Instituto Juan March
de Estudios e Investigaciones

56 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

# Programmed Cell Death in the Developing Nervous System

Organized by

R. W. Oppenheim, E. M. Johnson and J. X. Comella

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# INDEX

# INTRODUCTION

R.W.Oppenheim, E.M. Johnson and J.X. Comella

Although the existence of a large scale loss of cells during development has long been recognized, it is only in the last twenty years that the significance of programmed, naturally occurring cell death has been appreciated. Because, with the possible exception of the immune system, the programmed cell death of developing neurons has been studied longer and more extensively than that of any other cell type, it is perhaps not surprising that knowledge about the normal biology of cell death in the nervous system is also more complete. However, even in the nervous system our understanding of many aspects of cell death is still fragmentary. Although many aspects of cell death in the nervous system (especially molecular pathways) appear to be similar to that of cell death outside of the nervous system, there are also important differences that make neuronal cell death novel and unique. For example, control of cell death of neurons often involves interactions between neurons and interconnecting populations of synaptic targets and afferents that are absent in other cell types undergoing cell death. Another difference is that physiological activity, including synaptic transmission, plays a role in the regulation of cell death in the nervous system, whereas similar signals are probably not involved outside of the nervous system. Collectively, it is these unique and shared properties of cell death in nervous and non-nervous tissue that provided a major rationale for the first workshop on Programmed Cell Death in the Developing Nervous System held at the Juan March Foundation in Madrid. Because of similarities between cell death in nervous and other tissues, the information discussed at the meeting will be of interest to a wide spectrum of investigators in the cell death field. By focusing the meeting on the developing nervous system, however, it was assured that the many unique aspects of neuronal and glial cell death were the major topic of discussion.

The participants at the workshop represented an international group of prominent investigators with diverse interests, backgrounds, research strategies, animal models and viewpoints. Because of this diversity it was possible to address virtually all of the important issues in the field. These included the following topics: What is the biological significance of cell death in the nervous system and does this differ for cell types, brain regions and species; What role do targets and afferents play in the regulation of cell death and does this differ between vertebrates and invertebrates; What are the similarities and differences between cell death of neurons and glia; What neurotrophic agents and growth factors (and receptors) regulate survival in the nervous system and how do these act at the biochemical and molecular levels to promote survival; how are other cell-cell signals such as activity and hormones involved in regulating death and survival; What are the biochemical/molecular pathways that result in cellular degeneration and do these differ for different cells, brain regions and species; What genes are involved in mediating death and survival in the nervous system and how are these similar or different across species.

Although consensus was reached on our present understanding of many of these issues, for others there were differences of opinion and a general agreement that more information is needed for a final resolution. Despite the lingering differences of opinion on some key issues, however, all of the participants felt that the meeting was a great success in assessing the present state of knowledge in the field and in identifying the critical important issues that remain for future investigations. The organizers and participants would like to thank Andres Gonzalez and the staff of the Juan March Foundation for their hard work, generosity and perception in supporting this timely inaugural meeting on Programmed Cell Death in the Developing Nervous System.

# Vertebrate neuronal death I

## Chairman: Alun M. Davies

**The In Vivo Development and Survival of Embryonic Sensory and Motor Neurons Following the Removal of their Peripheral Targets**

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The early removal of synaptic (or innervation) targets is a well established model for studying the role of target-derived signals in the development and survival of avian neurons. Cell death following target-deprivation has been assumed to be a valid model of naturally-occurring neuronal death. By removing the hindlimb bud on embryonic day (E) 2, one can examine the development of lumbar spinal sensory (SS) and motor neurons (MN) that have never contacted peripheral target cells. Observations and experimental results that will be discussed using this model include: 1) Virtually all target-deprived MNs (90%+) undergo apoptotic programmed cell death (PCD) during the same developmental period as normal MN death. 2) Despite an almost complete loss of their peripheral targets, and in contrast to MNs, a large proportion (40-50%) of SS neurons survive up to late stages of embryogenesis. 3) The loss of target-deprived SS and MNs is associated with an increase in the number of degenerating (apoptotic) cells in each population. 4) Target deletion has no apparent effect on the proliferation, migration or early differentiation of MN or SS precursor cells. MNs do not enter S-phase of the mitotic cycle prior to normal or induced PCD. 5) Following limb-bud removal, many of the surviving lumbar SS neurons project peripheral axons to abnormal targets in the tail-bud. Combined tail-bud and limb-bud removal results in the loss of many of these SS neurons. 6) Agents that block transcription or translation rescue MNs and SS neurons from both normal and target-deprived PCD. 7) The PCD of target-deprived MNs is not prevented by certain agents that are effective in rescuing them from later normal PCD (e.g., ICE family protease inhibitors; anti-oxidants). 8) Treatment of limb-bud removal embryos from E4.5 to E8.5 with NGF alone or with combinations of NGF, BDNF and NT-3 rescues many, but not all, SS neurons. 9) Treatment of limb removal embryos with extracts from E9-10 embryonic limb muscles (MEX) rescues MNs (but not SS neurons) from both normal and induced cell death. 10) With the exception of GDNF, several other trophic factors (BDNF, NGF, NT-3, IGF, CNTF, bFGF) fail to prevent the loss of MNs following limb removal, whereas some of these are effective in preventing normal MN PCD (e.g. IGF, CNTF, BDNF). Similar to MEX, GDNF rescued MNs on both the ipsilateral (limb removal) and contralateral (control) sides of the spinal cord. GDNF may be a muscle-derived survival factor for MNs during early stages of the normal cell death period. In conclusion, because the PCD of target-deprived SS neurons and MNs exhibit several interesting differences from normal PCD in these populations, target-deprivation may not be a completely valid model of naturally-occurring PCD. The normal trophic requirements of developing avian MNs remain to be clarified.

# HOW ELECTRICAL ACTIVITY AND TROPHIC SIGNALS COMBINE TO REGULATE NEURONAL DEATH IN DEVELOPMENT

**Peter G.H. Clarke,** Institute of Anatomy, University of Lausanne, 1005, Switzerland.

Naturally occurring neuronal death is regulated by anterograde and retrograde signals, both of which are influenced by electrical activity. We are studying the nature of these signals and their interaction, in chick embryos.

## Anterograde signals

Our study of anterograde signals (Catsicas et al., 1992) focused on the retino-tectal projection at embryonic day (E) 16. Blocking activity by means of intraocularly injected tetrodotoxin induced the death of many neurons in the deep tectal layer (SGC) but only slightly increased cell death in the superficial layers (upper SGFS). In contrast, blocking axoplasmic transport with intraocular colchicine greatly increased cell death in both the superficial and deep layers. This suggests that the survival of different tectal neurons is determined, to varying degrees, by activity-dependent and activity-independent influences from the retino-tectal axon terminals. The influences may be mediated by transmitters and trophic factors. The latter may include NT-3, since intraocularly injected NT-3 has been shown to be transported to the optic tectum and released there, where it is taken up by tectal neurons (von Bartheld et al., 1996). NT-3 is known to be produced in the avian retina.

## Retrograde signals

Our current emphasis is on the study of *retrograde* signals, which we study in the projection from the isthmo-optic nucleus (ION) to the contralateral retina (Clarke, 1992). The ION undergoes 55% neuron death between E12 and E17. It becomes laminated at E14 owing to dendritic reorganization (Clarke & Kraftsik, 1996). The ION cell death can be reduced by the intraocular injection of BDNF (but not of NGF or NT-3), and we can show that this is by a retrograde signal due to the direct action of BDNF on the isthmo-optic terminals, since the protective effects of BDNF occur even after elimination of the ION's target cells (Primi & Clarke, 1996).

But we have evidence also for an activity-mediated retrograde signal that may be due to a different mechanism. The only means by which electrical activity is currently believed to initiate retrograde trophic signals is through postsynaptic events: modulated synthesis or release of trophic factors. However, we have several lines of evidence for the *presynaptic* initiation by action potentials of retrograde signals from retina to isthmo-optic nucleus (ION) (Primi & Clarke, 1995; Posada et

al., 1996). Intraocular injections of saxitoxin just before E14 reduce neuron death and prevent lamination in the ION within as little as 6h or even less. This is too fast to be mediated postsynaptically, and there is no such effect after a selective reduction, by glutamate receptor antagonists, in the electrical activity of the ION's amacrine target cells. Moreover, the rapid effects in the ION can be demonstrated even after pharmacological destruction of the target cells. It can be shown not to be mediated anterogradely via the tectum.

The chain of events between presynaptic action potential and retrograde message are unclear. Action potentials may lead to a series of second messenger events in the axon terminal that leads to retrograde signaling via the transport of a long-lived second messenger. We think the first step is calcium entry, because intraocular injection of calcium channel blockers leads to effects in the ION 6 and 12h later resembling those due to saxitoxin (Posada et al., 1996).

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CONTROL OF SEXUALLY DIMORPHIC NEURON DEATH IN THE AVIAN BRAIN.

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In several regions of the vertebrate nervous system programmed cell death is regulated by sexual differentiation to create robust sex differences in neuron number. This is exemplified dramatically in the development of brain regions controlling song behavior in birds. In some songbirds, several of these song control nuclei possess more neurons in adult males (who sing) than in females (who do not normally produce song), and recent studies have implicated programmed neuron death in sculpting these sex differences. Although the hormonal and cellular mechanisms that mediate these sex differences in cell death are not yet known, it is likely that sexual differentiation influences some of the same cellular interactions that regulate neuron survival more generally. This hypothesis will be evaluated in regard to the avian song system through an examination of studies investigating how neurotrophic effects associated with sexual differentiation may be mediated by and/or interact with trophic effects exerted by glia cells and/or afferent inputs.

# Receptors and Intracellular Pathways Initiating Deprivation-Induced Cell Death in Brainstem Auditory Neurons

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We have examined cell death of second order auditory neurons in the brainstem of neonatal chicks and mammals following manipulations of excitatory synaptic stimulation via the auditory nerve. Removal of the cochlea or blocking eighth nerve electrical activity results in rapid cell death of 30-50% of the postsynaptic neurons in the cochlear nucleus, and atrophy of the remaining neurons. Examination of the early events associated with neuronal death has revealed that afferent activity deprivation leads to a rapid decrease in protein synthesis associated with a rise in intracellular $Ca^{2+}$. Using an *in vitro* brainstem slice we show that both protein synthesis and $Ca^{2+}$ homeostasis are maintained by orthodromic stimulation via the eighth nerve. Antidromic stimulation, no stimulation or contralateral nerve stimulation do not maintain normal translational activity or $Ca^{2+}$ homeostasis. Pharmacological investigations suggest that glutaminergic activation of a metabotropic glutamate receptor plays a central role in maintaining normal $Ca^{2+}$ levels, through inhibition of both calcium influx and release from intracellular stores. When this inhibitory role of glutamate is prevented by afferent activity deprivation, intracellular calcium may achieve toxic levels, thereby initiating a cascade of events culminating in neuronal death.

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## REGULATION OF SCHWANN CELL DEATH IN DEVELOPING VENTRAL NERVE ROOTS OF THE CHICK EMBRYO.

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The present study shows that, in the chick embryo, Schwann cells (SCs) die by apoptosis both during normal development and following axonal degeneration induced with neurotoxins. SC apoptosis during development takes place in a period roughly coincident with naturally occurring motoneuron death. Administration of *N*-methyl-D-aspartate (NMDA) to E7 chick embryos results in an extensive excitotoxic motoneuronal damage in the spinal cord without any apparent effects on neurons in the dorsal root ganglia. Dying SCs in ventral nerve roots from NMDA treated embryos display ultrastructural features of apoptosis and exhibit *in situ* detectable DNA fragmentation. Conversely, NMDA-treatment does not increase the death of SCs in dorsal nerve roots. The combination of *in situ* detection of DNA fragmentation with the avian SC marker 1E8 antibody demonstrates that dying cells in ventral nerve roots belong to the SC lineage. Treatment with cycloheximide does not prevent the toxic effects of NMDA on motoneurons, but dramatically reduces the number of pyknotic SCs and DNA fragmentation profiles in the ventral nerve roots. *In ovo* administration of a variety of tissue extracts (muscle, brain and spinal cord) from the chick embryo or of motoneuron conditioned medium fails to prevent SC apoptosis in NMDA-treated embryos. Intramuscular administration of the snake toxin β-bungarotoxin induces a massive death of both lateral motor column motoneurons and dorsal root ganglia neurons resulting in a substantial increase in the number of pyknotic SCs in both ventral and dorsal nerve roots. Chronic blockade of neuromuscular activity during development with pharmacological agents such as (+)-tubocurarine (Tc) in the chick embryo rescues motoneurons from naturally occurring cell death. In our study, treatment of chick embryos with Tc from E3 results in a reduction of SC apoptosis on E9-E10, but not in earlier stages. By contrast, limb-bud ablation performed on E2, which leads to degeneration and death of up to 90% of motoneurons, determines an important increase in SC apoptosis coincident with the period of maximal motoneuron loss. It is concluded that, during development, axonal derived trophic signals are involved in the regulation of SC survival in peripheral nerves.

# Vertebrate neuronal death II

## Chairman: Ronald W. Oppenheim

ACTIVITY-DEPENDENT SURVIVAL OF A CNS NEURON. A. Meyer-Franke, M. Kaplan, F. Pfrieger, and B. Barres. Stanford University School of Medicine, Stanford, CA 94305-5401.

The signaling mechanisms that control the survival of CNS neurons are poorly understood. When highly purified postnatal rat retinal ganglion cells are cultured in serum-free medium they rapidly die in conditions that promote the survival of PNS neurons, including peptide trophic factor stimulation, cAMP elevation, or depolarization. Their survival is promoted by peptide trophic factors, however, if their intracellular cAMP is simultaneously increased, either by membrane permeable cAMP analogs or by depolarization with $K^+$ or glutamate agonists. Astrocyte and tectal-derived factors, such as BDNF, CNTF, IGF-1, and an additional protein made by oligodendrocytes, together with cAMP elevation, collaborate to promote the long-term survival of the majority of purified retinal ganglion cells in vitro. Both the cAMP- and depolarization-induced responsiveness to survival factors are blocked by protein kinase A inhibition. These results suggest that electrical activity may be necessary for optimal survival and growth of retinal ganglion cells and raise the question of whether other CNS neurons have similar survival requirements.

# The role of the bcl-2 family of proteins in regulating neuronal survival

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In the developing vertebrate peripheral nervous system, neurons depend for survival on one or more neurotrophic factors provided by the tissues they innervate [1]. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-3 (NT-4) comprise a family of neurotrophic factors called neurotrophins. Other neurotrophic factors like ciliary neurotrophic factor (CNTF) and growth-promoting activity (GPA) are unrelated to the neurotrophins. Previous work has shown that over-expression of the intracellular protein bcl-2 in certain cytokine-dependent myeloid and lymphoid cells prevents death following cytokine withdrawal [2]. To assess the role of bcl-2 in neuronal survival, we used microinjection to introduce vectors expressing sense and antisense *bcl-2* RNA into embryonic neurons [3, 4]. Overexpression of bcl-2 prevented the death of neurotrophin-deprived neurons, but did not prevent the death of neurons deprived of CNTF or GPA. Antisense *bcl-2* prevented the long-term survival response of neurons to neurotrophins, but did not affect the survival response of the same neurons to CNTF or GPA. These findings suggest that Bcl-2 is required for the continued maintenance of embryonic neurons by neurotrophins and suggest that alternative, Bcl-2-independent, survival mechanisms operate in neurons exposed to CNTF.

*In vitro* studies of neurons from *bcl-2* null mutant animals suggest that the role of bcl-2 in regulating neuronal survival changes with age. Some populations of neurons, like the sensory neurons of the trigeminal ganglion, switch their survival requirements from BDNF to NGF during the early stages of target field innervation [5, 6]. Whereas early trigeminal neurons from *bcl-2*$^{-/-}$embryos survive less well with NGF than wild type neurons, bcl-2-deficient neurons demonstrate a prolonged survival response to BDNF. This suggests that bcl-2 selectively modulates the survival response to different neurotrophins at particular stages of neuronal development. During the mid-fetal period when cell death is taking place, trigeminal and other sensory neurons from *bcl-2*$^{-/-}$ animals do not survive as long with neurotrophins as wild type neurons. However, by the late fetal period, bcl-2-deficient and wild type neurons survive equally well with neurotrophins, demonstrating that endogenously expressed bcl-2 is not required for the survival of neurons in response to neurotrophins at later stages of development. This decreased requirement for endogenous bcl-2 correlates with

the decreased effectiveness with which overexpressed bcl-2 rescues neurotrophin-deprived late fetal and postnatal neurons.

Bcl-2 is the founder member of a family of homologous proteins that includes Bcl-x [7] and Bax [8]. Whereas increased expression of Bcl-2 or Bcl-x promotes cell survival following withdrawal of survival factors, increased expression of Bax is thought to suppress survival [8]. To investigate the potential roles of these proteins in regulating the survival of developing neurons, we compared the effects of overexpressing these proteins in embryonic neurons deprived of different neurotrophic factors *in vitro*. Surprisingly, overexpression of Bax rescued populations of sensory neurons deprived of NGF or BDNF, as did overexpression of Bcl-2 and two Bcl-x variants, Bcl-x$_L$ and Bcl-x$\beta$. Bax also enhanced the survival of ciliary neurons deprived of CNTF, although this effect was short-lived. Whereas Bcl-2 overexpression did not affect the survival response of neurons to neurotrophic factors, Bax overexpression partially inhibited the action of neurotrophic factors. Co-injection of Bcl-2 and Bax expression vectors promoted the survival of neurotrophic factor-deprived neurons if either was in excess, but failed to rescue neurons if they were injected at a 1:1 ratio. Our findings demonstrate that Bax can promote the survival of neurotrophic factor-deprived neurons and that its effect on survival is dominant to that of neurotrophic factors. Our results also argue that the relative amounts of Bcl-2 and Bax are critical in regulating neuronal survival [9].

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**How do neurotrophic factors regulate motoneuron numbers during development?**

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Since the original demonstration by Hamburger that motoneurons die in the absence of their target tissues, much effort has been devoted to identifying the neurotrophic factors produced by the limb. Over the last few years, we and others have identified several candidates from different protein families (neurotrophins, cytokines, TGFßs, FGFs...) whose precise roles are now under study. We recently developed a new long-term culture system for purified motoneurons from E14 rat embryos, and have used it to analyse quantitatively the survival effects of the above factors tested singly or in combination. We observe two phenomena that we believe may be of importance in understanding the developmental regulation of motoneuron numbers:

*Fractional survival effects:* no single factor is capable of keeping all motoneurons alive in long-term culture. Rather, each factor maintains a characteristic fraction of motoneurons. This is reminiscent of recent results from knockout mice for GDNF or CNTF/LIF-receptor genes, and suggests that different sub-populations of embryonic motoneuron may respond to different trophic factors.

*Synergistic interactions between factors:* the combined effect of some factors is greater than the effect of each factor tested singly. This result (also reported by others in mouse models of motoneuron degeneration) implies that some single motoneurons can respond to more than one factor at once. Such synergy provides an attractive possible mechanism for selective survival of those motoneurons which have successfully contacted all their cellular partners. As an example, our data suggest that expression of GDNF and cytokines is highly compartmentalized during cell death to Schwann cells and muscle, respectively. If these factors are produced at sub-optimal levels by each tissue, and if there are synergistic interactions between them, then a combination of Schwann cell and muscle influences may be absolutely required to prevent death of a given motoneuron.

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# REGULATION OF CELL SURVIVAL AND CELL DEATH PROCESSES IN THE MOTONEURON POPULATION OF THE CHICK EMBRYO SPINAL CORD.

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During development, most neuronal populations undergo a process usually referred to as naturally occurring cell death. For motoneurons (MTNs) of the lumbar spinal cord of chick embryo, this process takes place in a well defined period of time, between embryonic days 6 and 10. For their survival, neuronal populations depend on the availability of specific neurotrophic factors derived form their innervation target tissue. Thus neurons that are unable to gain access to sufficient quantities of thophic factor die by apoptosis. The current interests of our groups are to study the mechanisms that control and regulate the processes of survival and death of this population of neurons. From a cell dissociate derived form entire spinal cord, by means of a gradient centrifugation, a 90% pure population of MTNs can be obtained and set up in primary culture. In this model we have performed the following studies:

**1.- Characterization of the death process subsiding the loss of trophic support (Comella et al., 1994).**

Upon deprivation of trophic factor MTNs enter a process of neuronal death with the typical characteristics of apoptosis. These include, morphological appearance, condensation of chromatin and internucleosomal DNA fragmentation. The process requires the synthesis of new macromolecules since it can be prevented by inhibiting either RNA or protein synthesis.

**Development of survival responsiveness to several neurotrophins during the culture period (Becker et al., 1996).**

Neurotrophins are the best characterized family of neurotrophic factors and include NGF, BDNF, NT3 and NT4/5. Their survival effects are mediated through specific high affinity receptors encoded by the trk gene family. These include trk A (for NGF), trk B (for BDNF and NT4/5) and trk C (for NT3). It was repeatedly reported that primary cultures of chicken MTNs do not respond to any of the neurotrophins when isolated and cultured from embryos at the beginning of the naturally occurring neuronal death period, i.e. E5-E6. However, some other labs have reported that daily injections of BDNF during the period of neuronal death are able to rescue a fraction of cells that, otherwise, would die. Our results are consistent with the former reports in primary cultures. However, when these MTNs are maintained alive in culture by a yet uncharacterized trophic factor from skeletal muscle extract for two days, a selective and specific neurotrophic response to BDNF, NT3 and NT4/5 develops. This phenomenon is correlated with an increased expression of the corresponding functional receptors, i.e. trk B and trk C which could explain the in vivo results.

**3.- Role of intracellular calcium in regulating the survival of MTNs (Soler et al., 1996).**

It is known that an increase in the intracellular calcium levels allows to maintain primary cultures of a wide variety of neuronal populations
in spite of the absence of neurotrophic factors. This can be achieved by increasing the extracellular potassium concentration that in turn depolarizes the cytoplasmic membrane and voltage dependent channels open. We have found that the same phenomenon can be induced in MTNs by adding 30 mM potassium to the culture media. Survival is dependent on extracellular calcium since addition of the calcium chelator EGTA blocks the potassium effect. Calcium entry is mediated by L-type voltage dependent calcium channels and by using FURA-2 intracellular calcium measurements it can be demonstrated that potassium increases chronically the concentration of calcium from a resting value of ca. 50 nM to a Ca. 150 nM. We have also devoted efforts to characterize the intracellular pathways that could be activated by this increased concentrations of intracellular calcium. We have observed that calcium is able to specifically activate the ras/MAPK pathway, which is also activated by neurotrophins through trk receptors. Finally, the activation of this pathway by calcium is mediated in some point by calmodulin since specific inhibitors (W13) but not the inactive chemically-related analogue (W12) blocks, both survival effects and activation of the ras/MAPK pathway.

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Cell death and microglial migration during quail retina development

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The distribution of macrophages/microglial cells revealed by QH1 immunocytochemistry during quail retina development indicates that microglial precursors first move tangentially, from the pecten/optic nerve head to the periphery of the retina along the nerve fiber layer (NFL), and afterwards migrate radially towards the sclera, in order to reach their definitive location within the retinal layers (Navascués et al., 1995). These migrations may depend on specific factors guiding the cells.

To analyze the possibility that cell death might be one of these factors, we are studying the cell death pattern during normal retinal development in quail embryos using TUNEL labeling both on paraffin sections and retinal wholemounts. In retinas of incubation day 6 (E6) quails, few dead cell fragments were seen, scattered throughout the entire retina. At E7 the number of labeled cell fragments greatly increased, appearing mainly concentrated in the central retina. The quantity of cell dead fragments increased progressively from E8, and extended to more peripherical regions. The number of labeled fragments decreased quickly after E12, so that on E16 (the last incubation day) few fragments were present in the retina, scattered in most peripheral regions. Cell death was present in both the developing ganglion cell layer (GCL) and the inner nuclear layer (INL).

In the wholemounts, microglial precursors migrating tangentially were seen in regions with intense cell death; transversal sections, however, revealed that these precursors migrated on Müller cell endfeet, thus outside the retinal layers in which cell death took place. Radially migrating microglial precursors only entered the INL after the number of apoptotic fragments had greatly decreased; electron micrographs showed that many fragments were phagocytosed by Müller cells before the arrival of microglial precursors. Our current results suggest that retinal cell death influences the migration of microglial precursors, not in a simple direct way, but in an indirect way, probably through Müller cells.

Navascués et al (1995) J Comp Neurol 354:209-228.

# Molecular mechanisms of vertebrate neuronal death I

## Chairman: H. Robert Horvitz

## RECEPTORS AND EFFECTORS IN THE NEURAL CELL DEATH PROGRAM

D. Bredesen

Pioneering studies by Hamburger and Levi-Montalcini, studying nerve growth factor, Johnson, studying neural cell death induced by NGF withdrawal, and Oppenheim, studying developmental motor neuron cell death, led to the concept that apoptosis is induced in developing neurons by the withdrawal of trophic factors during a critical developmental period. A search for the molecular basis of this phenomenon led to the identification of two neuronal "death receptors": one is p75NTR, the low-affinity neurotrophin receptor, and the other is a glycosphingolipid expressed by neocortical neurons.

The expression of p75NTR in neural and non-neural cell lines induces apoptosis in the absence of binding by neurotrophin, but survival is enhanced by the binding of neurotrophins. This thesis has been controversial, in part because it requires that an apoptosis-inducing signal be generated by a naked receptor; however, it is compatible with the finding of Barrett and Bartlett, who showed that the antisense inhibition of p75NTR expression by developing dorsal root ganglion neurons at E19-P4 inhibited apoptosis induced by NGF withdrawal.

The induction of apoptosis by p75NTR in some (but, surprisingly, not all) cell lines requires a region of the intracytoplasmic domain that has turned out to be similar structurally to the "death domain" of tumor necrosis factor receptor I, a region required for apoptosis induction by TNFR I.

The expression of p75NTR may be associated with both physiological and pathological neural cell death: expression of p75NTR has been shown to sensitize cells to toxicity from beta-amyloid peptide. This is of interest because the cholinergic basal forebrain neurons, which are the predominant cells expressing p75NTR in the adult brain, represent a selectively vulnerable region in Alzheimer's disease.

A second neuronal cell death receptor has also been identified, which is expressed by neurons of the neocortex. This has turned out to be a glycosphingolipid. Interestingly, it is the first one with a portable nature, that is, it can move from cell to cell, and then upon triggering, induce the death of the cell to which it has migrated.

MECHANISMS OF PROGRAMMED CELL DEATH

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We have found that staurosporine (STS), in the presence of cycloheximide (CHX) to inhibit protein synthesis, induces apoptotic cell death in a large variety of nucleated mammalian cell types, including blastomeres, suggesting that all nucleated mammalian cells constitutively express all of the proteins required to undergo programmed cell death (PCD) [1] . The mechanisms by which cells execute the death programme, however, remain unclear. We have previously shown that the nucleus, an intact mitochondrial respiratory chain, or reactive oxygen species (ROS) are not required for the death programme to run, or for Bcl-2 to be able to suppress it. To determine whether the nucleus is required for cells to undergo PCD, we studied enucleated cells (cytoplasts) [2]. To investigate the role of mitochondrial respiration, we studied cells that had been entirely depleted of mtDNA (termed $\rho^0$ cells) so that the cells cannot carry out oxidative phosphorylation [3]. To investigate the role of reactive oxygen species (ROS), we cultured cells in very low oxygen (<10 ppm) so that they could not make ROS [4]. In all of these experiments cells were able to undergo PCD and Bcl-2 protected them. These findings suggested that the death programme is orchestrated by a cytoplasmic regulator that acts on multiple intracellular targets in parallel, and is constitutively expressed in all nucleated mammalian cells.

The reliability of this conclusion depends on the evidence that STS-induced, and (STS+CHX)-induced, cell deaths are *bone fide* examples of PCD. There is rapidly accumulating evidence that some members of the Ced-3/Interleukin-1beta Converting Enzyme (ICE) family of cysteine proteases are part of the basic machinery of PCD. We have found that Z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk), a cell-permeable, irreversible, tripeptide inhibitor of some of these proteases, suppresses STS-induced and (STS+CHX)-induced cell death in a wide variety of mammalian cell types, including anucleate cytoplasts, providing strong evidence that these are all *bona fide* examples of PCD [5]. We have also found that, in STS-induced PCD, the Ced-3/ICE family member CPP32 is cleaved into its active form, and that Bcl-2 inhibits this activation. Furthermore, the nuclear protein poly-(ADP ribose) polymerase (PARP) is cleaved, in a pattern consistent with its cleavage by a CPP32-like protease, at the same time as PCD occurs. Most important, intracellular injection of Ac-DEVD-CHO (an inhibitor of CPP32-like proteases) but not Ac-YVAD-CHO (an ICE inhibitor) blocks STS-induced PCD. These findings indicate that STS-induced PCD in some cells at least requires CPP32, or a CPP32-like member of the Ced-3/ICE-family of proteases, but not ICE itself. Finally, we describe the use of zVAD-fmk to suppresses PCD in various developmental processes.

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Pathways regulating neuronal cell death. *Lloyd A. Greene, Carol M. Troy, Stephen E. Farinelli, David S. Park, Irene Yan, Leonidas Stephanis and Michael L. Shelanski.* Department of Pathology, Columbia University College of Physicians and Surgeons, New York, NY USA

We have compared the pathways that regulate death of rat PC12 cells and sympathetic neurons caused by trophic factor deprivation and by oxidative stress consequent to down-regulation of Cu/Zn superoxide dismutase (SOD1). Our findings suggest that trophic factor deprivation leads neuroblasts and neurons to attempt inappropriate entry/re-entry into the cell cycle which in turn triggers an apoptotic pathway. For example, drugs that selectively block cyclin dependent kinases rescue neuronally differentiated PC12 cells and neurons from death caused by withdrawal of trophic factors. In contrast, death due to SOD1 down-regulation is initiated by an entirely different mechanism that includes the formation of peroxynitrite. Despite these divergent initiating mechanisms, death in the two paradigms converges on an evolutionarily conserved general pathway that includes activation of the aspartase family of proteases. However, even for this general pathway, distinct aspartases appear to be involved, depending on the initiating cause of death. In particular, activation of the interleukin-1β converting enzyme (ICE) and consequent formation of IL-1β is involved in apoptosis caused by SOD1 down-regulation whereas a different family member is required for death caused by withdrawal of trophic support.

**GENETIC AND PHARMACOLOGICAL MANIPULATION OF NEURONAL DEATH AFTER TROPHIC FACTOR DEPRIVATION.**

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Deprivation of neurotrophic factor leads to a series of macromolecular synthesis-dependent events ultimately leading to neuronal apoptosis. We have rather extensively studied this programmed cell death in sympathetic neurons after removal of nerve growth factor (NGF). Published data describing the biochemical, morphological, and genetic events that occur will be briefly reviewed, with special emphasis on changes that appear critical to the ultimate death of the cell.

As in other cells, critical genes involved in the terminal apoptotic phase of programmed cell death include those encoding a family of cysteine proteases (the ICE- family) and the bcl-2 family of pro- and anti-apoptotic proteins. In collaborative efforts with other labs we have identified protease inhibitors and genetic deletions that prevent apoptosis of sympathetic neurons after NGF deprivation. Particularly in the case of the protease inhibitor, we have systematically examined the effect of blocking death on the biochemical and genetic events associated with NGF withdrawal. Despite the failure of the neurons to die, the catabolic and genetic events associated with NGF withdrawal are not prevented, and in some cases are amplified. The resulting "saved" neurons are markedly atrophic and presumably hypofunctional. However, these atrophic neurons are still capable of responding to NGF with reversal of atrophy and renewed growth.

A similar behavior is seen in sympathetic neurons derived from BAX knockout mice. Such neurons do not die in response to NGF deprivation. They become markedly atrophic and similarly respond to NGF readdition by reversal of atrophy and renewed growth. Naturally occurring neuronal death of sympathetic and motor neurons is reduced or abrogated in these animals. These experiments demonstrate that BAX is required for trophic factor deprivation and naturally occurring death of sympathetic and motor neurons.

**MOLECULAR MECHANISMS OF PROGRAMMED CELL DEATH INDUCED BY NERVE GROWTH FACTOR DEPRIVATION IN SYMPATHETIC NEURONS.**

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Sympathetic neurons depend on nerve growth factor (NGF) for survival. When deprived of NGF, they undergo programmed cell death (PCD). Our goal is to identify the differents components of the cell death program initiated in these neurons after NGF deprivation. Three components have been identified: oxidation, interactions between Bcl-x and Bax and protease activation.

1)We have shown that, after NGF deprivation, the redox potential of neurons is altered probably because of a decrease in glutathione levels. Compounds that sustain high levels of glutathione, such as N-acetylcysteine, can delay neuronal cell death. As a consequence of glutathione depletion, free radicals are probably generated. Different free radical scavengers can also delay neuronal death induced by NGF deprivation. Thus, the control of redox potential is crucial for neuronal survival. Glutathione may be an effector of cell death.

2) Bax and Bak proteins were found to accelerate the death of sympathetic neurons following NGF deprivation. Moreover, we found that overexpression of these proteins induce death of sympathetic neurons cultured in the presence of NGF, that is in the absence of any cell death stimulus. Finally Bax protein increases specifically in sympathetic neurons, in the hours following NGF deprivation. Taken together, these results suggest that Bax is a key regulator of programmed neuronal death.

3) ICE-like proteases also play a role in programmed cell death. The death of sympathetic neurons can be prevented by overexpression of crmA, a viral protein that inhibits ICE and others proteases of this family. We have concentrated our efforts on a member of this family called Nedd-2/Ich-1. Nedd-2 is expressed constitutively in sympathetic neurons and is activated at a post-transcriptional level after NGF deprivation. Different isoforms of Nedd-2, with or without the catalytic site, have been identified. Only one splice variant induces neuronal death. The function of this protease will be discussed.

# Molecular mechanisms of vertebrate neuronal death II

## Chairman: Eugene M. Johnson

Survival signalling in naive and injured sympathetic neurons - what's the crucial step?

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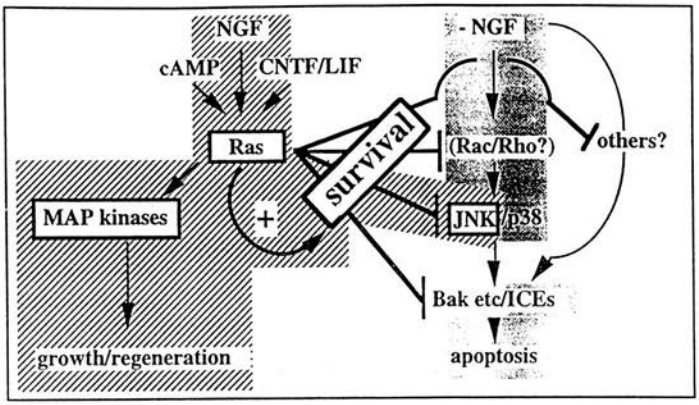
Understanding the mechanisms by which survival factors suppress cell death in the nervous system is a major challenge. Of particular interest are the mechanisms that might be mobilised for protection against injury. In cultured rat sympathetic neurons which depend on NGF for survival it appears that apoptosis caused by withdrawal of NGF can be suppressed by NGF and other survival factors at the post-translational level (1). Thus, signalling systems, rather than gene expression, may lie at the core of the acute phase of cell death suppression (2).

Analysis of death curves suggest that passage through the death commitment point of each individual neuron is a highly-cooperative process as opposed to a process comprising a sequential series of steps. This behaviour best fits a catalytic mode of death similar to what might be expected if ICE-like proteases were auto-activated to reach a critical threshold, thereby unleashing apoptosis by simultaneous cleavage of numerous cellular targets. In light of such a death mechanism, it would seem most unlikely that convergence on a single intracellular event would be sufficient for survival factors to suppress apoptosis. Yet, we have found such a site of convergence in p21Ras, which is critical in mediating survival induced by three survival factors - NGF, the cytokines LIF or CNTF, and the cAMP analogue CPTcAMP, as evidenced by neutralising p21Ras function using anti-Ras Fab antibody fragments (3). Moreover, activated p21Ras can sustain the survival and neurite regeneration of these neurons in the absence of NGF (4), suggesting that activation of p21Ras is both necessary and sufficient for survival of rat sympathetic neurons. Thus, p12Ras appears to be a critical co-factor for survival in SCG neurons.

Intrigued by this finding, we asked which factors might couple p21Ras to suppression of apoptosis? We first examined a role for the MAP kinase (or ERK) cascade but find that contrary to the reported critical role of ERKs in mediating differentiation induced by NGF, ERKs do not appear to mediate the Ras-to-survival pathway by any of the three survival agents (5). Examining other activities which have been reported to lie downstream of p21Ras or to act as co-stimulants, we have investigated a role for phosphatidyl inositol 3 kinase (PI-3-K) using the pharmacological inhibitor LY294002. Whilst LY294002 exerts no apparent effect on survival (over 1-2 days) it dramatically abolishes initiation of neurite outgrowth in response to NGF and the other survival factors. This lack of initiation of neurite growth is not surprising since PI-3-K is a critical mediator of vesicle trafficking. More interestingly, however, LY294002 begins to inhibit survival in response to NGF after longer periods of incubation, raising the possibility that internalisation of TrkA and vesicle traffic play an important role in mediating the survival effects of NGF. Indeed, the question of which signals reach the cell body from the targets which produce NGF is still unanswered. A role for serum factors in enhancing the long-term rescue of injured neurons by NGF has also been noted.

Withdrawal of NGF may not necessarily lead to a simple reversal of the positive signals induced by NGF but rather could lead to the activation of an alternative pathway which is kept inhibited by NGF. We have been focusing for some time on the stress-activated signalling pathways (comprising c-Jun N terminal kinase (JNK) and p38) since these kinases are differentially activated in response to specific kinds of cellular stress in many cell types. Indeed, JNK is activated after NGF-deprivation and is suppressed by NGF and cyclic AMP even after several hours of increased activity (6). However, it is clear that JNK activity per se is not sufficient to promote neuronal death and we think it unlikely that suppression of JNK activity is sufficient to rescue SCG neurons.

The hatched part of the scheme summarises our findings on forward signalling induced by the survival factors in SCG neurons, the dotted part of the scheme summarises the signalling events associated with NGF withdrawal. The remaining elements are important targets for future investigations:

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## CHANGES IN GENE EXPRESSION DURING EXCITOTOXIC NEURONAL CELL DEATH.

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Excitotoxicity refers to the process of neuronal death induced by excitatory amino acids. Abnormal accumulation of glutamate in the extracellular space after trauma, hypoxia-ischemia, hypoglycemia and sustained epilepsy triggers neuronal injury that can be attenuated by selective glutamate receptor antagonists (for review see Lipton and Rosenberg, 1994). Intracellular $Ca^{2+}$ overload, depressed mitochondrial function and concomitant generation of free radicals are thought to be the early cytoplasmic events that cause the neuronal damage in excitotoxic processes (Choi, 1995; Coyle and Puttfarcken, 1993; Ankarcrona et al., 1995). Although the early induction of transcriptionally competent genes has previously been associated with excitotoxic neuronal death, only recently has it been demonstrated that internucleosomal DNA fragmentation and the requirement of protein synthesis, features of apoptosis, are also involved in some models of glutamate-triggered delayed neuronal death.

Efforts to characterize death-related target genes for transactivation after early gene induction have failed to identify a common target in different models of cell death. Genes that have been shown to be induced in cell death processes include the heat shock *hsp70* (Buttyan et al., 1988), glycoprotein S-sulphate *TRPM-2* (Buttyan et al., 1989), *calmodulin* (Dowd et al., 1991), the non-characterized *RP-2* and *RP-8* genes (Owens et al., 1991) and the genes for ß-galactoside binding protein (Goldstone and Lavin, 1991) and glutathione S-transferase (Briehl and Miesfeld, 1991). Induced genes related to neuronal death include *ubiquitin* (Schwartz et al., 1990), *cyclin D1* (Freeman et al., 1994), the rat hairy-like gene *rhl* and genes for the extracellular matrix proteases transin and collagenase (Estus et al., 1994) and for acidic 40kDa protein (Montemayor et al., 1990). None of these genes has been implicated in NMDA-induced neuronal death, and so far no death-related target gene has been characterized during excitotoxicity. Here we will present the characterization by differential screening of the induction of *gas1* (growth arrest specific gene 1) during NMDA-induced neuronal death.

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# Naturally occurring (programmed) and radiation-induced apoptosis are associated with c-Jun expression in the developing rat brain

Isidro Ferrer and Anna M. Planas

ABSTRACT

Expression of the different members of transcription factors Fos and Jun is examined in the developing rat brain. Constitutive expression of c-Fos, Fos-related, Jun B and Jun D, as revealed with immunohistochemistry, is higher and more widely distributed in the developing rat brain than in the adult. Selective strong c-Jun expression is observed in the cytoplasm and nucleus of apoptotic cells during the whole process of naturally occurring (programmed) cell death. Cells expressing strong c-Jun immunoreactivity are undetermined cells, neurons and astrocytes. Selective c-Jun expression is also observed following ionizing radiation in rats aged 3 days. Induction of c-jun mRNA, as revealed with *in situ* hybridization, occurs between 5 and 15 min following gamma-irradiation. Strong c-Jun protein expression appears at 2 h, peaking at 6 h and decreasing thereafter to reach normal levels 48 h after gamma-ray exposure. Strong c-Jun protein expression is coincidental with endonuclease activation, as revealed with the method of *in situ* labelling of nuclear DNA fragmentation, and is restricted to apoptotic cells. Cycloheximide injection at the time of irradiation blocks c-Jun expression, thus indicating that c-Jun immunoreactivity is attributable to *de novo* protein synthesis. These observations demonstrate *in vivo* selective strong c-Jun expression associated with programmed cell death and ionizing radiation-induced apoptosis in the developing rat brain.

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## Induction of a DEVD-AFC/PARP cleavage activity in naive and neuronal PC12 cells following withdrawal of trophic support

Inhibitors of ICE and a related group of aspartases of the ICE/ced3 family inhibit apoptotic cell death in a variety of settings. However, the particular member(s) of the ICE/ced3 family that are relevant to cell death in these paradigms remain to be determined. We have previously shown that zVAD-FMK, a pseudosubstrate ICE inhibitor, and vICE, a novel ICE/ced3 peptide inhibitor, block apoptotic death in naive and neuronal PC12 cells and in sympathetic neurons following withdrawal of trophic support or oxidative stress. In the current study, we have measured the enzymatic activity of two representative members of the ICE/ced3 family, ICE itself and CPP32/Yama/apopain, before and during the apoptotic death of naive and neuronally differentiated PC12 cells that have been deprived of trophic support. To this end, we have used as substrates radioactively labelled pIL1-β and PARP, the natural substrates of ICE and CPP32/Yama/apopain respectively, and the synthetic fluorogenic substrates YVAD-AFC and DEVD-AFC, that mimic the cleavage sites on pIL1-β and PARP respectively. There was no detectable cleavage of YVAD-AFC or pIL1-β in cells undergoing apoptosis. By contrast, DEVD-AFC and tPARP cleavage activities were readily detected prior to apoptotic cell death and correlated with the rate of death. The kinetic properties of this activity resembled those reported for CPP32/Yama/apopain. A variety of agents that promote survival in our model, including a number of cell cycle blockers, ATA and bcl-2 blocked the activation of the DEVD-AFC/PARP cleavage activity, placing them at a point further upstream in the apoptotic cascade. Parallel assessement of jun kinase activity revealed that its activation temporally preceeded that of DEVD-AFC/PARP cleavage. These results argue for the induction of DEVD-AFC cleavage activity being a relatively late event in the apoptotic cascade. The ICE inhibitor zVAD-FMK proved to be a superior inhibitor of the DEVD-AFC cleavage activity in vitro compared to YVAD-CMK, reflecting its superiority as a promoter of survival in our model and raising the possibilty that it is a more potent inhibitor of the family of ICE/ced3 aspartases in general. We tested the ability of zVAD-FMK to inhibit the activity/activation of DEVD-AFC/PARP cleavage in vivo, in PC12 cells following withdrawal of trophic support. We found that the relative ability to block the activity/activation occured at doses about one order of magnitude lower than the ones required to promote survival. Based on this dissociation, we conclude that the DEVD-AFC/PARP-cleaving aspartase is not the main death effector in our model. It may instead serve a homeostatic function. Blockade of the activation and promotion of survival in neuronally

differentiated PC12 cells by actinomycin, an inhibitor of transcription, argues for ongoing or novel transcription as a prerequisite for the induction of DEVD-AFC/PARP cleavage in neuronal cells following trophic factor deprivation. This may also apply to the aspartase(s) that is (are) more directly related to cell death in this model.

# DISRUPTION OF THE *bcl-2* LOCUS BY REPLACEMENT WITH THE *lacZ* GENE AND NEURONAL CELL DEATH

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Bcl-2 is a major regulator of programmed cell death, a process which is critical in shaping the developing nervous system. It is a membrane-associated protein, expressed in various places in the nervous system. This molecule, selectively protects cultured neurons from apoptosis induced by neurotrophic factor deprivation and its overexpression in transgenic animals prevents lesion-mediated motoneuron degeneration. However, its physiological function in the nervous system remains elusive. To assess whether and to what extent Bcl-2 is involved in regulating neuronal survival and in the neuroprotective action of neurotrophic factors after axotomy, we generated transgenic mice carrying a null mutation of the *bcl-2* gene by homologous recombination. The second exon of *bcl-2* which encodes most of the Bcl-2 protein was replaced by the E. coli *lacZ* gene. In this way, the *lacZ* was placed under the transcriptional regulatory sequences of the *bcl-2* gene (including its 5' untranslated sequence, which might also have a regulatory role in its expression), providing a genetic marker of the mutation which allows us to monitor the developmental time course and the sites of *bcl-2* expression. The basic phenotype of the *bcl-2*$^{-/-}$ mice was similar to that described earlier. In our analysis we have focused on the effects of the absence of the *bcl-2* gene in both the central and peripheral nervous systems, at different developmental stages under physiological and experimental pathophysiological conditions. This study revealed that *bcl-2* is an essential component of the mechanisms controlling neuronal survival during the early postnatal development.

# Mechanisms of invertebrate neuronal death

## Chairman: Lloyd A. Greene

## GENETIC CONTROL OF PROGRAMMED CELL DEATH IN THE NEMATODE *CAENORHABDITIS ELEGANS*

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Of the 1090 nuclei generated during normal development of the *Caenorhabditis elegans* hermaphrodite, 131 undergo programmed cell death. We have characterized develop–mentally, genetically and molecularly the roles of 15 genes that function in some or all of these programmed cell deaths. Four or five of these genes control the death process, six act in the phagocytosis of dying cells by their neighbors, and one functions in the digestion of the DNA of cell corpses. Three additional genes specify which cells will or will not express this cell death program. Some of these genes show structural and functional similarities to genes that act in cell death in vertebrates. For example, the nematode gene *ced-9,* which protects against programmed cell death in *C. elegans*, is similar to the human oncogene *bcl-2*, which also protects against cell death. The nematode gene *ced-3*, which causes cell death in *C. elegans*, is similar to the human gene that encodes the enzyme interleukin 1-beta converting enzyme (ICE), which can cause the programmed death of mammalian cells. The nematode gene *ces-2*, which controls the decision of specific neurons to survive or undergo programmed cell death, is similar to the human gene that encodes the bZIP transcription factor HLF (hepatic leukemia factor), which has been implicated in acute lymphoblastic leukemia and suggested to affect programmed cell death. The baculovirus protein p35, which protects cells from undergoing programmed cell death in nematode, insect and mammalian cells, acts by inhibiting the aspartate-specific cysteine protease activity of CED-3/ICE-like enzymes. These findings indicate that molecular mechanisms responsible for programmed cell death are broadly conserved among organisms as diverse as nematodes and humans.

# The other side of death: molecular genetics of necrotic-like cell death in *C. elegans*

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We study the molecular genetics of necrotic-like cell death in the nematode *C. elegans*. Unusual dominant mutations in two genes, *mec-4* and *deg-1*, induce swelling and degeneration of the neurons that express these genes. *mec-4* and *deg-1* are members of the degenerin gene family related to vertebrate genes encoding subunits of the epithelial $Na^+$ channel. Molecular analyses support a degeneration model in which the initial destructive signal in *mec-4(d)*-expressing neurons is generated via an aberrant channel which, when "locked" into an open conformation, allows toxic excess ion influx.

Serial thin section electron microscopy has revealed that the earliest neuropathological changes in *mec-4(d)*-expressing neurons occur at the plasma membrane, where dramatic whorls of membrane appear. Membrane whorls move into the cytoplasm and large vacuoles appear. In the final stages of cellular demise, chromatin clumps and cytoplasm and organelles are degraded. The resultant degenerative death corpses are removed via the activity of several *ced* genes that function to eliminate programmed cell death corpses.

Some evidence suggests that necrotic-like swelling death can be induced by diverse injury signals. We are interested in identifying genes that might act downstream of damaging injury to execute degenerative cell death. Toward this end we are screening for death-suppressing mutations and we are testing candidate genes for involvement in the process.

**Control of Apoptosis in *Drosophila***

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During *Drosophila* development, large numbers of cells undergo natural cell death. Even though the onset of these deaths is controlled by many different signals, most of the dying cells display common morphological and biochemical changes that are characteristic of apoptosis in vertebrates (1, 2). We have identified two genes, *reaper* and *head involution defective (hid)*, which play a central control function for the activation of apoptosis in *Drosophila* (3, 4). A small deletion that includes both genes protects against cell death in response to many different death-inducing signals. The *reaper* gene is specifically expressed in cells that are doomed to die and is transcriptionally activated in response to many different death-inducing signals (3). This indicates that the integration of different signals occurs, at least in part, by a transcriptional mechanism. Ectopic expression of either *reaper* or *hid* activates apoptosis in cells that would normally live (4, 5). These ectopic deaths can be completely blocked by co-expression of the anti-apoptotic baculovirus p35 protein, suggesting that *reaper* and *hid* kill by activating a *ced-3*/ICE protease pathway. We have identified several ced-*3*/ICE-like genes in *Drosophila*. One member of this family, DCP-1, is closely related to the mammalian CPP32 gene and is ubiquitously expressed during embryonic development. Finally, we have designed a very sensitive and powerful genetic screening system to identify novel cell death genes. A large number of mutations that either enhance or suppress *reaper* and/or *hid*-mediated apoptosis have been identified, and we are in the process of characterizing several of the corresponding genes. We expect that this work will make a fundamental contribution to understanding both the molecular nature of the cell death program, and how that program is regulated by a variety of distinct signalling pathways.

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## INSECT SYSTEMS FOR EXAMINING THE STEROID REGULATION OF CELL DEATH

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In insects, the steroid hormones, the ecdysteroids are potent agents for inducing programmed cell death. In moths and flies, insects with complete metamorphosis, the surges of ecdysteroids that direct metamorphosis also bring about extensive degeneration of larval tissues so that they can later be replaced by the tissues of the adult. These surges of steroid may kill cells outright or they may work in combination with other signaling systems to evoke cell death. The talk will examine the role of ecdysteroids in causing programmed cell death in various tissues in *Drosophila* and in the moth *Manduca sexta*. Emphasis will be on experimental and *in vitro* approaches that help identify cellular interactions that participate with ecdysteroids in inducing death.

A primary interest is understanding the molecular pathways that lead from reception of the steroid to the subsequent death of the cell. The talk will then deal with the ecdysone receptor (EcR) and various transcription factors that are members of the ecdysone-induced cascade. It will explore whether there are patterns of EcR and transcription factor expression that prestage the onset of degeneration and how these factors may then interact with genes that control cell death such as the *reaper* gene of *Drosophila*.

# POSTERS

## PROGRESSIVE MOTOR NEURON DEATH IN AN ANIMAL MODEL OF FAMILIAL AMYOTROPHIC LATERAL SCLEROSIS.

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Mutations of Cu,Zn superoxide dismutase (SOD) cause an autosomal dominant form of familial amyotrophic lateral sclerosis (ALS). An animal model of the disease has been produced by expressing mutant human Cu,Zn SOD containing a glycine$^{93}$ -> alanine substitution (G93A) in transgenic mice (1). In order to quantify the dysfunction of the motor unit in G93A transgenic mice and to identify the origin and the specificity of the motor defects, electromyographic recordings were performed during the course of the disease. The first alterations in neuromuscular function appeared between P63 and P90, i.e before the clinical symptoms. The deficits became even more striking after P100: Compound muscle action potential (CMAP) in the hindlimb decreased by 80 % of initial value. Signs of collateral reinnervation were found at P100, but disappeared at later stages. Denervation signs also appeared progressively during the study and were measured in more than 50% of transgenic mice; these spontaneous fibrillation potentials (SFP) were frequent and appeared before fasciculation potential (FP), as is usually the case in a deficit of neurogenic origin. The number of motor units in the gastrocnemius muscle was progressively reduced over time, down to 28% of the initial value at P130. This decrease was concommittent with the decrease of CMAP and the appearance of SFP. The dysfunctions measured by EMG recording became more severe, in parallel with the intensification of the clinically significant paralysis. However, motor unit potential (MUP) did not vary significantly during the study, confirming that the disease was not of myogenic origin. On the other hand, CMAP measured after stimulation of the facial nerve were not modified, suggesting that the function of upper motor neurones was not affected despite previously documented vacuolar changes. Neither was the conduction velocity in sensory nerves altered. This study showed a biphasic progression of the motor unit dysfunctions in these G93A SOD1 mice, with a transition point at P100. It suggests that at least two components may be involved in the progression of the disease.

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**Pharmacological agents that increase intracellular glutathione arrest the programmed and injury-induced death of motor, but not sensory neurons in the developing chick embryo.**

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In the developing chick embryo large numbers of sensory and motoneurons undergo programmed cell death (PCD) when these cells fail to access target-derived trophic factors essential for their survival; an ontogenetic process that matches the number of sensory and motoneurons to the size of their efferent targets. Since PCD may also be induced in these neuronal populations by injury and/or disease we are interested in characterizing the molecular mechanisms by which sensory and motoneurons undergo PCD. One hypothesis which has received considerable recent attention is that an increase in intracellular reactive oxygen species (ROS) directly or indirectly triggers the programmed demise of developing neurons. To test this hypothesis we determined whether manipulations which increase the cellular anti-oxidant glutathione (GSH) can attenuate the programmed and induced death of sensory and motoneurons. GSH counteracts ROS by reducing the hydroxyl radical, and also by serving as a co-substrate for the detoxification of $H_20_2$ by glutathione peroxidase. We have found that administration of n-acetylcysteine (NAC) and glutathione ethyl ester (GSH-EE), compounds which increase GSH, arrest the programmed death of motoneurons *in vitro* and *in vivo*. In addition, these compounds also protect motoneurons from death induced by deafferentation and axotomy. These observations indirectly implicate oxidants in motoneuron death and suggest that, in addition to neurotrophic factors (e.g. GDNF), amino acid analogs such as NAC may be of therapeutic value for reducing the pathology-induced demise of motoneurons *in vivo*. In contrast, since pharmacological agents that increase GSH failed to rescue sensory neurons from programmed, deafferentation, and axotomy-induced cell death *in vivo*, it suggests that some forms neuronal death may occur independent of the cellular redox state. To address this issue we are currently measuring changes in ROS during the *in vitro* PCD of both sensory and motoneurons. Also, experiments are under way that will discern whether oxidants induce motoneuron PCD directly, by causing cellular damage, or indirectly by signaling other molecular events critical for neuronal death (such as activation of the redox sensitive transcription factor NF-kB).

**ACCUMULATION OF C-*JUN* PROTEIN IS ASSOCIATED WITH THE EARLY PHASES OF NORMAL AND INDUCED APOPTOTIC CELL DEATH IN DEVELOPING NERVOUS SYSTEM**

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The association of inducible transcripcion factor c-*jun* with normal or pathologically induced cell death at developing neuromuscular system was investigated. A polyclonal antibody against an amino terminal peptide sequence of mouse c-*jun* were used. In the chick embryo, motoneurons with c-*jun* positive immunoreactivity were transiently observed in the spinal cord lateral motor column in a period (E7-E8) coincident with the maximum accumulation of pyknotic motoneurons during physiologically-ocurring motoneuron death. Fluorescent double labeling demonstrates that motoneurons with positive c-*jun* immunolabeling also contained fragmented DNA evidenced by TUNEL procedure. Immunoreactivity to c-*jun* in dying (apoptotic) motoneurons sharply delineates the somata in continuity with varicose profiles corresponding to dendrites and axons in a Golgi-like staining pattern. Positive labeling was also seen inside the nucleus, but in areas in which fargmented DNA is accumulated forming dense spherical masses, c-*jun* immunoreactivity was excluded. However, a number of apoptotic bodies displaying positive TUNEL reaction are devoid of c-jun immunoreactivity. Similar finding were observed in dying neurons of dorsal root ganglia. Excitotoxic motoneuron death was elicited by *in ovo* administration of NMDA; in this situation dying motoneurons display morphological features of necrotic form of cell death instead of apoptosis and they do not shown detectable c-*jun* immunoreactivity. During normal or altered chick embryo development, Schwann cells and muscle cells also undergo a physiologically-regulated process of programmed cell death with morphological and histochemical characteristics of apoptosis (Ciutat et al, *J. Neurosci* in press, and Calderó et al, *J. Comp Neurol* submitted); in these cases apoptotic Schwann or muscle cells also displayed *c-jun* positive immunoreaction. Western blot analysis of spinal cord extracts from chick embryos, demonstrated that the antibody recognizes a 39 kDa band that was present in maximum amount on E7-E8. Motoneurons from young rats die by apoptosis when were disconnected from their peripheral targets by perpheral nerve axotomy (Casanovas et al, *Neuroscience* 71:313-325, 1996) and positive c-*jun* immunoreactivity was also evidenced in these cells. It is concluded that c-*jun* accumulation is associated with the early stages of apoptosis in a variery of neuronal and non-neuronal cells.

**BDNF CAN PREVENT EARLY PROGRAMMED CELL DEATH IN NEUROBLASTS FROM EMBRYONIC CHICKEN RETINA RESULTING IN A HIGHER PRODUCTION OF RETINAL GANGLION CELLS.**

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Neurotrophins are known to be trophic factors for neurons during the period of target innervation. Nevertheless, there is a previous period of programmed cell death (PCD) which takes place in neuroblasts undergoing differentiation. Although known for decades, it is not clear how this kind of early PCD is regulated. To address this question we are currently looking for the implication of neurotrophins.

Embryonic chicken retinas, as expected, showed two temporal peaks of laddering (i.e. apoptotic PCD) when genomic DNA isolated from different retinal stages was fractionated in agarose gels, the first peak corresponding to the main onset of differentiation which takes place at embryonic day 6 (E6) and the second one related to programmed retinal ganglion cell death (E12). Apoptotic nuclei, revealed by means of TUNEL staining, showed also a temporal pattern which was consistent with that of DNA fragments one in agarose gels. $^{125}$I- BDNF was shown to bind retinal dissociated cells also in a double-wave temporal pattern that resembles that of apoptotic cell death. Moreover, trkB message, detected by Northern blot and *in situ* hybridization, displayed a two-peak temporal pattern as well. E6 retinas from *in vivo* BDNF-, but not NGF-treated chicken embryos were totally prevented of apoptotic PCD as revealed by both agarose gel laddering and TUNEL staining. Furthermore, four-fold more retinal ganglion cells (RGC) were generated in the former ones, as revealed by the increasing of an specific marker and the total number of axons presented in the optic nerve. Moreover, TUNEL-positive/RGC-specific-marker-positive double labeled neuroblasts could be readily detected as they were migrating to the ganglion cell layer, suggesting that the increasing in RGC numbers is due to neuroblast survival in presence of BDNF.

These data strongly suggest that early cell death is implicated in the RGC's neurogenesis and that BDNF is able to rescue neuroblasts from cell death *in vivo* in central nervous system.

*Genetic convergence of different transduction pathways regulating neuronal survival.*

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In the nervous system different metabotropic pathways are able to mediate survival signals. For example, neurotrophin-activated signaling systems as well as Ca2+- or cAMP-dependent pathways exert neuroprotective effects. In primary cultures of cerebellar neurons, neuronal apoptosis induced by K+ deprivation is reversed by BDNF or PACAP treatments, which activate respectively the trk- or cAMP-dependent pathways. At the molecular level, neuroprotective activities are correlated with an increase in the expression of several immediate early genes, including c-fos and c-jun. We focused our attention on the transcriptional regulation of the c-fos promoter by the neurotrophin-, Ca2+- and cAMP-dependent pathways. These regulatory pathways are largely independent at the cytoplasmic level. Indeed, only PACAP (as forskoline) increases cAMP levels, BDNF or high level of K+ being without effect. Similarly, only K+-mediated depolarization significantly increases intracellular free Ca2+. Finally, inactivation of the cAMP dependent kinase (PKA) with a dominant inhibitor mutant of this enzyme suppress PACAP or forskolin mediated c-fos transcription but does not modify the stimulation effects of BDNF or K+. However, at the nuclear level, BDNF, cAMP and Ca2+ appears to operate by similar trans-activation mechanisms. We show that they ultimately recruit the same cis-acting regulatory elements: CRE, SRE and FAP present in the 5' regulatory flanking region of the c-fos promoter. Moreover, using a Gal4-CREB fusion protein system, we demonstrate that although the BDNF- and Ca2+-activated pathways are independent of PKA, both activate the transcription factor CREB by phosphorylation of ser 133. Our results suggest that trk, Ca2+ and cAMP dependent pathways may regulate neuronal survival through similar nuclear targets.

**Modulation of Bcl-2, Bcl-X, Bax, and c-Fos cell death effector protein expression during neuronal cell death *in vivo* and survival-promoting effects of c-*fos* antisense oligodeoxynucleotides *in vitro***

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Permanent occlusion of the middle cerebral artery (MCA) in rats was used to assess the effects of focal cerebral ischemia on the expression of members of the *bcl-2* gene family which have been implicated in the regulation of programmed cell death. After 6 hours of intraluminal MCA occlusion ischemic cell damage was detected within the ipsilateral caudate putamen, basolateral cortex and parts of the thalamus using magnetic resonance imaging and nitro blue tetrazolium staining of brain sections. In the infarcted basolateral cortex and thalamus fragmentation of DNA was frequently observed in neuronal nuclei using *in situ* end-labeling of DNA breaks by terminal transferase, whereas only scattered labeled nuclei were visible in the infarcted caudate putamen. Immunohistochemical analysis revealed activation of c-Fos in neurons of the infarcted cortex and thalamus and in the non-infarcted cingulate cortex. A decrease in immunoreactivity for Bcl-2 and Bcl-X and an increase in immunostaining for Bax was observed exclusively in neurons within the ischemic cortex and thalamus. Within the infarcted caudate putamen however, protein levels of Bcl-2 family members declined and c-Fos remained absent. RT-PCR revealed a pronounced decrease in *bcl-2* mRNA levels in the ischemic hemisphere, whereas the amount of *bax* mRNA was significantly elevated despite an ischemia-induced suppression in total RNA synthesis. Taken together, a decrease in immunoreactivity for Bcl-2, Bcl-X, Bax, and absence of c-Fos was observed within the ischemic caudate putamen where blood flow is lowest during MCA occlusion and overall protein synthesis is severly suppressed. Low incidence of nuclear DNA fragmentation in the infarcted caudate putamen may indicate that the majority of neurons in the infarct core die by necrosis. In the basolateral cortex and the thalamus however, there was a strong spatial correlation between ischemic cell damage, appearance of nuclear DNA breaks, activation of c-Fos, and modulation of Bcl-2, Bcl-X, and Bax expression. In these brain regions, a shift in the ratio of cell death repressors Bcl-2 and Bcl-$X_L$ to cell death effectors Bax and c-Fos following ischemic cell damage may lead to a derepression of the cell death programme and subsequent neuronal apoptosis.

Amyloid β protein (Aβ) has been implicated in the pathogenesis of Alzheimer´s disease (AD) since mutations in its precursor protein lead to the overproduction of Aβ in some cases of familial AD and since Aβ is directly toxic to neurons in culture. Although the molecular mechanisms by which Aβ causes neuronal death are not completely understood, several *in vitro* studies suggest an Aβ-mediated increase in oxidative stress or destabilization of calcium homeostasis. Since both oxidative stress and calcium influx induce a rapid expression of c-*fos* proto-oncogene in various cells in culture, and since increased immunoreactivity for Fos protein has been reported in AD brains, we investigated whether c-Fos is induced in cultured neurons following exposure to Aβ and whether its activation may play a causal role in Aβ neurotoxicity. Following treatment of a hippocampal neuronal cell line (HT-22) with Aβ (2 μM) number of neurons showing nuclear c-Fos immunoreactivity increased to maximal levels at 1-2 h after addition of Aβ, remained elevated up to 4 h and declined thereafter. Pretreatment with partially phosphorothioated c-*fos* antisense ODNs (5 μM) significantly attenuated the increase in c-Fos immunolabeling 2 h after exposure to Aβ, whereas random sequence control ODNs did not show any inhibitory activity. Inhibition of Aβ-induced c-Fos expression by antisense ODNs significantly ($P<0.01$, student´s t-test) increased cell survival following Aβ challenge compared to control cultures as assessed by MTT assay. Thus, activation of c-Fos may not merely reflect an Aβ-mediated deregulation of signal transduction pathways and may not be involved in a protective cellular stress response following exposure to Aβ, but may be causally linked to Aβ neurotoxicity.

**Neurodegeneration-associated expression of cathepsin D**

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Programmed cell death, including that occurring in the nervous system, is thought to require activity of proteases. However, little is known about the engagement of lysosomal proteolytic machinery in the neuronal cell death. We have investigated expression of cathepsin D (CatD), major lysosomal aspartic protease, in two models of neurodegeneration. Kainic acid, agonist of glutamate receptors, evokes prolonged seizures followed by neuronal death in the limbic system of the rat. MK-801, NMDA receptor antagonist, causes neurodegeneration in rat retrosplenial cortex. We found that, CatD mRNA level increased in limbic structures of rats treated intraperitoneally with 10 mg/kg of kainate and in retrosplenial cortex of animals receiving intraperitoneal injection of 5 mg/kg of MK-801. This increase was observed 24 hours - 7 days after each kind of treatment, i.e. at the time when neuronal cell death occurred. Immunohistochemistry performed 72 hours after the treatments, demonstrated elevated immunoreactivity for CatD localized mainly to degenerating neurons appearing in response to both MK-801 and kainate. Whereas apoptotic DNA fragmentation was found in kainate-evoked brain degeneration, no clear evidence for this alteration was provided in MK-801-evoked retrosplenial damage. Thus, kainate- and MK-801- evoked neuronal degenerations, although apparently differentially engaging endonucleolytic machinery, seem to share the upregulation of cathepsin D expression

P53 EXPRESSION INDUCES APOPTOSIS IN HIPPOCAMPAL PYRAMIDAL NEURON CULTURES.

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The death of populations of neurons occurs naturally during the development of the nervous system. It is clear that during this process neurons die by programmed cell death (PCD) or apoptosis. However, it is now also believed that the death of neurons in certain diseases, including stroke Alzheimer's disease and AIDS-associated dementia, may be due to events related to or identical to those occurring during development. It is clearly important therefore to understand the molecular basis of apoptosis in neurons and how these events can be manipulated.

Studies on a variety of cells have started to define the steps that are involved in apoptosis under different conditions. It is believed that the final common pathway that produces PCD operates in the cytoplasm and is probably common to most if not all cells. Several classes of proteins have powerful modulatory effects on apoptosis. Prominent among these are a group of "tumor suppressor proteins " that include the p53 gene product, the retinoblastoma gene product and several inhibitors of the cyclin dependent kinases, particularly the p21/WAF1/CIP1 protein (p21). The p53 protein plays a central role in the cellular response to DNA damage. It is believed that following DNA damage p53 is responsible for arresting the cell cycle so that DNA repair can occur and that it is also responsible for activating apoptotic pathways if repair seems impossible. Consistent with this idea is the finding that induction of overexpression of p53 following irradiation or DNA damaging ("genotoxic") drugs causes cell cycle arrest and/or apoptosis in a variety of cell types. It is therefore interesting that excitotoxic stimulation has been shown to stimulate p53 production by neurons and it has been suggested that this is responsible for triggering apoptosis under these circumstances. Although this seems to be an interesting possibility, it should be noted that virtually all studies on the role of tumor suppressor genes in apoptosis have been carried out on dividing cells. It is not known whether p53 actually causes highly differentiated, post-mitotic cells such as neurons to die and if so, precisely how this process is manifest in such cells. In the present series of studies we therefore set out to answer this question Our studies examined this issue and provide support for a role for p53 in neurodegenerative disease. Our data show that overexpression of the tumor suppressor gene p53 in cultured rat hippocampal pyramidal neurons using a adenoviral vector caused widespread neuronal death. Cell death under these circumstances exhibited features typical of apoptosis. X-irradiation of these neurons also produced cell death associated with features typical of apoptosis and was accompanied by the induction of p53 immunoreactivity. Overexpression of the retinoblastoma gene product blocked irradiation induced neuronal death, whereas overexpression of the cyclin dependent kinase inhibitor p21 did not. Treatment of neurons with transforming growth factor-β1 or overexpression of the enzyme superoxide dismutase also protected them from irradiation. These results are consistent with a possible role for p53 in neurodegenerative disease.

INSULIN, ACTING AS AN EMBRYONIC GROWTH FACTOR, PREVENTS APOPTOSIS IN CHICK EMBRYOS.

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Development proceeds orderly as a result of the modulation of the instrinsic developmental stage of the cells by signals coming from the cellular environment, including multiple growth factors and cytokines. In response to this orchestrated signalling, the embryonic cells proliferate, differentiate and survive. It is becoming evident that many events triggered by growth factors during vertebrate development may use apoptosis as a default pathway. Among the signalling factors acting on a broad range of cell types, insulin has been exploited over decades to support the growth of cultures of almost any cell type. Paradoxically, its recognition as an embryonic growth factor is very limited. We have previously demonstrated that insulin is expressed in the very early, prepancreatic, chick embryo and promotes growth in early organogenesis. Insulin, as well as insulin-receptor mRNAs are widespread in neurulating chick embryos, as shown by *in situ* hybridization. To further characterize the role of insulin in the neurulating chick embryo, we have studied apoptotic cell death in whole HH10 embryos (10 somites) cultured in defined medium, a growth factor-deprived situation. In these cultures, the embryonic cells undergo progressive apoptosis, evident by their pyknotic nuclei. Exogenous insulin ($10^{-8}$ M) added to the culture rescued by itself about half of the cells. Conversely, oligonucleotides against insulin mRNA and insulin receptor mRNA induced in the cultured embryos up to two-fold increase in the proportion of apoptotic cells. The insulin antisense oligonucleotides indeed decreased the biosynthesis of insulin-immunoreactive material by the embryo. Further more, *in ovo* treatment with AR provoked a 55% increase in apoptotic cells. These facts confirm the fisiological relevance of insulin signaling events on cell survival during early development.

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# TIMING OF NEURONAL DEATH IN *trkA*, *trkB* and *trkC* MUTANT EMBRYOS DEMONSTRATES NEUROTROPHIN SWITCHING IN DEVELOPING TRIGEMINAL NEURONS

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The sensory neurons of the embryonic mouse trigeminal ganglion are supported in culture initially by BDNF and NT3 and later switch to becoming NGF-dependent. To determine if this in vitro switch in neurotrophin responsiveness is physiologically relevant, we studied the timing of neuronal death in the trigeminal ganglia of embryos that have null mutations in the *trkA*, *trkB* and *trkC* genes which encode receptor tyrosine kinases for NGF, BDNF and NT3, respectively. In wild type embryos, the number of pyknotic nuclei increased from E11 to peak between E13 and E14 and decreased gradually at later ages. Neuronal death in the trigeminal ganglia of *trkA*$^{-/-}$ embryos also peaked between E13 and E14, but was almost three-fold greater than in wild type embryos at this stage.There was a great increase in the number of pyknotic nuclei in the trigeminal ganglia of *trkB*$^{-/-}$ at E11 and E12. Counts of the total number of neurons in E13 trigeminal ganglia revealed a marked decrease in *trkB*$^{-/-}$ but not *trk*A$^{-/-}$ or *trkC*$^{-/-}$ embryos. Consistent with the later onset of excessive neuronal death in *trkA*-/- embryos, there was a marked decrease in the neuronal complement of the trigeminal ganglia of *trk*A$^{-/-}$ embryos at E15. These results demonstrate that TrkB signalling is required for the in vivo survival of many trigeminal neurons during the early stages of target field innervation before they become NGF-dependent.

**SPHINGOSINE-1-PHOSPHATE, A NOVEL LIPID SECOND MESSENGER IN NEOROTROPHIN SIGNALING PATHWAYS IN PC12 CELLS**

**Grisha G. Pirianov, Ricardo A. Rius and Sarah Spiegel, Department of Biochemistry and Molecular Biology, Georgetown University, Washington DC**

Members of the tumor necrosis factor superfamily, TNF-α , Fas ligand and nerve growth factor (NGF) have been shown to stimulate the activity of sphingomyelinase, which catalyzes sphingomyelin degradation and results in the formation of ceramide. Breakdown products of membrane sphingolipids, including ceramide and sphingosine-1-phosphate (SPP), are now emerging as a new class of lipid second messengers. Although most of the effects of NGF are mediated by $p140^{trk}$, the high-affinity NGF receptor, some effects may involve the low affinity NGF receptor $p75^{NTR}$, which has structural homology with the TNF-α receptor and activates the sphingomyelin cycle (Dobrowsky et al., Science, 265:1596, 1994).However,we have previously shown in rat pheochromocytoma PC12 cells that NGF activates cytosolic sphingosine kinase, which catalyzes the phosphorylation of sphingosine to produce SPP.Further studies also indicate that activation of TrkA kinase is required for NGF-induced activation of sphingosine kinase.To further substantiate a potential role of SPP in the pleiotropic cellular response induced by NGF, we examined its effect on survival of PC12 cells. SPP not only mimicked the effects of NGF on cellular proliferation, it also prevented programmed cell death (apoptosis) and decreased genomic fragmentation caused by serum deprivation. SPP also attenuated apoptosis induced by UNC-01, a selective inhibitor of calcium-dependent protein kinase C isoenzymes or by dimethylsphingosine. Thus, similar to other growth factors, such as insulin and IGF-1, SPP is a survival factor for neurons. We propose that different signaling pathways regulate the dynamic balance between levels of sphingisine metabolites, ceramide and SPP, may determine whether a cell survives or dies.The beneficial effect of SPP in preventing apoptosis might be important for those diseases in which apoptosis may contribute to the pathology, including AIDS, neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, ischemic stroke, and even normal aging.

Opposing Effects of ERK and JNK-p38 MAP Kinases on Apoptosis

Zhengui Xia, Martin Dickens, Joël Raingeaud, Roger J. Davis, Michael E. Greenberg*

Apoptosis plays an important role during neuronal development and defects in apoptosis may underlie various neurodegenerative disorders. To characterize molecular mechanisms that regulate neuronal apoptosis, the contributions to cell death of mitogen-activated protein (MAP) kinase family members, including ERK (extracellular signal regulated kinase), JNK (c-Jun $NH_2$-terminal protein kinase) and p38, were examined after withdrawal of nerve growth factor (NGF) from rat PC-12 pheochromocytoma cells. NGF withdrawal led to sustained activation of the JNK and p38 enzymes and inhibition of ERKs. The effects of dominant-interfering or constitutively activated forms of various components of the JNK-p38 and ERK signaling pathways demonstrated that activation of JNK and p38 and concurrent inhibition of ERK are critical for induction of apoptosis in these cells. Therefore, the dynamic balance between growth factor-activated ERK and stress-activated JNK-p38 pathways may be important in determining whether a cell survives or undergoes apoptosis.

# List of Invited Speakers

**Workshop on**

## PROGRAMMED CELL DEATH IN THE DEVELOPING NERVOUS SYSTEM

**List of Invited Speakers**


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**Workshop on**

**PROGRAMMED CELL DEATH IN THE DEVELOPING NERVOUS SYSTEM**

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