Molecular Neurobiology

ISSN0893-7648/01/23(2/3): 137–153/$14.25

Intracellular, Nonreceptor-Mediated Signaling by Adenosine

Induction and Prevention of Neuronal Apoptosis

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Abstract

Inhibitory effect of adenosine on the isolated heart muscle and vascular system were first described in 1929 *(1).* Since then, numerous reviews have been published on the diverse actions of this nucleoside on a wide variety of cell types. Essentially all effects of adenosine in neurons and nonneuronal cells are mediated by activation of nucleoside membrane receptors coupled to specific intracellular second messenger pathways. This brief review describes two novel actions of adenosine in peripheral sympathetic neurons, which are not mediated by adenosine receptors. First is described how adenosine and related nucleosides are able to induce apoptosis during the initial stages of neuronal growth and development in vitro and in vivo. Second is discussed how adenosine is able to prevent or delay apoptosis in more mature sympathetic neurons subjected to nerve growth factor deprivation in culture. Both the induction and prevention of apoptosis are independent of receptor activation, and totally dependent on the intracellular accumulation and subsequent phosphorylation of adenosine. The physiological significance and mechanisms by which adenosine can induce apoptosis in one situation, and rescue from apoptosis in another, are described in this article.

Index Entries: Apoptosis; sympathetic neurons; nucleoside; adenosine.

Introduction

Numerous actions of adenosine in nonneuronal and neuronal cells are mediated by stimulation of different types of nucleoside cell membrane receptors. These receptors, coupled through G proteins, produce final effect through activation of various second-messenger pathways. However, there is growing evidence that adenosine gets internalized, then produces its toxic effects via intracellular mechanisms in lymphocytes. It seems that, in

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some cells, the nucleoside and its analogs act through membrane receptors, as well as intracellular sites. This article discusses toxic and protective actions of adenosine and 2′-deoxyadenosine (2′-dA) in neuronal cells. Both these actions seem to be mediated by internalized nucleosides.

All types of neurons, including those of the sympathetic nervous system, depend on specific growth factors for their survival and development. However, even in the presence of growth factors, neurons are susceptible to agents or procedures that can cause cell death. Neuronal death from neurotoxic injury can be either necrotic or apoptotic, depending on the nature of the toxic insult. Neuronal cell death, as studied in this project, occurs under two very different sets of circumstances, but, in both cases, is apoptosis. In one case, when freshly plated neurons are exposed to some neurotoxic agents, the cell bodies do not extend neurites, and remain undifferentiated for 12–24 hs, and eventually fragment and die by apoptosis, even in the presence of nerve growth factor (NGF). In the other case, sympathetic neurons, grown in the presence of NGF for a few days, then deprived of this trophic factor, undergo neurite fragmentation, shrinkage of cell bodies, leakage of mitochondrial enzymes, and loss of uptake/storage of their transmitter, and eventually die. This trophic factor deprivation-induced death has been well-characterized as apoptotic, and is extensively used to study programmed cell death in a variety of neuronal models.

What the authors have found in recent years is that the endogenous nucleoside, adenosine, induces apoptotic cell death in freshly plated sympathetic neurons, even in the presence of NGF. However, when NGF is removed from 2–3-d-old matured sympathetic neurons, and replaced with adenosine, apoptosis is prevented, and sympathetic neurons continue to survive for a few days in the absence of NGF. Thus, adenosine has the unique ability to influence neuronal survival in a positive or negative manner at different stages of development.

Induction of Apoptosis by Adenosine

Neurotoxic Actions of Nucleosides

A voluminous literature exists on the toxic effects of nucleosides in lymphocytes, in the production of severe combined immunodefficiency syndrome (SCIDS). It was realized that adenosine and 2′-dA have potential to be toxic agents in different types of cells. There is evidence from in vivo experiments and clinical studies that adenosine and 2′-dA do produce neurological disorders. Inhibition of adenosine deaminase (ADA) in mouse, by 2′-deoxycoformycin (dCF) produced a 50-fold increase in 2′-dA levels and massive cellular degeneration in neuroepithelium *(2).* High incidence of tremors in hands, trunk, and legs normally, observed in SCIDS patients, was significantly reduced after ADA therapy, and the neurologic examination was within normal limits *(3).* In another case study, neurological abnormalities, such as failure to focus and follow light, nystagmus, and absence of reflexes, in addition to retarded personal, social, language, and gross motor functioning, were recorded *(4).* There was a diffuse central nervous system (CNS) dysfunction and possible cortical blindness. All these clinical reports clearly suggest that both nucleosides are potentially toxic, not only to lymphocytes, but to many other types of cells, including neuronal cells.

The authors' studies provided more direct evidence for the toxic actions of adenosine and 2′-dA in cultured neuronal model. When adenosine is added to freshly plated sympathetic neurons of 10-d-old embryonic chick, it produces dose-dependent inhibition of cell growth, and eventually kills most of the neurons in 1–2 d *(5).* This effect was shared by 2′-dA but not by any other nucleosides or metabolites of their products (*see* the following section). Clearly, the toxic effects of nucleosides cannot be attributed to some anti-NGF action, because sympathetic neurons, supported in culture by factors other than NGF, such as phorbol esters or excess K^+, are also

susceptible to nucleoside-induced toxicity *(5–7).* These results indicate that the nucleosides are affecting survival of sympathetic neurons, not by interfering with neurotrophic action of NGF, or perhaps of other agents, but by inducing cell death by blocking some metabolic pathway of the neurons specifically linked to neuronal development and survival.

Generality of Nucleoside Toxicity and Nature of Cell Death

If the novel action of adenosine that the authors have discovered is indeed involved in controlling survival of neurons *in vivo,* then several other important points should be considered. One is that neuronal death should be physiological, and proceed perhaps by apoptosis, rather than by necrosis. Apoptosis, or programmed cell death, accounts for the removal of as many as half of the neurons formed during embryonic development. Unlike necrosis, which involves loss of cell membrane integrity and an inflammatory response, apoptosis causes the cell to digest and package itself for clean disposal by macrophages. Nuclear hallmarks of apoptosis include chromatin condensation and endonuclease digestion of DNA to nucleosomal-sized fragments *(8,9).* Bisbenzimide, a nuclear dye capable of detecting chromatin condensation, and TUNEL staining for DNA fragments, were used to identify these markers of apoptosis, and to determine whether adenosine-mediated neuronal death was a necrotic or apoptotic. Quantifying apoptotic cells, identified by either bisbenzimide or TUNEL, showed apoptosis-positive cells in >30% of neurons in adenosine-treated cultures, compared to ~1% apoptotic cells in control cultures *(5).*

A second point that needed to be examined was whether nucleoside-induced apoptosis could be observed in the whole-animal model, as well as in the culture model. To determine this, effects of adenosine and 2′-dA were monitored in developing avian embryos. Both nucleosides produced cell loss, and were teratogenic at sublethal doses *(10).* There was a significant reduction of neuronal cells in sympathetic ganglia, and, equally important, the loss of sympathetic neurons was accompanied by an increased number of cells positive for apoptosis, using TUNEL staining. The nucleoside-induced increase in apoptosis and loss of neuronal cells was not limited to sympathetic ganglia, but was also observed in optic tectum *(10).*

The effects of nucleosides were also examined on primary cultures of rat chromaffin cells, since chromaffin cells and sympathetic neurons are derived from the same precursor cells. 2′-dA but not adenosine, caused cell death *(11).* These results again show the generality of nucleoside toxicity in neurons and neuron-like cells.

Finally, if adenosine-induced apoptosis regulates neuronal survival, the phenomena should not be restricted to peripheral sympathetic neurons, but should be observed in other neurons, as well. The well-established model for study of CNS apoptosis, cerebellar granule neurons (CGN) cultured from neonatal rats, was used to test this possibility. Several of the following unpublished results point out some similarities and differences between adenosine-induced toxicity in rat CGN and chick sympathetic neurons. Adenosine-treated CGN showed large increases in TUNEL-positive cells, suggesting death by apoptosis. 2′-dA was also toxic to these mammalian neurons, and this effect was enhanced by ADA inhibitors. As observed in avian neurons, adenosine neurotoxicity was not mediated by membrane receptors, but required intracellular accumulation. However, there were several major differences between CGN and sympathetic neurons. For example, 2′-dA inhibited DNA turnover/repair by >90%, but adenosine had no effect at all in CGN. A nonmetabolizing nucleoside, 2′-chlorodeoxyadenosine, killed all the CGN at 10 μ*M* concentration. Nucleoside transport inhibitor, nitrobenzylthioinosine (NBTI), did not protect against adenosine toxicity, as it did in sympathetic neurons. Taken together, all the above findings show generality and broad spectrum of toxic nature of nucleosides in different types of neurons and neuron-like cells.

Nonreceptor-Mediated Actions

Generally, toxic effects of nucleosides on dividing immune cells are mediated by an intracellular action. However, there are also reports that activation of membrane receptors linked to various second-messenger pathways, like phosphatidyl inositol metabolism and cyclic adenosine monophosphate (cAMP) stimulation, are responsible for lymphocyte toxicity by nucleosides *(12,13).* There is also evidence that activation of adenosine receptors can stimulate apoptotic cell death in several other cell types *(14–16).* More recently, A1-type adenosine receptors were implicated in ischemia-induced death of CNS neurons *(17).* Thus, based on such literature, one might expect that the neurotoxic actions of nucleosides in postmitotic sympathetic neurons are a result of activation of membrane receptors.

Using appropriate pharmacologic agonists of A1 and A2 receptors (N^6-cyclopentyladenosine and CGS21680, respectively) and antagonists (aminophylline), the authors have ruled out the participation of extracellular site of action of adenosine and 2′-dA. More recently, A3 receptors have been implicated in the actions of adenosine in nonneuronal cells. For example, activation of A3 receptors by specific A3 agonist, 2-chloro-N6-iodobenzyl-5-N-methylcarboxamidoadenosine (CI-IB-MECA), caused apoptosis in cardiac myocytes *(18).* Activation of human A3 receptors in transfected Chinese hamster overy cells impaired cell cycle progression *(19).* For additional adenosine-mediated actions via A3 receptors, *see* a recent review *(20).* Based on this new evidence, the authors tested the effects of A3 adenosine receptor agonist CI-IB-MECA on freshly plated chick sympathetic neurons, in two separate experiments. This A3 agonist did not kill the neurons, even up to 50 μ*M* concentrations. Furthermore, ADA inhibitor, together with CI-IB-MECA, also had no deleterious effect on neuronal survival (unpublished results). Kull et al. *(21)* have reported that P2X purinoceptor is not necessary for thymocyte apoptosis. Thus, it is becoming clear that nucleoside receptors are not always associated with all the actions of nucleosides, especially apoptosis.

A number of pieces of additional evidence, including protection by nucleoside transport inhibitors (*see* the following section), also rule out the role of receptors in this novel action of nucleosides. The toxicity of nucleosides described in sympathetic neurons clearly seems to be independent of membrane receptors. Lack of receptor involvement strongly suggests an intracellular role of nucleosides in the apoptotic death of sympathetic neurons.

Adenosine Transporter in Sympathetic Neurons

If the nucleosides exert their toxic effects by their intracellular action, then these agents must enter sympathetic neurons. Clearly, the adenosine transporter has been identified in the CNS, and is known to be responsible for intracellular accumulation of adenosine *(22).* Using radiolabeled adenosine, the authors have also found a specific nucleoside transporter in chick embryo sympathetic neurons, which produces a time- and concentration-dependant accumulation of adenosine *(23).* Adenosine transport was almost 10× greater than that of 2′-dA, and did not saturate over a 2-h period. 2′-dA uptake reached its maximum within a few minutes. Furthermore, the transport of [^{3}H]adenosine was almost completely blocked by specific inhibitors of the nucleoside transporters, NBTI and dilazep *(5,6,23).* Thus, comparison of adenosine transport in chick sympathetic neurons, or in other types of neurons and cells, shows significant variations in their kinetic properties. It may be that different types of cells adopt different mechanisms for handling the nucleosides.

A major difference exists between the catecholamine transporter and the nucleoside transporter, in sympathetic neurons. Catecholamines are predominantly taken up and retained in neurites, but are poorly retained in somatic regions of sympathetic neurons *(24).* Adenosine, on the other hand, is taken up and

retained in large amounts in freshly plated neurons (i.e., predominantly in cell bodies), before the growth of neurites. These results indicate the importance of the adenosine transporter in neuronal cell bodies, and its possible critical role in modulating survival of neurons. Possibly adenosine reuptake is essential in all regions of neurons, for different uses.

By far the most important observation, offering direct evidence for the intracellular site of action of the nucleosides in sympathetic neurons, is that the nucleoside transport inhibitors almost completely prevented adenosine toxicity *(5).* A similar type of protection against adenosine and 2′-dA toxicity, by transport inhibitors, has been reported in nonneuronal cells *(25).* However, a major difference is that, in the authors' model, NBTI and dilazep prevented neuronal death by adenosine, but not by 2′-dA *(23).* This difference in the protective action of transport inhibitors is consistent with the different transport properties of the two nucleosides (*see* the previous section). Of course, lack of protection against 2′-dA toxicity by nucleoside transport inhibitors does not mean that 2′-dA exerts its lethal action by an extracellular site of action. It is entirely possible that 2′-dA enters sympathetic neurons by some mechanism other than the classic nucleoside transporter, and therefore transport inhibitors fail to protect neurons from 2′-dA toxicity.

If adenosine worked by an extracellular mechanism, blockade of transport would be expected to increase the extracellular concentration, and to facilitate, rather than inhibit, toxicity. Neurons kept in high concentrations of adenosine were rescued by pyrimidine supplementation with uridine or 2′-deoxycytidine, or by inhibiting adenosine kinase (AK) with iodotubercidin (*see* the following section). Taken together, all these experiments reinforce the conclusion that membrane receptors and their second-messenger pathways do not participate in the toxic actions of adenosine or 2′-dA in sympathetic neurons.

Some of the investigators who first reported that adenosine-induced apoptosis occurs by activation of membrane receptors, through specific signaling pathways, have recently deviated from this conclusion. 2-chloroadenosine and deoxy-D-ribose are known to cause apoptosis in mammalian astroglial cells, by activation of extracellular receptors, but the signaling pathway is not known *(26).* On the other hand, in human astrocytoma cell line, adenosine analogs were found to induce toxicity by intracellular accumulation, followed by its phosphorylation *(27).* Finally, apoptosis by 2-chloro-2′-dA and 2-chloroadenosine, in human peripheral blood mononuclear cells, was suggested to proceed by three pathways: activation of A2A-like extracellular membrane receptors; activation of A3 extracellular membrane receptors; and entry of nucleosides into cells, followed by activation of intracellular events leading to apoptosis *(28).* These different conclusions, coming from the same laboratory, imply that each cell type has a unique pathway for initiating the process of apoptosis, and there is not a uniform mechanism of nucleoside toxicity.

Intracellular Site of Action

Intracellular adenosine or 2′-dA is metabolized by multiple pathways involving different enzymes in different tissues. The various metabolic pathways and enzyme kinetics are well-established, and have been derived from a variety of cell types. Although nucleoside metabolism in sympathetic neurons is expected to be similar to other cells, studies of nucleoside biochemistry have not been carried out in sympathetic neurons *per se.* Among the different metabolic pathways, the most dominant involves deamination of adenosine and 2′-dA by ADA to their metabolites, which are excreted in the urine. Actually, it has been reproted that AK, another enzyme, which converts adenosine to nucleotides, has a much lower Km than ADA. However, the total amount of ADA is much higher than AK, and therefore intracellular adenosine is primarily degraded to inosine *(29,30).* Conversion of adenosine by AK is considered to be a salvage pathway that produces excess AMP, adenosine

diphosphate (ADP), and adenosine triphosphate (ATP). Another pathway for removal of adenosine is its conversion to S-adenosylhomocystine (SAH) by S-adenosylmethyonine (SAM) hydrolase and L-homocystine. SAH potently inhibits SAM-mediated methylation involving DNA, RNA, and protein. Which of these pathways contribute to generation of metabolites, capable of inducing neuronal death by the nucleosides, was an important question to be addressed.

Inhibition of ADA by specific and potent inhibitors, 2′-deoxycoformycin (dCF) and erythro-9-(2-hydroxy-3-nonyl)adenine, resulted in marked potentiation of adenosine toxicity, but not of 2′-dA, in sympathetic neurons. There was almost 10-fold less concentration required to obtain the same extent of toxicity in the presence of ADA inhibition *(5).* These results provided two important pieces of evidence. First, and most important, the deamination pathway is not responsible for neurotoxicity. Second, ADA is very active in removal or metabolic degradation of intracellular adenosine in sympathetic neurons, as in other cells. Elimination of this pathway in the neurotoxicity directed attention to the remaining two routes of nucleoside metabolism. The pathway involving SAH/SAM was investigated using L-homocysteine thiolactone, a membrane permeant analog of SAH. The toxic action of adenosine was not potentiated by up to 300 μ*M* L-homocysteine thiolactone, and this agent also had no adverse effects on its own *(5).* Thus, the authors found no evidence to support impairment of methylation in the mechanism of adenosine toxicity in the model.

Elimination of the above pathways leads to the last possibility, phosphorylation of adenosine by AK to nucleotides. First, AK activity in chick sympathetic neurons (2.59 ± 0.35 nmol/min/mg protein) *(5)* was even higher than that reported for human liver (1.48 nmol/min/mg protein) *(31).* Blockade of this pathway by appropriate AK inhibitors completely protected sympathetic neurons against adenosine toxicity. The neurotoxicity of 2′-dA was also found to be completely prevented by blockade of AK. 5′-iodotubercidin (ITu) and 5′-amino-5′-dA were used as AK inhibitors, and detailed dose-response studies showed that adenosine and 2′-dA may be phosphorylated by different types of nucleoside kinases in sympathetic neurons *(23).* The authors also showed that ITu completely inhibits the enzyme activity of AK *(6).* Results of these experiments led the authors to conclude, without hesitation, that phosphorylated products of intracellular adenosine and 2′-dA are responsible for death of sympathetic neurons. Treatment of HL-60 cells with adenosine analogs produced apoptosis that was caused by phosphorylation of internalized nuleosides, rather than by activation of membrane receptors *(32).*

Additional proof for this pathway's contribution in nucleoside toxicity came from analysis of ATP and the effect of AK inhibition on neuronal ATP content. ATP content of sympathetic neurons increased in a time-dependent manner, after adenosine exposure: Significant increases were observed within 4 h, and maximum levels were reached by 18 h. As would be expected, this increase in ATP was almost completely abolished by ITu treatment *(23).* In the case of 2′-dA, phosphorylation results in large increases in dATP in a time- and concentration-dependent manner *(6).* Excess dATP is a known inhibitor of ribonucleotide reductase, and is accompanied by depletion of endogenous ATP. The authors have suggested that the dramatic reduction of ATP by dATP could lead to neuronal death *(23).* ITu prevented dATP formation, and protected sympathetic neurons from 2′-DA toxicity *(6).* The authors provided additional support for the idea that ATP depletion is a cause of neuronal death by 2′-dA, by increasing ATP content with adenosine. Clearly, ATP content of a tissue, after nucleotide treatment, could change in either direction, and still cause apoptosis, because of several other mechanisms working simultaneously to initiate the cascade of apoptosis, which may be unrelated to ATP content. In fact, recent studies showed that phosphate uptake was decreased, without any change in

ATP content, during apoptosis of PC12 cells *(33).*

The most vivid example of the key role of AK in nucleoside toxicity was obtained from experiments carried out in another neuronal cell type. In primary cultures of chick embryo dorsal root ganglion cells, neither adenosine nor 2′-dA had any adverse effect on these sensory neurons *(34).* Sensory neurons did express nucleoside transport, and retained large quantities of [^{3}H]adenosine and [^{3}H]2′-dA. However, AK activity of these neurons was only one-tenth that in sympathetic neurons. These findings clearly show the importance of AK in inducing toxic effects of nucleosides in neurons, and perhaps in other cells. This housekeeping enzyme is mostly downregulated in sensory neurons, compared to sympathetic neurons, when both types of neurons are derived from the same embryonic precursor cells.

The above data supported the idea that ITu-sensitive phosphorylation of adenosine or dA to AMP, ADP, and ATP, or to the deoxynucleotides, respectively, plays an important role in nucleoside toxicity in sympathetic neurons.

Interaction Between Pyrimidine Nucleotides and Adenosine Toxicity

Adenosine and its phosphorylated metabolites are known to affect the synthesis of pyrimidine nucleotides in dividing cells, including HeLa cells, fibroblasts, and lymphoblasts. The depletion of pyrimidines directly affects the synthesis of DNA and RNA. Thus, depletion of pyrimidine pools may play a role in adenosine toxicity. To determine whether the depletion of pyrimidine nucleotides was involved, sympathetic neurons were supplied with excess uridine or 2-deoxycytidine, to maintain pyrimidine pools, and thus prevent impaired synthesis of DNA and/or RNA. Uridine and 2-deoxycytidine almost completely and dose-dependently prevented the toxic effect of adenosine *(5).* Neither agent by itself affected the survival or growth of neurons, nor did they interfere with the transport of adenosine. The rescue from adenosine toxicity by pyrimidines indicates that the mechanism of toxicity results from accumulation and phosphorylation of adenosine, and consequent impairment of pyrimidine nucleotide synthesis. This would be expected to impair both DNA and RNA synthesis, but, since these neurons are postmitotic, impaired DNA synthesis may not be as important as RNA, particularly new message or transiently expressed message and proteins necessary for growth and survival.

Adenosine Inhibits RNA and Protein Synthesis

To test the hypothesis that phosphorylated products of adenosine cause depletion of pyrimidine pools, which in turn inhibit mRNA and protein synthesis, the incorporation of [^{3}H]uridine into RNA was monitored in freshly plated control and adenosine-treated cultures of sympathetic neurons. Adenosine reduced [^{3}H]uridine incorporation by ~30%, in as little as 1 h, and had a maximum effect of ~60% at 8 h. Pretreatment with ITu successfully reversed the inhibitory effect of adenosine *(35).* An obvious consequence of inhibition of RNA synthesis would be inhibition of synthesis of new proteins. Therefore, protein synthesis was studied using [^{35}S]methionine. Adenosine inhibited radiolabeled methionine incorporation by 25% in as little as 2 h, and almost 50% inhibition occurred over a 12-h period. Since labeled methionine uptake was measured over extended periods, it could be argued that protein turnover, rather than synthesis, was inhibited by adenosine in the above experiments. Additional experiments carried out, in which neurons were treated with adenosine for 3–4 h, then pulsed with [^{35}S]methionine for only 10 min, also showed a significant inhibition (47 ± 3%, n = 5) of the synthesis of new proteins (unpublished results).

The above studies showed for the first time that adenosine has the unique action of inhibiting both RNA and protein synthesis in chick embryo sympathetic neurons. The neurotoxic

actions of adenosine, as well as the inhibition of RNA and protein synthesis, are completely prevented by AK inhibitors. These results imply that, among the various metabolic routes for adenosine in the cell, phosphorylation appears to be responsible for inhibition of RNA and protein synthesis, and for the toxicity. Very likely, excess formation of AMP and ADP (as evidenced by elevated ATP concentrations) inhibits phosphoribosyl pyrophosphate (PRPP), and thereby causes depletion of pyrimidine pools in sympathetic neurons. Depletion of this important biochemical intermediate would clearly interfere with other key cell functions, such as DNA and RNA synthesis. Indeed, depletion of PRPP and pyrimidine pools, and consequent impairment of DNA synthesis, is a major pathway for adenosine toxicity in human erythrocytes *(36).* In postmitotic sympathetic neurons, inhibition of DNA synthesis would be expected to have minimal effects. Thus, the authors propose that inhibition of new mRNA and protein synthesis would be a major cause of neuronal death. In dividing cells, such as rat liver hepatocytes, adenosine has been reported to inhibit protein synthesis *(37).* However, the inhibitory action was not mediated by the phosphorylation pathway, but by adenosine-driven accumulation of SAH, and consequent SAH impairment of methylation reactions and decreased protein synthesis *(37,38).* Still another level of mechanism of action of adenosine was reported, in which tyrosine phosphatase plays a vital role in the actions of adenosine by decreasing p38α activity *(39).* The significance of such diverse pathways, leading to inhibition of protein synthesis by adenosine in different types of cells, remains unclear.

Time Window for Adenosine Toxicity Is Limited

One of the intriguing findings in this study was the limited time window in which adenosine exerts its toxic effects in sympathetic neurons. Sympathetic neurons, cultured from 10–12-d-old embryos, die in 1–2 d, when adenosine or 2′-dA is added to cultures at the time of seeding of the cells. Neurons continue to be susceptible to adenosine toxicity over the first 16 h in culture. After that point, even high concentrations of adenosine do not kill sympathetic neurons. On the other hand, 2′-dA is toxic to sympathetic neurons at any time during culture, from a few hours to several days. Clearly, the inability of adenosine to induce neurotoxicity is not a result of lack of transport of adenosine into sympathetic neurons, or its subsequent phosphorylation, which are the essential steps leading to apoptosis. Both of these pathways are working as efficiently in 1- to several-day-old sympathetic neurons as they are in freshly plated neurons, yet only freshly plated neurons are sensitive to adenosine toxicity. This time window for adenosine toxicity is consistent with the idea that adenosine inhibits the expression of some protein necessary for the initial growth and survival of sympathetic neurons.

Studies using differential display polymerase chain reaction (DDPCR) have indeed revealed a transient message that appears in freshly plated sympathetic neurons, but not in 2-d-old cultures (Fig. 1). The message is drastically reduced or eliminated by adenosine, but other messages are not. This message may well represent a critical molecule that is necessary for the initial growth and development of sympathetic neurons, and, when inhibited by accumulation of excess adenosine, is responsible for the induction of apoptosis. Ongoing work to identify this and other transient messages and their proteins, which are inhibited by adenosine, should offer new insights into the mechanism of apoptosis in general and the mechanism of adenosine-induced neuronal death in particular.

Summary: Mechanism of Adenosine-Induced Apoptosis

At this point, clearly, adenosine is able to induce apoptosis in both central and peripheral neurons in birds and mammals. The neurotoxic action is mediated by nucleoside transport, and

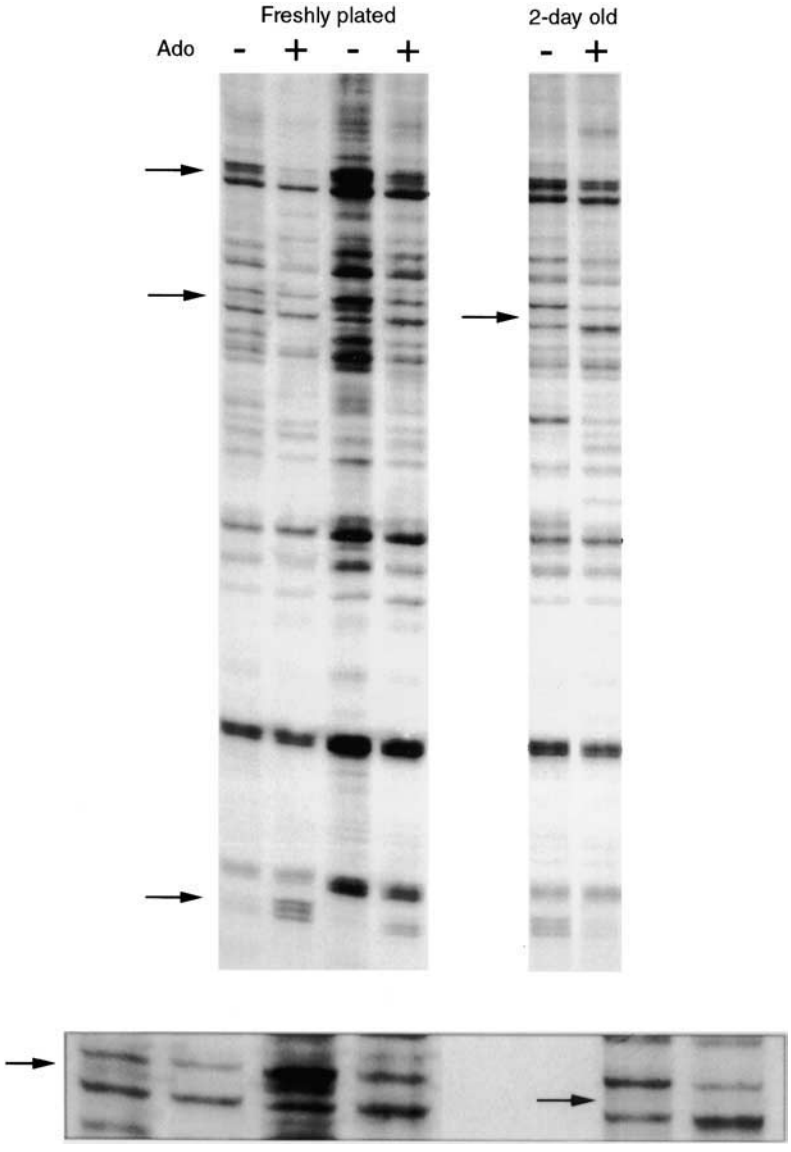

Fig. 1. DDPCR was used to search for proteins that might be unique to initial growth and development of neurons, and also be inhibited by adenosine *(40).* Sympathetic neurons were grown for 4 h in medium, with or without 100 μ*M* adenosine plus dCF. Total RNA was extracted, and DDPCR performed, using CT-11 primer for reverse transcription, and HAP-1 and CT-11 for forward and reverse PCR primers, respectively. The autoradiograph shows messages in the presence of adenosine (lanes labeled [+]) in 4-h-old neurons (freshly plated), and in 2-d-old neurons (2-day old), compared to control medium (lanes labeled [–]). Arrow 1 points to a most intriguing band (enlarged, and marked by arrow heads in the bottom insert). This message was present in freshly plated cultures in control medium, but was absent in 2-day old cultures. The message was inhibited in adenosine-treated neurons, even when other messages were increased by adenosine (2, e.g., 4-h and 2-d neurons).

Reamplification of the control and test RNAs at different dilutions, to overcome potential differences in cDNA synthesis, gave the same results. More important, independent assay of the RNA, prepared from a different batch of control and adenosine-treated cultures, resulted in the same transient band being inhibited by adenosine.

phosphorylation is the major pathway responsible for neuronal death. Furthermore, the reversal of apoptosis by pyrimidine replacement suggests that accumulation of phosphorylated metabolites (AMP, ADP, and ATP) causes inhibition of DNA and RNA synthesis by adenosine. This was confirmed by demonstrating adenosine-induced inhibition of tritiated uridine incorporation into RNA. Similarly, adenosine caused a decreased synthesis of proteins measured by tritiated methionine incorporation. Thus, inhibition of mRNA synthesis and proteins essential for cell survival provide a likely mechanism of adenosine-mediated apoptosis in sympathetic neurons.

Adenosine Protects Against NGF-Deprivation-Induced Apoptosis

From the above discussion, two facts emerge concerning some of the unique actions of adenosine in sympathetic neurons: First, adenosine is neurotoxic only during the early stages of development, but has no adverse effect on more developed neurons; second, regardless of the developmental stage, adenosine continues to be a partial inhibitor of protein synthesis in sympathetic neurons. These facts prompted the authors to explore an additional property of adenosine that has not been considered in the past. The rationale for this thinking was as follows. Well-developed sympathetic neurons are known to undergo apoptosis when NGF is removed from the culture medium. NGF deprivation activates the cell death program, requiring synthesis of new message and proteins for execution of neuronal death. Since adenosine has an unique ability to act as a protein synthesis inhibitor, and to not be neurotoxic in developed sympathetic neurons, the authors considered that adenosine may be an endogenous molecule capable of rescuing sympathetic neurons from NGF-deprivation-induced apoptosis.

Here, the authors discuss data showing that adenosine inhibits NGF-deprivation-induced cell death, and significantly prolongs the period within which readdition of NGF can rescue the neurons. Here again, the neuroprotective action of adenosine is not affected by membrane-receptor-blocking drugs. However, block of nucleoside transport by NBTI, or inhibition of AK by ITu, prevents adenosine protection of NGF-deprived sympathetic neurons, supporting an intracellular mechanism similar to that involved in the previously discussed toxicity. Adenosine also causes an approx 20% inhibition of RNA and protein synthesis in more mature neurons, consistent with the idea that specific proteins, necessary for apoptosis, are inhibited by adenosine. The activity of caspase-3 (CPP32, apopain) is completely suppressed in NGF-deprived, adenosine-rescued neurons. Thus, adenosine, known to have diverse effects on neuronal function, has the additional ability to prevent apoptosis of sympathetic neurons by inhibiting expression of the death program. This mechanism could have an important physiological role in situations (e.g., ischemia) in which excess adenosine is known to be neuroprotective.

Adenosine Prevents NGF-Deprivation-Induced Cell Death

Sympathetic neurons, supported by NGF in culture, exhibit dense neurite outgrowth, and bright, light refractory cell bodies. When these neurons are deprived of NGF by washout and addition of NGF antibodies, the cells are induced to undergo apoptosis *(41,42),* and show fragmentation of neurites and disintegration of cell bodies within 24–48 h. However, if adenosine is added at the time of NGF withdrawal, neurons exhibit healthy neurites, and cell bodies are indistinguishable from NGF-supported neurons.

Although adenosine-supported sympathetic neurons are morphologically similar to neurons maintained by NGF, it is important to determine whether neurons grown under the two culture conditions are functionally simi-

lar. A hallmark of healthy, functional sympathetic neurons is the uptake, retention, and exocytotic release of their neurotransmitter, norepinephrine (NE). The authors have previously shown that the number of live neurons corresponds closely to the functional property of [^{3}H]NE uptake *(23)*. Therefore, the characteristic uptake and retention of [^{3}H]NE is monitored, to assess the functional integrity of neurons rescued by adenosine. A significant increase in [^{3}H]NE uptake, compared to NGF-deprived controls, was observed with 10 µ*M* adenosine, and the maximum effect of 100 µ*M* adenosine restored [^{3}H]NE uptake to >80% of NGF-supported controls. The maximum uptake of label in the presence of 100 µ*M* adenosine was as good as that in NGF-supported neurons grown in sister dishes. To assure that adenosine was not affecting uptake properties, [^{3}H]NE uptake was determined in neurons maintained in NGF with and without adenosine plus dCF. Adenosine and dCF, alone or in combination, had no stimulatory or inhibitory effect on the uptake properties of these neurons (unpublished results).

Rat sympathetic neurons can be rescued (50% survival) by readdition of NGE, within ~1 d of NGF withdrawal, but after this time the cells are committed to die *(42)*. Similarly, in embryonic chick sympathetic neurons, NGF-deprivation-induced cell death is prevented, when NGF is reintroduced within 15–18 h *(43)*. The authors questioned whether adenosine could rescue neurons with a similar time-course, and found that both adenosine and NGF rescued >50% of NGF-deprived neurons, when added within 9 h of NGF withdrawal. After 15 h NGF withdrawal, survival was significantly reduced, compared to NGF-supported cultures, whether adenosine or NGF was added as the rescuing agent. However, although NGF was able to rescue ~50% of the neurons after 15 h NGF withdrawal, adenosine was ineffective at this time. By 24 h NGF was also ineffective in rescuing NGF-deprived cultures (unpublished data).

Adenosine Prolongs Survival and Duration of Rescue Period in NGF-Deprived Neurons

Time-course studies showed that adenosine prevented death in >50% of NGF-deprived neurons for at least 48 h after NGF withdrawal. Even after 96 h, NGF-deprived cells, supplemented with adenosine, showed significantly more [^{3}H]NE uptake than those deprived of NGF without adenosine. From the change in [^{3}H]NE uptake over time, it was clear that NGF-treated controls were growing and NGF-deprived cultures were dying, as reflected by increasing and decreasing [^{3}H]NE uptake, respectively. However, adenosine-treated cultures showed relatively less change in [^{3}H]NE uptake over the experimental period, consistent with inhibition of proteins necessary for cell death, as well as those necessary for cell growth. If this interpretation is correct, then adenosine should also extend the time within which NGF-deprived cells can be rescued by readdition of NGF. Indeed, adenosine prolonged the rescue period to >48 h after NGF deprivation, and significantly increased the ability of NGF to rescue NGF-deprived neurons at all times over a 4-d experimental period.

In cultures supported for 2 d by NGF, then deprived of NGF for 24 h, <25% of the cells survived. Reintroduction of NGF to 24h-deprived cultures caused a slight but significant increase in survival, to ~40%. Thus, ~60% of chick sympathetic neurons were committed to die after 24-h NGF withdrawal. However, in cells exposed to adenosine during NGF-deprivation, reintroduction of NGF rescued >80% of the neurons within 24 h.

Adenosine also improved the ability to rescue neurons after longer periods of NGF deprivation. In cultures not exposed to adenosine, reintroduction of NGF had no effect on survival after 48-h NGF deprivation. However, in adenosine-treated cultures, almost 60% of the neurons could be rescued by addition of NGF, after 48 h. Adenosine caused a significant increase in survival, even after 96-h NGF deprivation, when the majority of cells were dead. These findings are consistent with adenosine block of protein

synthesis, and consequent suspension of the death program.

Adenosine Rescues by an Intracellular Mechanism

Neuroprotection by adenosine is certainly not a new phenomenon. Several investigators have demonstrated that adenosine affords neuroprotection by activation of its membrane receptors. More specifically, CA3 pyramidal neurons were protected from kainic acid by adenosine, through activation of A1 receptors *(44).* activation of A2B-subtype purinergic receptors by adenosine increased dopamine uptake and prevented dopamine neuronal death in cultured neurons *(45).* Adenosine and ADP prevent apoptosis of cultured rat cerebellar granule cells by activation of A1 and P2X receptors *(46).* Adenosine also inhibited lymphoma cell proliferation by binding to A3 receptors *(47).* However, the authors' work with adenosine and 2'-dA very clearly showed that neuroprotective action of these compounds in NGF-deprived sympathetic neurons is executed by intracellular site, and depends on phosphorylation of nucleosides.

A variety of agents were used to confirm the authors' hypothesis that adenosine rescues NGF-deprived neurons by a mechanism that involves intracellular accumulation of the nucleoside, and not by activation of membrane receptors. Block of nucleoside transport by NBTI, or inhibition of AK by ITu, effectively prevented adenosine protection of NGF-deprived sympathetic neurons. Consistent with these findings, and with an intracellular mechanism, block of adenosine receptors by aminophylline had no effect on adenosine protection from NGF deprivation (unpublished results).

Adenosine Blocks RNA and Protein Synthesis in Mature Sympathetic Neurons

The ability of excess uridine to prevent adenosine effects is consistent with the idea that depletion of pyrimidine pools underlies an inhibition of mRNA and protein synthesis necessary for execution of the death program during NGF deprivation. [^{3}H]uridine incorporation was monitored in control and in cultures treated with adenosine, to determine if adenosine caused a decrease in RNA synthesis in neurons cultured for 2 d, as it did in freshly plated neurons. Adenosine significantly reduced 8-h [^{3}H]uridine incorporation by ~20%. Actinomycin-D, which served as a positive control, inhibited [^{3}H]uridine incorporation by almost 95%. An obvious consequence of inhibition of RNA synthesis would be inhibition of synthesis of new proteins. To study protein synthesis, the authors used [^{35}S]methionine incorporation in adenosine-treated sympathetic neurons. Adenosine significantly inhibited (25%) [^{35}S]methionine incorporation over a 12-h period. Cycloheximide, a positive control, inhibited [^{35}S]methionine incorporation by >90% (Table 1). These results clearly show that adenosine has a small but significant inhibitory effect on RNA and protein synthesis, which are necessary for execution of the death program.

Adenosine Prevents, but Does Not Reverse, Expression of Caspase-3

Activation of interleukin-1β-converting enzyme (ICE) family proteases, particularly caspase-3 (CPP32), has been proposed to occur late in the death program in NGF-deprived sympathetic neurons *(42).* Because adenosine blocks RNA and protein synthesis, it should be able to inhibit the NGF-withdrawal-induced elevation of apoptosis-related caspases. Caspase-3 activity was monitored by its ability to cleave the fluorescent 4-aminomethylcoumarin from the peptide substrate, DEVD-4-aminomethylcoumarin. Caspase-3 activity increased more than sixfold, following 6–9 h NGF withdrawal, and this was completely prevented when adenosine was included from the time of NGF withdrawal. When caspase-3 activity was elevated by NGF withdrawal for 6 h prior to adenosine addition for 3 h, adenosine failed to reduce caspase-3 activity. Under these same conditions, readdition of NGF caused a complete reversal of cas-

Table 1
Adenosine Inhibits RNA and Protein Synthesis in 2-d-old Sympathetic Neurons

	Incorporation of radioactivity (cpm/mg protein)	
Treatment	[^{3}H]uridine	[^{35}S]methionine
None	57,467 ± 3,267	$8.35 \times 10^6 \pm 3.71 \times 10^5$
Adenosine + dCF	43,467 ± 3,600[a]	$6.72 \times 10^6 \pm 2.53 \times 10^5$ [a]
Actinomycin-D	3933 ± 533[a]	–
Cycloheximide	–	$0.995 \times 10^6 \pm 1.15 \times 10^5$ [a]

2-d-old sympathetic neurons were incubated in 100 µ*M* adenosine plus 3 µ*M* dCF, 0.1 µg/mL actinomycin-D or 1 µ*M* cycloheximide, as indicated, and labeled with 1 µCi/mL [^{3}H]uridine or [^{35}S]methionine, plus 10 µ*M* unlabeled compound, for 8–12 h, respectively. [a] $p < 0.05$ compared to untreated cultures (none); $n = 4–6$.

pase-3 activity. These findings are consistent with adenosine inhibition of NGF-withdrawal-induced expression of caspase-3, which has a broad peak in activity between 5 and 10 h after trophic factor withdrawal *(48,49),* and with mRNA levels peaking within ~4 h *(50).* A complete reversal of caspase-3 activity by NGF is consistent with the author's recent finding that NGF replacement causes destruction of transiently expressed death proteins via the proteasome *(43).* More recently, ATP and adenosine have been shown to inhibit activation of caspases in apoptotic AKR-2B fibroblasts, by different pathways *(51).*

Adenosine, added at the time of NGF withdrawal, prevents the induction of caspase-3, protects NGF-deprived neurons up to 48 h, and prolongs the time within which readdition of NGF can rescue the neurons. Adenosine prevented cell death, even when added up to 9 h after NGF withdrawal, but did not reverse caspase-3, which is maximally induced during this time (unpublished results). The fact that adenosine is able to rescue NGF-deprived neurons long after caspase-3 induction has apparently reached maximal levels, and, without reversing caspase-3 enzyme activity, suggests the existence of other transiently expressed death effectors downstream of caspase-3. The authors speculate that adenosine inhibits the induction of postcaspase-3 signals, by its ability to inhibit new mRNA. To the authors' knowledge, only one postcaspase-3 death protein has been reported: DNA fragmentation factor *(52).* However, more than one transient signal downstream of caspase-3 is likely to be involved in exposing DNA and activating its cleavage by endonucleases. Whether adenosine inhibits mRNA induction for DNA fragmentation factor, or other death signals, remains to be elucidated.

Conclusions

Many exogenous substances have been found to delay apoptosis in trophic factor-deprived neurons. There is strong evidence that agents, which enhance or mimic cAMP, delay NGF-deprivation-induced cell death in sympathetic neurons and PC12 cells *(42,53–55),* and K^+/serum deprivation-induced death in central neurons *(56,57).* Inhibitors of RNA and protein synthesis, such as actinomycin-D and cycloheximide, are also known to delay execution of the death program *(42,58).* However, there has been little evidence for an endogenous substance that could protect the neurons in the absence of NGF. One such substance, pituitary adenylate cyclase activating polypep-

tide (PACAP), has been found to be protective against K^+/serum-deprivation-induced apoptosis in central neurons *(57,59),* and against NGF-deprivation in sympathetic neurons *(43).* How the protective action of PACAP comes into play under physiologic or pathologic situations remains to be determined. Accumulation of excess adenosine, on the other hand, is known to occur in a variety of situations, particularly during ischemia, and the protective action of adenosine, acting via its membrane receptors in these situations, is well-established *in vitro* and *in vivo (60,61).*

The present work suggests that adenosine has an additional intracellular mechanism to halt expression of the death program. The mechanism of adenosine protection is via inhibition of protein synthesis. Adenosine has been reported to inhibit protein synthesis in rat liver hepatocytes *(37).* However, in these dividing cells, adenosine-driven accumulation of SAH, and consequent impaired methylation reactions, caused the decreased protein synthesis *(37,38).* The mechanism by which adenosine accumulation inhibits protein synthesis in postmitotic cells has been examined in freshly plated sympathetic neurons *(35),* and the reduction in RNA and protein synthesis was found to be a result of excess formation of phosphorylated adenosine metabolites (AMP and ADP), which inhibit PRPP, causing depletion of pyrimidines and impaired nucleotide synthesis. Thus, impaired synthesis of mRNA leads to decreased protein synthesis.

Although the mechanism of protein synthesis inhibition by adenosine in immature (freshly plated) and mature (2 d or more in culture) chick sympathetic neurons is the same, the extent and consequences are totally different. Adenosine inhibits RNA and protein synthesis ~20–25% in mature neurons, and has no detrimental effect on survival (unpublished data), but in immature neurons the inhibition is 40–60%, and results in cell death *(5).* Regarding this, consider the authors recent results with cycloheximide. When protein synthesis was inhibited with this agent by 20–30%, there was no neuroprotection, as with adenosine. Cycloheximide was effective in preventing apoptosis only when protein synthesis was blocked by >80%. Adenosine appears to completely inhibit specific proteins, rather than cause a general decrease in all proteins. Depletion of pyrimidines would be expected to cause an across-the-board decrease in nucleotide synthesis. Decreased DNA synthesis is probably of little consequence in postmitotic neurons, over the short time in culture. However, impaired mRNA synthesis would be most deleterious to inducible, transiently expressed proteins. This interpretation is consistent with the actions of adenosine on both freshly plated and mature neurons. In freshly plated neurons, there is a high requirement for new proteins involved in initial growth and development, and for the extension of neurites. Excess adenosine arrests growth in these neurons, and eventually leads to death, consistent with inhibition of transient proteins specifically involved in survival, growth, and neurite development. In neurons allowed to develop for 2 d in culture, excess adenosine had no effect on their survival over the next several days in culture, consistent with a general low level of synthesis and a low level of inhibition. However, when NGF deprivation is used to induce proteins involved in executing the death program, excess adenosine prevented neuronal death, consistent with the interpretation that transient, inducible proteins are most susceptible to inhibition by adenosine. Furthermore, adenosine not only prevented death in >50% of NGF-deprived neurons for up to 48 h, but it also prolonged the commitment-to-die point to more than 48 h, which suggests that delay of the death program by adenosine is efficient, and supports the idea that adenosine inhibits the synthesis of death-effector proteins. Thus, adenosine is a good physiologic candidate for the temporary rescue of sympathetic neurons or other types of neurons during trophic factor deprivation.

The data here suggest that adenosine, already known to have protective receptor-mediated effects, may offer another line of defense by delaying onset of the death pro-

gram by an intracellular mechanism. Thus, adenosine provides a new and powerful tool for dissecting out the proteins associated with apoptosis induced by NGF withdrawal, as well as those involved in the initial growth and development of neurons *in vitro.*

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