed in the ipsilateral striatum 12 hrs following QA administration with cell counts of 152 ± 27. The number of ISEL positive cells peaked at 24 hrs with cell counts of 297 ± 23, and was less abundant at 72 hrs with cell counts of 165 ± 26 (Fig. 14). No ISEL labeling was observed in the contralateral striatum at any time point.

Fig. 13. Quinolinic acid lesions cause DNA fragmentation in striatal neurons. QA-induced DNA fragmentation was assessed using *in situ* end labeling . ISEL labeling was detected in the striatum at 12 hours following QA lesion (B). The number of ISEL labeled cells was greatest at 24 hours (C) and by 72 hours had subsided and was less intense and less abundant (D). Labeling was not seen in the contralateral striatum at any time point (A) (n=2 per time point). Scale bar = 75μm

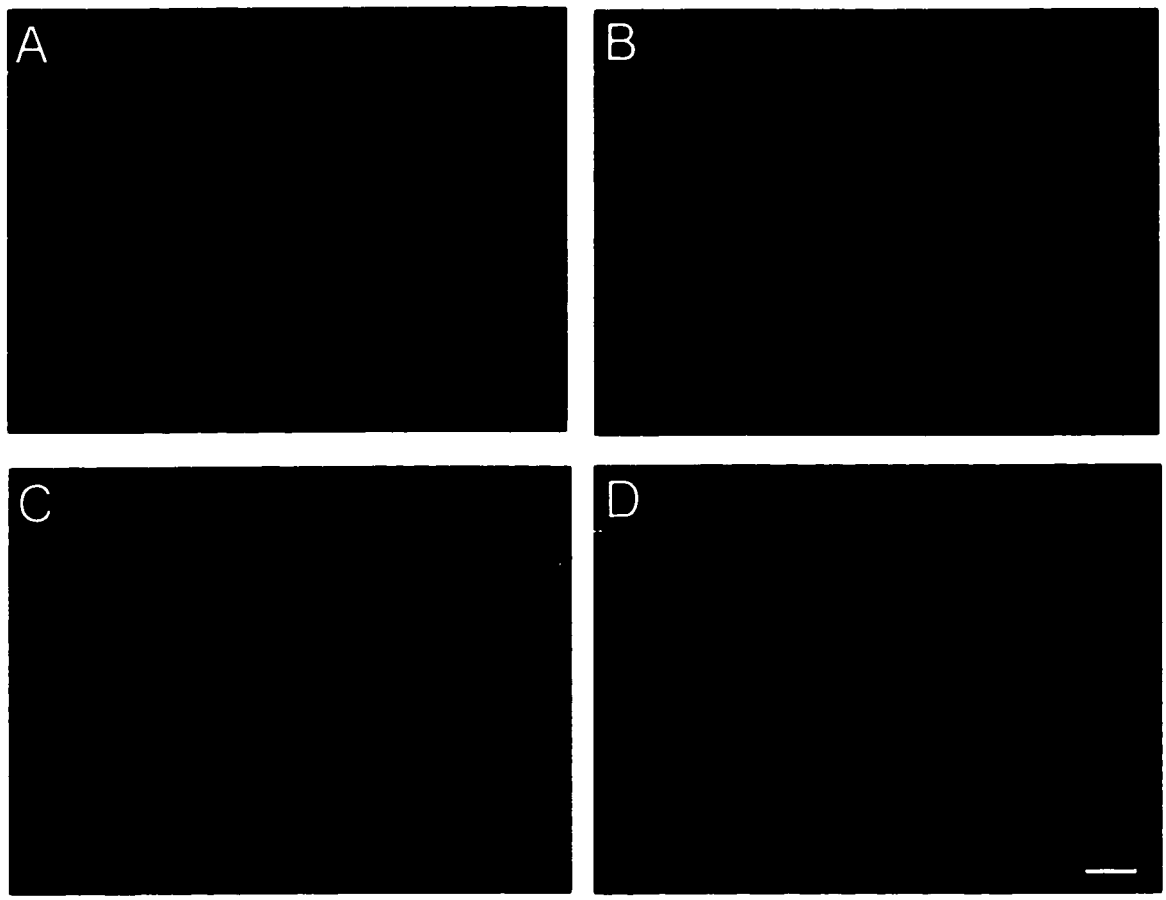
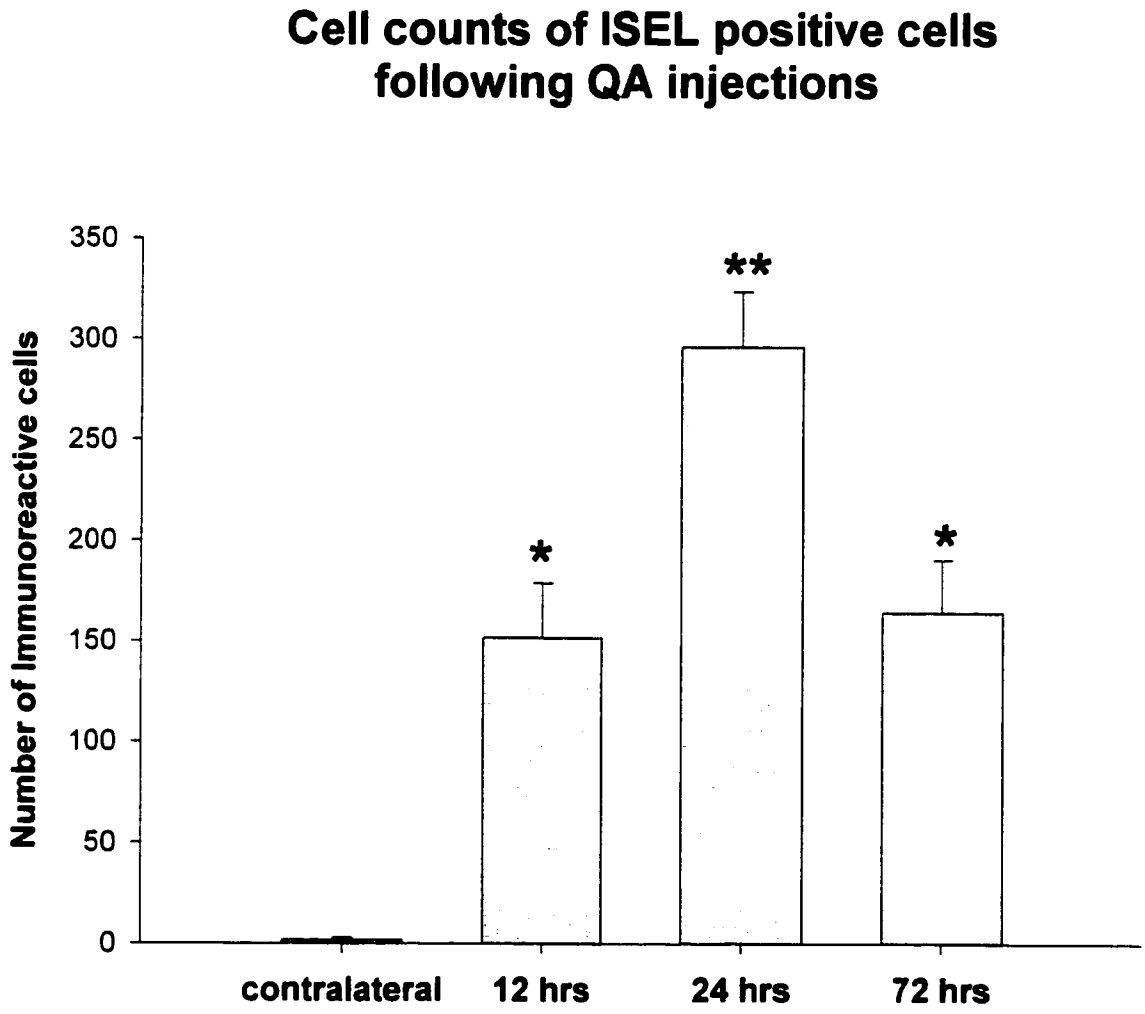

A
B
C
D

Fig. 14. Quinolinic acid lesions cause DNA fragmentation in striatal neurons. Cell counts of ISEL labeled neurons revealed that ISEL labeling was first observed at 12 hours, peaked at 24 hours, and had subsided by 72 hours with cell counts of 162 ± 29, 297 ± 33, and 165 ± 23, respectively (n=2 per time point).

Statistical analysis was performed using an ANOVA followed by a Newman-Keuls test.

** = significantly different from contralateral, 12 hrs, and 72 hrs

* = significantly different from contralateral


Cell counts of ISEL positive cells following QA injections
Number of Immunoreactive cells
350
300
250
200
150
100
50
0
*
**
*
contralateral
12 hrs
24 hrs
72 hrs

B. Effects of intrastriatal QA administration on caspase-3 activation in the striatum

Unilateral QA injections resulted in elevated caspase-3 immunoreactivity in the striatum (Fig. 15). Caspase-3 labeling was not detected in the striatum 1 or 3 hrs following injection of QA, cell counts of 1 ± 0.5 and 1.3 ± 0.9, respectively. Caspase-3 immunoreactivity was first observed in the ipsilateral striatum at 6hrs following QA administration, with cell counts of 373 ± 24 immunoreactive cells (Fig. 16). Maximal staining of active caspase-3 was observed 12 hrs following QA with neuronal cell counts of 405 ± 8. Active caspase-3 staining was less abundant by 24 hrs, with neuronal cell counts of 15 ± 9. No caspase-3 labeling was observed in the contralateral striatum at any time point.

Figure 15: Quinolinic acid (QA) lesions activate caspase-3 in the striatum. Catalytically active caspase-3 was not detected in the striatum of sham treated animals (A). At 1 (B) and 3 (C) hours following QA injections no caspase-3 labeling was found. Active caspase-3 immunoreactivity was first observed at 6 hours following QA injection (D). The number of active caspase-3 labeled cells was greatest at 12 hours following QA injection (E). By 24 hours caspase-3 labeling had subsided(F). Scale bar = 75μm.

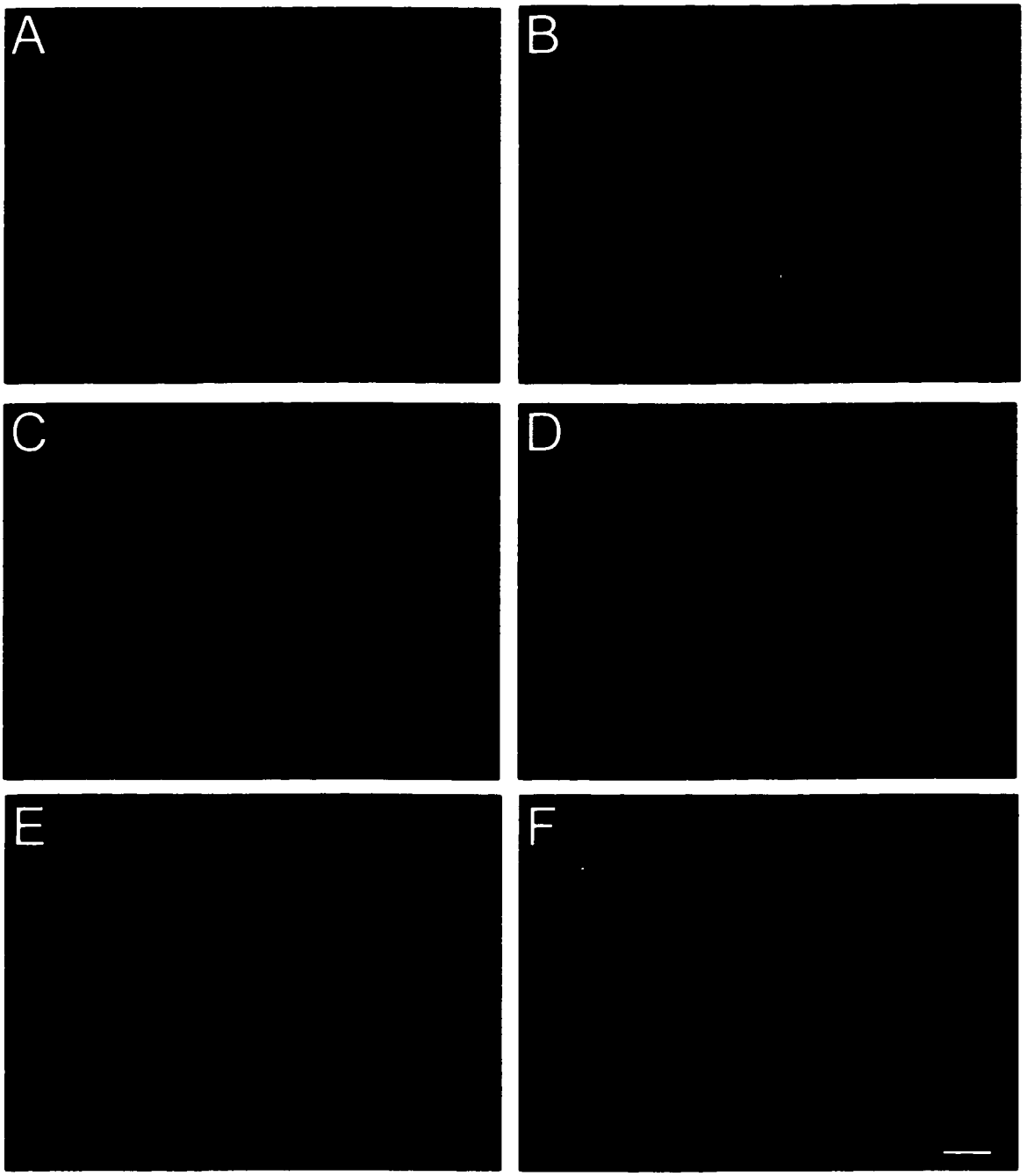
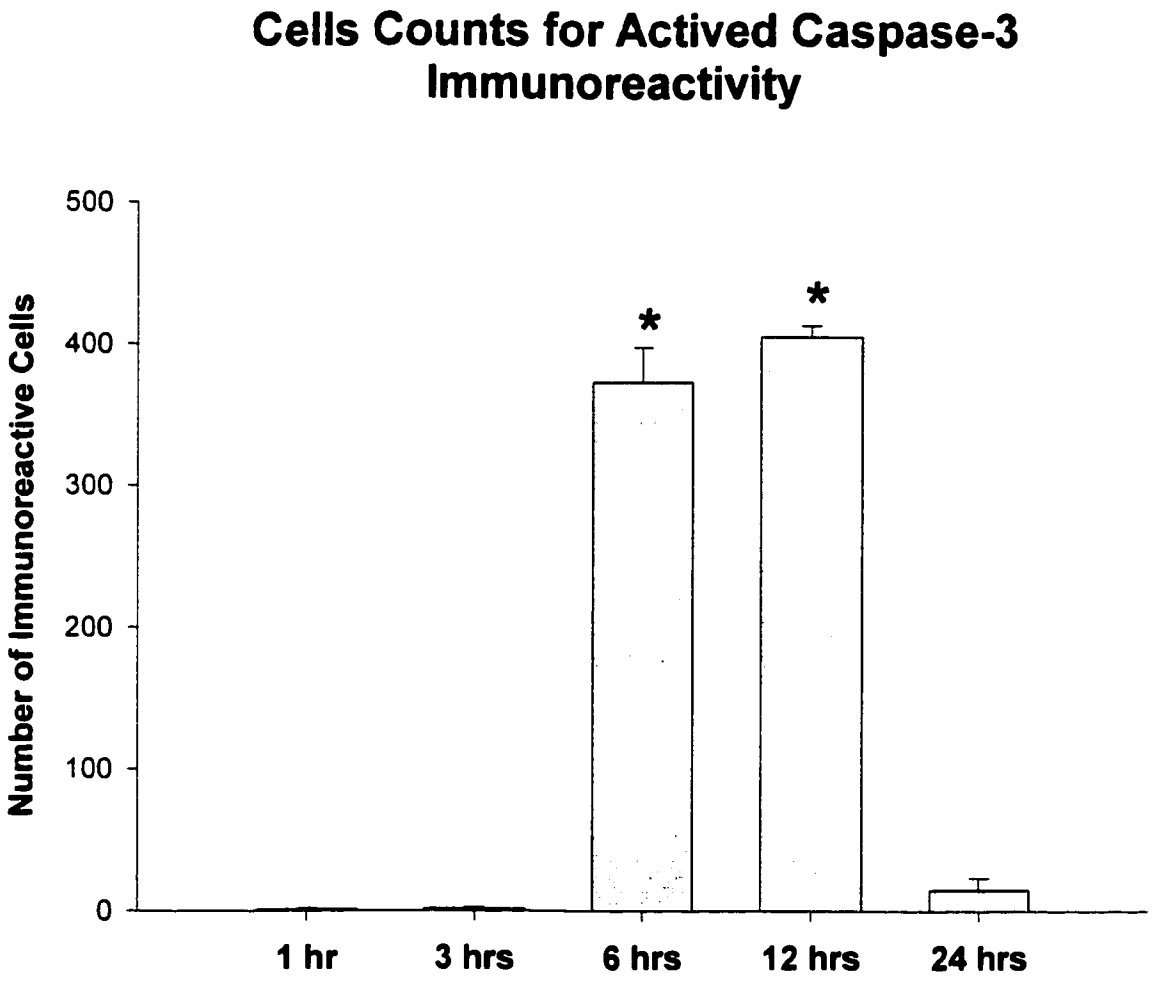

A
B
C
D
E
F

Fig. 16. Quinolinic acid lesions activate caspase-3 in the striatum. Cell counts of activated caspase-3-immunoreactive neurons revealed that very little active caspase-3 was present at one or three hours, 1 ± 0.6 and 1.3 ± 0.9, respectively. Active caspase-3 immunoreactivity was first observed 6 hours following QA injections with cell counts of 373 ± 24, was greatest at 12 hours with cell counts of 405 ± 8, and had subsided by 24 hours with cell counts of 15 ± 8. Statistical analysis using an ANOVA followed by a Newman-Keuls test revealed that significantly more labeled neurons were present at 6 and 12 hrs when compared to 1, 3 and 24 hours ($p < 0.005$).


Cells Counts for Actived Caspase-3 Immunoreactivity
Number of Immunoreactive Cells
500
400
300
200
100
0
*
*
1 hr
3 hrs
6 hrs
12 hrs
24 hrs

C. Effects of intrastriatal QA administration on amyloid-β precursor protein (APP) cleavage in the striatum

Intrastriatal injection of QA resulted in expression of the caspase-3 cleaved form of APP (designated $\alpha\Delta C^{Csp}$-APP). $\alpha\Delta C^{Csp}$-APP immunoreactivity was observed in the ipsilateral striatum as shown in Fig. 17. $\alpha\Delta C^{Csp}$-APP immunoreactivity was first detected at 6 hrs following QA administration with cell counts of 28 ± 5. $\alpha\Delta C^{Csp}$-APP immunoreactivity was less abundant at 12 and 24 hrs with cell counts of 20 ± 7 and 14 ± 8, respectively (Fig. 18). No $\alpha\Delta C^{Csp}$-APP was observed in the contralateral striatum at any time point.

Fig. 17. Intrastriatal QA injections result in $\alpha\Delta C^{Csp}$-APP labeling in the ipsilateral striatum. $\alpha\Delta C^{Csp}$-APP labeling was present at 6hrs and had subsided by 24 hrs. No $\alpha\Delta C^{Csp}$-APP immunoreactivity was observed in the contralateral striatum at any time point.

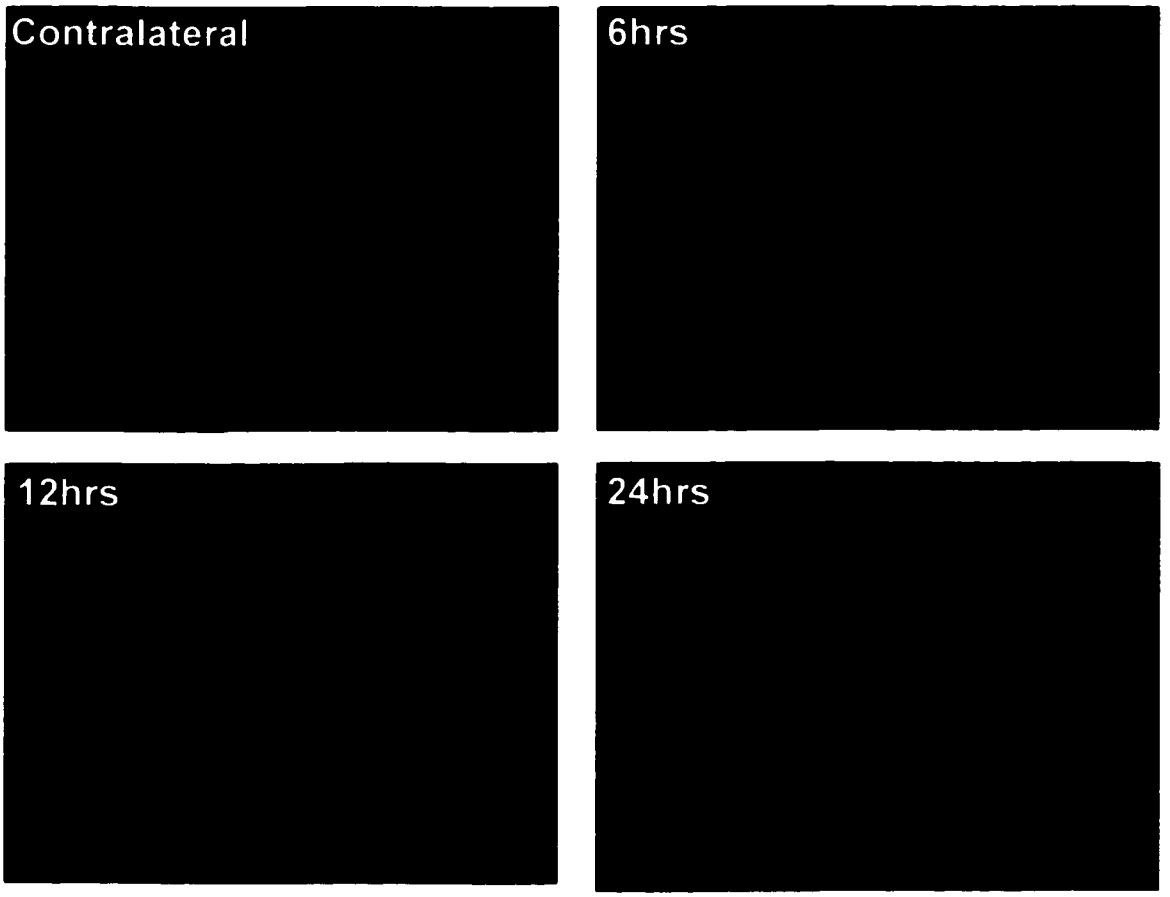
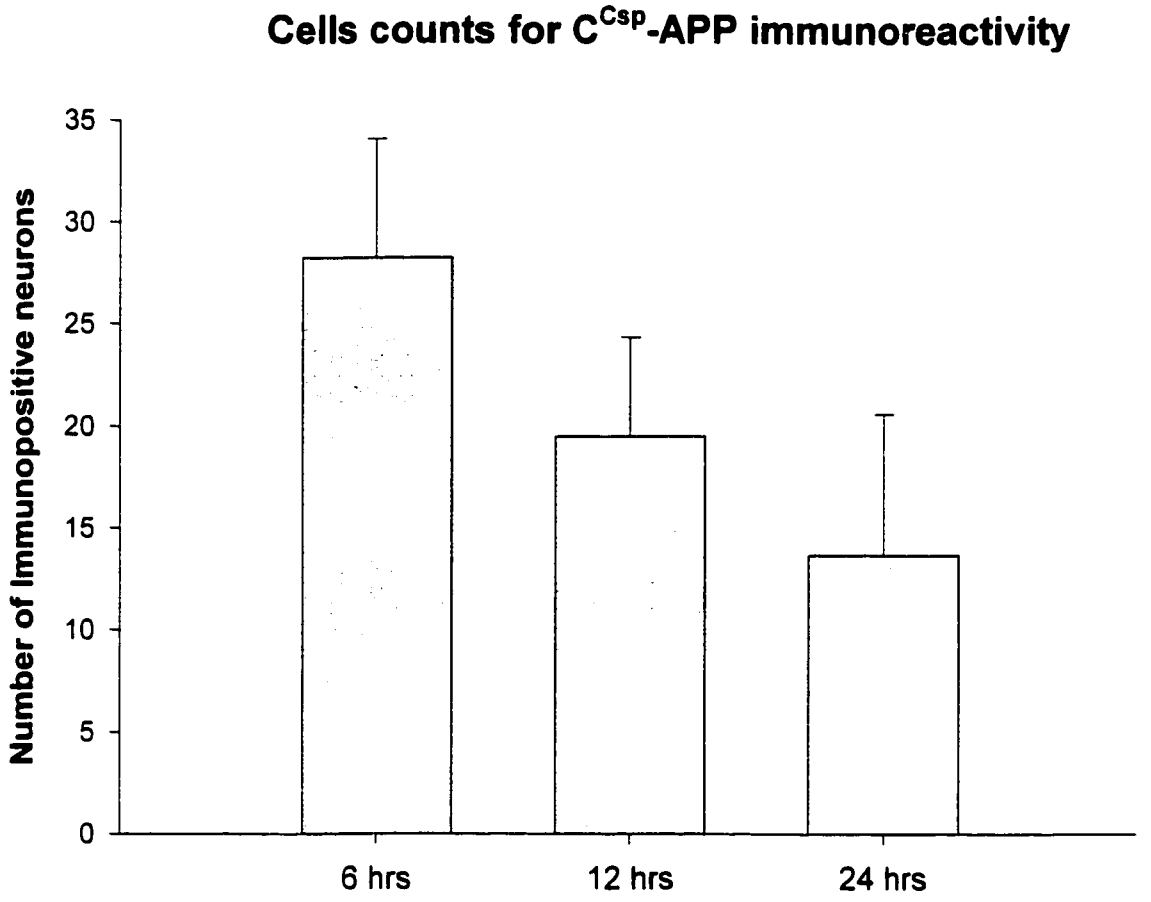

Contralateral
6hrs
12hrs
24hrs

Fig. 18. Intrastriatal QA injections result in $\alpha\Delta C^{Csp}$-APP labeling in the ipsilateral striatum. $\alpha\Delta C^{Csp}$-APP immunoreactivity was first detected 6 hrs following QA administration with cell counts of 28 ± 5. $\alpha\Delta C^{Csp}$-APP immunoreactivity was less abundant at 12 and 24 hrs with cell counts of 20 ± 7 and 14 ± 8, respectively. No $\alpha\Delta C^{Csp}$-APP was observed in the contralateral striatum at any time point.


Cells counts for C^Csp-APP immunoreactivity
Number of Immunopositive neurons
35
30
25
20
15
10
5
0
6 hrs
12 hrs
24 hrs

DISCUSSION

I. UPREGULATION OF IAPs USING OF ADENOVIRAL CONSTRUCTS CONTAINING IAP GENES

A. Intrastriatal injection of the adenovirus containing the *xiap* gene leads to overexpression of the XIAP protein by western blot analysis

Injection of the adenoviral construct containing XIAP into the striatum resulted in a 10 fold increase in XIAP protein levels in the ipsilateral striatum relative to the contralateral striatum which received no injection. This indicates that the XIAP adenovirus was able to elevate expression of this anti-apoptotic protein in the striatum.

B. Intrastriatal injection of the adenovirus containing the *xiap* gene leads to overexpression of the XIAP protein as determined by immunohistochemistry

Immunohistochemistry was used to confirm the upregulation of XIAP observed by Western blot analysis. XIAP immunoreactivity was observed in the ipsilateral striata of animals which had been injected with the XIAP adenoviral construct. The number of neurons that were protected against QA-induced neuron death by overexpression of the XIAP adenovirus (approximately 200) exceeded the number of neurons infected by the adenovirus (60 ± 27). XIAP infected neurons were found most abundantly around the injection cannula tract and within close proximity to the injection site.

There are several possible explanations as to why the total number of neurons that

were rescued exceeded the number that were infected. Firstly, immunohistochemistry may not be sensitive enough to detect the XIAP protein within neurons: The amount of XIAP present in the neurons may be below the level of detection by immunohistochemical methods. Secondly, adenoviral vectors are notorious for infecting more than just neurons. They have been shown to also infect glial cells and astrocytes (Slack & Miller, 1996). Thus, the protection of neurons by the XIAP adenovirus may be due to a bystander effect whereby support cells and neurons which were infected by the XIAP protein may be providing some form of protection to their neighbouring neurons which are most vulnerable to the excitotoxin (Baumgartner & Shine, 1997). This protection may be through the modulation of calcium or glutamate efflux, or through trophic support (Beal, 1988 #107). Finally, the XIAP adenovirus may be causing some form of barrier at the site of injection which impedes the infusion of QA into the striatum, thus blocking the delivery of the neurotoxin to the striatal neurons. The existence of such a barrier could be determined by labeling the QA with a radioisotope and by measuring its distribution within the striatum.

II. EFFECT OF ADENOVIRALLY-MEDIATED OVEREXPRESSION OF THE *iap* GENES ON QA-INDUCED EXCITOTOXIC LESIONS

A. Adenovirally-mediated XIAP overexpression reduces neuronal loss following intrastriatal QA administration

Overexpression of the IAPs reduced the destruction of striatal neurons following QA lesions. Animals which had undergone striatal QA lesions showed significant neuronal loss,

as determined by NeuN immunohistochemistry. Animals that were injected with the adenoviral constructs containing either XIAP or NAIP one week prior to QA showed a significant reduction in neuronal loss. XIAP produced significantly more protection against QA-induced neuron loss than did NAIP.

The protection conferred by the IAPs in the present study is in accordance with research which suggests that QA lesions result in apoptotic cell death. Studies by Portera-Cailliau (1995) used DNA fragmentation (as indicated by TUNEL labeling) and ultrastructural analysis to show that neurons subjected to intrastriatal injections of the excitotoxin QA die by apoptosis. Furthermore, the IAPs have been shown to protect against cell death induced by a variety of apoptotic triggers, both *in vitro* and *in vivo* (Liston *et al*, 1996; Xu *et al*, 1999; Xu *et al*, 1997a). It has also been demonstrated that adenovirally-mediated overexpression of NAIP (Xu *et al*, 1997a) and XIAP (Xu *et al*, 1999) decreased the loss of hippocampal CA1 neurons following transient forebrain ischemia produced by 4 vessel occlusion (4-VO).

Protection against neuronal loss and reduction of lesion size following QA lesions have been observed following overexpression of NGF, CNTF or GDNF (Anderson *et al*. 1996; Araujo & Hilt, 1997; Davies & Beardsall, 1992). Virally-mediated overexpression of GDNF has also been shown to ameliorate QA-induced deficits in behavioural tests of striatal function. Intrastriatal administration of GDNF 30 minutes prior to injection of QA at the same site significantly decreased both amphetamine-induced ipsilateral rotation and lesion size (Araujo & Hilt, 1997).

Recent experimentation suggests that glutamate-induced apoptosis in cultured

cerebellar granule neurons (CGNs) may occur through a caspase-3 dependent mechanism (Du *et al*, 1997). Exposure of cultured CGNs to a relatively low concentration of glutamate (30 μM, 24 hrs) resulted in significant cell death. When these CGNs were coincubated with 30 μM glutamate and the specific caspase-3 inhibitor Ac-DEVD-CHO (200 μM) cell death was attenuated by 75% (Du *et al*, 1997). However, Simons et al. (1998) demonstrated that the IAPs do not block neuronal apoptosis following exposure to a severe excitotoxic stimulus produced by high concentrations of glutamate (Simons *et al*, 1999). This study was done with cultured CGNs which were infected with adenoviral vectors containing either NAIP, XIAP, HIAP 1, or HIAP 2 for 24 hours and treated with 100 μM and 1 mM glutamate for 24 hrs. Simons et al. (1998) failed to observe activation of caspase-3 and the IAPs did not reduce excitotoxic cell death produced by either dose of glutamate. Taken together these studies suggest that treatment of CGNs with low concentrations of glutamate may result in apoptotic neuronal death, while treatment with higher doses of glutamate may produce necrotic cell death. Given that selective caspase-3 inhibitors block cell death at 30 μM, it would be interesting to determine whether IAP overexpression can reduce death produced by 30 μM of glutamate.

B. Effects of adenovirally-mediated IAP overexpression on QA-induced deficits in the Morris Water Maze

Bilateral intrastriatal QA lesions have been shown to produce deficits in spatial memory which can be assessed behaviourally using the Morris Water Maze (Block *et al*, 1993; Furtado & Mazurek, 1996). In this task, animals are required to locate a submerged

platform that is hidden from view. With training, rats rapidly learn the location of the platform using visual and spatial cues. Deficits in the Morris Water Maze produced by bilateral QA lesions are thought to be due to the disruption of spatial memory rather than visual, locomotor, or motivational deficits. We assessed the ability of XIAP or NAIP overexpression to reduce the deleterious effects of bilateral QA lesions of the striatum on spatial learning performance in the Morris Water Maze.

QA administration produced a modest deficit in spatial memory that was only significant on the first test day. On the remaining two test days, QA lesioned and sham treated animals performed at comparable levels. This indicates that the QA lesioning paradigm that we used had only minor effects on spatial memory performance in the Morris Water Maze (Fig. 11). Nevertheless, the XIAP adenovirus did prevent the deficits in spatial learning observed on the first day. In contrast, the NAIP adenovirus did not prevent this deficit indicating that the XIAP adenovirus had greater neuroprotective effects than the NAIP adenovirus. This observation is supported by the greater number of surviving neurons seen in the XIAP adenovirus injected animals compared to those which received the NAIP or LacZ vectors.

C. Adenovirally-mediated XIAP overexpression attenuates QA-induced seizure activity

Intrastriatal QA results in clonic-tonic movements of the contralateral limbs and episodic barrel rotations beginning approximately 30 minutes after QA injections and lasting for several hours (Marrannes & Wauquier, 1988). In the present study, bilateral administration of QA into the striatum resulted in episodic barrel rotations, excessive head

waving and chewing, rearing, and clonic-tonic movements of the limbs. Animals which received bilateral injections of the LacZ adenovirus one week prior to QA showed this seizure-like behaviour including barrel rotations. In contrast, animals which received the XIAP adenovirus failed to display seizures or barrel rotations after QA injections. NAIP injected animals showed a slight attenuation of QA-induced seizure-like activity and seldom displayed barrel rotations.

There are differing opinions as to whether barrel rotations are indicative of brain seizures or are simply a manifestation of seizure-like activity. According to one school of thought, barrel rotations are the result of a dominance in skeletal muscle tone on one side of the body. Contraction of the neck muscles on one side may lead to a deviation of the head towards the side of the body with higher muscle tone and to a twisting of the rostral body segment in that direction. The rest of the body may follow suit resulting in complete rotation about the long axis (Marrannes & Wauquier, 1988).

Evidence that intrastriatal QA injections produce brain seizures, and not simply seizure-like activity, is provided by a model of temporal lobe epilepsy. In this model, intrahippocampal injection of QA results in repetitive seizure episodes which are associated with changes in cortical and hippocampal EEGs approximately 30 minutes following injection (corresponding to the appearance of seizure-like activity) (Vezzani *et al*, 1986). Barrel rotations, clonic-tonic movements of the limbs, and chewing are all characteristic of QA-induced seizures. Chewing is also a form of seizure seen in patients with temporal lobe epilepsy (Vezzani *et al*, 1986). Furthermore, antiepileptic agents such as carbamazepine, diazepam, phenobarbital, diphenylhydantoin, ethosuximide, and sodium valproate reduce the

number of barrel-like rotations in QA-lesioned animals (Marrannes & Wauquier, 1988). Marrannes and Wauquier (1987) also demonstrated that administration of sabeluzole, desipramine (tricyclic antidepressant), etomidate, meprobamate, and the analgesic ketamine, a potent NMDA antagonist, reduced the number of QA-induced barrel rotations in a dose dependant manner. Moreover, intrastriatal QA administration also causes changes in EEG voltage amplitudes (Popoli *et al*, 1994). These observations suggest that intrastriatal injections of QA do, in fact, result in seizures.

The mechanisms by which XIAP blocks QA-induced barrel rotations are unclear. Given that quinolinic acid results in excessive NMDA receptor activation, it is possible that XIAP overexpression attenuates signaling through this system. This hypothesis could be tested in a number of ways. For instance, XIAP may play a role in ion conductance. Whole-cell patch clamp experiments could be performed to determine whether changes in QA-activated current are affected by the presence of XIAP. The effect of coapplication of XIAP and the NMDA receptor antagonist MK-801would also serve to determine whether XIAP has a direct effect on NMDA channels.

III. MECHANISM OF NEURONAL DEATH FOLLOWING INTRASTRIATAL QA INJECTIONS

A. Effect of intrastriatal QA administration on DNA fragmentation in the striatum

As in previous studies, intrastriatal QA lesions resulted in DNA fragmentation in the ipsilateral striatum. In the present study, ISEL labeling was used to detect DNA

fragmentation. DNA fragmentation was not observed in the striatum 1, 3 or 6 hours following QA administration. ISEL labeling was first observed in the ipsilateral striatum 12 hrs following QA administration with cell counts of 152 ± 27 /area of measurement (450 x 600 μm); the labeling peaked at 24 hours with cell counts of 297 ± 23 /area of measurement and appeared to be less abundant by 72 hours 165 ± 26 /area of measurement (Fig. 13, Fig. 14). No ISEL labeling was observed in the contralateral striatum at any time point.

B. Effect of intrastriatal QA administration on caspase-3 activation in the striatum

Caspase-3 labeling was not detected in the ipsilateral striatum at 1 or 3 hrs following QA injections, cell counts of 1 ± 0.5 and 1.3 ± 0.9, respectively. Caspase-3 immunoreactivity was first observed in the ipsilateral striatum at 6hrs following QA administration, with cell counts of 373 ± 24 immunoreactive cells. Maximal staining of active caspase-3 was observed 12 hrs following QA with neuronal cell counts of 405 ± 8. Active caspase-3 staining was less abundant by 24 hrs, with neuronal cell counts of 15 ± 9 (Fig. 15, Fig. 16). No caspase-3 labeling was observed in the contralateral striatum at any time point.

Caspase-3 activation therefore appears to precede DNA fragmentation. These observations fit with research that shows caspase-3 to be upstream of DNA fragmentation in the apoptotic pathway: Caspase-3 activates CAD, thus triggering DNA fragmentation (Enari *et al*, 1998). A similar sequence of events has been demonstrated following transient forebrain ischemia (Xu *et al*, 1999). Xu et al. (1999) reported that caspase-3 activation in hippocampal CA1 neurons peaked 24-48 hours after ischemia while DNA fragmentation

peaked at 3-5 days post injury. Hence, caspase-3 activation precedes DNA fragmentation in both QA lesions and ischemia.

Xu et al. (1999) also found that adenovirally-mediated overexpression of XIAP one week prior to ischemia prevented activation of caspase-3, and consequently DNA fragmentation. Thus, the protection conferred to striatal neurons by XIAP overexpression is also likely to function by inhibiting caspase-3 activation. Future experiments could examine whether animals pretreated with XIAP do in fact show a lack of caspase-3 activation following QA injections.

If XIAP does in fact confer protection against QA-induced neuronal loss by blocking activation of caspase-3, additional studies would be needed to clarify the mechanism by which QA induces neuronal apoptosis. One such study could examine whether the preferential caspase-3 inhibitor Ac-DEVD-CHO confers protection from QA lesions in a similar manner to XIAP.

In summary, the presence of caspase-3 activation further confirms that the excitotoxin QA results in apoptotic cell death. In addition, overexpression of the IAPs, specifically XIAP, appears to confer protection against QA-induced neuronal cell death.

C. Effects of intrastriatal QA administration on caspase-3 mediated cleavage of amyloid-β precursor protein (APP) in the striatum

Intrastriatal injection of QA resulted in expression of $\alpha\Delta C^{Csp}$-APP in the ipsilateral striatum. $\alpha\Delta C^{Csp}$-APP was first detected 6 hours following QA injection, the same time that active caspase-3 was first observed. These results are consistent with the observations of

Gervais et al. (1999) that $\alpha\Delta C^{Csp}$-APP staining occurs in apoptotic hippocampal neurons following excitotoxic injury with kainic acid or transient forebrain ischemia.

Gervais et al. (1999) have shown that the toxic Aβ fragment of APP is more readily generated from $\alpha\Delta C^{Csp}$-APP than APP. Consequently, caspase-3 activation may promote apoptosis in neurons by increasing the production of Aβ which is toxic to neurons. The generation of $\alpha\Delta C^{Csp}$-APP further suggests that striatal neuron death following QA administration is apoptotic.

To conclude, adenovirally-mediated overexpression of the IAPs resulted in a reduction in QA-induced neuronal loss. Both XIAP and NAIP reduced QA-induced neuronal death, however, XIAP produced a greater degree of neuroprotection than NAIP. Consistent with this observation, XIAP overexpression produced a larger attenuation of QA-induced seizures than NAIP. These results (i.e. ISEL labeling, active caspase-3 staining, and $\alpha\Delta C^{Csp}$-APP staining) suggest that QA lesions result in apoptotic cell death which is mediated by a caspase-3 dependent mechanism.

IV. FUTURE EXPERIMENTS

In order to confirm that IAP overexpression results in the attenuation of seizure activity, EEG recordings may be performed on animals following intrastriatal administration of QA. In this way, it would be possible to determine whether XIAP actually reduces seizure activity in the brain. Further experiments to determine the role of XIAP in blocking seizures could be performed using transgenic animals which selectively overexpress XIAP in neurons.

Transgenic mice have recently been created in which XIAP expression is driven by a neuron specific enolase (NSE) promoter (Dr. Peter Liston, personal communication).

The intrastriatal QA lesion model has been used in the past because of its pathological and behavioural similarities to HD. There are, however, genetic models now available which more closely resemble the human condition. For example, Reddy et al. (1998) generated a transgenic mouse model using a full-length cDNA with normal and expanded CAG repeats (48 and 89 repeats) behind a heterologous cytomegalovirus (hCMV) promoter. Expression of the transgene is observed in peripheral and brain tissues at equal or higher expression than that of the endogenous mouse huntingtin. These transgenic mice display a progressive neurological phenotype, beginning with feet clasping and/or trunk curling within the first two months and progressing to hyperactivity, (circling, backflips and excessive grooming), and eventually hypoactivity and akinesia days before death. Neurodegeneration was observed selectively in the striatum and cortex of these animals, with mild damage in the thalamus and hippocampus (Reddy *et al*, 1998). Cell counts indicated that there was a striatal neuronal loss of approximately 20% in the transgenic animals compared to wild-type litter mates.

One way to test the therapeutic potential of the IAPs, more specifically XIAP, in HD would be to breed XIAP transgenic mice with those which express expanded CAG repeats. Successive crossings of the offspring would be required to generate animals that express high levels of XIAP and CAG repeat transgenes.

V. THERAPEUTIC POTENTIAL OF THE IAPs

Do the IAPs have potential to be used as a clinically useful tool in the treatment of HD? Several issues warrant further consideration prior to the use of adenovirally-mediated XIAP overexpression in a clinical setting. Firstly, the route of administration needs to be less invasive. Intracerebral injections of adenoviral constructs are not a feasible option for humans [Crocker, 1999 #145]. Current vector technology cannot target specific cells such as neurons. Thus, non-selective apoptosis inhibitors, such as the IAPs, can not be used over the long term due to their potential to harm other cells. In addition, intracerebral administration of the currently used adenoviral vectors can trigger inflammation in the brain (Byrnes *et al*, 1995). The ideal vector for gene therapy would be able to both cross the blood brain barrier and selectively target those neurons which are affected in HD (i.e. medium spiny neurons). Therefore, if adenoviral vectors are to be used clinically, they must be modified to ensure safe and selective delivery to the affected neurons.

A drug, or small molecule, which upregulates the IAPs in specific regions of the brain is one approach. Recent findings have demonstrated that there are drugs, such as K252a, capable of upregulating NAIP in the CNS (Xu *et al*, 1997a). Unfortunately, this compound is likely to alter the expression of many other genes calling into question the feasibility of a transcriptional approach.

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