Title
Extracellular nucleotide signaling in neuronal differentiation and survival: Multiple roles of the P2Y₂ receptor

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Extracellular nucleotide signaling in neuronal differentiation and survival: Multiple roles of the P2Y$_2$ receptor.

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biology by

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2006
The dissertation of David Benjamin Arthur is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

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2006
# Table of Contents

Signature Page...........................................................................................................iii

Table of Contents.....................................................................................................iv

List of Symbols.........................................................................................................v

List of Figures...........................................................................................................vi

List of Tables.............................................................................................................ix

Acknowledgements................................................................................................x

Curriculum Vita.......................................................................................................xiii

Abstract..................................................................................................................xvi

Chapter 1: Introduction..............................................................................................1

Chapter 2: Characterization of the System: Extracellular nucleotide-NGF: 
interaction in regulation of differentiation of PC12 cells .......................................15

Chapter 3: P2Y$_2$ receptor activates NGF/TrkA receptor signaling to enhance 
neuronal differentiation.............................................................................................53

Chapter 4: Interaction with Src is necessary for P2Y$_3$ and TrkA receptor-
mediated enhancement in neuronal differentiation..................................................80

Chapter 5: Inhibition of apoptosis by P2Y$_2$ receptor activation: novel 
pathways for neuronal survival............................................................................93

Chapter 6: Changes in nucleotide-stimulated norepinephrine release in 
response to neuronal differentiation by nerve growth factor...............................113

Chapter 7: Conclusion..............................................................................................126

Appendix...................................................................................................................135

References...............................................................................................................137
List of Symbols

ATP, adenosine triphosphate
ATPγS, adenosine 5′-O-(3-thio)triphosphate
DRG, dorsal root ganglion neurons
ERK1/2, early response kinase 1/2
GPCR, G-protein coupled receptor
GAP-43, growth associated protein-43
HUVEC, human umbilical vein endothelial cells
IP, immunoprecipitation
NT’s, nucleotidase inhibitors
PC12, pheochromocytoma cell 12
P-ERK1/2, phosphorylated ERK1/2
P-p38, phosphorylated p38
PLC, phospholipase C
P-TrkA, phosphorylated TrkA
p38, protein 38
P2, purinergic receptor 2
P2Y<sub>2</sub><sup>−/−</sup>, P2Y<sub>2</sub> knock out mouse
QRT-PCR, quantitative reverse transcription polyclonal chain reaction
RASMC, rat aortic smooth muscle cells
RCF, rat cardiac fibroblasts
Src, Src family protein tyrosine kinase
siRNA, small interfering ribonucleic acid
TrkA, tyrosine kinase receptor A
UTP, uridine triphosphate
wt, wild type
List of Figures

Figure 1-1: Nucleotide/Purinergic Receptor Classifications………………………………3
Figure 1-2: P2 Receptor Ligands and Signal Transduction Molecules…………………..5
Figure 1-3: Neurotrophin Receptors and Ligands………………………………………9
Figure 1-4: Signal Transduction Pathway for the NGF/TrkA Receptor………………….10
Figure 1-5: NGF-dependent and Independent Pathways of Neuronal Survival………12

Figure 2-1: PC12 morphology………………………………………………………………38
Figure 2-2: Morphological measurements of NGF-promoted PC12 cell differentiation..39
Figure 2-3: ATPγS increases the fraction of PC12 cells expressing neurites at submaximal NGF concentrations…………………………………………………………..40
Figure 2-4: The number of neurites per cell correlates with cell body size in NGF and NGF+ATPγS treated PC12 cells…………………………………………………………….41
Figure 2-5: Effect of NGF and lower temperature on ATP release from PC12 cells following a media change………………………………………………………………….42
Figure 2-6: Differentiated PC12 cells have altered responses to nucleotide-stimulated nucleotide release…………………………………………………………………43
Figure 2-7: UTP- and UDP-stimulated ATP release changes with NGF-promoted differentiation………………………………………………………………………..44
Figure 2-8: Cardiac and endothelial cells are a source of nucleotide release………………………………………………………………………………..45
Figure 2-9: Cardiac and vascular endothelial cells mediate PC12 cell differentiation independent of NGF…………………………………………………………….46
Figure 2-10: PC12 cells differentiate in co-culture with HUVEC independent of NGF…47
Figure 2-11: PC12 cells express 7 of 8 P2Y receptor mRNA……………………………48
Figure 2-12: P2X expression in PC12 cells………………………………………………49
Figure 2-13: ATPγS, NGF, and ATPγS+ATP alter expression of P2Y_6, P2Y_11, P2Y_12, and P2Y_13…………………………………………………………………………………………50
Figure 2-14: NGF differentiation alters relative P2 receptor mRNA expression in PC12 cells………………………………………………………………………………52

Figure 3-1. ATPγS increases NGF-stimulated neurite formation via TrkA and ERK1/2 activation. …………………………………………………………………………………71
Figure 3-2. P2Y agonists increase NGF-mediated neurite formation by increasing sensitivity to NGF…………………………………………………………………………72
Figure 3-3. Neurite growth, P2Y_2-TrkA co-localization and signaling in DRG neurons..73
Figure 3-4. siRNA reduction of P2Y_2 eliminates ATPγS-enhancement of NGF-induced neurite formation and signaling………………………………………74
Figure 3-5. P-TrkA and P2Y_2 receptors co-immunoprecipitate upon NGF induction…..75
Figure 3-6. ATPγS increases GAP-43, NGF increases P2Y_2 levels in vivo……………..76
Figure 3-7. ATPγS increases GAP43 expression via ERK1/2 activation…………………77
Figure 3-8. Model of interaction of P2Y_2 with TrkA receptors…………………………78
Figure 3-9. P2Y_2 receptors mediate increased NGF-stimulated neurite formation……..79

Figure 4-1: Src inhibition prevents ATPγS-mediated enhancement of NGF-promoted neuronal differentiation in PC12 cells………………………………………………90
Figure 4-2: Src inhibition blocks ATPγS-mediated enhancement of axonal growth in DRG neurons.................................................................91
Figure 4-3: Src co-immunoprecipitates with TrkA and P2Y2........................................92

Figure 5-1: ATPγS inhibits serum starvation-induced PC12 apoptosis via P2Y2 receptors independent of NGF/TrkA signaling.................................................................107
Figure 5-2: ATPγS/NGF interact to enhance inhibition of apoptosis.........................108
Figure 5-3: ATPγS inhibits apoptosis via activation of ERK1/2 and Akt....................109
Figure 5-4: ATPγS inhibits apoptosis via Src activation of Akt and B-Raf-mediated ERK1/2 activation.................................................................110
Figure 5-5: ATPγS inhibits serum starvation-induced DRG apoptosis via P2Y2 independent of NGF/TrkA signaling.................................................................111
Figure 5-6: Model of P2Y2-mediated inhibition of neuronal apoptosis....................112

Figure 6-1: ATPγS and ATP promote NE release.................................................122
Figure 6-2: NGF-differentiated PC12 cells have greater sensitivity to ATPγS and greater ATPγS-stimulated NE release.................................................................123
Figure 6-3: NGF-differentiated PC12 cells have higher NE reuptake than undifferentiated cells.................................................................124
Figure 6-4: ATPγS-stimulated NE is not depleted by Ba²⁺ in NGF-differentiated versus naïve PC12 cells.................................................................125
Figure 7-1: Summary Model of P2Y$_2$ Receptor Neuronal Signal Transduction Pathways
List of Tables

Table 2-1: P2Y Primer Sequences. .................................................................21
Table 2-2: P2Y receptor expression in PC12 cells........................................51
Table Appendix-1: P2 receptor agonist/antagonist profiles..........................136
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Note

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Extracellular nucleotide signaling in neuronal differentiation and survival:

Multiple roles of the P2Y\textsubscript{2} receptor.

by

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Doctor of Philosophy in Biology

University of California, San Diego, 2006

Professor Paul A. Insel, Chair

Extracellular nucleotides signaling through P2 nucleotide receptors expressed throughout the nervous system regulate a variety of neuronal functions. The characterization of nucleotide effects on neuronal differentiation, growth and survival are potentially important targets that may facilitate regeneration and survival of neurons after injury, disease, or age-related loss of function. Work in this thesis tested the hypothesis that extracellular nucleotides, acting through P2 receptor(s), alter neuronal differentiation, growth and survival. I characterized morphological changes that occur with nerve growth factor (NGF) differentiation and corresponding changes in ATP release. Target tissues, such as vascular endothelial and cardiac cells, were identified as sources of ATP
release and promoters of differentiation of PC12 cells independent of NGF. The altered expression of P2X and P2Y receptors in differentiated versus non-differentiated PC12 cells, combined with the enhancement of NGF-stimulated differentiation by ATP and UTP led to the identification of P2Y_2 receptors as mediating nucleotide-promoted neuronal differentiation and growth in primary cultured neurons and PC12 cells. Such effects occurred via increased NGF sensitivity and enhanced activation of the NGF receptor, TrkA. Endogenous co-localization and co-immunoprecipitation of P2Y_2 and TrkA receptors requiring activated Src as a physical intermediate. In vivo treatment with ATPγS treatment enhanced a marker of neuronal growth. P2Y_2 receptor activation, independent of NGF/TrkA, also mediated inhibition of neuronal apoptosis by ATP/UTP. In addition to effects on differentiation, P2Y_2 inhibition of apoptosis occurred through Src-mediated activation of ERK and Akt. In other experiments I found that undifferentiated, compared to NGF-differentiated, PC12 cells had lower, briefer ATPγS-stimulated norepinephrine release and reuptake; only NGF-differentiated PC12 cells were capable of desensitization to ATPγS. These results identify a role for P2Y_2 receptors in neurotrophin-dependent differentiation as well as neurotrophin-independent inhibition of neuronal apoptosis. P2Y_2 receptors thus regulate neuronal differentiation, survival, and function in part via interaction with neurotrophin/receptor tyrosine kinase receptors. As such, P2Y_2 receptors provide a new target for therapeutic intervention in neuropathological conditions.
Chapter 1:

Introduction
The emergence of the study of nucleotides in biological systems, beyond the classical role they play in cellular metabolism and storage of cellular energy, has revealed a plethora of functions through a group of receptors known as the purinergic (now nucleotide) receptors (Ralevic and Burnstock, 1998). Nucleotides, in addition to their intracellular functions as sources of energy or in intracellular regulatory events, have a profound effect as extracellular signals. Nucleotides are released by cells in response to injury, apoptosis, mechanical stimulation, rises in intracellular Ca\(^{2+}\), vesicular neuronal release or co-release with neurotransmitters at \(\mu\)M to mM concentrations (Neary et al., 1996; Rathbone, 1999; Burnstock, 2000; Fields and Stevens, 2000; Burnstock, 2001; Ostrom et al., 2001). Identification of the receptors that mediate the functions of, and cellular responses to, released nucleotides has led to a greater understanding of the mechanisms by which extracellular nucleotides exert their effects.

Nucleotide receptors fall into two classes, P1 and P2. P1 receptors, of which there are four subtypes, respond primarily to adenosine. The A\(_1\) and A\(_3\) receptors have a high affinity for adenosine and inhibit adenylyl cyclase activity and cyclic adenosine monophosphate (cAMP) formation (Fleming and Mogul, 1997; Fredholm et al., 1997), while the A\(_{2a}\) and A\(_{2b}\) receptors have a lower affinity for adenosine and stimulate adenylyl cyclase activity and cAMP synthesis (Stone, 1991; Fredholm et al., 1994). While these receptors respond to adenosine and adenosine analogues, adenine and uridine nucleotide signals are mediated through a different class (Fig. 1-1).
Figure 1-1: Nucleotide/Purinergic Receptor Classifications.

The P2 class of receptors, which are activated by nucleotides, is of two classes. The ionotropic P2X receptors are ligand-operated ion channels that are permeable to cations such as Na⁺, K⁺, and Ca²⁺ (Burnashev, 1998). Seven identified P2X receptor subtypes (P2X₁-7), which respond to ATP and have selectivity for specific ATP analogues (Lambrecht, 2000; North and Surprenant, 2000) (Table Appendix-1). These receptors (P2X₁, P2X₂, P2X₆, P2X₇), with the exception of P2X₇, are capable of forming heteromultimers to form a cation pore (Radford et al., 1997; Le et al., 1998a; Torres et al., 1998; Torres et al., 1999; Kim et al., 2001). P2X receptors, which can be further classified into fast (P2X₁, P2X₃) and slow (P2X₂, P2X₄) desensitizing (Radford et al., 1997), are responsible for membrane permeability to cations when cells are stimulated by...
extracellular ATP, and in the case of P2X7 (originally termed P2x) can lead to apoptosis (Surprenant et al., 1996).

The other P2 class, P2Y receptors, are seven transmembrane G-protein coupled receptors (GPCR) that are activated by both adenine and uracil nucleotides (Burnstock, 2000). Eight functional human P2Y receptors have been identified (P2Y1, 2, 4, 6, 11-13). The P2Y1, P2Y2, P2Y4, and P2Y6 couple to Gq and activate phospholipase C (PLC) while P2Y11 couples to both Gq and Gs, the latter G-protein stimulating adenylyl cyclase and cAMP formation; the P2Y12-14 receptors couple to Gi and inhibit of adenylyl cyclase activity (Communi et al., 1996b; Communi et al., 1996a; Barnard and Simon, 2001; Communi, 2001; Abbracchio et al., 2003; Burnstock and Knight, 2004) (Fig. 1-2). These receptors mediate a variety of functions depending upon the cell type in which they are expressed. The unique ability of P2Y receptors to respond to both ATP and UTP led me to focus on these receptors as mediators of ATP/UTP functional responses (Chapter 2-4), and led to the identification of the role of P2Y2 receptors in neuronal differentiation and survival.
A comprehensive overview of extracellular nucleotide signaling is not complete without mentioning the roles of ecto-nucleotidases. Ecto-nucleotidases are enzymes responsible for the hydrolysis/degradation of nucleotides from tri- to di- to monophosphates and nucleosides (Zimmermann, 2000). These enzymes are expressed on the plasma membrane surface and rapidly degrade ATP or UTP to adenosine and uridine, respectively (Dunwiddie, 1997; Zimmermann, 2000). The degradation of extracellular nucleotides is thus a regulatory mechanism for controlling stimulation of P2 receptors. This degradation, however, adds a complication to the study of nucleotide signaling in that ATP or UTP can be broken down into hydrolysis products that can stimulate other P2 receptors and P1 receptors (adenosine). An additional concern is an enzyme that can remove a phosphate group from one nucleotide and add it to another, i.e. ectonucleotide
diphosphokinase, which catalyzes extracellular phosphorylation of AMP or UMP from
phosphate donors (Lazarowski et al., 1997a; Yegutkin et al., 2001). The use of non-
hydrolysable nucleotide analogues helps minimize the role of such enzymes that
metabolize nucleotides.

P2 receptors are expressed in nearly every tissue and cell type in the body. The
current studies are primarily concerned with the expression and activity of P2 receptors in
the central and peripheral nervous system. P2X receptors are expressed throughout the
nervous system. Examples include the following: P2X1 in the cerebellum; P2X2 in the
cortex, hippocampus, and spinal cord; P2X3 in dorsal root ganglion (DRG) neurons and
brainstem; P2X4 in the hippocampus, cerebellum and brainstem; and P2X7 in the
hippocampus, cerebellum and spinal cord (Chen et al., 1995; Lewis et al., 1995; Collo et
al., 1997; Vulchanova et al., 1997; Loesch and Burnstock, 1998; Kanjhan et al., 1999;
Deuchars et al., 2001; Diaz-Hernandez et al., 2001; Bo et al., 2003). P2Y receptors are
also expressed throughout the nervous system: P2Y1 receptors in the cerebral and
cerebellar cortices, hippocampus, nucleus accumbens and midbrain; P2Y2 and P2Y4
ubiquitously at low levels in the brain; P2Y6 in the amygdala and nucleus accumbens;
P2Y11 in the parahippocampal gyrus, nucleus accumbens, and striatum; P2Y12 in the brain
and microglia; and P2Y13 and P2Y14 in whole brain (Chambers et al., 2000; Moore et al.,
2000; Moran-Jimenez and Matute, 2000; Moore et al., 2001; Zhang et al., 2002; Sasaki et
al., 2003). The wide distribution of these receptors suggests an array of physiological
effects on nervous system function in response to nucleotide release and receptor
occupancy.
Glial cells, consisting of astrocytes, Schwann cells, oligodendrocytes, and microglia, express an array of P2 receptors that influence glial function. ATP is released by glial cells (Ferrari et al., 1997; Verderio and Matteoli, 2001). Glial cells express a number of different P2 receptors, including $\text{P2Y}_{1,2,4,6,12,14}$ and $\text{P2X}_{1,5,7}$ (King et al., 1996; Idestrup and Salter, 1998; Jimenez et al., 2000; Fumagalli et al., 2003; Moore et al., 2003). Extracellular nucleotides stimulate glial cell arachidonic acid release and prostaglandin synthesis (Pearce et al., 1989; Bruner and Murphy, 1990), and increase intracellular calcium leading to calcium wave propagation (Guthrie et al., 1999; Sun et al., 1999; James and Butt, 2002), neurotransmitter release (Wang et al., 2002; Duan et al., 2003), and proliferation of astrocytes (Abbracchio et al., 1994). These effects on glial cells by extracellular nucleotides influence the physiology and signaling by neurons. One example is the impact of glial calcium waves on neurotransmitter release that alters synaptic transmission (Sun et al., 1999; Duan et al., 2003). ATP release after ischemia can lead to astrogliosis that can exacerbate neuronal injury and death (Franke et al., 2001; James and Butt, 2002). A summary of the expression of P2 receptors on glia (and dorsal root ganglion neurons) appears in a review by Fields and Stevens (Fields and Stevens, 2000). These influences on neuronal physiology are not limited to effects of extracellular nucleotides mediated through glial cells.

Neuronal function is affected by extracellular nucleotides. Neurons express many P2X and P2Y receptors that are involved in presynaptic modulation of neurotransmitter release and postsynaptic responses to released nucleotides (Le et al., 1998b; Kanjhan et al., 1999; Norenberg and Illes, 2000). Presynaptic P2 receptors can modulate release of dopamine, norepinephrine, glutamate, serotonin, GABA, glycine, and acetylcholine
P2X receptors expressed pre- and postsynaptically can facilitate NMDA-mediated long term potentiation (Pankratov et al., 2002). Hippocampal presynaptic P2X receptors inhibit transmission by stimulating GABA or by inhibiting glutamate release (Inazu et al., 2003). P2Y receptors can inhibit (via P2Y₃) or stimulate (P2Y₂) NMDA receptors (Wirkner et al., 2002; Luthardt et al., 2003). Both P2X (in particular P2X₃) and P2Y receptors have demonstrated effects on pain transmission (Vulchanova et al., 1997; Vulchanova et al., 1998; Okada et al., 2002; Gerevich et al., 2004), the former enhancing, the latter inhibiting synaptic transmission of pain signals.

The many and varied effects of extracellular nucleotides on the function of different portions of the nervous system is still being discovered and refined.

At the time work on this thesis began, the role of nucleotides in neuronal developmental, particularly in neurotrophin-mediated differentiation, had not been defined. Neurotrophins are a class of proteins that influence neuronal differentiation and survival. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and neurotrophin 4 (NT4) are four classes of such molecules, the first being the best studied (Crain and Wiegand, 1961; Sara et al., 1978; Hohn et al., 1990; Hallbook et al., 1991). These proteins are secreted as precursor neurotrophins (proneurotrophins) that can act to inhibit neurite outgrowth and initiate apoptosis (Teng et al., 2005). The cleaved, mature-form neurotrophins are released by neurons, glia, and neuronal target tissues to promote differentiation, growth, synaptic formation, and survival of neurons (Lu et al., 2005). Mice with heterozygous knockout of NGF have reduced hippocampal innervation, memory function, and a loss of peripheral innervation (Crowley et al., 1994; Chen et al., 1997). Loss of BDNF causes loss of
mechanosensitivity, peripheral innervation and impairs long-term potentiation (Ernfors et al., 1994a; Carroll et al., 1998; Bartoletti et al., 2002); loss of NT3 causes cardiovascular defects, and loss of mechanoreceptors and peripheral innervation (Ernfors et al., 1994b; Airaksinen et al., 1996; Donovan et al., 1996). These defects reveal the important roles for neurotrophins in the development and maintenance of the nervous system.

The receptors responsible for mediating the effects of neurotrophins are the tyrosine receptor kinase (Trk) family of receptors and the p75 neurotrophin receptor. The Trk receptors have 3 identified members, TrkA, TrkB, and TrkC, each of which responds to specific neurotrophin molecules (Fig. 1-4).

**Neurotrophin Receptors**

<table>
<thead>
<tr>
<th>Ligands:</th>
<th>NGF</th>
<th>BDNF, NT4</th>
<th>NT3</th>
<th>NGF, BDNF, NT3, NT4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptors:</td>
<td>TrkA</td>
<td>TrkB</td>
<td>TrkC</td>
<td>p75</td>
</tr>
</tbody>
</table>

**Figure 1-3: Neurotrophin Receptors and Ligands.**

The best-characterized signal transduction pathway is that of TrkA. Receptor activation leads to phosphorylation and dimerization of TrkA, causing dual activation of early response kinase 1/2 (ERK1/2) via a Ras/B-Raf pathway, which promotes neuronal differentiation and survival, and Akt/phosphokinase B (PKB) activation via phosphoinositol-3-kinase (PI3K), which promotes survival (Fig. 1-5) (Chao, 2003b).
These signal transduction pathways are the key intracellular events that enable neurotrophins to affect neuronal physiology.

**Figure 1-4**: Signal Transduction Pathway for the NGF/TrkA Receptor. (adapted from Chao, 2003)

Molecules capable of enhancing neurotrophin signaling represent potential treatments for enhancing neuronal regeneration, growth and survival after loss of innervation due to age, injury, or disease. Two molecules known to act through TrkA pathways are pituitary adenylate cyclase activating peptide (PACAP) and adenosine (Lee and Chao, 2001; Lee et al., 2002b); both of which do not enhance differentiation, but instead function to enhance survival. Prior to work on this thesis, ATP had been reported to enhance neuronal differentiation and activate ERK1/2, though the exact mechanism for its effects has yet to be determined (D'Ambrosi et al., 2000; D'Ambrosi et al., 2001).
Molecules and signaling mechanisms that interact with neurotrophins to promote survival as well as growth and development represent an elusive target for discovery.

Promotion of neuronal survival through neurotrophins is a well-established phenomenon. Neurotrophins mediate survival through their activation of Trk receptors and subsequent activation of survival-promoting kinases such as ERK1/2 and Akt (Lu et al., 2005), thereby preventing programmed cell death, or apoptosis. Apoptosis differs from necrotic cell death, in that the former is a highly regulated set of intracellular events leading to a set of hallmark morphological and biochemical events resulting in cell death, whereas the latter does not display these regulated characteristics. Programmed cell death results from a complex series of “death” kinases leading to activation of one or more caspases (Saunders, 1966; Twomey and McCarthy, 2005), molecules originally identified as pre-aspartate proteases activated during apoptosis (Black et al., 1989). The activation of caspases such as caspase 8, 9, and 3 leads to such events as DNA fragmentation, plasma membrane inversion, and membrane “blebbing” (Batistatou and Greene, 1993; Fernandes-Alnemri et al., 1994; Martin et al., 1995; Zhou et al., 1997).

Regulation of neuronal apoptosis is critical for the development of the central and peripheral nervous systems (Lasky and Wu, 2005). The inhibition of the signals that cause apoptosis are important for facilitating neuronal regeneration after neuropathological trauma as well as sustaining neuronal populations at risk for programmed cell death as a result of loss of trophic factors.

Molecular inhibition of apoptosis through neurotrophin-dependent and independent pathways is an important target for neuronal cell survival and rescue from apoptosis. Recent data have identified PACAP, adenosine and nitric oxide (NO) as
inhibitors of neuronal apoptosis. PACAP and adenosine cause a transactivation of TrkA receptors, independent of NGF, leading to downstream activation of Akt and ERK1/2 (Lee and Chao, 2001; Lee et al., 2002a; Rajagopal et al., 2004). While none of these molecules require NGF, they are dependent upon the TrkA pathway for their anti-apoptotic effects. NO, however, has been shown to cause activation of cGMP andPKG, leading to TrkA-independent activation of Akt and ERK1/2 for the inhibition of apoptosis (Culmsee et al., 2005). Extracellular nucleotides have also been suggested to show NGF-independent inhibition of apoptosis, though the mechanism remains unknown (Fig. 1-6) (D'Ambrosi et al., 2001).

**Figure 1-5:** NGF-dependent and Independent Pathways of Neuronal Survival.

The pathways illustrated represent the known effector and signal elements prior to work on this thesis. (adapted from Akassoglou, 2005)
Molecules and receptor signal transduction pathways that can enhance neuronal differentiation, axonal extension, synaptogenesis, and survival provide potentially important targets for the treatment of a host of neuropathological conditions. Loss of neuronal connectivity and survival in Alzheimer’s and Parkinson’s Disease, injury-related loss of peripheral connectivity, and age-related loss of peripheral neuronal input may be reduced or reversed by neuropharmacological facilitation of neurotrophin signaling or independent activation of pathways parallel to neurotrophin signaling that enhance differentiation, growth and survival. Extracellular nucleotides signaling through P2 receptors affecting these physiological events may be such a class of molecules.

**Hypothesis**

This thesis tests the hypothesis that extracellular nucleotides and their receptors modulate neuronal differentiation, growth and survival. I first tested whether extracellular nucleotides alter markers of neurotrophin-induced morphological differentiation (Chapter 2). I further assessed the changes in nucleotide release and P2 receptor expression that occur as a result of NGF differentiation in PC12 cells and assessed the ability of potential neuronal targets, such as vascular endothelium and cardiac cells, to promote differentiation and release ATP (Chapter 2). Based upon results from Chapter 2, studies in Chapter 3 address the hypothesis that P2Y receptors promote enhanced NGF-stimulated differentiation. I identified P2Y$_2$ receptors as necessary for these effects and assessed the mechanisms and interactions of this receptor in the enhancement of NGF-promoted neuronal differentiation in primary neurons and PC12 cultures, as well as tested the hypothesis that *in vivo* administration of a non-hydrolyzable
nucleotide could promote neuronal growth (Chapter 3). The endogenous co-immunoprecipitation of P2Y$_2$ and TrkA receptors found in Chapter 3 combined with results from the literature prompted studies to test whether Src was an intermediate in this receptor interaction (Chapter 4). Studies in Chapter 5 address the hypothesis that nucleotides inhibit neuronal apoptosis by P2Y$_2$ receptors and assess the mechanisms behind these effects. Experiments in Chapter 6 assessed a secondary hypothesis with respect to interaction of neurotrophin-stimulated differentiation and nucleotides effects on neuronal communication by assessing whether neuronal differentiation by NGF alters nucleotide-stimulated catecholamine release (Chapter 6). The results and potential implications from studies conducted in this thesis are discussed in Chapter 7.
Chapter 2:

Characterization of the System: Extracellular nucleotide-NGF interaction in regulation of differentiation of PC12 cells
Abstract

Nerve growth factor, NGF, differentiates PC12 cells into a sympathetic-like neuron with formation of neurites. In the present study, I tested the hypothesis that extracellular nucleotides interact with NGF in promoting this differentiation by assessing 4 morphological changes: 1. fraction of PC12 cells expressing neurites, 2. neurite size, 3. number of neurites per cell, and 4. size of the cell body. I found that ATP, UTP, or nonhydrolyzable ATP analogs enhanced NGF-promoted formation of the number of PC12 cells expressing neurites, while ADP, UDP, and adenosine reduced this number. Nucleotide treatment reduced neurite size and number and decreased cell body size. NGF-differentiated PC12 cells released more ATP into the extracellular medium in response to mechanical stimulation or addition of UTP. Incubation of cells at 20°C (vs. 37°C) inhibited UTP-stimulated ATP release, implying that carrier-mediated exocytosis is the mechanism of ATP transport. ATP release was also found in cardiovascular target tissues of sympathetic neurons. In addition, I found that PC12 cells express mRNA for 7 of the 8 known P2Y receptors (all except P2Y₁₄); expression of 2 of these, P2Y₆ and P2Y₁₁, decreased in PC12 cells incubated with NGF. Addition of ATPγS alone increased P2Y₁₂ receptor expression; ATPγS in the presence of NGF increased expression of P2Y₆, P2Y₁₁ and P2Y₁₃ receptors. Thus, nucleotides modulate NGF response and in turn, NGF can regulate expression of several P2Y receptors. These results implicate extracellular nucleotides and P2Y receptors as factors that influence neuronal differentiation and development, in particular, of sympathetic neurons that are modeled by PC12 cells.
Introduction

Nucleotides, in addition to their roles in intracellular metabolism and motility, function as extracellular regulators of cell physiology. Cellular release of nucleotides can result in extracellular nucleotide concentrations that alter cell function (Lazarowski et al., 2000; Ostrom et al., 2001; Schwiebert et al., 2002). Cells release substantial levels of nucleotides in response to shrinkage, swelling, exocytotic co-release with classic neurotransmission, or cellular injury (Wilkin, 2001; Boudreault and Grygorczyk, 2002; Schwiebert et al., 2002; Kubista et al., 2003; Loomis et al., 2003; Tanaka et al., 2003). In neuronal systems, extracellular nucleotides modulate neurotransmitter release in a negative feedback manner (Mongin and Kimelberg, 2002) and also regulate neuron-to-glial neurotransmitter reuptake (Fields, 2000). Nucleotide signaling in astrocytes and glial cells promote Ca\textsuperscript{2+} wave propagation and amino acid release (Salter and Hicks, 1994, 1995; Mongin and Kimelberg, 2002). Moreover, ATP is co-stored and co-released with neurotransmitter in several types of neurons and acts as a modulator of neurotransmission when released by glial cells (Cotrina, 2000; Stjarne, 2001; Newman, 2003).

Nucleotides and their metabolites exert their effects by P1 and P2 receptors. P1 receptors respond primarily to adenosine, while P2 receptors are activated by purine and pyrimidine nucleotides. The P2 family is divided into the P2X and P2Y subfamilies. P2X receptors (of which 7 isoforms have been identified) are ionotrophic receptors that facilitate entry of extracellular calcium. P2Y receptors (of which 8 isoforms have been identified) are metabotropic receptors that couple to heterotrimeric G-proteins to regulate
enzyme systems, including phospholipase C and adenylyl cyclase, and ion channels (for reviews see (Burnstock, 2000; Nicholas, 2001)).

Some data have suggested a role for extracellular nucleotides in neuronal development and differentiation (Bogdanov et al., 1997; D'Ambrosi et al., 2001; Paes-De-Carvalho, 2002; Pearson et al., 2002), but key aspects of such effects are not well understood, in particular, the precise role of different P2 receptors and the ability of nucleotides to interact with specific neurotrophic factors.

PC12 cells provide an excellent system to explore the potential for P2 receptors to modulate neuronal cell fate and to interact with a specific growth factor. These pheochromocytoma-derived cells, originally isolated from a rat adrenal medulla, differentiate into sympathetic neuron-like cells upon treatment with nerve growth factor (NGF), thereby providing a model for neuronal differentiation during early development (Greene, 1976; Kaplan and Stephens, 1994; Vaudry et al., 2002).

The current studies tested the hypothesis that extracellular nucleotides would alter NGF-induced PC12 cell differentiation. Accordingly, I measured the impact of nucleotides on NGF-promoted morphological differentiation and assessed PC12 cells for ATP release and P2Y receptor mRNA expression. The results demonstrate a regulatory role for extracellular nucleotides (detectable at both the mRNA and morphological levels) and provide evidence for interaction of nucleotides and NGF in PC12 cell differentiation.

**Materials and Methods**

**Reagents** ATP, ATPγS, αβMeATP, ADP, UTP, UDP, AMP, adenosine, adenosine deaminase, apyrase, and thapsigargin, collagen, poly-D-lysine, anti-NGF blocking
antibody were all obtained from Sigma (St. Louis, MO). Nerve Growth Factor (NGF) was obtained from Invitrogen (Carlsbad, CA).

**Cell culture conditions** PC12 cells (gift of David Schubert, Salk Institute as described in (Taupenot et al., 1999)) were grown in PC12-specific media: Dulbecco’s Modified Eagles Medium, high glucose with L-glutamine, 5% fetal bovine serum (Omega Scientific, Inc., Tarzana, CA), 10% heat-inactivated horse serum (Omega), and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). Cells were grown to 60-80% confluency in 5% CO₂ at 37°C in Falcon T75 filter flasks. Cells were split 1:2 every 48-72h up to 18 passages. HUVEC were grown to 60% confluency in MDBC131, 10%FBS, 1%NaHCO₃, 1% hydrocortisone, 10% heparin, 1% endothelial cell growth supplement, and 10µg/L epidermal growth factor at 95% air/5% CO₂ and 37°C. Primary rat cardiac fibroblasts were cultured in DMEM containing 10%FBS, 1% pen/strep and 1% fungizome. Primary rat cardiac myocytes were cultured in Hanks199 medium. Primary rat aortic smooth muscle cells (RASMC) were cultured in DMEM containing 16% FBS and 1%pen/strep. Rat cardiac myocytes and RASMC cells were incubated at 95% air/5% CO₂ and 37°C.

**Cell co-cultures** HUVEC, RASMC, and primary rat cardiac myocytes were grown to a 60% confluent monolayer, washed twice with PBS, and PC12 cells were then added in co-culture along with PC12 media.
**Conditioned Media** HUVEC, RASMC or primary rat myocyte conditioned media was generated using HUVEC flasks at either 10% or 85% confluency. Conditioning cells were washed twice with PBS and then incubated with PC12 media for 72h. The conditioned PC12 media was then added to naive PC12 cells.

**Morphology** PC12 cells were plated at 60% confluency and incubated with 100ng/ml NGF in combination with other treatments for 72h. ATP, ADP, UTP, UDP, AMP, UMP, and adenosine, all at 100µM, and apyrase (2u/ml), were added every 12h. ATPγS and αβMeATP were added once at 100µM. Images were taken every 24h for 3 days, each condition using a SPOT CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and a LD Achrostigmat 32X (0.4 Ph1; ∞/0.5-1.5) objective. All conditions were assessed in triplicate. Images were analyzed using Adobe Photoshop.

**ATP release assay** ATP release was measured by plating PC12 cells on Poly-D-Lysine (Sigma)-coated, 24-well plates in serum-free DMEM buffered media for 1h. All conditions were tested in triplicate. Media was aspirated, fresh serum-free media was reapplied and then sampled at 1, 3, 5, 10, 30, and 60 min. In other experiments, 100µM UTP or UDP was applied and media was sampled after 5 min. In some experiments, thapsigargin was added 20 min prior to media sampling. Bioluminescence was measured using the Bioluminescence Assay Kit HS II (Roche, Indianapolis, IN) on a single-channel setting on a TD20/20 luminometer (Turner Designs, Sunnyvale, CA).
Quantitative RT-PCR PC12 cells were grown in PC12-specific media alone, with NGF, or with NGF in combination with 100µM ATPγS for 72 hours. Total RNA was isolated using RNA-Stat 60 (Teltest, Friendswood, TX). The reverse transcriptase (RT) reaction was conducted using random hexamers and Superscript II (Invitrogen). The quantitative PCR reaction was carried out using equal cDNA loading and the Quantitect SYBR green kit (Qiagen) on the ABI Prizm 7700 thermacycler (95°C for 15min, 94°C for 30s and 58°C annealing for 45s and 72°C for 40s repeated 40 cycles). The following primers were used:

**Table 2-1:** P2Y Primer Sequences.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward*</th>
<th>Reverse*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5’AACATCATCCCTGCTTCCAC3’</td>
<td>5’GACCACCTTGCTCCTCAGTGT3’</td>
</tr>
<tr>
<td>P2Y1</td>
<td>5’ACCCTGTACGGCAGATTCCTTCTTCCAC3’</td>
<td>5’AGGWAGSAGASGGCGAAGAC3’</td>
</tr>
<tr>
<td>P2Y2</td>
<td>5’AGTCCCCCGTGCTTACTTT3’</td>
<td>5’GTCAGTCTCCTGCCACCTGT3’</td>
</tr>
<tr>
<td>P2Y4</td>
<td>5’CATCAGYGTGACCCGTCACM3’</td>
<td>5’GGGGHCGAGTCACYTTRAR3’</td>
</tr>
<tr>
<td>P2Y6</td>
<td>5’TTACCCTGGCGCATCTGCTACC3’</td>
<td>5’TAAACATGGGAAGGGCAGGAAG3’</td>
</tr>
<tr>
<td>P2Y11</td>
<td>5’CTGGTGGTTAGAGTGCTGCTG3’</td>
<td>5’GGTGCAAGGTAAGAGGAAGC3’</td>
</tr>
<tr>
<td>P2Y12</td>
<td>5’CAGGTTCTCTTCCACATTT3’</td>
<td>5’CAGCAATGATGATGAAACC3’</td>
</tr>
<tr>
<td>P2Y13</td>
<td>5’CTGSTRTCCCCGGYYCTCTA3’</td>
<td>5’TGAGGTAGAYGATRAAGGTGAR3’</td>
</tr>
<tr>
<td>P2Y14</td>
<td>5’TCAATNNCCGGGNNTCCNT3’</td>
<td>5’TCCGNNCCAGNTCNTTNTTNTN3’</td>
</tr>
</tbody>
</table>

*(degenerate primers: W=A or T; S=C or G; Y=C or T; R=G or A; M=A or C; K=G or T; D=A, G, or T; H=A, C, or T; N=A, C, G, or T).

All PCR products were verified by dissociation curves, which indicated a single product, and run on ethidium bromide gels for correct product size. The bands were
purified using a mini-prep (Qiagen, Valencia, CA) and sequenced for additional verification (Retrogen, San Diego, CA). Changes in cycle threshold (Ct) were calculated by subtracting GAPDH expression within each data set and then using $2^{\Delta \text{Ct}}$ for fold mRNA change (Pfaffl, 2001). Cycle thresholds, or the cycle at which the PCR reactions become linear, were assigned using the Applied Biosystems Sequence Detection Systems version 1.9.1 analysis software. Relative levels of receptors were calculated from cycle threshold values: a receptor with a lower cycle threshold value relative to the same receptor in another treatment group thus has a greater abundance of mRNA present in the cell from which RNA was isolated.

**Statistical analysis** Data were subjected to a one-way analysis of variance, followed by Tukey’s multiple comparison test, or linear regression. Significance was assigned to p<0.05. For quantitative RT-PCR, we used an 8-fold change as a significant response between treatments.

**Results**

**Morphology**

Treating PC12 cells with 100ng/ml NGF for 72h differentiates the cells by promoting neurite formation, thereby yielding a sympathetic neuron-like morphology ((Greene, 1976; Kaplan and Stephens, 1994; Vaudry et al., 2002) and Fig. 2-1). Addition of extracellular nucleotides alone was unable to stimulate this differentiation (data not shown), but when added together with NGF, nucleotides altered NGF-promoted changes. We assessed four measures of differentiation: 1. the fraction of PC12 cell expressing
neurites, 2. neurite size, 3. number of neurites per cell, and 4. cell body area. Cells were examined under phase contrast and digitally imaged to assess the four variables. The fraction of cells expressing neurites is a measure of the overall extent of differentiation; a PC12 cell was considered “differentiated” if it expressed at least one neurite as long as its cell body. ATP, ATPγS, αβMeATP and UTP significantly (p<0.001 for ATPγS, p<0.01 for all others) increased the fraction of NGF-treated PC12 cells that expressed neurites (Fig. 2-2A). By contrast, ADP, UDP, and adenosine (a P1 agonist) significantly (p<0.001) reduced the fraction of cells expressing neurites in response to NGF (Fig. 2-2A). Growth of PC12 cells on collagen, a more endogenous substrate than plastic, did not alter the fraction of neurite bearing cells (data not shown). ATPγS, a non-hydrolyzable ATP analog, enhanced sensitivity of PC12 cells to NGF, increasing the fraction of PC12 cells expressing neurites in the presence of submaximal concentrations of NGF (Fig. 2-3).

By contrast with effects of trinucleotides on the fraction of PC12 cells expressing neurites (Fig.2-2A), nucleotides did not enhance the size of neurites (Fig. 2-2B). However, the average size (area) of the neurites was significantly (p<0.001) altered by the nucleoside adenosine, which increased neurite size about 2-fold (Fig. 2-2B).

Another measure of PC12 cell morphological differentiation, the number of neurites per cell, showed a different response compared to neurite size and fraction of cells expressing neurites. All nucleotides and nucleosides tested, ATP, ATPγS (p<0.05), αβMeATP, UTP, UDP, ADP, as well adenosine, significantly (p<0.001) reduced this measure of differentiation (Fig. 2-2C).

As an additional morphological measurement of PC12 cell neuronal differentiation, we assessed the area of the cell body. Upon NGF treatment, PC12 cells
flattened, increasing the area of the soma (Fig. 2-1). ATP, ATPγS, αβMeATP, UTP, ADP, UDP, and adenosine all significantly (p<0.001) reduced the NGF-induced increase in cell body area to a size similar to that of control cells not treated with NGF (Fig. 2-2D). The number of neurites per cell significantly correlated (p<0.0001) with the size of the cell body, such that larger numbers of neurites were found on cells with larger areas. Figure 2-4 shows the correlation for neurite number with cell area for cells treated with NGF alone or NGF and ATPγS.

The ability of extracellular nucleotides to modulate NGF-promoted differentiation in PC12 cells led me to test the enzyme apyrase, which hydrolyzes ATP to adenosine, as a means to evaluate whether PC12 cells constitutively release nucleotides and alter NGF-induced neuronal differentiation in an autocrine/paracrine manner. By itself, apyrase did not have a significant effect on any of the morphological measurements of NGF-stimulated PC12 cell differentiation in our studies (Fig. 2-2A-D). However, in the presence of ATP and NGF, apyrase decreased the fraction of cells expressing neurites and number of neurites per cell (p<0.001), increased the size of the neurites (p<0.001), and decreased the cell area (Fig. 2-2A-D). Results obtained with apyrase plus ATP results are likely attributable to the generation of adenosine from the catalysis of ATP by apyrase, since adenosine had similar effects on these measures of NGF-induced PC12 neuronal morphology.

Taken together, the data indicate that trinucleotides but neither dinucleotides, mononucleotides, adenosine, nor endogenously released ATP, increase the fraction of PC12 cells whose differentiation is promoted by NGF. The action of the exogenously added trinucleotides likely results from a sensitization of PC12 cells to NGF. By
contrast, all the nucleotides tested, as well as adenosine, reduce the number of neurites, which correlates with the soma flattening that occurs with NGF treatment.

**ATP Release in undifferentiated and NGF-treated PC12 cells**

Since exogenous nucleotides and adenosine were able to alter patterns of NGF-induced PC12 cell differentiation and other cell types are known to release nucleotides (Harden and Lazarowski, 1999; Lazarowski and Harden, 1999; Ostrom et al., 2001; Schwiebert et al., 2002), we asked whether PC12 cells were capable of releasing ATP when stimulated by mechanical stress (Fig. 2-5), βγMeATP, a potent P2X₃ agonist (Burnstock, 2000) (Fig. 2-6), or uridine nucleotides, a P2Y₂ and P2Y₄ agonist (Burnstock, 2000) (Fig. 2-7). Removal and replacement of serum-free media on undifferentiated PC12 cells resulted in >25-fold increase in ATP release into the extracellular media, with a return to basal (1.7nM) ATP levels within 1h (Fig. 2-5). Differentiated PC12 cells (NGF-treated for 72 hours) had a 75-fold increase in extracellular [ATP] after the first minute of a media change, thus causing a 3-fold higher level than with undifferentiated cells. Both NGF-differentiated and undifferentiated PC12 cells showed a similar decline in extracellular ATP levels (Fig. 2-5).

As one means to determine the mechanism of ATP extracellular transport, we used a decrease in temperature to distinguish carrier-mediated cytoplasmic exocytosis from vesicular transport. Previous data indicate that a threshold temperature of 17°C substantially (~80%) inhibits carrier-mediated cytoplasmic release from neurons while potentiating vesicular release (Vizi, 1998). We found that a temperature of 20°C inhibited ATP release in PC12 cells treated with NGF to a level similar to that of NGF-
untreated PC12 cells (Fig. 2-5). The reduced temperature had minimal effect on ATP release in undifferentiated PC12 cells. This ability of decreased temperature to blunt the increase in ATP release implies that carrier-mediated exocytotic release may be the mechanism for transport of ATP in NGF-differentiated PC12 cells.

We also assessed βγMeATP-, UTP-, and UDP-stimulated ATP release in differentiated versus undifferentiated PC12 cells, as a means of testing possible regulation of release by P2Y receptor activation. βγMeATP, an agonist associated with P2X receptors (Burnstock, 2000) (see Table Appendix-1 for P2 receptor agonist/antagonist profiles), was more effective at causing ATP release from undifferentiated, rather than NGF-differentiated PC12 cells (Fig. 2-6C and D). βγMeATP-stimulated release was sensitive to inhibition by suramin (P2 inhibitor) and of both intracellular (thapsigargin) and extracellular (Gd³⁺) calcium stores (Fig. 2-6C), indicating potential signaling by both P2X and P2Y receptors (Burnstock, 2000). UTP, an agonist that only activates P2Y, and not P2X, receptors was a more effective stimulator of ATP release from NGF-differentiated, rather than undifferentiated PC12 cells in both a time (Fig. 2-6A) and concentration-dependent manner (Fig. 2-6B). UTP (an agonist of P2Y₂ and P2Y₄ receptors (Burnstock, 2000)) stimulated significantly (p<0.05) higher levels of ATP release from NGF-differentiated than undifferentiated PC12 cells (Fig. 2-6B). This effect was abolished in both untreated and NGF-treated PC12 cells by the inhibitor of intracellular calcium stores, thapsigargin (Gysbers, 2000) (Fig. 2-7). This sensitivity of UTP-evoked ATP release to depletion of intracellular (rather than extracellular calcium) calcium stores suggests a P2Y-mediated mechanism (Burnstock, 2000). UDP, a P2Y₆ receptor agonist, was able to stimulate release of ATP in untreated
PC12 cells, but differentiation by NGF significantly (p<0.05) decreased UDP-evoked ATP release (Fig. 2-7).

Previous studies in other cell types demonstrate that the addition of UTP in the presence of ecto-nucleoside diphosphokinase and ADP can result in the formation of UDP and ATP (Lazarowski et al., 1997b). Thus, when UTP is added to cells this enzyme can act on a pool of extracellular ADP to generate ATP. In order to test whether this conversion might account for the increase in extracellular [ATP] when UTP was used, we added ADP to the media and quantified [ATP] using a luciferin/luciferase assay. Adding ADP (>10nM) in serum-free media resulted in bioluminescence levels at intensities well above undisturbed control PC12 cells in serum-free media (data not shown), and thus should have yielded a readily detectable signal of conversion of UTP to ATP were the mechanism accounting for the observed UTP-mediated ATP release. Therefore, we believe that UTP, presumably acting via P2Y receptor activation and not enzymatically generating ATP, is responsible for the UTP-stimulated ATP release.

The results for ATP release and inhibition of release, in particular the change in response to UTP and UDP with NGF treatment, imply a possible change in receptor expression that would contribute to regulation of ATP release in response to NGF-promoted differentiation.

**ATP and neurotrophic factor release from sympathetic neuronal target tissues**

In addition to autocrine release of ATP from PC12 cells, a model of sympathetic differentiation, paracrine release from target tissues might also serve as a source of extracellular nucleotide signaling. In particular, sympathetic innervation of both cardiac
and vascular cells represent important loci for regulation of cardiovascular function (Esler et al., 2003; Schlaich et al., 2003). I assessed $\beta\gamma$MeATP-, and UTP-, and mechanically-stimulated ATP release from human umbilical vein endothelial cells (HUVEC), rat aortic smooth muscle cells (RASMC), and rat cardiac fibroblasts (RCF) (Fig. 2-8). Media change induced the greatest ATP release in HUVEC, but lower levels of release also resulted from UTP (blocked by suramin) and $\beta\gamma$MeATP (blocked by thapsigargin). The UTP and $\beta\gamma$MeATP release results implicate P2Y receptors and mobilization of intracellular calcium stores as mechanisms of ATP release by these agonists, respectively (Fig. 2-8A). RASMC cells demonstrated release of ATP in response to media change or UTP stimulation, the latter blocked by the P2 antagonist, suramin (Fig. 2-8B). RCF cells released ATP only in response to media change (Fig. 2-8C). A comparison of ATP release across these cell types indicated that HUVEC release of ATP by media change was the largest, with levels 50-fold higher than PC12 cells (+/-NGF), 23-fold higher than RCF, and 11-fold higher than RASMC (Fig. 2-8D). These results implicate endothelial cells as potential sources of extracellular nucleotides for sympathetic neurons.

Studies conducted with PC12 cells in co-culture with HUVEC, RASMC, and RCF demonstrated differentiation of PC12 cells in the absence of NGF (Fig. 2-9A). This effect was not due to a release of NGF by any of these cell types as identified using an NGF-blocking antibody (Fig. 2-9A and 2-10A-D). Conditioned media from HUVEC and RASMC was also capable of inducing differentiation, the former most significantly ($p<0.001$) (Fig. 2-9B). HUVEC conditioned media (CM) was able to induce differentiation, an effect that was enhanced by supplementation with ATP$_{\gamma}$S at the 24 h
time point (Fig. 2-9C). The effect of HUVEC CM was both time and dose-dependent (Fig. 2-9D). These data indicate that morphological differentiation of sympathetic neurons may occur both via a neurotrophic factor released by vascular and cardiac cell types, as well as be enhanced by nucleotide release from these target tissues. Though potentially interesting, I did not choose to further pursue this co-culture work in my thesis studies.

**Quantitative RT-PCR of P2Y receptor mRNA: Effects of NGF and ATPγS**

In order to determine the relative mRNA expression levels of P2Y receptors, I employed quantitative RT-PCR with untreated, NGF-treated, and NGF-ATPγS co-treated PC12 cells. There are 8 known P2Y receptors: P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃ and P2Y₁₄ (Adrian, 2000; Burnstock, 2000; Communi, 2001; Nicholas, 2001; Abbracchio et al., 2003). I found that PC12 cells express all these receptors except P2Y₁₄ (Fig. 2-11). In addition, PC12 cells express all 7 P2X receptor transcripts, most prominently P2X₁-₄, and most modestly P2X₅-₇ (Fig. 2-12), as measured by QRT-PCR.

To assess the impact of NGF-promoted differentiation on P2Y receptor expression, PC12 cells were treated with NGF (100ng/ml) or NGF and ATPγS (100µM) for 72h. Treatment of PC12 cells with NGF altered the expression of several P2Y receptor mRNA’s (Fig. 2-13 and Table 2-2). Real time PCR analysis (Table 2-2) facilitated quantification of changes in different P2Y receptor subtypes in response to NGF treatment. NGF decreased expression of P2Y₆ and P2Y₁₁ receptor mRNA significantly (>8-fold, Table 2-2) with the decrease in P2Y₆ mRNA being the most
prominent. By contrast, treatment of PC12 cells with ATPγS increased P2Y_{12} mRNA expression.

Addition of ATPγS together with NGF altered expression of several P2Y receptor mRNAs with significant increases in P2Y_{6}, P2Y_{11}, and P2Y_{13} (≥8-fold) in PC12 cells co-incubated with NGF and ATPγS compared to cells incubated only with NGF. P2Y_{13} receptor mRNA levels were dramatically increased in cells co-treated with NGF and ATPγS; the decreased expression of P2Y_{6} and P2Y_{11} receptor mRNA in cells incubated with NGF returned to levels similar to those of untreated PC12 cells when cells were incubated with ATPγS together with NGF. ATPγS in the presence of NGF also produced a modest change in P2Y_{4} receptor mRNA (~6-fold) but did not decrease P2Y_{12} receptor mRNA (in contrast with what was observed in the absence of NGF). QRT-PCR amplification plots across 40 cycles, showing the amplification of specific receptor cDNA from control PC12 cell mRNA and in cells incubated with NGF, and NGF plus ATPγS are shown in Fig. 2-13. Additionally, measurements by QRT-PCR demonstrate changes in expression of P2Y and P2X transcripts in response to NGF-differentiation (Fig. 2-14). NGF-differentiation increases expression of P2X_{1,3,5} and decreases expression of P2Y_{6,11}. These receptor changes in response to differentiation by neurotrophins may alter cellular responses to extracellular nucleotide signaling.

Thus P2Y receptor mRNA expression changes in response to NGF-promoted differentiation and the pattern of mRNA expression is altered in the presence of a non-hydrolyzable ATP derivative, ATPγS.
Discussion

In this work, I provide several new observations regarding the action of ATP and other nucleotides on PC12 cells, in particular the interaction of nucleotides with NGF, which differentiates PC12 cells: 1. PC12 differentiation promoted by NGF (neurite formation) is enhanced by nucleotide triphosphates; 2. PC12 cells release ATP in response to mechanical stimulation and uridine nucleotides; the extent of UTP-, UDP- and mechanically-induced ATP release changes with NGF-promoted differentiation; 3. PC12 cells express 7 of 8 known P2Y receptors and NGF alters expression of several of certain P2Y receptors. Below I discuss each of these findings.

PC12 cells treated with NGF differentiate into sympathetic neuron-like cells with formation of neurites and cell body expansion (Greene, 1976; Kaplan and Stephens, 1994; Vaudry et al., 2002). Addition of nucleotides increases the extent of differentiation in the presence of NGF (fraction of cells expressing neurites) while reducing the number of neurites per cell and cell body area. These results imply that trinucleotides interact with NGF to promote a “switch” in cellular fate to a neuronal phenotype but in parallel the nucleotides decrease the number of neurites. Increased [Ca\textsuperscript{2+}]\textsubscript{i} can result in neuronal differentiation via a pathway that does not require NGF (Boglari and Széberényi, 2001). The morphological modulation of PC12 cell differentiation in which ATP sensitizes PC12 cells to NGF (Fig. 2-3) might result from a potentiation by NGF of ATP-induced [Ca\textsuperscript{2+}]\textsubscript{i} increases (Huang and Kao, 1996).

Both the hydrolysis of ATP to adenosine by apyrase and addition of adenosine, a P1 receptor agonist, increased the size of the neurites in PC12 cells, implicating P1 receptors in this morphological response. ATP is rapidly degraded to adenosine by ecto-
nucleotidases present on PC12 cells (Vollmayer et al., 2001). Among several known adenosine (P1) receptor subtypes, PC12 cells only express A$_{2\alpha}$ receptors (Thevananther et al., 2001; Cheng et al., 2002). Adenosine was reported to increase the fraction of PC12 cells expressing neurites at sub-optimal (<50ng/ml) NGF concentrations, but no change was observed in this measure of differentiation at 100ng/ml NGF (Cheng et al., 2002). I obtained somewhat different results at 100ng/ml NGF (Fig. 2-2), in that adenosine reduced measures of neuronal differentiation. Differences between the previous results and current findings may relate to one or more methodological differences in the studies: the quantitation of neurite-bearing cells (Cheng et al. quantified neurites of 2 cell body lengths while I used a 1 cell body length threshold), the use of an A$_{2\alpha}$-selective agonist (CGS21680 by Cheng et al.) rather than adenosine, or a higher [CO$_2$] during cell incubation (10% by Cheng et al. vs. 5% in the current study). I found that treatment with adenosine deaminase, which hydrolyzes adenosine to inosine (thereby removing adenosine produced from ATP hydrolysis) was cytotoxic to PC12 cells and thus could not be used to eliminate adenosine from the cultures (data not shown).

Although the morphologic studies conducted here primarily involved assessment of the impact of addition of exogenous compounds, PC12 cells release ATP. As a consequence, ATP and its metabolic products have the potential to be autocrine/paracrine signals. Incubation of PC12 cells with apyrase does not produce morphological changes, but when added with ATP, yielded NGF-induced morphological changes similar to those seen with adenosine treatment (Fig. 2-2). These findings suggest that constitutive release of ATP by PC12 cells may not be occurring at high enough levels for autocrine/paracrine regulation. However, it is possible that PC12 cells respond to nucleotides (or other
factors) that are released from other cell types, perhaps post-synaptic sympathetic target cells (Arthur, 2003) (Fig. 2-8, 2-9).

These data regarding release of ATP from PC12 cells are consistent with previous findings indicate that cells release ATP in response to mechanical stress (Lazarowski and Harden, 1999; Ostrom et al., 2001; Schwiebert et al., 2002). I found that mechanical stimulation (media change) induces higher levels of ATP release from NGF-differentiated than from control PC12 cells. Because lowering the assay temperature abolished the NGF-induced component of ATP release, the mode of release may be carrier-mediated exocytosis, which is more sensitive than vesicular release to lower temperature (Vizi, 1998). PC12 cells are known to store and release norepinephrine and acetylcholine in discrete vesicles (Schubert and Klier, 1977). ATP may be stored/released with these neurotransmitters in PC12 cells as in native chromaffin cells (Kuwashima et al., 2000). The inhibition by lower temperature of a large component of ATP release suggests that vesicular release may not be the primary mechanism of ATP exocytosis in NGF-differentiated PC12 cells.

P2Y receptor activation may be the mechanism by which UTP stimulates release of ATP from PC12 cells. This conclusion is supported by results with thapsigargin because P2Y receptors, unlike P2X receptors, utilize internal calcium stores (Burnstock, 2000). This idea is supported by the decreased accumulation of ATP released by UDP, a P2Y₆ receptor agonist (Hou et al., 2002), in NGF-differentiated PC12 cells, which show a decrease in expression of P2Y₆ receptor mRNA (Fig. 2-5, 2-7B).

P2Y receptors may also be responsible for paracrine release of nucleotides involved in sympathetic neuronal development and function. Release of nucleotides from
vascular endothelial cells, cardiac fibroblasts and aortic smooth muscle cells (Fig. 2-8) identifies potential sources of nucleotide signaling that may contribute to the morphological enhancement of NGF-stimulated differentiation observed in PC12 cells (Fig. 2-1, 2-2). In addition, these cells may release other neurotrophic factors that may contribute to differentiation and growth of sympathetic neurons.

Slowly developing cellular responses to agonists, such as growth and morphologic effects, can be transduced by G-protein coupled receptors, GPCR’s (Cameron et al., 1998). Such actions, coupled with the results from nucleotide release studies, led me to ask if NGF treatment of PC12 cells changes P2Y (and P2X) receptor expression. In previous work PC12 cells were shown to have altered P2Y receptor expression in response to ATP, 2-Cl-ATP, and NGF (D'Ambrosi et al., 2001). The authors tested regeneration (as opposed to de novo propagation of neurites in the current studies), and assessed a limited subset of P2Y receptor subtypes (P2Y2 and P2Y4), finding an upregulation of expression of P2Y2 receptors. Other authors have probed for P2Y receptor mRNA expression in differentiated versus undifferentiated PC12 cells and have detected P2Y2, P2Y4, P2Y6, and P2Y12, with an increase in P2Y12 mRNA in response to NGF-promoted differentiation (Unterberger et al., 2002). By using quantitative real time-PCR, which provides a more sensitive and complete assessment of mRNA expression in PC12 cells, I found that, compared to control cells, NGF-differentiated PC12 cells express significantly lower levels of P2Y6 and P2Y11 mRNA (and no change in P2Y12). Additional upregulation of P2X receptor transcripts (1-3, and 5) in response to NGF were also found (Fig. 2-14). The use of different primers or greater cycle numbers (40 in our QRT-PCR vs. 30 by RT-PCR by Unteberger et al.) may account for the previous RT-
PCR studies reporting absence of P2Y\textsubscript{11} receptor in the earlier studies, and perhaps our differing results for P2Y\textsubscript{12}. The data shown here are the first to demonstrate expression of P2Y\textsubscript{13} in PC12 cells. P2Y\textsubscript{13} is a G\textsubscript{i}-coupled receptor which, when stimulated by ADP, results in the inhibition of adenylyl cyclase activity and a decrease in intracellular cAMP levels (Communi, 2001).

Addition of ATP\textsubscript{γS} in the absence of NGF increased expression of P2Y\textsubscript{12} receptor mRNA and in the presence of NGF significantly increased P2Y\textsubscript{6}, P2Y\textsubscript{11}, and P2Y\textsubscript{13} mRNA expression, P2Y\textsubscript{6} and P2Y\textsubscript{13} most dramatically relative to NGF alone. Increases in P2Y\textsubscript{6} and P2Y\textsubscript{11} with NGF and ATP\textsubscript{γS} resulted in a near return to control levels of mRNA expression for these receptors relative to what was observed with NGF alone, while P2Y\textsubscript{13} was prominently increased compared to either ATP\textsubscript{γS} alone or NGF alone. The ability of ATP\textsubscript{γS} to increase mRNA expression for several P2Y receptors contrasts with actions of agonists to down-regulate expression of many GPCR’s (Claing et al., 2002). Further studies will be needed to define the precise mechanism by which P2Y receptor mRNA changes in response to NGF and ATP\textsubscript{γS}. It is of interest that akin to the effect of ATP\textsubscript{γS} on several P2Y receptors, cytokine treatment increases adenosine A\textsubscript{2a} receptor expression in PC12 cells (Trincavelli et al., 2002).

Changes in P2Y receptor mRNA expression may result in altered PC12 cell responses to extracellular nucleotides. NGF and ATP\textsubscript{γS}-promoted changes in P2Y receptor mRNA correlated with the biochemical and morphological data in PC12 cells. Thus increases in P2Y\textsubscript{11} mRNA in response to ATP\textsubscript{γS} (vs. NGF alone) suggest a role for this receptor in the enhancement of differentiation when PC12 cells were incubated with NGF. P2Y\textsubscript{11} is the only known subtype of P2YR’s that is coupled to G\textsubscript{i}, stimulating
cAMP production through adenylyl cyclase and leading to downstream effects via phospholipase C/adenylyl cyclase and protein kinases A and C (Wilkin, 2001). The ability of ATP in the presence of NGF to up-regulate P2Y_11 receptor levels suggests that ATP provides a feed-forward mechanism for enhancing PC12 cell differentiation. Lower levels of mRNA expression of P2Y_6 in NGF-differentiated PC12 cells may account for the reduction of UDP-stimulated ATP release in differentiated PC12 cells (Fig. 2-14).

Overall, the data presented suggest a role for extracellular nucleotides in the regulation of neuronal differentiation, in particular of sympathetic neuronal differentiation. Neuronal development through nucleotides and P2Y receptor activity may constitute a paracrine system for the “decision” to initiate a neuronal fate and may help modulate neurite formation early in neuronal development, especially via interaction with growth factors, such as NGF.
Acknowledgements

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Figure 2-1: PC12 morphology. Undifferentiated PC12 cells in PC12-specific media (see Methods) (A) and after 72h incubation with 100ng/ml NGF (B). Arrows indicate neurites.
Figure 2-2: Morphological measurements of NGF-promoted PC12 cell differentiation. PC12 cells were incubated in the absence or presence of 100ng/ml NGF for 72h along with the indicated treatments. A concentration of 100µM was used for all nucleotides and adenosine. The concentration of apyrase was 2u/ml. ∧p<0.05, *p<0.01, **p<0.001 v. NGF treatment alone. (A) The fraction of total PC12 cells expressing one or more neurites as long as a cell body diameter was assessed. (B) The area of each neurite was measured (arbitrary units). (C) The number of neurites per neurite-bearing PC12 cell was counted. (D) The area of PC12 cell bodies was measured (arbitrary units).
Figure 2-3: ATPγS increases the fraction of PC12 cells expressing neurites at submaximal NGF concentrations. PC12 cells were incubated for 72h in the presence (triangles) or absence (squares) of 100µM ATPγS together with the indicated NGF concentrations. **p<0.001.
Figure 2-4: The number of neurites per cell correlates with cell body size in NGF and NGF+ATPγS treated PC12 cells. Morphological measurements for the number of neurites per cell and the cell body area were paired. NGF treated (---, ■, r=0.93) and NGF+ATPγS (—, ▲, r=0.89) both had highly significant (p<0.0001) correlations.
**Figure 2-5**: Effect of NGF and lower temperature on ATP release from PC12 cells following a media change. PC12 cells were plated on Poly-D-Lysine coated plates and incubated in the presence or absence of 100ng/ml NGF for 72h. Cells were then incubated in serum-free media at 37°C or 20°C and subjected to a media change. ATP released in the extracellular media was sampled and measured in a luciferin/luciferase assay (see Methods) at the indicated time points. (control PC12 cells 37°C ■; control PC12 cells 20°C ▼; NGF-treated PC12 cells 37°C ▲; NGF-treated PC12 cells 20°C ★)
Figure 2-6: Differentiated PC12 cells have altered responses to nucleotide-stimulated nucleotide release. PC12 cells grown for 72h +/- NGF (control-black; NGF-red) were stimulated with 100µM UTP for up to 1h (A), were stimulated for 1min with the indicated concentration of UTP (B), with 100µM βγMeATP for 1min and suramin, apyrase, gadolinium (Gd³⁺, channel blocker), or thapsigargin (thapsi) where indicated (C), and with 100µM βγMeATP for up to 60 min (D) and assessed for ATP release.

*p<0.05, **p<0.01, ***p<0.001 v. control.
Figure 2-7: UTP- and UDP-stimulated ATP release changes with NGF-promoted differentiation. PC12 cells were grown for 72h in the presence or absence of 100ng/ml NGF, pre-incubated in serum-free media and treated with the indicated compounds. Media samples were taken and measured in a luciferin/luciferase assay (see Methods). Thapsigargin (Thapsi) was added 20min prior to sampling, UTP and UDP were added 5min prior to media sampling. (^p<0.05 v. UTP alone, *p<0.05 v. UDP alone, **p<0.001 Thapsi/UTP v. UTP and Thapsi/UTP+NGF v. UTP+NGF)
**Figure 2-8:** Cardiac and endothelial cells are a source of nucleotide release. Human umbilical vein endothelial cells (HUVEC) (A), rat aortic smooth muscle cells (RASMC) (B), and rat cardiac fibroblasts (C) were vehicle-treated (mock), treated with a bulk media change, UTP, αβMeATP, thapsigargin (thapsi) and suramin where indicated and ATP release was assessed. HUVEC ATP release is measured in nM (A), and RASMC and rat cardiac fibroblast ATP release is measured relative to mock release (B and C). Levels of released ATP were compared between these cells and PC12 (+/- 72h NGF) cells (D). (*p<0.05, **p<0.01, ***p<0.001 v. basal)
**Figure 2-9:** Cardiac and vascular endothelial cells mediate PC12 cell differentiation independent of NGF. PC12 cells co-cultured with rat cardiac myocytes, HUVEC, RASMC, or RCF in the presence or absence of ATPγS or an anti-NGF blocking antibody (where indicated) and scored for the fraction of neurite-bearing PC12 cells (A). PC12 cells were plated using conditioned media (CM) from HUVEC, RASMC, or RCF and the fraction of neurite-bearing cells were scored (B). The fraction of PC12 cells bearing neurites when grown in HUVEC-CM and ATPγS (where indicated) for the time points shown (C). PC12 cells grown in HUVEC-CM generated using a 10% versus 85% confluent HUVEC flask were scored over the tampons indicated for the fraction of neurite-bearing cells (D). (*p<0.05, **p<0.01, ***p<0.001, unless otherwise indicated)
**Figure 2-10:** PC12 cells differentiate in co-culture with HUVEC independent of NGF. PC12 cells were cultured for 72 h with NGF (A), NGF together with an anti-NGF blocking antibody (B), co-cultured with HUVEC (C), and co-cultured with HUVEC together with anti-NGF blocking antibody (D). Arrows indicate neurites.
**Figure 2-11:** PC12 cells express 7 of 8 P2Y receptor mRNA. PC12 cells were grown for 72h in the presence of absence of 100ng/ml NGF. mRNA was isolated and subjected to reverse transcription. The resulting cDNA was amplified in a quantitative PCR reaction. The resulting products were run on ethidium bromide gels.
Figure 2-12: P2X expression in PC12 cells. Ethidium Bromide gel of RT-PCR products for P2X receptor transcripts from PC12 cells (A). Real-time RT-PCR relative quantitation of GAPDH-normalized P2X receptor expression in PC12 cells.
Figure 2-13: ATPγS, NGF, and ATPγS+NGF alter expression of P2Y$_6$, P2Y$_{11}$, P2Y$_{12}$, and P2Y$_{13}$. PC12 cells were grown for 72h in media alone, or in the presence of ATPγS (100μM), NGF (100ng/ml), or ATPγS+NGF. mRNA was isolated, and assayed for P2Y receptors mRNA levels using QRT-PCR. Amplification plots for P2Y$_2$ (A), P2Y$_6$ (B), P2Y$_{11}$ (C), P2Y$_{12}$ (D), and P2Y$_{13}$ are shown. P2Y$_2$ mRNA expression did not change with NGF treatment (+ ATPγS) but ATPγS enhanced expression of P2Y$_6$, P2Y$_{11}$, and P2Y$_{13}$. The initial phase of the graph where the plot becomes linear is selected at the CT value (see Table 1 for the actual values).
Table 2-2: P2Y receptor expression in PC12 cells.

<table>
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<tr>
<th>Treatment</th>
<th>GAPDH</th>
<th>P2Y1</th>
<th>P2Y2</th>
<th>P2Y4</th>
<th>P2Y5</th>
<th>P2Y7</th>
<th>P2Y11</th>
<th>P2Y12</th>
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<tr>
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<td>22.15</td>
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<td>40.00</td>
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<tr>
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<td>32.08</td>
<td>29.41</td>
<td>30.42</td>
<td>26.77</td>
<td>18.30</td>
<td>40.34</td>
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<tr>
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<td>35.14</td>
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<td>29.37</td>
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<td>22.87</td>
<td>40.00</td>
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<tr>
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<td>40.00</td>
<td>30.23</td>
<td>22.81</td>
<td>38.27</td>
<td>35.08</td>
<td></td>
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<tr>
<td>NGF + ATPgammaS</td>
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<td>29.79</td>
<td>26.55</td>
<td>27.62</td>
<td>26.71</td>
<td>23.60</td>
<td>27.54</td>
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Relative Fold mRNA Expression Change (Compared to Control)*

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<th>P2Y2</th>
<th>P2Y4</th>
<th>P2Y5</th>
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<tr>
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<td>-1.10/</td>
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<td>-0.85/</td>
<td>-0.85/</td>
<td>-1.20/</td>
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<td>-3.89/</td>
<td>-3.89/</td>
<td>-2.110</td>
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</table>

Quantitative RT-PCR cycle threshold and relative expression values of P2Y receptor and GAPDH mRNA. Cycle threshold values were assigned using ABI Prizm software and expression changes were calculated using $2^{\Delta\Delta CT}$. A change ≥ 8-fold was considered significant. *Data for relative fold mRNA expression (compared to control) were calculated (using equation in methods) relative to GAPDH expression for each mRNA in the experiments shown.
Figure 2-14: NGF differentiation alters relative P2 receptor mRNA expression in PC12 cells. Quantitative RT-PCR of PC12 cells treated for 72h +/- NGF and assayed for P2X (A) and P2Y (B) expression. All values were GAPDH adjusted and normalized to control. * >8-fold change in expression.
Chapter 3:

P2Y$_2$ receptor activates NGF/TrkA receptor signaling to enhance neuronal differentiation
Abstract

Neurotrophins are essential for neuronal differentiation but the onset and the intensity of neurotrophin signaling within the neuronal microenvironment are poorly understood. We tested the hypothesis that extracellular nucleotides and their cognate receptors regulate neurotrophin-mediated differentiation. We found that ATPγS activation of the G-protein-coupled receptor, P2Y₂, in the presence of NGF leads to the co-localization and association of TrkA and P2Y₂ receptors and is required for enhanced neuronal differentiation. Consistent with these effects, ATPγS promotes phosphorylation of TrkA, ERK1/2 and p38, thereby enhancing sensitivity to NGF and accelerating neurite formation in both PC12 cells and dorsal root ganglion neurons. Genetic or siRNA depletion of P2Y₂ receptors abolished the ATPγS-mediated increase in neuronal differentiation. Moreover, in vivo injection of ATPγS into the sciatic nerve increased GAP-43, a marker for axonal growth, in wild-type but not P2Y₂⁻/⁻ mice. The interactions of tyrosine kinase- and P2Y₂⁻ signaling pathways provide a novel paradigm for the regulation of neuronal differentiation and suggest a role for P2Y₂ as a morphogen receptor that potentiates neurotrophin signaling in neuronal development and regeneration.
Introduction

Neurite formation, a process extending from the cell soma and led by a growth cone, is a primary morphological event in neuronal differentiation, ultimately facilitating synaptic connections by neurons (Yamamoto et al., 2002). Neuronal regeneration depends on the formation of neurites to repair injured or lost connections. These neuronal growth events require appropriate spatial and temporal expression and action of both initiation signals and promoter molecules (Terenghi, 1999). Neurotrophins, such as nerve growth factor (NGF), are key regulators of neuronal differentiation. NGF is released by target tissues, initiates neurite generation, maintains neuronal survival, prevents apoptosis and promotes synapse formation (Gavazzi and Cowen, 1996; Boglari and Szeberenyi, 2001; Chuang et al., 2001). NGF signaling via the tyrosine kinase receptor A (TrkA) leads to stimulation of early response kinase 1/2 (ERK1/2) via Ras/Raf pathways, which is required for differentiation, as well as the activation of the survival kinase, Akt (Chao, 2003a). The regulation of NGF/TrkA signaling is determined by availability of NGF, as well as TrkA activation, characterized by receptor autophosphorylation, internalization and retrograde transport from axons to cell bodies (Yano and Chao, 2004). Recent evidence has shown receptor crosstalk is an important mechanism for regulation of neurotrophin signaling (Chao, 2003a). The NGF/TrkA signaling pathway converges with pain-related ion channels to regulate NGF-mediated heat sensitivity of sensory neurons (Chuang et al., 2001) and with adenosine A2A receptors to regulate neuronal survival (Chi et al., 2001). The contributions of other signal transduction pathways integrated with NGF/TrkA signaling to modulate neuronal differentiation remain largely unknown.
It has been suggested that extracellular nucleotides may influence neuronal differentiation, migration and survival (Neary et al., 1996), such as enhancement of NGF-dependent neurite outgrowth from PC12 cells (Rathbone et al., 1999). Nucleotides in the nervous system are stored in and released from synaptic vesicles and glial cells at mM concentrations, thereby affecting neurotransmitter release and glial calcium wave propagation, respectively (Neary and Zhu, 1994; Lechner et al., 2004). ATP has been proposed as an activity-dependent signaling molecule that regulates glial-glial and glial-neuron communication (Neary and Zhu, 1994; Fields, 2000).

Extracellular nucleotide signaling occurs via P2 ionotropic (P2X) and metabotropic (P2Y) G-protein-coupled receptors (GPCRs). Seven P2X receptors (1-7) and eight P2Y (1, 2, 4, 6, 11-14) receptors, each with unique agonist response profiles, have been identified (Burnstock and Knight, 2004). Both P2Y and P2X receptors are expressed in the nervous system. P2X receptors regulate synaptic transmission (Khakh, 2001), pain (Cockayne et al., 2000) and respiration (Gourine et al., 2005). The role of P2Y receptors in the nervous system is less well characterized, but they can couple to neuronal ion channels (Boehm, 2003) and modulate pain responses (Molliver et al., 2002). The contribution of individual P2 receptor subtypes to neuronal functions and their interaction with signal transduction pathways in the nervous system remain largely unknown. In this study we used several complementary approaches to assess the role of P2Y receptors in neuronal differentiation. The results, which indicate an important contribution for P2Y$_2$ receptors in this differentiation via interaction between the TrkA and P2Y$_2$ signaling pathways, define novel mechanisms for merging the interaction of neurotrophins and extracellular nucleotides.
Materials and methods

Reagents ATPγS, AMPCP, and ARL67156 (Sigma, St. Louis, MO). Nerve Growth Factor (NGF) (Invitrogen, Carlsbad, CA). K252a (Calbiochem, San Diego, CA) and U0126 (Cell Signaling, Beverly, MA).

Morphology PC12 cells were plated at 60% confluency on collagen/poly-D-lysine coated plates and incubated with 100ng/ml NGF with other treatments for 72h. ATPγS, AMPCP and ARL67156 were added once for a 3-day interval at 100µM, 250µM, and 50µM, respectively. K252a (10nM) and U0126 (10µM) were applied once. Images were taken every 24h for 3 days, using an Axiocam CCD camera on an Axiophot Zeiss microscope (Zeiss, Germany). The fraction of PC12 cells expressing a neurite that was at least one cell body in length were counted. All conditions were assessed in triplicate.

Immunoblot Analysis Protein samples, loaded at equal concentrations, were separated on 10% or 12% precast SDS polyacrylamide gels (Invitrogen) and then transferred to polyvinylidene difluoride membranes. Membranes were blocked in 20mM PBS, 1% Tween with 1.5% nonfat dry milk and then incubated with primary antibody at 4°C overnight. Antibodies used were: P-TrkA, P-ERK1/2, ERK1/2, P-p38, p38, Myc, and HA (Cell Signaling), P2Y₂, P2Y₄ (Alomone Labs, Jerusalem, Israel), actin, TrkA (Santa Cruz Biotech, Santa Cruz, CA), GAPDH (Novus Biologicals, Littleton, CO), GAP-43 (Chemicon, Temecula, CA). Secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotech) were visualized with ECL reagent (Amersham, Piscataway, NJ).
**Immunoprecipitation**  PC12 and DRG cells were treated with the indicated pharmacological/neurotrophic agents for 1h. Cell lysate was pre-cleared by with Protein G agarose beads (Roche, Indianapolis, IN) for 1h, incubated with the primary immunoprecipitating antibody for 3h, and Protein G agarose bead was added for another 3h. The beads were isolated, washed and the immunoprecipitated protein was used in immunoblot analysis.

**Immunofluorescence Analysis**  Cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton and were incubated with P2Y₂ (Alomone), P-TrkA 496 (Santa Cruz Biotech) and SMI31 (Sternberger Monoclonals) (1:100) antibodies overnight at 4°C. Cells were incubated with FITC or Cy3-conjugated antibody (1:250; Jackson Immunoresearch) for 2 h. Confocal images of cells were taken at 40X on a Nikon E600FN microscope with a Radiance 2000 (BioRad) confocal. Cells were imaged in a Z-series stack in 0.5µM slices. Images were processed and co-localization measurements were made using ImageJ software (NIH).

**Immunostaining**  Sciatic nerves were immunostained as previously described (Akassoglou et al., 2002)

**PC12 cell Transfection**  PC12 cells were transfected with pcDNA HA-tagged TrkA (gift of Dr. Moses Chao, NYU), pLXSN Myc-tagged P2Y₂ (gift of Sam Wolff and Ken Harden, UNC) constructs and with pre-designed, commercially synthesized, non-
overlapping siRNA (Ambion, Austin TX; ID# 50110, 143692) for P2Y₂ with Lipofectamine2000 (Invitrogen) according to the manufacturer’s instructions.

**Cell isolation and culture** DRG neurons were dissected and trypsin dissociated from P1 wt or P2Y₂⁻/⁻ mice (Cressman et al., 1999) (gift of Dr. Beverly Koller, UNC). Dissociated cultures were grown on laminin/poly-D-lysine/collagen coated 8-chambered slides for 24h in Modified Eagles Medium with 10% heat-inactivated FBS, L-glutamine and glucose in the presence of 50ng/ml NGF. PC12 cells were grown as described by Taupenot et al. (Taupenot et al., 1999).

**Sciatic Nerve Injection** Sciatic nerves were injected using as previously described (Cosgaya et al., 2002; Aoki et al., 2004). The left sciatic nerves of P1 mice were injected with 6µg ATPγS in 6µl of PBS, 2µg NGF in PBS, or the combination using a 5µl Hamilton syringe with a 26-gauge needle. The right sciatic nerve of each mouse served as a control and was injected with 6µl PBS. Results of 3 independent experiments of 3 animals per condition are shown.

**Statistical analysis** Data were analyzed by a paired t-test, one-way analysis of variance followed by Tukey’s multiple comparison test or linear regression. Significance was assigned to p<0.05. For quantitative RT-PCR, an 8-fold change was used as a significant response between treatments. Neurite length and branch point measurements were performed in a blinded fashion by 2 independent observers.
Results

Genetic or siRNA depletion of P2Y$_2$ receptors eliminates ATP$_\gamma$S-enhanced neurite formation and signaling in DRG neurons and PC12 cells

Neurite formation is a hallmark event in neuronal differentiation, neurodevelopment and regeneration after injury (Park et al., 2002; Bareyre et al., 2004). ATP$_\gamma$S enhances neuronal differentiation in PC12 cells ((D'Ambrosi et al., 2001) and (Fig. 3-1A-D). A general P2 inhibitor (suramin) but not a P2X inhibitor (PPADS) suppressed ATP$_\gamma$S-enhanced NGF-mediated neuronal differentiation in PC12 cells (Fig. 3-2A). Since selective antagonists do not exist for P2Y$_2$ receptors, we used both a mouse knock-out and siRNA to deplete P2Y$_2$ and examine its contribution in ATP$_\gamma$S-mediated enhancement of neuronal differentiation.

DRG neurons express both TrkA (Smeyne et al., 1994) and P2 receptors (Sanada et al., 2002) and neurite formation in DRG neurons is regulated by TrkA (Tuttle and O'Leary, 1998). Because neonatal DRG neurons undergo apoptosis in the absence of NGF, neurite growth experiments were conducted in the presence of the neurotrophin (Adler et al., 1984). In order to assess the signaling effects of ATP$_\gamma$S independent of NGF, we used DRG neurons derived from 4-week old mice, which do not require NGF for survival (Lindsay, 1988).

DRG neurons from wt and P2Y$_2^{-/-}$ mice were immunostained for activated TrkA (P-Trk) and P2Y$_2$ receptors to assess their co-localization along with the neurofilament marker SMI31 after 24h growth with either NGF alone or NGF with ATP$_\gamma$S. P-Trk (red) and P2Y$_2$ (green) co-localized at the SMI31-positive cell bodies and neurites (blue) in wt
DRG neurons in both treatment groups (Fig. 3-3A and B, white). Neurites expressed P-Trk along the neurite processes and in the cell body, while P2Y₂ expression was greater in the soma, co-localizing with P-Trk (Fig. 3-3A and B). P₂Y₂⁻/⁻ DRG neurons stained positive for SMI31 (blue) and P-Trk (red) but not P₂Y₂ receptor (Fig. 3-3C and D, purple). There was no measurable difference between numbers of TrkA-expressing DRG neurons in wt versus P₂Y₂⁻/⁻ mice (data not shown), suggesting the changes in P-Trk expression do not result from a decrease in TrkA-positive neurons. ATPγS in the presence of NGF enhanced neurite growth in control DRG neurons (Fig. 3-3B), when compared to DRG neurons treated with NGF alone (Fig. 3-3A).

By contrast, DRG neurons from P₂Y₂⁻/⁻ mice did not show changes in neuronal differentiation when treated with ATPγS in the presence of NGF compared to NGF treatment alone (Fig. 3-3C and D). Quantification demonstrated a 3-fold increase (p<0.001) in neurite length (Fig. 3-3E) and 5-fold increase in neurite branching (p<0.001) (Fig. 3-1F) in wt DRG but no change (p>0.1) in P₂Y₂⁻/⁻ DRG neurite length (Fig. 3-3E) and branching (Fig. 3-3F). We obtained similar results in 4-week old DRG cells from wt and P₂Y₂⁻/⁻ mice (data not shown). Thus, the enhancement by ATPγS of NGF-induced neuronal differentiation requires P₂Y₂ receptors.

Immunoblot analysis of murine DRG neurons also demonstrated the requirement of P₂Y₂ receptors for enhanced NGF signaling. ATPγS-mediated P-ERK1/2 formation and increase in NGF-promoted P-Trk occurred in DRG neurons from wt but not P₂Y₂⁻/⁻ mice (Fig. 3-3G). Phosphorylation of p38 was similar in wt and P₂Y₂⁻/⁻ DRG neurons (Fig. 3-3G). Abolition of ATPγS–mediated signaling changes in the P₂Y₂⁻/⁻ DRG neurons is in accord with results from siRNA P₂Y₂ receptor depletion from in PC12 cells (Cf. Fig.
3-4B-D vs. 3-3A-G). Use of 2 distinct P2Y<sub>2</sub> receptor siRNAs in PC12 cells decreased P2Y<sub>2</sub> receptor mRNA (Fig. 3-4A) and significantly (p<0.001) reduced P2Y<sub>2</sub> receptor protein compared to cells treated with empty vector (Fig. 3-4B). No change in P2Y<sub>4</sub> occurred in either treatment group. Reduced expression of P2Y<sub>2</sub> abolished the enhanced P-TrkA formation in response to ATPγS in the presence of NGF, the increase in P-ERK1/2 in response to ATPγS alone (Fig. 3-4B) and inhibited ATPγS-promoted enhancement of NGF-mediated neurite formation (Fig. 3-4C). Thus, use of P2Y<sub>2</sub>-KO animals and P2Y2 receptor siRNA with PC12 cells both demonstrate that P2Y<sub>2</sub> is the nucleotide receptor that activates TrkA phosphorylation and signaling to enhance neuronal differentiation.

Inhibitors of TrkA (K252a) and ERK1/2 (U0126) can abolish their downstream signaling effects (Xie et al., 2000; MacInnis et al., 2003). To assess the involvement of TrkA and ERK1/2 in the enhancement by ATPγS of NGF-promoted neurite formation, we treated PC12 cells with either K252a or U0126; K252a abolished neurite formation by NGF alone or together with ATPγS while U0126 blocked the enhancement of NGF-promoted neurite formation by ATPγS (Fig. 3-4C). K252a did not inhibit the enhanced P-ERK1/2 formation by ATPγS independent of NGF (data not shown). The results showing enhanced neurite formation by ATPγS are consistent with findings demonstrating MAP kinase-dependent enhancement of neurite outgrowth from PC12 cells (Behrsing and Vulliet, 2004) and further support the notion that ATPγS mediates enhanced NGF-promoted neurite formation through increased activation of both TrkA and ERK1/2.
P2Y-specific receptor agonists enhance neurite formation in PC12 cells through increased NGF sensitivity

Extracellular ATP is degraded to ADP, AMP, and ultimately adenosine by endogenous ecto-nucleotidases and 5’nucleotidases expressed on the surface of many cell types, including PC12 cells (Vollmayer et al., 2001; Joseph et al., 2003). The ability of ATPγS to enhance neurite formation in the presence of NGF led us to test whether nucleotides sensitize PC12 cells to NGF. As shown in Fig. 3-4D, ATPγS (100µM) significantly (p<0.01) increased the fraction of neurite-bearing PC12 cells and left-shifted the NGF concentration-response curve, indicating sensitization to NGF by ATPγS (similar results shown in Fig. 2-3). PC12 cells express multiple ecto-nucleotidases that hydrolyze ATP and UTP and 5’nucleotidases that generate adenosine from AMP and uridine from UMP (Vollmayer et al., 2001). Inhibition of ecto-nucleotidase activity by ARL67156 (ecto-nucleotidase inhibitor) and AMPCP (5’nucleotidase inhibitor) (the combination did not by themselves cause neurite formation or mimic ATP action on NGF response, data not shown), to prevent the hydrolysis of ATP and UTP (Schwann et al., 1994; Sesti et al., 2002) significantly (p<0.05) left-shifted the NGF concentration-response curve (Fig. 3-4D).

P2Y2 and TrkA receptors co-immunoprecipitate in PC12 and DRG cells

Because the data suggested P2Y2-TrkA signaling pathway interaction in the regulation of neuronal differentiation, we examined the cellular localization of P2Y2 and TrkA as well as their physical interaction by co-immunoprecipitation. In accord with the data from immunoblot studies (Fig. 3-1E), we detected low levels of P-TrkA in the
absence of NGF (Fig. 3-5A and B). Addition of NGF caused co-localization of the P-TrkA (red) with P2Y$_2$ receptors (green) (Fig. 3-5C and D, yellow). NGF-treated cells (Fig. 3-5C and D) had 4-fold greater fluorescence intensity of individually labeled receptors compared to untreated or ATP$\gamma$S-treated cells, consistent with enhanced P-TrkA and P2Y$_2$ expression. Cells treated with NGF together with ATP$\gamma$S had 76% co-localization while untreated, ATP$\gamma$S-, and NGF-treated cells, demonstrated 3.2%, 14%, and 52% co-localization, respectively. By contrast, P2Y$_{12}$ receptors and P-TrkA did not co-localize (data not shown).

To further assess the interaction of the P2Y$_2$ and TrkA receptors, we transfected PC12 cells with Myc-tagged P2Y$_2$ and HA-tagged TrkA expression vectors. Immunoblot analysis of co-immunoprecipitated proteins demonstrated that the TrkA and P2Y$_2$ receptors physically interact when activated by ATP$\gamma$S, NGF or the combination (Fig. 3-5E). Moreover, immunoprecipitation of untransfected PC12 cell lysates using a phospho-specific TrkA antibody (to assess co-localization of the endogenous proteins) revealed that NGF-activated TrkA (P-TrkA) physically interacts with P2Y$_2$, an interaction that was increased by ATP$\gamma$S and abolished by treatment with K252a (Fig. 3-5F). Endogenous co-immunoprecipitation of P2Y$_2$ and TrkA receptors from P1 rat DRG neurons treated with NGF alone or together with ATP$\gamma$S (1h) also showed physical interaction of these receptors (Fig. 3-5G). Ours are the first data of which we are aware to document that TrkA co-immunoprecipitates with a GPCR.
ATPγS increases GAP-43 expression in sciatic nerves

To test whether P2Y2 activation increases a marker of neuronal growth in vivo, we treated neonatal P2Y2−/− and wt mouse sciatic nerves (which express TrkA, (Delcroix et al., 2003)), with NGF, ATPγS, or the combination. After 48h, we assessed expression of GAP-43, a marker of neuronal growth, extension and plasticity (Van der Zee et al., 1989; Verge et al., 1990). Untreated sciatic nerve from wt (Fig. 3-4A) or P2Y2−/− mice (Fig. 3-6C) showed similar basal levels of GAP-43 (green) although P2Y2−/− mice lacked expression of P2Y2 receptor (Fig. 3-6E). Injection of ATPγS induced an increase in GAP-43 in sciatic nerves of wt (Fig. 3-6B) but not P2Y2−/− mice (Fig. 3-6D). Sciatic nerves from wt treated with ATPγS had a 1.8-fold increase of GAP-43 compared to controls; treatment with ATPγS in combination with NGF further increased GAP-43 expression (Fig. 3-6E). No changes were observed with treatment of sciatic nerves from P2Y2−/− mice (Fig. 3-6E). GAP-43 immunostaining of ATPγS-injected wt sciatic nerves showed an increase in GAP-43 expression as early as 12 h post-injection, which was sustained for 24 h (Fig. 3-7A). Bielschowsk silver axon staining did not reveal morphological differences of control versus treated sciatic nerve axons (Fig. 3-7B). Western blots demonstrated ERK1/2-dependent upregulation of GAP-43 expression by ATPγS in DRG neurons, an effect blocked by the ERK kinase inhibitor, U0126 (Fig. 3-7C). Overall, these results suggest that P2Y2 regulates expression of GAP-43 in the sciatic nerve.

Discussion

The current data provide evidence from a variety of complementary approaches for interaction of NGF and extracellular nucleotides acting at P2Y2 receptors in the
enhancement of neuronal differentiation in PC12 cells, DRG neurons and sciatic nerve. The TrkA and P2Y2 receptors co-immunoprecipitated in cells stimulated with NGF or ATPγS, indicating a physical association regulated by receptor activation. In vivo treatment of P2Y2-expressing (but not P2Y2-null) sciatic nerves with ATPγS increased levels of GAP-43, a marker for neuronal growth and differentiation (Strittmatter et al., 1995).

A model for the interaction between NGF/TrkA and P2Y2 signaling (Fig. 3-8) begins with TrkA activation, which enhances its physical association with and protein levels of P2Y2 receptors. The convergence of NGF/TrkA with ATP/P2Y2 signaling results in increased ERK1/2 activation by P-TrkA leading to increased neurite formation. Activation of P2Y2 receptors increases P-ERK1/2 formation independent of NGF, although the increase is not sufficient to induce neurite formation in the absence of neurotrophin signaling (Fig. 3-1F, 3-4C). Overall, our results suggest that enhancement of P-TrkA formation by P2Y2 activation is a crucial interaction between NGF/TrkA and P2Y2 receptor signaling. It is intriguing that during embryonic development both TrkA (Martin-Zanca et al., 1990) and P2Y2 (Cheung et al., 2003) are expressed by sensory spinal ganglia. Such findings, together with the current data, implicate P2Y2-NGF/TrkA interaction in neuronal development in vivo.

Release of nucleotides occurs in both neurons and glia and can modulate neurotransmission (Newman, 2003; Brockhaus et al., 2004) but little is known regarding the role of released nucleotides in the developing nervous system. Our data suggest that the enhanced NGF-mediated neuronal differentiation may represent a physiological role for extracellular nucleotide signaling, a proposal consistent with results we obtained from
inhibition of nucleotidases (Fig. 3-2). Autocrine/paracrine action of released nucleotides thus may modulate neurotrophin-promoted neuronal differentiation during development.Expression of mRNA for multiple P2Y receptors, including P2Y₂, has been detected in DRG neurons (Sanada et al., 2002); in situ hybridization demonstrates P2Y₂ mRNA in 77% of such neurons in vivo (Molliver et al., 2002). Furthermore, P2Y receptor transcripts are ubiquitously found in human central and peripheral nervous tissue samples (Moore et al., 2001); P2Y receptors thus are likely to be important physiologically as well as potentially involved in neurological injury and disease (Koles et al., 2005).

I hypothesize that released nucleotides function as neuronal morphogens, akin to the action of another extracellular acting nucleotide, cAMP, in Dictyostelium (Kim et al., 2002). If one were to equate nucleotide release as the molecular “gas pedal,” then the enzymes that hydrolyze these molecules would be the “brake.” Multiple ectonucleotidases have been identified on neurons and glia during development (Braun et al., 2003; Braun et al., 2004). The temporal and spatial abundance of nucleotidases have the potential to influence extracellular nucleotide signaling and, as suggested here, neurite formation and neuronal differentiation.

Multiple types of neurons and cells in the nervous system express P2Y receptors. While we assessed functions of the P2Y₂ receptor in vitro and in vivo, the roles of other P2 receptors in vivo cannot be discounted. PC12 cells express 6 of the 8 known P2Y receptors (Fig. 3-9B-E), results that confirm and extend previous findings (D'Ambrosi et al., 2001; Moskvina, 2002) (Fig. 2-11). Only P2Y (and not P2X) receptors respond to UTP, e.g., P2Y₂ (ATP=UTP) and P2Y₄ (UTP) (Burnstock, 2000). The enhancement in NGF-promoted neurite expression (Fig. 3-2B-D) by ATP and UTP together with the
siRNA and knockout data (Fig. 3-4, 3-3) implicates P2Y$_2$ receptors as a primary mediator of this effect. P2Y$_2$ receptors mediate excitation of DRG neurons, perhaps in the activity-dependent regulation of pain (Molliver et al., 2002). Interestingly, TrkA knock-out mice show extensive loss of DRG neurons and fail to respond to painful stimuli (Smeyne et al., 1994). Our data showing co-localization and co-immunoprecipitation of P2Y$_2$ and TrkA in DRG neurons provides the first evidence of physical interaction between these receptors, suggesting involvement of P2Y$_2$ and TrkA in neurite extension, neuropathic pain and nociception.

Nucleotide release occurs after injury, which via P2Y receptor activation may modulate axonal regeneration (Neary and Kang, 2005). Our study shows P2Y$_2$/TrkA receptor interaction in nociceptive (TrkA) neurons (Smeyne et al., 1994). Additional tyrosine kinase receptors, TrkB and TrkC, are expressed by motor and proprioceptive neurons (Snider, 1994), which may also crosstalk with P2Y$_2$ receptors. Future work will address these interactions and their roles in nerve injury and regeneration.

Pharmacological inhibition of P-TrkA (by K252a) abolished neurite formation and co-localization of P-TrkA with P2Y$_2$, while partial inhibition of P-ERK1/2 (by U0126) blunted the enhancement of neurite formation by ATP$_7$S without inhibiting ATP$_7$S-enhanced increased in P-TrkA in the presence of NGF. These results identify two independent activities of the P2Y$_2$ receptor for enhancing neurotrophin-mediated differentiation: 1) increase in level of activated neurotrophin receptor and 2) increased activation of ERK1/2. In undifferentiated PC12 cells, P2Y$_2$ receptors increase intracellular calcium levels (Arslan et al., 2000), which may activate PKC, Ras, and Raf, thereby activating ERK1/2 (Bouschet et al., 2003). Activation of Raf by P2Y$_2$ receptors
and TrkA could represent a convergence point for the two signaling pathways.

Our study provides the first in vivo evidence for involvement of GPCRs in neuronal differentiation. In previous work, PACAP (via its GPCR) and adenosine (via A$_{2A}$, also a GPCR) enhanced neuronal survival in vitro independent of NGF (Lee and Chao, 2001). Here we show that ATP$_{\gamma}$S (via P2Y$_2$ receptors) enhances NGF-dependent neuronal differentiation and neurite extension in vivo as well as in vitro. The interaction of P2Y$_2$ signaling on neurotrophin-mediated action identifies a mechanism for enhanced neuronal differentiation by extracellular nucleotides. Although our work emphasizes neuronal differentiation, selective P2Y$_2$ receptor agonists might have potential as pharmacological agents to aid in neuronal regeneration after injury or disease.
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Figure 3-1: ATPγS increases NGF-stimulated neurite formation via TrkA and ERK1/2 activation. PC12 cells were grown for 72h untreated (A), with 100μM ATPγS (B), 100ng/ml NGF (C), or ATPγS and NGF (D). Cell bodies were stained with Neurotrace (red) and nuclei with DAPI (blue). Immunoblot and densitometric quantitation of P-TrkA and TrkA receptor protein in PC12 cells treated with 100μM ATPγS, 100ng/ml NGF, or the combination with or without 10nM K252a for 10min (E). Immunoblot and corresponding densitometric analysis of PC12 cells treated as in (A-D) with or without U0126 for the indicated MAP kinases at the indicated timepoints (F).
Figure 3-2: P2Y agonists increase NGF-mediated neurite formation by increasing sensitivity to NGF. (A and B) PC12 cells were scored after 72h for fraction of neurite-bearing cells in the presence of 100ng/ml NGF and the indicated treatments. All nucleotides and adenosine were at a concentration of 100µM and apyrase was at 2u/ml. All nucleotides except ATPγS were applied every 24h. (C) PC12 cells were grown in the presence of 100ng/ml NGF alone, or with 10ng/ml NGF alone and/or together with increasing concentrations of ATPγS, ATP, or UTP. ATPγS. (D) PC12 cells were treated with 10ng/ml NGF alone or with the ecto-nucleotidase and 5’ nucleotidase (NT) inhibitors ARL67156 (50µM) and AMPCP (250µM). PC12 cells were treated with 10ng/ml NGF and ATPγS, ATP, or UTP in the presence of NT inhibitors.
**Figure 3-3:** Neurite growth, P2Y₂-TrkA co-localization and signaling in DRG neurons.

Dissociated DRG neurons from wt and $P2Y_2^{-/-}$ P1 mice grown for 24h with 50ng/ml NGF alone (A and C, respectively), or 100µM ATP₇S and 50ng/ml NGF (B and D, respectively) and labeled with SMI31 (blue), and antibodies for P-Trk (red) and P2Y₂ receptor (green) (A). Triple-localization appears white. Neurite length (E) and branch point (F) measurements. Immunoblot of wt and $P2Y_2^{-/-}$ DRG neurons treated as indicated for 10min (G). Bar = 30 µm.
Figure 3-4: siRNA reduction of P2Y₂ eliminates ATPγS-enhancement of NGF-induced neurite formation and signaling. QRT-PCR of PC12 cells independently treated with 2 non-overlapping siRNAs (seq.1 orange, seq.2 orange checkered) against the P2Y₂ receptor or a scrambled sequence (A). Immunoblot of receptor and signaling proteins in PC12 cells treated with P2Y₂ siRNA (seq.1/seq.2) or scrambled sequence (B). Fraction of PC12 cells expressing neurites treated with P2Y₂ siRNA (seq.1 solid, seq.2 checkered), K252a, U0126 for 72h or a scrambled sequence and the indicated treatments (C). (D) PC12 cells treated with 100µM ATPγS at the indicated NGF concentrations.
Figure 3-5: P-TrkA and P2Y₂ receptors co-immunoprecipitate upon NGF induction. Confocal immunofluorescence images of PC12 cells incubated with P-TrkA (red) and P2Y₂ (green) antibodies. The merged images show co-localization (yellow). PC12 cells untreated (A), treated with 100μM ATPγS (B), 100ng/ml NGF (C), or the combination (D) for 1h. (E) Transfected PC12 cells treated for 1h with indicated treatments, lysated, and immunoprecipitated. (F) Native PC12 cells treated for 1h with indicated treatments. K252a pretreated 15min prior to agonist treatment and cell lysates immunoprecipitated. (G) P1 rat DRG neurons treated for 1h with indicated treatments and cell lysates immunoprecipitated.
Figure 3-6: ATPγS increases GAP-43, NGF increases P2Y₂ levels in vivo. Untreated (A, C) or ATPγS-injected (B, D) sciatic nerves stained for SMI31 (blue) and GAP-43 (green). (E) Sciatic nerves probed for indicated protein markers. Densitometries calculated as fold increase over un-injected contra-lateral sciatic nerve from within each treatment group. Bar = 100 µm.
**Figure 3-7:** ATPγS increases GAP43 expression via ERK1/2 activation. Sciatic nerves from wild-type P1 rat pups were injected with ATPγS for either 12 or 24 hours. Sciatic nerves were immunostained for GAP43 (A) and axons were Bielschowski stained (B); images (right column, scale bar=230µm) and magnified insets (left column, scale bar=50µm) are shown. Immunoblots of DRG neurons treated with ATPγS for 24 hours +/- the ERK kinase inhibitor, U0126 (C).
**Figure 3-8:** Model of interaction of P2Y$_2$ with TrkA receptors. Activation of TrkA by NGF (P-TrkA), leads to ERK1/2 (P-ERK1/2) activation and up-regulation of the P2Y$_2$ receptor. UTP or ATP activates the P2Y$_2$ receptor, which increases P-TrkA levels in the presence of NGF, leading to increased neurite formation and growth.
Figure 3-9: P2Y2 receptors mediate increased NGF-stimulated neurite formation. (A) PC12 cells were grown for 72h with 100ng/ml NGF and 100µM ATPγS, ATP, or UTP. Cells were treated with 10µM suramin or 100µM PPADS where indicated. (B) Ethidium bromide gel of P2Y receptor RT-PCR products from untreated PC12 cells. 100 bp ladders are at the extreme right of each gel. (C) Real-time PCR measurements of mRNA levels, normalized to GAPDH levels and P2Y4 expression levels, in untreated PC12 cells. (D and E) Immunoblot of P2Y2, P2Y4, and GAPDH (loading control) protein levels in each of the 4 treatments as in D and the corresponding densitometric quantitation (fold over basal).
Chapter 4:

Interaction with Src is necessary for P2Y$_2$ and TrkA receptor-mediated enhancement in neuronal differentiation
Abstract

Neuronal differentiation and growth are crucial steps in the formation and regeneration of connections with target tissues. The complex signaling that initiates and modulates these processes are important potential targets for treatments of neurodegenerative diseases or other injuries. In these studies, I sought to define whether Src is a component of the signaling mechanism, enabling ATPγS/P2Y\textsubscript{2} receptors to enhance NGF/TrkA receptor-mediated neuronal differentiation and growth. Inhibition of Src blocked the ATPγS enhancement of NGF-promoted neuronal morphological differentiation and downstream signaling in PC12 cells. The enhancement by ATPγS of axonal growth and branching in DRG neurons was also abrogated by the inhibition of Src. In addition, Src co-immunoprecipitated with both TrkA and P2Y\textsubscript{2} receptors and inhibition of Src phosphorylation blocked the ability of TrkA and P2Y\textsubscript{2} receptors to co-immunoprecipitate. These results identify Src as a crucial mediator of nucleotide-enhanced neurotrophin-dependent neuronal differentiation and growth and a potential target for yet undiscovered molecular interactions between receptor tyrosine kinases and GPCRs.
**Introduction**

Neuronal differentiation and growth is initiated and modulated by an ever-expanding array of identified signals. Neurotrophins, and in particular nerve growth factor (NGF), are amongst the best characterized. Autocrine/paracrine NGF release prevents apoptosis and initiates neuronal differentiation of precursor cells, resulting in neurite extension and growth (Greene, 1976; Greene, 1978). NGF acts via its cognate receptor, TrkA, which transduces the NGF signal by activating a series of kinases, in particular ERK1/2 and Akt (Chao, 2003a). These signal transduction pathways, particularly with respect to activation of ERK1/2 and Akt, both stimulate differentiation and promote survival, respectively (Chao, 2003a).

Nucleotide signaling has emerged as both ubiquitously present and multifaceted in its effects across tissues (Burnstock, 2000). With respect to the nervous system, P2 (nucleotide) receptor signaling via signaling can modulate astrocytic calcium waves and synaptic transmission (Neary and Zhu, 1994; Lechner et al., 2004). Many of these effects are mediated through the ionotropic P2X receptor subclass of P2 receptors, but a second class of P2 receptor, metabotropic P2Y receptors, are also expressed in the nervous system and may contribute to neuronal function (Illes, 1996).

Previous work has demonstrated an interaction between the TrkA neurotrophin receptor tyrosine kinase (RTK) and the P2Y<sub>2</sub> nucleotide G-protein coupled receptor (GPCR) (Arthur et al., 2006). ATP<sub>γ</sub>S activation of P2Y<sub>2</sub> receptors leads to increased activated/phosphorylated TrkA (P-TrkA) receptors in the presence of NGF that results in enhanced neuronal differentiation and growth (Arthur et al., 2006). TrkA and P2Y<sub>2</sub> receptors were also found to immunoprecipitate, implicating a physical interaction in the
nucleotide enhancement of neurotrophin-stimulated signaling leading to neuronal differentiation (Arthur et al., 2006). Work presented in this chapter sought to identify a novel role of a well-known signaling kinase, Src, as a path-limiting component in the TrkA/P2Y_2 effects on neuronal differentiation. In addition, endogenous immunoprecipitation identifies Src as a physical component of the interaction between these TrkA and P2Y_2 receptors.

**Materials and methods**

**Reagents** ATPγS (Sigma, St. Louis, MO). Nerve Growth Factor (NGF) (Invitrogen, Carlsbad, CA). PP2, SU6656 (Calbiochem, San Diego, CA), U0126 and LY294002 (Cell Signaling, Beverly, MA).

**Cell isolation and culture** DRG neurons were dissected and trypsin dissociated from neonatal P1 C57/Black wt mice. Dissociated cultures were grown on laminin/poly-D-lysine/collagen coated plates for 96 h in Neurobasal A (Gibco, Carlsbad, CA) with B27 supplement (Gibco) and 5-Fluoro-2’-deoxyuridine (FUDR, Sigma). PC12 cells were grown as described by Taupenot et al. (Taupenot et al., 1999).

**Morphology** PC12 cells were plated at 60% confluency on collagen/poly-D-lysine coated plates and incubated with 30ng/ml NGF with other treatments for 72h. ATPγS, was added once for a 3-day interval at 100µM. PP2 (10µM) or SU6656 (5µM) was applied once. Images were taken every 24h for 3 days, using an Axiocam CCD camera on an Axiophot Zeiss microscope (Zeiss, Germany). The fraction of PC12 cells
expressing a neurite that was at least one cell body in length were counted. All conditions were assessed in triplicate.

**Immunoprecipitation** PC12 and DRG cells were treated with the indicated pharmacological/neurotrophic agents for 1h. Cell lysate was pre-cleared by with Protein G agarose beads (Roche, Indianapolis, IN) for 1h, incubated with the primary immunoprecipitating antibody for 3h, and Protein G agarose bead was added for another 3h. The beads were isolated, washed and the immunoprecipitated protein was used in immunoblot analysis.

**Immunofluorescence Analysis** Cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton and were incubated with P2Y$_2$ (Alomone), TrkA (Santa Cruz Biotech) and SMI31 (Sternberger Monoclonals) (1:100) antibodies overnight at 4°C. Cells were incubated with AMPA, FITC, or Cy3-conjugated antibody (1:250; Jackson Immunoresearch) for 2 h.

**Immunoblot Analysis** Protein samples, loaded at equal concentrations, were separated on 10% or 12% precast SDS polyacrylamide gels (Invitrogen) and then transferred to polyvinylidene difluoride membranes. Membranes were blocked in 20mM PBS, 1% Tween with 1.5% nonfat dry milk and then incubated with primary antibody at 4°C overnight. Antibodies used were: P-TrkA, P-ERK1/2, ERK1/2, P-Src, Src, (Cell Signaling), P2Y$_2$ (Alomone Labs, Jerusalem, Israel), TrkA (Santa Cruz Biotech, Santa Cruz, CA), GAPDH (Novus Biologicals, Littleton, CO). Secondary antibodies
conjugated to horseradish peroxidase (Cell Signaling) were visualized with ECL reagent (Amersham, Piscataway, NJ).

**Statistical analysis** All experiments were performed in triplicate. Data were analyzed by a paired t-test, one-way analysis of variance followed by Tukey’s multiple comparison test or linear regression. Significance was assigned to p<0.05.

**Results**

**Src inhibition blocks the ATPγS-mediated enhancement of NGF-promoted differentiation**

Previous work in our lab demonstrated a physical interaction between the TrkA and P2Y_2 receptors that enhances neuronal differentiation through enhanced TrkA signaling (Arthur et al., 2006). Here, I tested the hypothesis that Src is a factor mediating the interaction of TrkA with P2Y_2 receptors during neuronal differentiation. To test this hypothesis I sought to inhibit Src with 2 chemically distinct selective antagonists PP2 and SU6656 (Nagao et al., 1998; Blake et al., 2000). PC12 cells show enhanced NGF-promoted differentiation, as measured by the fraction of cells bearing a neurites, when co-treated with NGF and ATPγS (Fig. 2-2, 3-1, 4-1A). ATPγS, PP2, or SU6656 alone does not initiate neurite formation (Fig. 3-1 and data not shown). The enhancement of neurite formation by ATPγS in the presence of NGF was blocked by either PP2 or SU6656 (Fig. 4-1A).

**Src inhibition blocks ATPγS-mediated TrkA signaling enhancement**
Immunoblot analysis of key signaling molecules enhanced by ATPγS/ P2Y₂ in the TrkA pathway demonstrated a dependence upon Src. Src activation has been previously shown to increase, while Src inactivation was shown to decrease signaling molecules (e.g. TrkA and ERK1/2 activation) in the NGF pathways, a finding consistent with the reduced TrkA and ERK1/2 activation shown in Fig. 4-1B (Ohmichi et al., 1991; Ohmichi et al., 1992; Tsuruda et al., 2004; Tucker et al., 2005). NGF activation of TrkA (P-TrkA) was enhanced by ATPγS (2.3 fold); however, this enhancement was abolished by the Src inhibitors by PP2 or SU6656 (Fig. 4-1B). The NGF-mediated increase in P2Y₂ expression was also blocked PP2 and SU6656 (Fig. 4-1B). Verification of the action of PP2 and SU6656 was shown by its ability to block Src activation by NGF and ATPγS (Fig. 4-1B); ERK1/2 activation (P-ERK1/2) by ATPγS was blocked by PP2 and SU6656, but ERK1/2 activation by NGF was to a smaller extent reduced (40%, Fig. 4-1B). The prominent enhancement in P-ERK1/2 by NGF and ATPγS was also blunted by PP2 (Fig. 4-1B). These signaling results implicate Src as a key downstream signaling component in the enhancement of NGF-promoted neuronal differentiation by ATPγS.

**Inhibition of Src prevents ATPγS-enhanced axonal growth and branching in DRG neurons**

In order to test the role of Src, I assessed the growth of a primary cell, dorsal root ganglion (DRG) neurons. DRG neurons from post-natal day 1 mice (NGF required for survival, (Adler et al., 1984)) have enhanced axonal length and branching in response to ATPγS in the presence of NGF (Fig. 4-2, (Arthur et al., 2006)). Inhibition of Src via PP2
inhibited the ATPγS-mediated enhancement of both axonal length (Fig. 4-2B v. D, and E) and branching points (Fig. 4-2B v. D, and F).

**Src immunoprecipitates with TrkA and P2Y₂ and Src inhibition blocks TrkA/ P2Y₂ co-immunoprecipitation in DRG neurons**

Based on previous co-immunoprecipitation data demonstrating TrkA/P2Y₂ physical interaction (Arthur et al., 2006), I assessed the role of Src in this interaction. Studies with PC12 cells showed that inhibition of Src activation blocked the ATPγS-mediated increase in P-TrkA (Fig. 4-1B). Incubation of DRG neurons with PP2 blocked the co-immunoprecipitation of P2Y₂ and TrkA receptors by NGF and/or ATPγS, results in contrast with those obtained with untreated cells or cells treated with NGF and/or ATPγS without PP2 (Fig. 4-3). PP2 treatment alone blocked the immunoprecipitation of TrkA/P2Y₂ (Fig. 4-3). Lysates immunoprecipitated with a Src antibody and probed for TrkA or P2Y₂ demonstrated a physical interaction between these two receptors and Src, an interaction that was also blocked by PP2 (Fig. 4-3). Together, these data implicate Src as interacting with both TrkA and P2Y₂ receptors.

**Discussion**

The experiments presented here identify Src as a physical intermediate in the interaction between TrkA and P2Y₂ receptors as well as a signal-limiting kinase necessary for the ATPγS-enhanced NGF-promoted neuronal differentiation. Inhibition of Src activation blocked ATPγS-enhanced neurite formation and signal transduction via an inhibition of the physical interaction of TrkA and P2Y₂ in PC12 cells and DRG
neurons (Fig. 4-1, 4-2, 4-3). This work defines a role for Src in mediating the physical interaction and enhanced signaling of a RTK with a GPCR.

Previous data identified an interaction between TrkA and Src (Wooten et al., 2001), and enhanced TrkA and MAP kinase activation downstream of NGF signaling (Tsuruda et al., 2004). Src-mediated enhancement of neurotrophin signaling likely represents a link between neurotrophin signaling and other signal transduction pathways regulated by Src activation.

P2Y_2 receptors were characterized initially by agonist profiles demonstrating these receptors as the only P2Y subtype to have equal affinity for both ATP and UTP (von Kugelgen and Wetter, 2000). P2Y_2 receptors couple to G_q/11 proteins, activating phospholipase C and increasing intracellular Ca^{2+} and PKC (Burnstock, 2000; von Kugelgen and Wetter, 2000). This well-characterized pathway was not initially thought to include activation of Src or an interaction with RTKs. However, recent studies have shown that a Src homology domain within the P2Y_2 receptor may mediate interaction with Src (Liu et al., 2004). Furthermore, activation of P2Y_2 by UTP increased ERK1/2 activation and co-localization of the P2Y_2 receptor with the epidermal growth factor receptor (EGFR), itself an RTK; inhibition of Src abolished both of these effects (Liu et al., 2004). The interaction of the P2Y_2 receptor with a growth factor RTK receptor that is dependent upon Src is akin to our results demonstrating Src mediated interactions between P2Y_2 receptors and a neurotrophin RTK receptor.

The interaction of extracellular nucleotide signaling to enhance neurotrophin signaling and neuronal differentiation has been further elucidated with the identification of a key components in this pathway. The contribution of Src as a mediator between
different classes of receptors signaling through classical and non-classical pathways is an emerging idea. Src-mediated interactions between trophic factor signaling and the modulation of signaling events that alter neuronal physiology are thus targets for enhancing neuronal growth after injury or disease for the regeneration of central and peripheral neuronal connections.
Figure 4-1: Src inhibition prevents ATPγS-mediated enhancement of NGF-promoted neuronal differentiation in PC12 cells. PC12 cells were treated for 72 h with 30ng/ml NGF, 100µM ATPγS, 10µM PP2, 5µM SU6656 (as indicated). The fraction of cells neurite-bearing cells was scored (A). Immunoblots of PC12 cells treated for 1 h (except for P2Y2, 24 h) with NGF, ATPγS, PP2, SU6656 (as indicated) (B). *p<0.05 v. NGF-treated alone.
Figure 4-2: Src inhibition blocks ATPγS-mediated enhancement of axonal growth in DRG neurons. DRG neurons cultured for 24 h with NGF and ATPγS, PP2, or the combination were stained for P2Y₂ (green), TrkA (red), and SMI31 (blue). Triple localization appears white (A-D). Scale bar=30µM. DRG neuron axon length was measured (E) and branching points counted (F) for the conditions in A-D. *p<0.05, ***p<0.001 v. NGF-treated alone.
Figure 4-3: Src co-immunoprecipitates with TrkA and P2Y$_2$. DRG neurons treated for 1 h with NGF, ATP$_{i}$S, and PP2 (where indicated) were lysed and co-immunoprecipitated with P2Y$_2$, TrkA, Src and probed for the P2Y$_2$/TrkA, and Src where indicated.
Chapter 5:

Inhibition of apoptosis by P2Y$_2$ receptor activation: novel pathways for neuronal survival
Abstract

Cell survival is an essential function in the development and maintenance of the nervous system. We demonstrate here a previously unappreciated role for extracellular nucleotide signaling through the P2Y₂ receptor in the survival of neurons: PC12 cells and dorsal root ganglion (DRG) neurons are protected from serum-starvation-induced apoptosis by ATP, UTP, and ATPγS, an effect mediated via P2Y₂ receptors, as demonstrated by siRNA and genetic knockout models. This protection occurs independently of neurotrophin signaling but requires Src activation of ERK and Akt. Moreover, ATPγS and NGF act synergistically to enhance neuronal survival through enhanced TrkA signaling. The results, which define a novel mechanism for inhibition of apoptosis, implicate parallel, interacting systems--extracellular nucleotides/P2Y₂ receptors and neurotrophin/TrkA--to sustain neuronal survival.
Introduction

The regulation of cell development and organismal growth is a highly regulated process that involves maintaining a balance between proliferation and apoptosis (Duque-Parra, 2005). A large body of data document that both intrinsic and extrinsic apoptotic pathways result in distinct morphological and biochemical cellular changes (Saunders, 1966; Twomey and McCarthy, 2005). Injury, oxidative stress, and reduced extracellular levels of trophic factors are examples of inciting stimuli that can result in cells initiating apoptotic pathways (Twomey and McCarthy, 2005).

The maintenance of cell survival is crucial component of neuronal function. Survival of neurons, in particular the inhibition of apoptosis, is dependent upon the presence of trophic and non-trophic factors to maintain function (Akassoglou, 2005; Shaw, 2005). Nerve growth factor (NGF) is one of the best-studied examples of an extracellular stimulant that regulates neuronal survival by anti-apoptotic effects. NGF acts via its cognate receptor, TrkA, with subsequent activation of ERK1/2 and Akt kinases that inhibit apoptotic signaling in neurons (Greene, 1978; Chao, 2003b).

Extracellular nucleotide signaling via nucleotide (P2) receptors is a mechanism that may regulate apoptosis (Burnstock, 2000). P2 receptors, which are comprised of P2X (ionotropic) and P2Y (metabotropic, G-protein-coupled) subtypes, respond to a variety of nucleotide agonists. The role of P2 receptors, particularly P2X receptors, in apoptosis has been demonstrated in both non-neuronal and neuronal cells, including most prominently the P2X7 receptor in initiating glial cell apoptosis (Franke et al., 2004; Sim et al., 2004; Coutinho-Silva et al., 2005). By contrast, the role of P2Y receptors in neuronal apoptosis remains largely unexplored.
In this study, we tested the hypothesis that extracellular nucleotides, signaling through P2Y₂ receptors, modulate neuronal apoptosis. Using a series of complementary approaches, we demonstrate a role for P2Y₂-mediated inhibition of neuronal apoptosis through signaling pathways that are both neurotrophin-dependent and –independent, resulting in enhanced survival in response to trophic factor withdrawal.

Materials and methods

Reagents ATPγS, ATP, UTP (Sigma, St. Louis, MO). Nerve Growth Factor (NGF) (Invitrogen, Carlsbad, CA). K252a, PP2, Ro-31-8220, PD98059, SU6656, U71322, BAPTA-AM, Raf kinase inhibitor (Calbiochem, San Diego, CA), U0126 and LY294002 (Cell Signaling, Beverly, MA).

Cell isolation and culture DRG neurons were dissected and trypsin dissociated from adult wt or P2Y₂⁻/⁻ mice (Cressman et al., 1999) (gift of Dr. Beverly Koller, UNC). Dissociated cultures were grown on laminin/poly-D-lysine/collagen coated plates for 96 h in Neurobasal A (Gibco, Carlsbad, CA) with B27 supplement (Gibco) and 5-Fluoro-2’-deoxyuridine (FUDR, Sigma). PC12 cells were grown as described by Taupenot et al. (Taupenot et al., 1999).

PC12 cell Transfection PC12 cells were transfected with pre-designed siRNA (Ambion, Austin TX; ID# 50110, 143692) for P2Y₂ with Lipofectamine2000 (Invitrogen) according to the manufacturer’s instructions.
**Apoptosis** PC12 cells and DRG neurons were incubated in serum-free media for 12 h with ATPγS (10µM), ATP (100µM), UTP (100µM), NGF (10ng/ml), K252a (10nM), PP2 (10µM), Raf kinase inhibitor (50nM), SU6656 (5µM), U0126 (10µM), LY294002 (10µM) (unless otherwise noted) where indicated. Cells were lysed and were ELISA assayed for DNA fragmentation (Roche, Indianapolis, IN), Caspase 3 (Roche) and membranes inversion (APOPercentage, Biocolor Ltd., Newtonabbey, Northern Ireland). All conditions were assessed in triplicate.

**Immunoblot Analysis** Protein samples, loaded at equal concentrations, were separated on 10% or 12% precast SDS polyacrylamide gels (Invitrogen) and then transferred to polyvinylidene difluoride membranes. Membranes were blocked in 20mM PBS, 1% Tween with 1.5% nonfat dry milk and then incubated with primary antibody at 4°C overnight. Antibodies used were: P-TrkA, P-ERK1/2, ERK1/2, P-Src, Src, P-B-Raf, P-Akt, Akt (Cell Signaling), P2Y<sub>2</sub> (Alomone Labs, Jerusalem, Israel), actin, TrkA (Santa Cruz Biotech, Santa Cruz, CA), GAPDH (Novus Biologicals, Littleton, CO), B-Raf (Abcam, Cambridge MA). Secondary antibodies conjugated to horseradish peroxidase (Cell Signaling) were visualized with ECL reagent (Amersham, Piscataway, NJ).

**Statistical analysis** All experiments were conducted in triplicate. Data were analyzed by a paired t-test, one-way analysis of variance followed by Tukey’s multiple comparison test or linear regression. Significance was assigned to p<0.05.
Results

P2Y₂ activation inhibits apoptosis of PC12 cells

Removal of serum from the media in which PC12 cells are grown (i.e., serum-starvation for 12 h) significantly (p<0.001) increases DNA fragmentation, a result indicative of apoptosis (Fig 5-1A, (Batistatou and Greene, 1993)). NGF treatment prevents apoptosis produced in this manner (Fig. 5-1A). Because of our recent findings demonstrating interaction between nucleotide/P2Y₂ and NGF/TrkA signaling in enhancing neuronal differentiation and growth (Arthur et al., 2006), we tested whether nucleotide/P2Y₂ receptor activation might also promote neuronal survival. In initial studies, we treated cells with ATP, UTP or ATPγS, all agonists of P2Y₂ receptors (Burnstock, 2000)) and found that all 3 agonists reduced serum starvation induced DNA fragmentation, ATPγS more significantly (p<0.05) ATP or UTP (Fig. 5-1A and 5-2A).

As a more direct test of the role of P2Y₂ receptors, we transfected PC12 cells with each of 2 non-overlapping siRNA sequences directed against P2Y₂ (Arthur et al., 2006). Incubation with NGF reduced the level of DNA fragmentation in both scrambled and P2Y₂ siRNA (sequence 1)-treated cells; ATPγS only significantly (p<0.001) reduced serum-starvation induced DNA fragmentation in control (scrambled), but not P2Y₂ siRNA- treated, cells (Fig. 5-1A). These results implicate the P2Y₂ receptor as crucial in the protection by ATPγS from apoptosis. Similar results were obtained using P2Y₂ siRNA sequence 2 (data not shown). In addition to the effect on DNA fragmentation, ATPγS inhibited caspase 3 activation and membrane inversion, both measures of apoptosis (Fernandes-Alnemri et al., 1994; Martin et al., 1995; Zhou et al., 1997); these
responses were also independent of NGF and reversed by reduction of P2Y\textsubscript{2} receptor protein by the P2Y\textsubscript{2} siRNA (Fig. 5-1C-D).

**Inhibition of apoptosis of PC12 cells by P2Y\textsubscript{2} activation is independent of NGF/TrkA**

K252a, an inhibitor of TrkA phosphorylation (P-TrkA) (MacInnis et al., 2003), reversed the NGF-mediated, but not the ATP\textsubscript{γ}S-mediated, inhibition of DNA fragmentation (Fig. 5-1A). Immunoblotting of serum-starved PC12 cells showed activation of TrkA (P-TrkA) with NGF treatment (blocked by K252a), but not with ATP\textsubscript{γ}S (Fig. 5-1B). P2Y\textsubscript{2} siRNA-treated PC12 cells showed similar levels of P-TrkA with NGF and lack of activation with ATP\textsubscript{γ}S (data not shown).

**P2Y\textsubscript{2} and TrkA synergistically inhibit apoptosis**

Based on previous findings showing ATP\textsubscript{γ}S-enhanced P-TrkA formation in the presence of NGF (Arthur et al., 2006), we tested the potential interaction of nucleotide and neurotrophin signaling in the inhibition of apoptosis. Lower concentrations of NGF (3ng/ml) or ATP\textsubscript{γ}S (1μM) individually did not prevent DNA fragmentation in serum-starved PC12 cells (Fig. 5-2A) but the combination of these sub-optimal concentrations resulted in full inhibition of DNA fragmentation (Fig. 5-2A). Immunoblot analysis of serum-starved PC12 cells treated with the combination of lower concentrations of NGF and ATP\textsubscript{γ}S revealed that these cells expressed more P-TrkA than cells treated with a low concentration of NGF alone (Fig. 5-2B), implying that enhancement in TrkA signaling by
ATPγS contributes to the promotion of survival by the synergistic combination of ATPγS and NGF.

**P2Y<sub>2</sub> activation inhibits apoptosis via both ERK and Akt**

Agonists of P2Y<sub>2</sub> receptors in PC12 cells are able to activate ERK1/2 (P-ERK1/2; (D'Ambrosi et al., 2001; Arthur et al., 2006), a kinase that inhibits apoptosis (Xia et al., 1995). We thus tested the role of ERK1/2 in inhibition of apoptosis by activation of P2Y<sub>2</sub> receptors and found that PC12 cells serum-starved in the presence of ATPγS and the ERK1/2 inhibitor, U0126 (Xie et al., 2000), had similar levels of DNA fragmentation as did cells incubated with ATPγS alone (Fig. 5-3A). Serum-starved PC12 cells incubated with LY294002, an inhibitor of Akt activation (P-Akt formation), another kinase that can inhibit apoptosis (Crowder and Freeman, 1998), also showed no difference in the ability of ATPγS to reduce DNA fragmentation (Fig. 5-3A). However, combined inhibition of ERK1/2 and Akt by U0126 and LY294002 blocked ATPγS-mediated inhibition of serum-starvation induced DNA fragmentation (Fig. 5-3A).

Treatment of serum-starved PC12 cells with either ATPγS or NGF activated both Akt and ERK1/2 (Fig. 5-3B) and cells treated with low concentrations of NGF and ATPγS had a synergistic enhancement in P-Akt and P-ERK1/2 formation, consistent with the impact of the two classes of agonists on DNA fragmentation and P-TrkA expression (Cf. Fig. 5-2 and Fig. 5-3B). ATPγS treatment of serum-starved PC12 cells increased expression of both P-Akt and P-ERK1/2, responses that could be blocked by inhibition of the kinases by LY294002 or U0126, respectively, (Fig. 5-3C). Inhibition of both kinases blocked formation of both phosphorylated species, implying that both of these signaling
molecules function to prevent apoptosis (Fig. 5-3A) in response to activation of P2Y<sub>2</sub> receptors.

**ERK and Akt activation by P2Y<sub>2</sub> requires Src**

In order to further elucidate components involved in the inhibition of apoptosis via P2Y<sub>2</sub> activation, we assayed several additional signaling molecules that might contribute to the downstream responses. Inhibitors of PLC (U71322 (Nussenzveig et al., 1993)), PKC (Ro-31-8220 (Bacon and Camp, 1990)), and intracellular calcium (BAPTA-AM) had no effect on the ATPγS-mediated inhibition of serum-starvation induced apoptosis (data not shown). By contrast, we found that Src appears to play an important regulatory role in the signal transduction pathway that mediates this inhibition. PP2, an inhibitor of Src phosphorylation (Nagao et al., 1998), reversed the ATPγS/P2Y<sub>2</sub>-mediated inhibition of DNA fragmentation in both PC12 cells and dorsal root ganglion (DRG) neurons, a peripheral nerve cell known to express P2Y<sub>2</sub> receptors (Sanada et al., 2002; Arthur et al., 2006) (Fig. 5-4A). Another Src inhibitor, SU6656 (Blake et al., 2000), produced similar results (data not shown). Immunoblot analysis of serum-starved PC12 cells treated with ATPγS showed activated Src (P-Src) expression while inhibition of ATPγS-mediated P-Src formation by PP2 abolished P-Akt and P-ERK1/2 formation (Fig. 5-4B). Together, these results indicate that Src activation is a necessary step in the ATPγS/P2Y<sub>2</sub>-receptor-mediated inhibition of neuronal apoptosis.
ERK activation by P2Y2 requires B-Raf

Based on previous data showing that activation of ERK1/2 by TrkA occurs via B-Raf (Chao, 2003b), we tested the potential role of B-Raf in the regulation of ERK1/2 activation in the ATPγS/P2Y2 receptor-mediated inhibition of neuronal apoptosis. Inhibition of Raf kinase (by Calbiochem, cat. No. 553008) in ATPγS-treated serum-starved PC12 cells and DRG neurons did not alter the reduction in apoptotic DNA fragmentation (Fig. 5-4C) but decreased P-ERK1/2 formation by ATPγS in serum-starved PC12 cells (Fig. 5-4D). However, simultaneous inhibition of Raf and Akt activation (by LY294002) blocked formation of P-ERK1/2 and P-Akt (Fig. 5-4D) and inhibited DNA fragmentation ascribed to ATPγS activation of P2Y2 receptors (Fig. 5-4C). These results place Raf activation upstream of ERK1/2 activation, but not Src or Akt activation.

P2Y2 activation in DRG neurons inhibits apoptosis independent of TrkA

Because no specific antagonists exist for the P2Y2 receptor (Burnstock, 2000), we used two alternative approaches, siRNA (Fig. 1) and genetic knock-outs (KO) (Fig. 5-5) to evaluate the role of these receptors in the inhibition of apoptosis by extracellular nucleotides. Using P2Y2−/− mice, we assessed adult DRG neurons, which do not require NGF for survival (Lindsay, 1988). Serum-starvation of DRG neurons for 12 h in the absence of presence of NGF or ATPγS revealed that ATPγS inhibited DNA fragmentation and caspase 3 activation to levels similar to those produced by treatment with NGF (Fig. 5-5A-B). By contrast, DRG neurons derived from P2Y2−/− mice responded to NGF but, did not demonstrate inhibition by ATPγS of serum-starvation induced apoptotic DNA fragmentation or caspase 3 activation (Fig. 5-5A-B).
Inhibition of TrkA activation by K252a reversed NGF-promoted inhibition of DNA fragmentation in response to serum starvation in both wt and $P2Y_2^{-/-}$ DRG neurons (Cf. Fig. 5-5A and 5-5C) and blocked P-TrkA formation in both wt and $P2Y_2^{-/-}$ DRG neurons (Fig. 5-5D). Immunoblotting demonstrated that ATPγS did not activate TrkA in either wt or $P2Y_2^{-/-}$ DRG neurons (Fig. 5-5D), confirming results obtained with PC12 cells (Cf. Fig. 5-5D and 5-1B, D) and provides further evidence for the role of P2Y$_2$ receptors as mediating extracellular nucleotide inhibition of apoptosis in a TrkA-independent manner.

Discussion

The ability of ATPγS, ATP, or UTP to inhibit neuronal apoptosis through the P2Y$_2$ receptor, as demonstrated by both siRNA and genetic knock out mice, represents a previously unidentified receptor target for the modulation of programmed cell death in the nervous system. Our data also reveal key elements in the signal transduction pathway that mediate this anti-apoptotic effect (Fig. 5-6): P2Y$_2$ receptor activation leads to Src activation/phosphorylation, which, in turn, activates B-Raf and PI3 kinase. These events lead to activation of ERK1/2 and Akt, respectively. Activation of ERK1/2 and Akt inhibits apoptosis by suppression of molecules such as JNK, p38, and various caspases (Berra et al., 1998; Shimoke et al., 1999; Horn et al., 2005). In addition to its direct activation of anti-apoptotic events, agonist stimulation of P2Y$_2$ receptors can indirectly inhibit apoptosis by potentiating NGF-promoted activation of TrkA, leading to enhanced ERK1/2 and Akt activation. It is conceivable that Src activation contributes to the
enhanced P-TrkA formation by P2Y₂ receptor agonists; future experiments are required to explore this possibility.

P2Y, unlike P2X receptors, respond to UTP; P2Y₂ are the only P2Y receptors that respond to ATP and UTP with similar affinity (Burnstock, 2000). P2Y₂ receptors couple to G_{q/11} proteins which activate phospholipase C, leading to formation of inositol-1,4,5-phosphate that increases levels of intracellular Ca^{2+} and diacylglycerol, which activates protein kinase C (Gonzalez et al., 2005). Activation of ERK1/2 by P2Y₂ receptors has been shown to occur through elevated intracellular Ca^{2+} and PKC activation (Soltoff et al., 1998), while activation of Akt by P2Y₂ receptors has been demonstrated in renal mesangial cells (Huwiler et al., 2002), but not previously in neuronal cells.

The studies here thus present a novel pathway for P2Y₂ signaling and regulation of neuronal apoptosis by both neurotrophin-dependent and neurotrophin-independent mechanisms. Inhibition of “classical” components of P2Y₂ G-protein signal transduction (i.e. Ca^{2+} and PKC) did not affect the inhibition of apoptosis, as measured by DNA fragmentation, caspase 3 activation, and membrane inversion (data not shown). G-protein-coupled receptors, such as P2Y₂ receptors, are capable of transducing signals independent of G-proteins, particularly with respect to modulation of signals involved in neurotransmission (Heuss and Gerber, 2000; Pierce et al., 2002). Signal transduction by GPCRs independent of G-proteins can occur through molecules such as beta-arrestins, which scaffold and regulate kinases such as JNK, p38, Src, and ERK1/2 (Luttrell and Luttrell, 2004; Lefkowitz and Shenoy, 2005; Shenoy et al., 2005). Such non-traditional (i.e., G-protein-independent) signal transduction pathways may be involved in the ability of P2Y₂ receptors to activate Src, ERK, and Akt leading to the inhibition of apoptosis.
A possible mechanism for the linkage to Src may involve a unique property of the P2Y2 receptor itself: association with Src via a Src homology 3 (SH3) binding domain located in the C-terminal region of the P2Y2 receptor; mutations to this domain alter signaling and receptor association with tyrosine kinases (Zhang et al., 2001; Liu et al., 2004; Gonzalez et al., 2005; Weisman et al., 2005). Since Src is able to activate ERK1/2 (via Raf (Troppmair et al., 1994)) and Akt in PC12 cells and DRG neurons (Fig. 5-3, 5-4) as well as in other cell types (Zachary, 2003; Mehdi et al., 2005), activation of Src by P2Y2 receptors may provide a mechanism that contributes to the maintenance of neuronal survival.

Neurotrophins such as NGF are well-established inhibitors of neuronal apoptosis, but the current data suggest that ATP and UTP acting via P2Y2 receptors represents another physiologically relevant system for the regulation of survival of neurons. It is of interest that adenosine, a metabolic product of ATP hydrolysis, is able to inhibit neuronal apoptosis in a TrkA-dependent manner by activation of the A2A P1 receptors (Lee and Chao, 2001; Wakade et al., 2001; Lee et al., 2002a). Our results define a TrkA-independent mechanism for inhibition of apoptosis that does not require ATP hydrolysis. P2Y receptor transcripts are widely expressed in central and peripheral nervous tissue samples and P2Y2 receptors have been shown to play an important role in neuronal differentiation (Moore et al., 2001; Franke and Illes, 2005; Arthur et al., 2006). We propose that release of nucleotides from glia, neurons or perhaps other cell types (e.g. vascular elements) (Lazarowski and Boucher, 2001; Hansson and Ronnback, 2003; Newman, 2003; Brockhaus et al., 2004; Wang et al., 2005) may serve as autocrine-paracrine sources of extracellular nucleotides that promote survival, either acting alone or
through potentiation of neurotrophin signaling. As such, ATP (and perhaps UTP) may serve key extracellular regulators of neuronal development that protect developing neurons from pro-apoptotic stimuli. Moreover, since neurotrophin signaling and innervation of peripheral target tissues declines with age (Gavazzi and Cowen, 1996; Santer et al., 2002), based on the current findings, drugs that activate P2Y2 receptors would appear to have potential to prevent this age-related decline, as well as apoptosis triggered by disease or injury.

The current findings define a previously unappreciated aspect of function of nucleotides/P2Y2 receptors in the nervous system in addition to enhancement of neuronal differentiation by these receptors (Arthur et al., 2006). Together with the latter results and recent evidence obtained with P2Y2−/− mice indicating that P2Y2 receptors are critically involved in allodynia and processing of pain stimuli (Davis et al., 2005), the data described herein identify extracellular nucleotides and their activation of P2Y2 receptors as a physiologically important system for regulation of development, survival and function of neurons.
Figure 5-1: ATPγS inhibits serum starvation-induced PC12 apoptosis via P2Y₂ receptors independent of NGF/TrkA signaling. PC12 cells were transfected with P2Y₂ siRNA, then grown for 12 h in the presence or absence of serum, ATPγS (10µM), NGF (10ng/ml), and/or K252a (10nM), and analyzed for apoptosis by quantitation of DNA fragmentation (A). P2Y₂ receptor expression in cells transfected with P2Y₂ siRNA decreased by >70% vs. cells transfected with a scrambled siRNA sequence. Treatment with the P2Y₂-targeted siRNA’s failed to alter serum starvation-induced apoptosis. Immunoblot of PC12 cells treated as in (A) and probed for TrkA activation (B). PC12 cells transfected with P2Y₂ siRNA, serum-starved for 12 h with the indicated treatments were analyzed for apoptosis by Caspase 3 activation/expression (C) and an assay for plasma membrane inversion (D). (*** p<0.001 versus serum-starvation alone, values normalized to serum-starvation alone)
**Figure 5-2:** ATPγS/NGF interact to enhance inhibition of apoptosis. Serum-starved PC12 cells were treated with ATP (100µM), UTP (100µM), NGF, and/or ATPγS at the indicated concentrations. Apoptosis was quantitated using DNA fragmentation (A). Immunoblot analysis of cells treated with NGF (10 or 3ng/ml), ATPγS (10 or 1µM) or 3ng/ml NGF together with 1µM ATPγS and probed for TrkA activation (B). (*p<0.05, **p<0.01, *** p<0.001 versus serum-starvation alone, values normalized to serum-starvation alone)
Figure 5-3: ATPγS inhibits apoptosis via activation of ERK1/2 and Akt. Serum-starved PC12 cells were treated with U0126 (10µM) or LY2942002 (10µM) and the indicated treatments were analyzed for apoptosis by quantitation of DNA fragmentation (A). Immunoblot of serum-starved PC12 cells left untreated, treated with 10ng/ml NGF, 10µM ATPγS, or as indicated and probed for activated Akt and ERK1/2 (B). Immunoblot of serum-starved PC12 cells treated as in (A) and probed for Akt and ERK1/2 activation (C). (*** p<0.001 versus serum starvation alone, values normalized to serum-starvation alone)
**Figure 5-4:** ATPγS inhibits apoptosis via Src activation of Akt and B-Raf-mediated ERK1/2 activation. Serum-starved PC12 cells and DRG neurons were treated with 10µM PP2 and 10µM ATPγS. Cells were analyzed for apoptosis by quantitation of DNA fragmentation (A). Immunoblot of PC12 cells treated as in (A) and probed for Src, Akt, and ERK activation (B). Serum-starved PC12 cells and DRG neurons were treated with a Raf kinase inhibitor (50nM) and/or an Akt inhibitor (LY294002, 10µM). Cells were analyzed for apoptosis by quantitation of DNA fragmentation (C). Immunoblot of PC12 cells treated as in (C) and probed for B-Raf, Akt, and ERK activation (D). (*** p<0.001 versus serum-starvation alone, values normalized to serum-starvation alone)
**Figure 5-5:** ATPγS inhibits serum starvation-induced DRG apoptosis via P2Y2 independent of NGF/TrkA signaling. DRG neurons from wt and P2Y2-/− mice were serum-deprived for 12 h alone, with 10ng/ml NGF, or with 10µM ATPγS. Apoptosis was quantitated by DNA fragmentation (A) or caspase 3 expression (B). DRG neurons from wt and P2Y2-/− mice were serum-starved for 12 h in the presence of the TrkA inhibitor K252a (10nM) and the indicated treatments (C.f. (A)). Cells were lysed and analyzed for apoptosis by DNA fragmentation (C). Immunoblot analysis of DRG neurons treated as in (C) and probed for TrkA activation (D). (** p<0.001 versus serum starvation alone, values normalized to serum-starvation alone)
Figure 5-6: Model of P2Y₂-mediated inhibition of neuronal apoptosis. Agonists (e.g. ATP, ATPγS, or UTP) activate P2Y₂ receptors leading to Src activation/phosphorylation (+P). Src activates/phosphorylates B-Raf and PI3K leading to ERK1/2 and Akt activation/phosphorylation, respectively. Activation of ERK1/2 and Akt inhibits apoptosis. Activation of P2Y₂ receptors in the presence of NGF increases TrkA activation/phosphorylation, thereby increasing the activation of ERK1/2 and Akt, resulting in inhibition of apoptosis via a NGF-dependent pathway.
Chapter 6:

Changes in nucleotide-stimulated norepinephrine release in response to neuronal differentiation by nerve growth factor
Abstract

Modulation of synaptic transmission is an important regulatory mechanism involved in the control of neuronal communication. Extracellular nucleotides are modulators of neurotransmitter release. In the current study, I examined the ability of nucleotides the release and reuptake of norepinephrine (NE) from undifferentiated and NGF-differentiated PC12 cells. The non-hydrolyzable ATP analog, ATPγS, caused greater release of NE from NGF-differentiated PC12 cells compared to undifferentiated PC12 cells. NGF-differentiated cells were more sensitive to ATPγS-mediated NE release. In addition, NGF-promoted differentiation was associated with a decrease in NE uptake. These results indicate a functional change in P2 receptor activity in the regulation of neurotransmitter release as a result of neuronal differentiation.
Introduction

Extracellular nucleotides act as signaling molecules and modulators of a variety of biological functions. In the nervous system, extracellular nucleotides, signaling through P2 receptors, cause calcium wave propagation in glial cells and alter neurotransmission across synapses (Burnstock, 2000). Extracellular nucleotide receptors are present in cerebellar, cortical, and hippocampal neurons (Illes and Ribeiro, 2004), as well as postganglionic sympathetic neurons (Dunn et al., 2001). The multifaceted roles of extracellular nucleotides in neuronal transmission appear to depend not only on the cell type in which they are expressed, but also the P2 subtype(s) present in each cell. These differences can lead to altered cellular responses and changes in the release of specific neurotransmitters.

Catecholamines are involved in a variety of central and peripheral nervous system functions. For example, NE acting via α- and β-adrenergic receptors regulate autonomic responses in discrete nuclei of the brain and multiple functions in the cardiovascular, pulmonary, and endocrine systems functions in the periphery (Cooper et al., 2003). NE is co-stored and co-released with ATP in both chromogranin cells and sympathetic neurons (Westfall et al., 1978; Mahata, 1999), both of which derive from neural crest precursor cells (Lee et al., 1977). ATP is a potent stimulator of NE release in both of these cell types (Rhoads et al., 1993). The release of NE by chromaffin and neuronal cell types regulates post-synaptic target cell activation, and thus differences in the regulation of NE release can identify cellular changes that accompany neuronal differentiation and function.
In this study, I characterized the release of catecholamines by nucleotides in order to assess potential changes in P2 receptor mediated action that occur as a result of neuronal differentiation. I identified differences in NE release between undifferentiated and NGF-differentiated (i.e. sympathetic neuronal-like) PC12 cells, thus providing evidence for a functional response for a plasticity in the role of P2 receptors during neuronal differentiation.

**Methods and Materials**

**Reagents** ATP, ATPγS, UTP, poly-D-lysine, were obtained from Sigma (St. Louis, MO). Nerve Growth Factor (NGF) was obtained from Invitrogen (Carlsbad, CA).

**Cell culture conditions** PC12 cells (gift of David Schubert, Salk Institute as described in (Taupenot et al., 1999)) were grown in PC12-specific media: Dulbecco’s Modified Eagles Medium, high glucose with L-glutamine, 5% fetal bovine serum (Omega Scientific, Inc., Tarzana, CA), 10% heat-inactivated horse serum (Omega), and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). Cells were grown to 60-80% confluency in 5% CO₂ at 37°C in Falcon T75 filter flasks. Cells were split 1:2 every 48-72h up to 18 passages.

**Norepinephrine Release** PC12 cells (+/-NGF 72h) were incubated in [H³] NE for 2h at 37°C in DMEM containing 10% heat-inactivated horse serum, 5% fetal bovine serum and 1% penicillin/streptomycin. Cells were washed 2x with Ca²⁺ secretion buffer, incubated in serum-free media for 1h, and again washed with Ca²⁺ secretion buffer. Following a 20
min pre-incubation and removal of media (Incubation I), a second 20min incubation (Incubation II) occurred with the indicated treatments. The buffer from incubation II was collected separately along with whole cell lysates in both of which I measured [H\(^3\)] NE by scintillation counting with corrections for background radiation.

Statistical analysis  All experiments were conducted in triplicate. Data were subjected to a one-way analysis of variance, followed by Tukey’s multiple comparison test, or linear regression. Significance was assigned to p<0.05.

Results

Nucleotide-stimulated NE release in undifferentiated PC12 cells

PC12 cells release NE when stimulated with ATP (Rhoads et al., 1993). In order to prevent effects that might be caused by degradation products of ATP, I used a non-hydrolysable ATP analog, ATP\(\gamma\)S to stimulate [H\(^3\)] NE release. ATP\(\gamma\)S caused a greater release than ATP, while UTP (which acts on P2Y\(_2\) and P2Y\(_4\) receptors that are expressed in PC12 cells, see (Burnstock, 2000) and Fig. 2-11) did not cause release of NE (Fig. 6-1). Pre-incubation of undifferentiated PC12 cells in ATP\(\gamma\)S caused a desensitization of subsequent ATP\(\gamma\)S-stimulated NE release (Fig. 6-1).

Differences in NE release between NGF differentiated and non-differentiated PC12 cells

In order to determine if differences existed between a neuronal precursor and sympathetic neuronal cell state, we grew PC12 cells for 72 h in the presence of nerve
growth factor (NGF), a neurotrophin known to cause PC12 cells to differentiate into a sympathetic neuronal phenotype (Greene, 1976; Kaplan and Stephens, 1994; Vaudry et al., 2002). NGF-differentiated PC12 cells were both more sensitive to ATPγS-stimulated NE release (EC₅₀ ~3.4µM) than non-differentiated cells (EC₅₀ ~17µM) and had initially higher, more sustained NE release compared to undifferentiated cells (Fig. 6-2B).

**NE reuptake in differentiated versus non-differentiated PC12 cells**

The reuptake of NE by neurons contributes to the termination of signaling by the neuron (Cooper et al., 2003). I assessed differences in NE reuptake by NGF-differentiated and non-differentiated PC12 cells and found that differentiated PC12 cells had greater reuptake over a similar time course compared to undifferentiated cells (Fig. 6-3). PC12 cells treated with NGF and ATPγS had reduced overall reuptake relative to untreated cells or PC12 cells incubated with NGF alone (Fig. 6-3). Thus growth of cells with NGF and ATPγS appears to decrease NE reuptake, in particular relative to NGF treatment alone.

**Desensitization to ATPγS-stimulated NE release**

Differences in NE release by differentiated versus non-differentiated PC12 cells implies a differentiation-associated alteration in the cellular machinery responsible for neurotransmitter release. To assess whether differences in desensitization of ATPγS-stimulated NE release existed, we pre-stimulated undifferentiated and NGF-differentiated PC12 cells with ATPγS, and subsequently re-stimulated with ATPγS. No significant
differences in the capacity of undifferentiated, compared to NGF-differentiated, PC12 cells to desensitize to ATPγS-stimulated NE release were found (Fig. 6-4A).

**Ca\(^{2+}\) sensitivity of ATPγS-stimulated NE release**

The differences in NE release and reuptake between differentiated and undifferentiated cells led me to question the source of NE released. Barium (Ba\(^{2+}\)) is a potent stimulator of exocytosis of Ca\(^{2+}\)-sensitive pools of NE (Przywara et al., 1993). Incubation of NGF-differentiated and undifferentiated PC12 cells with ATPγS did not effect Ba\(^{2+}\)-induced NE release from either cell group (Fig. 6-4B and C). However, Ba\(^{2+}\)-induced NE release was far greater in NGF-treated compared to untreated PC12 cells (C.f. Fig. 6-4B and C).

**Discussion**

These studies demonstrate key changes in the release and reuptake of NE in response to extracellular nucleotides upon differentiation of PC12 cells. The transition between an undifferentiated neuronal precursor cell to one with a neuronal phenotype involves alterations needed for neurons to perform their biological roles. Differentiation of PC12 cells by NGF is a well-studied model system of neurotrophin-mediated conversion of undifferentiated cells to sympathetic presynaptic neurons (Greene, 1976).

The change in NE release by PC12 cells in response to NGF stimulated differentiation and in response to nucleotide stimulation indicates a potential change in a number of components involved in the action of nucleotides.
P2 receptor changes during differentiation are a potential mechanism responsible for the differences in ATPγS-stimulated NE release. The results demonstrating undifferentiated PC12 cells release NE in response to ATPγS, but not UTP (Fig. 6-1) are consistent with previous work demonstrating only transient rises in intracellular Ca\(^{2+}\) in response to UTP stimulation, which was not sufficient to cause NE release (Nikodijevic et al., 1994). The transient increase in Ca\(^{2+}\) (via release of intracellular pools) by UTP stimulation differs from the influx of extracellular Ca\(^{2+}\) by ATP-activated Ca\(^{2+}\) channels (Nakazawa and Inoue, 1992), implying only a small portion from intracellular Ca\(^{2+}\) pools, and thus implicating P2X receptors as the predominant P2 receptor subtype responsible for nucleotide-stimulated NE release (Inoue et al., 1989; Majid et al., 1992).

P2 nucleotide receptor changes have been demonstrated in the developing nervous system (Franke and Illes, 2005). The differences in the potency and efficacy of ATPγS to cause NE release as well as the increased duration of release observed in this study between undifferentiated and NGF-differentiated PC12 cells (Fig. 6-2) are consistent with the idea that differentiation leads to changes in expression of P2 receptors and/or the signaling mechanisms that follow receptor activation. Such results suggest change(s) in expression and/or properties of P2 receptors during differentiation. Receptor transcripts for all 7 P2X receptors were found in undifferentiated PC12 cells as well as their relative levels of expression (Fig. 2-12); NGF-stimulated differentiation caused an increase in P2X\(_{1,3,5}\) expression relative to untreated PC12 cells (Fig. 2-14A). Expression of 7 of 8 P2Y receptors were found in undifferentiated PC12 cells while NGF-differentiation caused decreased expression in P2Y\(_6\) and P2Y\(_{11}\) (Fig. 2-11, 2-14B). These changes in
P2Y expression along with potential changes in P2X expression may underlie the altered responses of these cell states to nucleotide-stimulated NE release.

Differentiation not only changed NE release, but also NE reuptake. The recycling of NE is a crucial step in rapid responses of chromaffin cells and sympathetic neurons to stimuli. Previous studies have revealed that NGF-promoted differentiation is associated with reduced NE reuptake and reduced expression of norepinephrine transporter (NET) protein (Ikeda et al., 2001). This is in contrast to another protein, chromagranin A, involved in vesicular packaging of NE, which does not change with NGF differentiation (Rausch et al., 1988). Extracellular ATP causes increased in a secondary reuptake (uptake 2) of NE (Hendley et al., 1988; Hardwick et al., 1989; Gliese et al., 1994). NGF-promoted differentiation increased, while the combination of NGF and ATPγS, reduced the percentage of NE reuptake (Fig. 6-3). The reduced reuptake by the combination of NGF and ATPγS may be a result of desensitization of the nucleotide receptor that mediates both release and reuptake observed in these cells.

The involvement of extracellular nucleotide signaling in the release of neurotransmitters, particularly in NE release, has the potential to act on many neuronal systems and regulate neuronal communication and effects on neuronal target tissues. The changes in P2 receptor expression that occur during neuronal differentiation likely helps regulate P2 receptor-mediated responses in the nervous system and their contribution to neuronal function.
Figure 6-1: ATPγS and ATP promote NE release. Undifferentiated PC12 cells were incubated (Incubation I) in Ca²⁺ buffer (mock), ATPγS, or UTP where indicated. Cells were then stimulated with the indicated treatment (Incubation II) and the [H³] NE released was calculated as a percent of the total [H³] NE per well. **p<0.01
Figure 6-2: NGF-differentiated PC12 cells have greater sensitivity to ATPγS and greater ATPγS-stimulated NE release. PC12 cells grown for 72 h +/- NGF were stimulated by ATPγS at the indicated concentrations for 20 min. The [H\(^3\)] NE released was calculated as a percent of the total available (A). PC12 cells grown for 72 h +/- NGF were stimulated by 100\(\mu\)M ATPγS. [H\(^3\)] NE released was measured at the indicated timepoints and calculated as a percent of the total available (B). (*p<0.05, **p<0.01, ***p<0.001)
Figure 6-3: NGF-differentiated PC12 cells have higher NE reuptake than undifferentiated cells. PC12 cells grown for 72 h +/- NGF were stimulated with ATPγS and the percentage of released [H³] taken back up into the cells NE was measured at the indicated timepoints. (*p<0.05, **p<0.01)
**Figure 6-4:** ATPγS-stimulated NE is not depleted by Ba^{2+} in NGF-differentiated versus naïve PC12 cells. PC12 cells +/- NGF for 72 h were pre-stimulated with Ca^{2+} buffer (mock) or ATPγS and subsequently stimulated with ATPγS and assessed for [H³] NE release (A). [H³] NE release was measured from naïve (B) or NGF-differentiated (C) PC12 cells pre-stimulated with Ca^{2+} buffer (mock) or ATPγS and subsequently stimulated with ATPγS or 2mM Ba^{2+}. The percent [H³] NE release was calculated.
Chapter 7:

Conclusion
The work from the studies reported in this thesis define a number of previously unappreciated roles for extracellular nucleotides in the enhancement of survival of neurons, the facilitation of differentiation and growth initiated by neurotrophins (see diagram below), and the regulation of NE release and reuptake.

**Figure 7-1:** Summary Model of P2Y$_2$ Receptor Neuronal Signal Transduction Pathways.

The role of nucleotide signaling in neuronal development is relatively unexplored. The release of extracellular nucleotides in a neuronal environment by glial cells as well as by neurons themselves can alter synaptic transmission via transmitter release (Burnstock,
and simultaneously act as a feed-forward mechanism for the continued promotion of survival and connectivity of a neuron. This autocrine/paracrine release of nucleotides may serve to further strengthen the idea that neurons that “fire” together “wire” together, in that activation of neuronal connections and release of extracellular nucleotides promotes the survival of pre- and post-synaptic neurons through P2Y₂ activation. In the presence of neurotransmitters such as NGF, the release of nucleotides can act to further promote the survival and connectivity of their networks.

Peripheral neurons are perhaps the most likely to benefit from the facilitation of neuronal growth and survival. Like the glial sources of ATP and UTP in the central nervous system, target tissues of neurons like the cardiovascular cell types explored preliminarily in this work (see Chapter 2), are sources of both neurotrophin-like differentiation and nucleotides. This combination of soluble signal release in the local environment by tissues in the process of being innervated, or already innervated, would sustain the survival of the existing neuronal input cell(s) while promoting the growth and connectivity of incoming neurons. Recent data from P2Y₂ knockout mice demonstrated reduced perception of thermally induced painful stimuli, implicating endogenous P2Y₂ receptors as important in the development and/or survival of peripheral neuronal connectivity (Davis et al., 2005).

In humans, as well as in animal models, neuroaxonal dystrophy develops with aging, a process that is distinct from any known neuropathy. For example, rats demonstrate an age-related degeneration in sympathetic neuronal populations isolated from the pelvic ganglion (Santer et al., 2002). The reduced sympathetic innervation associated with aging has consequences for sympathetic target tissues, particularly the
cardiovascular system. With increasing age, reductions have been measured in cardiac innervation and blood pressure regulation by the autonomic nervous system (Chow et al., 2001; Jones et al., 2001). Age-related neuronal dystrophy can be compounded by diabetes mellitus. Diabetes not only has been linked to decreased heart rate variability via autonomic denervation (Oida et al., 1999) but also accelerates age-associated sympathetic neuronal dystrophy in animal models and humans (Schmidt et al., 1997; Schmidt, 2002). Loss of target-derived nerve growth factor (NGF), a well-known sympathetic neurotrophin, was thought to explain the loss of sympathetic innervation but reduced production of NGF by sympathetic targets does not appear sufficient to account for age-related neuronal dystrophy (Gavazzi and Cowen, 1996). Nucleotides may play an endogenous role in the enhancement of signaling by low-level NGF, allowing the survival of neuronal connections despite reductions in neurotrophin levels. As the levels of neurotrophins fall off with age, continued autocrine and/or paracrine nucleotide release would sensitize neurons to lower levels of available neurotrophins and allow their continued connectivity and survival. Increasing levels of nucleotides or combining low levels of neurotrophins together with nucleotides may prevent, perhaps even reverse neuronal dystrophies.

Potential caveats to the use of nucleotide-neurotrophin therapies are their effects on pain. Therapeutic trials with NGF treatment of neuropathologies have resulted in patients experiencing increased pain sensation (Eriksdotter Jonhagen et al., 1998; Thoenen and Sendtner, 2002). The use of lower levels of neurotrophins in combination with nucleotides has the potential to mitigate the stimulation of pain pathways via high-concentration NGF treatment. Another problem is that extracellular ATP signaling
through P2X₃ also increases pain through neuronal stimulation (Chizh and Illes, 2001). Recent data has demonstrated that siRNA targeted against the P2X₃ receptor as well as a non-nucleotide antagonist specific to the P2X₃ receptor reduces neuropathic pain (Jarvis et al., 2002; Dorn et al., 2004). The use of a specific antagonist against P2X₃ activation in combination with lower dose NGF and nucleotides may provide a way to treat neuronal dystrophies and neuropathologies that cause a loss of innervation while minimizing unwanted side effects.

Nucleotide triphosphates, but not di- or monophosphates were the ligands that promoted neuronal differentiation and survival (Chapter 2). Rapid degradation of these molecules in the local environment may act as regulatory steps, allowing brief stimulation of the neuron without accompanying over-stimulation that may result in uncontrolled growth. From the perspective of therapeutic intervention, the conflicting effects on neuronal differentiation and growth by hydrolysis products of nucleotides (Chapter 1) as well as the rapid formation of these competing ligands (Dunn et al., 2001) may require the use of non-hydrolyzable ATP analogs or non-nucleotide P2 subtype-selective molecules. The use of non-nucleotide small molecules specific to the P2Y₂ receptor has the potential added benefit of circumventing the cross-activation of pain pathways while still reaping the benefits of enhanced neuronal growth and survival.

The expanding discoveries related to extracellular nucleotide signaling will undoubtedly lead to the further identification of roles for these natural ligands and their cognate receptors as regulators of neuronal development as well as potential roles in axonal growth and guidance. An increased understanding of the functions of the ubiquitously expressed purinergic receptor system, not just in the nervous system, but
across all tissues may yield important information concerning overall mechanisms of biological action as well as potential insight into targets for treatment of disease.

The studies on NE release and reuptake (Chapter 6) demonstrate altered responses of NGF-differentiated, versus undifferentiated, cells. The many cellular changes that accompany neuronal differentiation may regulate, or be regulated by extracellular nucleotides/P2 receptors. P2 receptor expression changes in response to NGF-stimulated differentiation (Fig. 2-14). The changes in P2 express that occur appear to be the basis for differences in NE release and reuptake observed in response to extracellular nucleotides. Differences in P2 receptor expression and the signal transduction machinery that accompany such changes may allow neuronal cells to respond to a similar extracellular signaling molecule (i.e. ATP/UTP) with different effects appropriate to altered biological functions of the cell. Future work will need to resolve such issues and may lead to the identification of abnormalities associated with P2 receptors associated with neuronal pathologies.

Future Directions

The work presented in this dissertation has brought forward previously unappreciated interactions of extracellular nucleotide and neurotrophin signaling in the enhancement of neuronal differentiation, growth and survival, and regulation of NE release and reuptake. Additional studies that extend the findings presented here may help to further define the signal transduction pathways involved as well as identify additional extracellular nucleotide-neurotrophin signaling interactions, and other yet unidentified roles for extracellular nucleotide signaling in neuronal function.
Studies of PC12 cell co-culture with cardiovascular cells (Chapter 2) revealed a soluble, non-NGF substance produced by HUVEC cells capable of differentiating PC12 cells. The identification of this neurotrophic factor may uncover a previously undiscovered function of cardiovascular cells in the maintenance of neuronal cells and perhaps a novel neurotrophic factor. Proteomic studies from HUVEC conditioned media would be required for the isolation and identification of the molecule involved.

Further studies that identify the effects on neuronal growth and development by *in vivo* administration of ATPγS may help characterize the potential for extracellular nucleotide treatment in neuropathologies. Comparative electron microscopy of ATPγS-treated vs. untreated sciatic nerves may identify whether the increased GAP-43 expression (Chapter 3) was indicative of increased axonal numbers or increased axonal size. Initial behavioral studies have identified differences in pain perception by *P2Y2*−/− mice (Davis et al., 2005). Behavioral studies on ATPγS-treated vs. untreated mice may reveal differences in motor function, proprioception, or pain perception as a result of administration of extracellular nucleotides during development. Additionally, the use of extracellular nucleotides in a sciatic nerve crush together with behavioral assessment of regeneration paradigm (wt vs. *P2Y2*−/−) may reveal functions of extracellular nucleotide signaling through P2Y2 receptors in the regeneration and growth of neurons after injury.

The effects of extracellular nucleotide signaling are counterbalanced by degradation of these molecules by ectonucleotidases. Inhibitors of ectonucleotidases may reveal a contribution of endogenous extracellular nucleotide release and signaling in the promotion of neuronal growth and maintenance of survival *in vivo*. Inhibition of nucleotide degradation may lead to aberrant neuronal growth due to unchecked
nucleotide signaling-enhancement of endogenous neurotrophin signaling. Such studies may be important in understanding the regulation of extracellular nucleotide signaling on neuronal development and survival.

The work presented in this thesis identified signaling components necessary for the effects of extracellular nucleotides on neuronal growth and survival. Other signaling components that have not been explored that may be involved in this pathway warrant consideration. In particular, the role of cAMP in nucleotide-enhanced neuronal growth and survival has not been studied. Increased cAMP can lead to differentiation and survival of PC12 cells (Yao et al., 1998; Boglari and Szeberenyi, 2001). Extracellular nucleotide signaling causes arachidonic acid release and prostaglandin synthesis, which leads to elevation of intracellular cAMP concentrations in a canine kidney endothelial cell line (Ostrom et al., 2001). This mechanism may play a role in the increased survival and differentiation of neurons by extracellular nucleotides.

The interaction between P2Y₂ and TrkA receptors may be indicative of similar interactions with other members of the Trk receptor family. The identification of the domains of the P2Y₂ and TrkA receptors necessary for their physical interaction may reveal common sites of interaction for other receptors with similar domains (i.e. TrkB and TrkC). The identification of P2Y₂ interactions with other Trk family members by immunoprecipitation may provide evidence for extracellular nucleotide signaling enhancements of other neurotrophin signaling molecules and pathways. These interactions may in turn lead to the identification of other potentially therapeutic targets for the survival, growth, and regeneration of neurons.
The role of P2 receptors in the regulation of NE release and reuptake is another area for studies to compliment those in this thesis. Using the identified changes in P2 receptors as a result of NGF-differentiation, defining those receptors necessary for the altered regulation of NE observed may identify additional roles for P2 receptors in neurotrophin-stimulated differentiation. The use of selective antagonists (where available) and/or siRNA to block the activity of specific P2 receptors involved in NE release and reuptake may demonstrate roles for specific P2 receptors in these functions.
Appendix
Table Appendix-1: P2 receptor agonist/antagonist profiles.

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<th>Antagonists</th>
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<td>P2Y₂</td>
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<td>Suramin</td>
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