ABSTRACT

Title of dissertation: ACTIVITY-DEPENDENT REGULATION OF SCHWANN CELL DEVELOPMENT BY EXTRACELLULAR ATP

Beth Stevens, Doctor of Philosophy, 2003

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During development, the importance of activity-dependent plasticity in neurons is widely appreciated, but comparatively less is known of the role of electrical activity in controlling glial development. Schwann cells (SCs)--the myelinating cells in the peripheral nervous system--are critically dependent on axons during the perinatal period, but axonal signals controlling SC development and myelination have remained elusive. The onset of high frequency action potential activity along developing peripheral nerves corresponds to the period when SCs are exiting the cell cycle and initiating myelination. We postulated that

neural impulse activity could play an instructive role in regulating SC gene expression and function during development.

To address these questions, a neuron/SC co-culture system equipped with stimulating electrodes was used to evoke action potentials in dorsal root ganglion neurons (DRGs), and study the ensuing effects in pre-myelinating SCs. We found that SCs can detect neural impulse activity in pre-myelinated axons, and the activity-dependent axon-Schwann cell signaling molecule was identified as extracellular ATP. Activity-dependent release of ATP activated multiple intracellular signaling pathways in SCs, and increased levels of several transcription factors, including CREB, *c-fos*, and *krox-24*. Importantly, we found that ATP has profound effects on SC development. Activity-dependent ATP release significantly inhibited SC proliferation, arrested SC differentiation, and completely prevented the formation of myelin.

Extracellular ATP can activate multiple types of purinergic receptors; therefore we explored the specific purinergic receptors and signaling pathways that could mediate this form of activity-dependent neuron-SC communication. Using a combination of pharmacological and molecular approaches, we found that pre-myelinating SCs express a far more complex array of ATP receptors (P2X and P2Y) that previously thought. Surprisingly, we discovered that pre-myelinating SCs also express a class of functional adenosine receptors (A2), which are positively coupled to cAMP. Extracellular adenosine, a breakdown product of ATP, regulated MAP Kinase signaling and proliferation in SCs independently of

ATP. Collectively, our findings suggest that ATP and adenosine released from electrically active axons activate a complex intracellular signaling network in SCs, in which ATP and adenosine act together to regulate SC function during development and nervous system plasticity.

ACTIVITY-DEPENDENT REGULATION OF SCHWANN CELL DEVELOPMENT BY EXTRACELLULAR ATP

by

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INTRODUCTION

Myelination is one of the most complex cell-cell interactions that exist in biology. The process requires recognition, association, ensheathment, and elaboration of glial membrane around the appropriate axon at precisely the right time in development. The end result is the formation the compact myelin sheath, which is essential for rapid propagation of nerve impulses along central and peripheral vertebrate axons. The significance of myelin can not be disputed. Its importance in nervous system function is especially highlighted in demyelinating diseases or pathologic conditions in which myelin has broken down.

Myelination is achieved by two glial cell types --oligodendrocytes in the central nervous system (CNS) and Schwann cells in the periphery (Figure 1). Schwann cells myelinate a single axon segment, where as oligodendrocytes can myelinate several. We have come far in our understanding of the cellular and molecular architecture of myelin, but incredibly, the extracellular signals that initiate the myelination program remain a mystery. It is well established that myelinating glia are dependent on continual communication with axons during embryonic and early postnatal development. The prevailing view is that myelination is controlled by the axon (Peles and Salzer, 2000). One hypothesis is that intrinsic axonal

properties determine whether it will become myelinated. A second is that myelination is induced by contact-dependent and/or soluble axonal signals. There is evidence to support both sides, and while this topic is highly contentious, there is general agreement that axonal signals are necessary, especially in the peripheral nervous system (PNS).

Myelination enables rapid propagation of neural impulses along developing axons, but could impulse activity regulate myelination? Evidence from several model systems have established a fundamental role for electrical activity in modifying developing neural circuits (Katz and Shatz, 1996; Zhang and Poo, 2001). Glial cells are intimately associated with developing neurons at synapses and along fiber tracts, yet the role of neural activity in controlling glial development has not been adequately explored. The onset of neural impulse activity along developing axons corresponds to the period when myelinating glia are undergoing dramatic changes in gene expression, proliferation, differentiation into myelin forming cells (Fitzgerald, 1987; Mirsky and Jessen, 1999). Could impulse activity also provide instructive signals to developing glia?

There is growing evidence that activity-dependent neuron-glia communication occurs in the mature nervous system. With the development of new techniques and tools with which to study glia, it is becoming increasingly clear that glial cells can actively communicate with neurons. Although glia lack the membrane properties to generate action potentials, they

express many of the same ion channels, and receptors systems as neurons, and thus are well equipped to receive and transmit neuroactive signals (Fields and Stevens-Graham, 2002). Indeed, activity-dependent calcium transients have been observed in glia at the synapse (Castonguay et al., 2001; Haydon, 2001), and along mature myelinated axons (Lev-Ram and Ellisman, 1995), but activity-dependent neuron-glia communication along developing axons has not been previously demonstrated.

The rodent sciatic nerve is an accessible mammalian model in which to study the effects of neural impulse activity on glial development and myelination *in vivo*, and *in vitro.* In contrast to oligodendrocytes, SCs are strictly dependent on axonal signals for induction of myelin gene expression and initiation of myelin. Each stage of Schwann cell development has been extensively characterized, both at the morphological and molecular levels, making the sciatic nerve an excellent model system for studying the signals that regulate SC development (Mirsky and Jessen, 1996, 1999). In addition, *in vivo* electrophysiological recordings in rodent sensory neurons have established that the onset of spontaneous and low frequency neural impulse activity corresponds to a critical period of SC development before the onset of myelination (around birth), when action potential activity could provide instructive signals to SCs (Fitzgerald, 1987). A major goal of this research is to determine whether developing SCs respond to neuronal activity, and to investigate possible candidate signaling molecules involved. A closer look at

the cellular and molecular mechanisms controlling Schwann cell development could lend important insight into these questions.

SCHWANN CELL DEVELOPMENT: AN OVERVIEW

Schwann cells are critically dependent on axonal signals throughout embryonic and postnatal development. SC precursor cells originating from the neural crest migrate along developing peripheral axons and undergo a series of well-defined, morphologically distinct developmental stages characterized by specific patterns of gene expression (Jessen and Mirsky, 2002)(see fig. 2). There are 3 major transitions: generation of SC precursors from migrating neural crest cells (~E 14 in rat), transition from SC precursor to embryonic/pre-myelinating SCs (~E16-birth), and finally the divergence of the SC lineage into mature myelinating or non-myelinating phenotypes from birth through the first 2-3 postnatal weeks (Jessen and Mirsky, 1999a; Mirsky and Jessen, 1999). These two cell types exist in approximately equal numbers in adult peripheral nerves. Myelinating Schwann cells wrap multiple layers of cell membrane around a single axon, whereas non-myelinating SCs ensheath multiple small diameter axons. In addition to morphological and functional differences, a set of molecular markers distinguish the two phenotypes (see fig. 2). Despite major progress in understanding the cellular and molecular differences between these two phenotypes, the identity of the signal(s)

Figure 1. Major categories of glia in the nervous system.

(a) Non-myelinating Schwann cells, labeled by fluorescence immunocytochemistry for the S-100 protein, appear spindle shaped when cultured without axons (left). Myelinating Schwann cells undergo dramatic changes in morphology, extending multiple wraps of myelin membrane around a single axonal segment. Myelinated segments are interrupted by regularly spaced Nodes of Ranvier, which serve to propagate action potentials over great distances. (b) A cultured oligodendrocyte, immunostained with myelin-basic protein (MBP) displays several elaborate extensions of myelin membrane. In contrast to Schwann cells, a single oligodendrocyte can myelinate multiple axons (right). (c) Astrocytes do not form myelin, but they communicate with neurons, glia, and blood vessels to regulate diverse functions in the CNS. Cultured rodent astrocytes, stained with the astrocytic marker GFAP are pictured on the left. (Drawings adapted from Figure 2-3, Kandel and Schwartz).

Figure 1

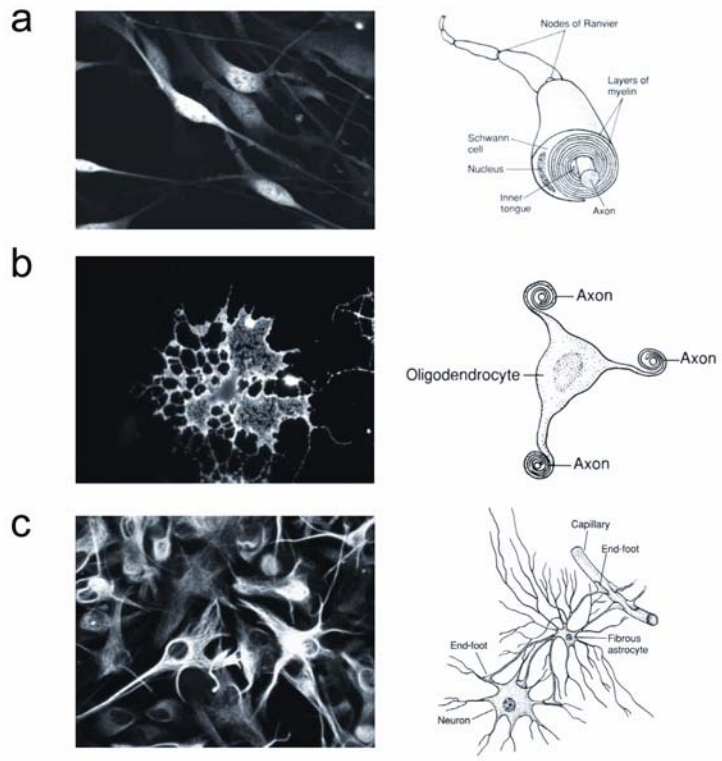

a
Nodes of Ranvier
Layers of myelin
Schwann cell
Nucleus
Inner tongue
Axon
b
Axon
Oligodendrocyte
Axon
Axon
c
Capillary
End-foot
End-foot
Fibrous astrocyte
Neuron

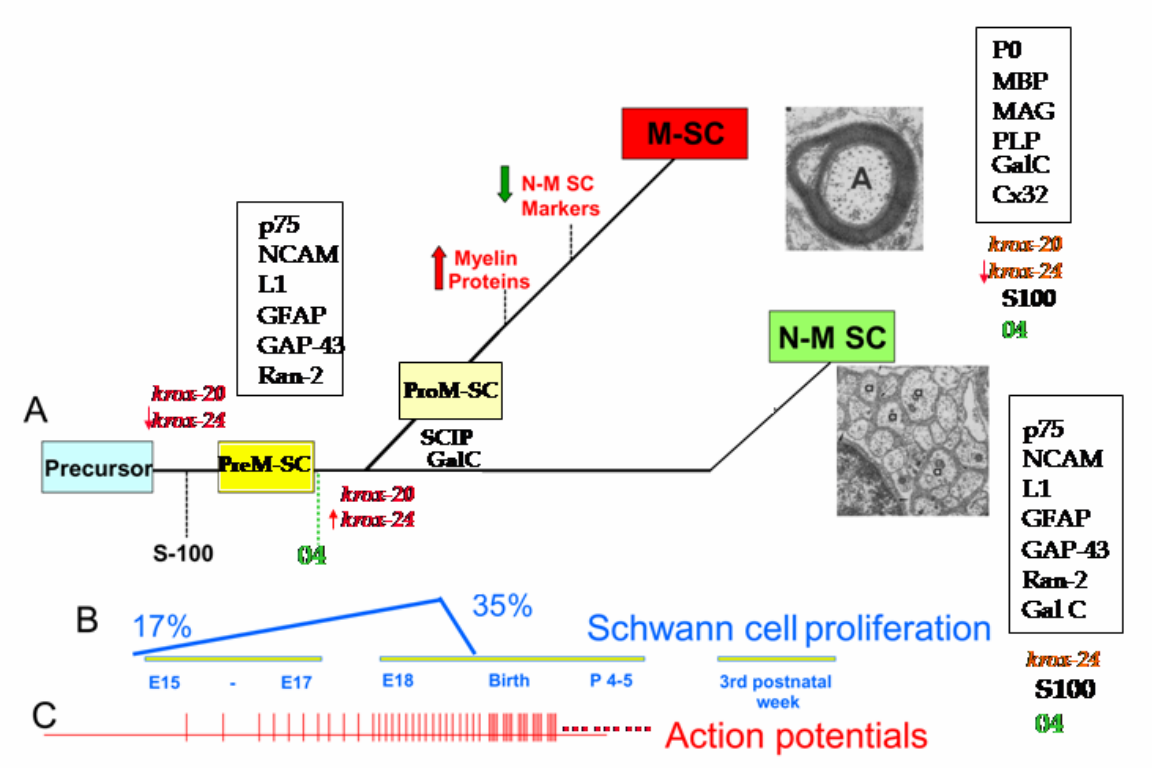
Figure 2. Correlation between Schwann cell development and changes in neural impulse activity in rat sensory neurons during the perinatal period.

A. An outline of Schwann cell development based on the rat sciatic nerve. PreM-SC, pre-myelinating Schwann cell. ProM-SC, promyelinating Schwann cell. M-SC, myelinating Schwann cell. N-M SC, non-myelinating Schwann cell. This diagram summarized the profile of markers used to define distinct developmental stages in both the myelinating and non-myelinating Schwann cell lineages.

B**.** The rate of Schwann cell proliferation increases in late fetal development and begins to decrease near the time of birth, just prior to the onset of myelination.

C. Action potentials from rat DRG neurons show the onset of active spontaneous and sensory-evoked firing begins ~E16 and steadily increases into the postnatal period.

Figure 2


M-SC
N-M SC Markers
Myelin Proteins
p75
NCAM
L1
GFAP
GAP-43
Ran-2
P0
MBP
MAG
PLP
GalC
Cx32
krox-20
krox-24
S100
04
N-M SC
ProM-SC
SCIP
GalC
A
krox-20
krox-24
Precursor
PreM-SC
krox-20
krox-24
S-100
04
p75
NCAM
L1
GFAP
GAP-43
Ran-2
Gal C
krox-24
S100
04
B
17%
35%
Schwann cell proliferation
E15
-
E17
E18
Birth
P 4-5
3rd postnatal week
C
Action potentials

that direct a Schwann cell toward the myelinating or non-myelinating fate has not been identified.

Schwann cells are exceptionally plastic cells. While the specific axon signal (s) that regulate each key stage of SC development are not known, a number of important growth factors and soluble signals have been described (Stewart et al., 1991; Zorick and Lemke, 1996; Jessen and Mirsky, 2002). Interestingly, the biological consequences of an extracellular signal appear largely dependent on the developmental period, and/or the environmental context. This is exemplified by the neuregulin family of growth factors (otherwise known as glial growth factor (GGF), heregulin/ARIA, neu differentiation factor), which have profound effects on SC development at multiple stages (Topilko et al., 1996; Jessen and Mirsky, 1997; Garratt et al., 2000; Jessen and Mirsky, 2002). Very early in SC development (i.e. E14), β-neureg-1/erbB3 receptors are essential for the generation SC precursors from neural crest cells. Neuregulins promote the survival and migration of SC precursor cells (Mirsky and Jessen, 1999), and have also been shown to play and instructive role early in SC development (Shah et al., 1996). The transition from precursor to pre-myelinating SCs is also dependent neuregulin (Dong et al., 1995). During the pre-myelinating stage (~E16), SCs initiate autocrine survival loops, and become independent of neuregulin for survival. However, neuregulins vigorously stimulate SC proliferation in premyelinating SCs *in*

vitro (Dong et al., 1995; Kim et al., 1997a). In addition, neuregulins can induce the expression of myelin-related transcription factors (Murphy et al., 1996; Wu et al., 2001). At later stages, neuregulin can dramatically inhibit SC alignment, ensheathment, and myelination (Zanazzi et al., 2001). In fact, treatment of mature, myelinated SC cultures with neuregulin can induce striking de-myelination (Zanazzi et al., 2001) . Together, these findings suggest that the same extracellular signal can have diverse functional effects in SCs depending upon the biological context.

Schwann cell plasticity is further demonstrated following peripheral nerve injury or pathology. Unlike oligodendrocytes, Schwann cells are capable of de-differentiating following denervation. This has been demonstrated *in vivo* following axotomy, as well as in cell culture models (Scherer, 1997; Jessen and Mirsky, 1999b). It is this remarkable plasticity that likely contributes to Schwann cells unique ability to promote regeneration and re-myelination in the PNS. Following axon degeneration, the myelin sheath breaks down and myelinating SCs down-regulate expression of myelin-related proteins and mRNAs. SCs de-differentiate into a phenotypes resembling pre-myelinating SCs, after which many developmental events including proliferation, differentiation and myelination are recapitulated. Thus, understanding the signals that regulate pre-myelinating SCs during development could also lend insight into the mechanisms that control remyelinaiton following injury or disease.

REGULATION OF SCHWANN CELL PROLIFERATION

SCs continue dividing after migration into peripheral nerves, and SC proliferation ceases around the time of birth. It is well established that SC proliferation is regulated by contact with axons, and axon-derived factors. Direct contact of SCs with the axolemma, sustains SC proliferation until initiation of axon ensheathment (Salzer et al., 1980; DeVries et al., 1982; Ratner et al., 1985). *In vivo* studies in the rat sciatic nerve demonstrated a rise in SC proliferation between E17-19, followed by a dramatic decline ~ 2 days before the onset of myelination (Stewart et al., 1993). Among the most convincing candidates for axonal mitogens is neuregulin (Morrissey et al., 1995; Maurel and Salzer, 2000). The mitogenic effects of β -neuregulin on glia have been observed in several species including humans (Kim et al., 1997; Levi et al., 1995; Morrissey et al., 1995; Rutkowski et al. 1995). In addition, axon-induced mitogenic responses can be inhibited with antibodies to neuregulin and to neuregulin receptors erbB2 in DRG/SC co-cultures (Morrissey et al.). *In vitro* models have identified a number of signals that promote SC proliferation, either alone or in concert with signals that elevate intracellular cAMP levels including bFGF, PDGF, IGF, TGF-β, endothelins (Davis and Stroobant, 1990; Eccleston et al., 1990; Schumacher et al., 1993; Guenard et al., 1995; Svenningsen and Kanje, 1996; Berti-Mattera et al., 2001).

While significant progress has been made in identifying factors that stimulate Schwann cells to divide, much less is known about the extracellular signals that negatively regulate SC mitosis *in vivo*, and *in vitro*. Just before birth, pre-myelinating SCs undergo a dramatic decline in their proliferation rate, which is a pre-requisite for induction of SC differentiation and myelination (Asbury, 1967; Stewart et al., 1993). Several tissue culture studies have established that only post-mitotic SCs are responsive to myelin-inducing axon signals. Other factor (s) likely over-ride pro-mitotic axonal signals to suppress the growth cycle in SCs, but the identity of these anti-proliferative signals in developing peripheral nerves is unknown. Thus, mechanisms that arrest SC proliferation could indirectly regulate myelination by enabling SCs to respond to myelin-inducing signals at the appropriate time. A major aim of this research will be to determine whether action potential activity can influence SC proliferation at the pre-myelinated stage.

SCHWANN CELL DIFFERENTIATION AND MYELINATION

Myelination involves a highly regulated sequence of molecular interactions between axons and Schwann cells. Significant progress has been made in identifying key intracellular signals and genes involved in myelination in the PNS. There is compelling evidence *in vitro* and *in vivo* that continual axon-SC signaling is essential for the formation and maintenance of the myelin sheath.

Cyclic AMP (Hutchinson et al.) has been implicated as an important second messenger, and is thought to mimic axon signals *in vitro* (Morgan et al., 1991). Agents that increase intracellular cAMP levels in non-dividing SCs dramatically up-regulate myelin gene expression, and induce morphological changes associated with the myelinating phenotype. In culture, exposure of SCs to forskolin or differentiating media induce the transition of bipolar, spindle-shaped Schwann cells to rounded flattened cells that intimately associated with axons (Sobue et al., 1986). Myelinating Schwann cells sequentially up-regulate a series of myelin genes involved in the formation and maintenance of the myelin sheath (P0, PMP22, MBP), and down-regulate several of the markers expressed by immature and mature non-myelinating Schwann cells (Zorick and Lemke, 1996; Scherer, 1997; Mirsky and Jessen, 1999)(see fig 2)**.**

Several transcription factors have also been shown to be differentially regulated in myelinating and non myelinating SCs. As shown in figure 2, Krox 24, c-jun and Pax 3 are expressed by non-myelinating and denervated SCs, while Krox 20, and SCIP (Oct6/tst-1) are expressed in myelinating SCs (Scherer, 1997). The zinc family transcription factor, Krox-20 is essential for PNS myelination (Topilko et al., 1994; Murphy et al., 1996; Zorick et al., 1996). Studies carried out *in vivo* have revealed Krox-20 is expressed in embryonic SCs before the onset of myelination, but its expression is later restricted to myelinating SCs (Topilko et al., 1997). The POU domain

transcription factor SCIP, is expressed transiently in the pro-myelinating stage of both phenotypes, and appears to play a role in determining the timing of cell cycle withdrawal and induction of myelination. Recent work by Zorick et al. (1996) demonstrated that SCIP is significantly up-regulated in *krox-20* mutants, suggesting a link between these two transcription factors. In contrast to Krox-20, Krox-24 expression is generally restricted to the non-myelinating, denervated, proliferating Schwann cells (Topilko et al., 1997). Interestingly, there is a narrow window at birth when the two genes are co-expressed in Schwann cells, and it has been suggested that *krox-24* may suppress the expression of myelin-related genes, or enhance the expression of non-myelinating genes during development and regeneration (Topilko et al., 1997).

Inhibition of SC proliferation and the subsequent induction of differentiation and myelin gene expression occur within a narrow window before birth. Identifying candidate axonal signal(s) that are present during this critical period in SC development may contribute a better understanding of how SC proliferation and myelination is regulated. Spontaneous and stimulus-evoked neural activity begins along developing sensory axons during this time, and as we shall see below, there is functional evidence that electrical activity can influence glial development and myelination in the CNS and PNS.

ACTIVITY-DEPENDENT REGULATION OF GLIAL DEVELOPMENT

Activity-dependent axon-glial communication has been proposed to influence glial proliferation in the developing optic nerve. Barres and Raff reported that proliferation of oligodendrocyte precursor cells (OPCs) depends on electrical stimulation in neighboring axons (Barres and Raff, 1993) . Intraocular injection of TTX in developing rat optic nerve significantly reduced OPC proliferation, and this TTX-dependent inhibition of proliferation was rescued with the growth factor, PDGF, indicating that electrical activity could stimulate the production and release of mitogens that stimulate OPC proliferation. The authors suggested that electrically active axons produce a signal that stimulates type 1-astrocytes to release PDGF, since astrocytes are a major source of PDGF in developing optic nerve. However, this hypothesis was not further explored, and the axonal signal(s) were not identified. In the same year, Kreigler and Chiu observed calcium transients in developing optic nerve glia in response to axonal firing, but neither the type of glial cell nor the activity-dependent signal were identified (Kriegler and Chiu, 1993).

Several lines of evidence from the central and peripheral nervous systems suggest that the onset of neural impulse activity can affect initiation of myelination. Earlier studies in the optic nerve demonstrated that mice reared in the dark developed fewer myelinated axons compared with control mice (Gyllensten et al., 1963). Hypomyelination was also observed in optic nerve of

the naturally blind cape-mole rat (Omlin, 1997), whereas myelination was accelerated by premature eye opening in rabbit optic nerves (Tauber et al., 1980). More recent experiments in CNS neurons revealed that electrical activity can promote myelination. Blockade of sodium-dependent action potentials with tetrodotoxin (TTX) significantly inhibited myelination *in vitro,* and in developing optic nerve *in vivo*, while increasing neuronal firing with α-scorpion toxin enhanced myelination (Demerens et al., 1996). The molecular mechanisms underlying these activity-dependent effects on myelination are not known. A number of molecules were proposed, including cell adhesion molecules, extracellular potassium, and the release of a soluble axonal signal (Demerens et al., 1996; Zalc and Fields, 2000).

Opposite effects of neural impulse activity on myelination have been reported in the PNS. Recent experiments from our laboratory have shown that electrical activity can inhibit myelination the PNS by changing membrane properties of developing axons (Stevens et al., 1998). Homophilic interactions between L1 on axons and Schwann cells are important for ensheathment and the initial stages of myelination (Seilhemer and Schachner 1988; Wood et al., 1990). We found that myelination of dorsal root ganglion (DRG) axons by Schwann cells was inhibited following electrical stimulation of axons at a low frequency (0.1 Hz) via down-regulating mRNA and protein levels of the cell adhesion molecule L1 on axons (Itoh et al., 1995; Stevens et al., 1998).

Given the expanding evidence that glial cells can actively respond to neuronal activity, it is plausible that action potential activity along developing axons could also influence SC myelination through the release of activity-dependent soluble signals. A closer look into the mechanisms underlying activity-dependent neuron-glia communication in the adult nervous system could provide insight into candidate activity-dependent signals regulating axon-Schwann cell communication during development.

GLIAL CELLS ARE EXCITABLE

The notion that glial cells are active, excitable cells emerged with the advent of new techniques with which to study neuron-glia communication. Glial calcium signaling is a measurable indicator of glial excitability, providing a powerful tool to study glial cells in action. Time-lapse calcium imaging in several model systems have recently demonstrated that glia located at synapses respond to neuronal activity with increases in intracellular calcium (Araque et al., 1999; Castonguay et al., 2001; Haydon, 2001). Likewise, chemical or mechanical stimulation of astocytes can elicit calcium responses in nearby neurons. Astrocytes can also actively communicate with one another. Stimulation of one astrocyte, can trigger a local elevation in astrocytic calcium that can subsequently spread to neighboring astrocytes in the form of a calcium wave, which can propagate at a rate of 100 μm/sec. The mechanisms underlying astrocytic calcium waves include local diffusion

of second messengers through gap junctions, and extensive chemical signaling along astrocytic networks.

Several important mechanisms underlie the excitability of glial cells. First, glial cells express most types of voltage-gated ion channels (Barres et al., 1990; Deitmer et al., 1998; Verkhratsky and Steinhauser, 2000). Potassium channels are by far the most prevalent, which is consistent with the critical role of glia in buffering extracellular K^+ during neural activity. Glial cells also express several types of calcium channels (predominantly L and T types), in addition to chloride channels. Ca^{2+} channels have been demonstrated in astrocytes *in vivo* and *in situ* (MacVicar et al. 1991; Duffy and McVicar 1994), as well as in perisynaptic Schwann cells at the neuromuscular junction (Robitaille et al., 1996). Although glia lack the membrane properties to generate action potentials, voltage –gated Na^+ channels have been found in astocytes (Barres et al., 1989; Steinhausser et al, 1994; Schaller et al. 1995), and in Schwann cells (Chiu, 1991). Na^+ channels are thought to be involved in the regulation Na^+ homeostasis and Na^+ – dependent transporters.

Glial cells also express functional receptors for most neurotransmitters, including ionotropic (non-NMDA) and metabotropic glutamate receptors, in addition to GABAergic, adrenergic, cholinergic, purinergic, and serotonergic receptors. Glial cells also express receptors for several neuropeptides, including substance P, and CGRP (Chiu and Kriegler, 1994, Kimelberg, 1995;

Shao and McCarthy, 1995; Vernadakis, 1996; Verkhratsky and Steinhauser, 2000). Finally, activity-dependent activation of glial receptors can induce the calcium-dependent synthesis and release of several neuroactive substances, including glutamate, ATP, and prostaglandins (Parpura et al., 1995; Castonguay et al., 2001). For example, cultured Schwann cells have been shown to release glutamate in response to extracellular ATP (Jeftinija and Jeftinija 1998). Through these mechanisms, activity-dependent release of neuroactive substances from glia could have broad implications in modulating both neurons and glia at the synapse and in extrasynaptic regions of axons.

ACTIVITY-DEPENDENT NEURON-SCHWANN CELL COMMUNICATION

Emerging evidence in the adult nervous system indicate the Schwann cells respond to neuronal activity. Activity-dependent axon-SC signaling has been observed *in situ* in the paranodal region of peripheral nerves during axonal activity (Lev-Ram and Ellisman, 1995). Axonal stimulation (5 min 20 Hz stimulation) induced calcium transients in myelinating SCs at the Node of Ranvier, presumably as a result of accumulation of extracellular potassium at the node (Lev-Ram and Ellisman, 1995).

Activity-dependent axon-SC communication has been most extensively studied at the neuromuscular synapse. Elegant electrophysiogical and imaging

studies performed at the in-tact frog and mouse NMJ have demonstrated that terminal Schwann cells surrounding neuromuscular junctions in the PNS, respond to synaptic release of neurotransmitters, such as glutamate and acetylcholine, with increases in intracellular calcium. These activity-dependent calcium fluxes can regulate glial intracellular signaling pathways, and gene expression. For example, tetanic stimulation of the presynaptic motor nerve axon was shown to elicit calcium transients in terminal Schwann cells, which down-regulated expression of GFAP via activation of acetylcholine (ACh) receptors on SCs (Georgiou et al., 1999).

Extracellular ATP can also mediate activity-dependent signaling between neurons and terminal Schwann cells. ATP is co-released with neurotransmitters at synapses in an activity-dependent manner, and terminal Schwann cells express several types of purinergic receptors. It was recently demonstrated that blockade of ATP receptors in SCs reduced the size and onset of activity-dependent Ca^{2+} transients in terminal SCs, suggesting that purinergic receptors on perisynaptic SC's are activated by ATP released by synaptic transmission (Robitaille, 1995).

Extrasynaptic release of ATP along axons has not been previously demonstrated, but activity-dependent non-synaptic release of adenosine has been shown in studies if the rabbit vagus nerve (Maire et al., 1984). There is evidence for non-synaptic release ATP from various cell types. In fact, ATP has been recently identified as a key signaling molecule in astrocytic calcium

waves (Cotrina et al., 1998; Guthrie et al., 1999). Extracellular ATP released from stimulated astrocytes activates metabotropic purinergic receptors on neighboring cells (Fam et al., 2000). Together these findings suggest that extracellular ATP could serve as an activity-dependent signal between axons and developing Schwann cells.

PURINERGIC RECEPTORS IN GLIAL CELLS

Recent evidence indicate that all glial cell types express membrane receptors for extracellular ATP (Fields and Stevens, 2000). ATP has several unique characteristics that make it a potent neuron-glia transmitter; it is highly diffusible in the extracellular space, it rapidly degrades into adenosine by ecto-nucleotidases, and it can activate a complex set of purinergic receptors which could have diverse biological outcomes depending on the cell type and environmental context.

Extracellular purines (ATP, adenosine, and ADP) and pyrimidines (UTP, and UDP) are important signaling molecules that mediate diverse biological function inside and outside of the nervous system (Ralevic and Burnstock 1998). Membrane receptors for extracellular purines are a complex class of receptors that can be grouped into two main families; the P2 receptors for ATP, and the P1 receptors for adenosine. The ATP (P2) receptor family can be subdivided into ionotropic (P2X) and metabotropic (P2Y) receptors, and each of these families has several members. Seven mammalian P2X

receptors ($P2X_{1-7}$) and eight P2Y ($P2Y_{1, 2, 4, 6, 11, 12, 13, 14}$) have been cloned and characterized (Ralevic and Burnstock, 1998) (see fig. 3).

Adenosine (P1) receptors have been characterized into four subtypes: A1, $A2_A$ and $A2_B$ and A3, all of which couple to G proteins. A1 and A3 adenosine receptors act through intracellular calcium and inhibit adenylate cyclase, where as the A2 class of receptors are positively coupled to cAMP (Fields and Stevens, 2000; Klotz, 2000). As shown in Fig 3**,** selective agonists and antagonists have been developed for many of P1 and P2 receptors, allowing pharmacological characterization of purinergic expression and function. The list of selective compounds continues to expand, and serve as useful tools for studying purinergic receptor function *in vivo* and *in vitro.* A combination of molecular, pharmacological, and functional approaches should be used to characterize a specific subtype, since the dose and selectivity of many of these compounds varies with species and cell type.

SCHWANN CELLS RESPOND TO EXTRACELLULAR ATP

Lyons & McCarthy first demonstrated that cultured Schwann cells respond to extracellular ATP with increases in intracellular calcium (Lyons et al., 1994, 1995). Similar findings were reported in cultured immortalized SCs (Berti-Mattera et al., 1996). Importantly, it was further shown that purinergic receptor expression declines after several days in culture, unless they are co-cultured with neurons or agents that mimic axonal signaling such as forskolin

(Lyons et al., 1994). Purinergic receptor expression can be rescued in long term SC monocultures by the addition of axon signals. Although the specific types of ATP receptors were not identified, these findings suggest that continuous neuron-glia signaling may be necessary for purinergic signaling in SCs.

There is also evidence that Schwann cells express ATP receptors *in situ* (Mayer et al., 1998). Interesting phenotypic differences in calcium responses have been observed between myelinating and non-myelinating SCs in adult nerve following application of selective ATP receptor agonists (Mayer et al., 1998). When and how this phenotypic divergence in receptor expression occurs in SCs is not known, and warrants further investigation. The presence of these receptors *in vivo* has also been shown by focal application of ATP, which stimulates calcium increases in paranodal Schwann cells of mammalian nerve (Wachteler et al., 1996; Lyons et al., 1995; Mayer et al., 1997), and perisynaptic Schwann cells at the amphibian neuromuscular junction (Jahromi, Robitaille, Charlton 1992; Robitaille 1995).

Most cells, including glia, express more than one type of purinergic receptors. For example, astrocytes express multiple adenosine ATP receptor subtypes, often in the same cell (King et al., 1996; Neary et al., 1998). A similar picture is beginning to emerge for cells of the oligodendrocyte lineage (Stevens et al., 2002a). It is not yet known which types of purinergic receptors

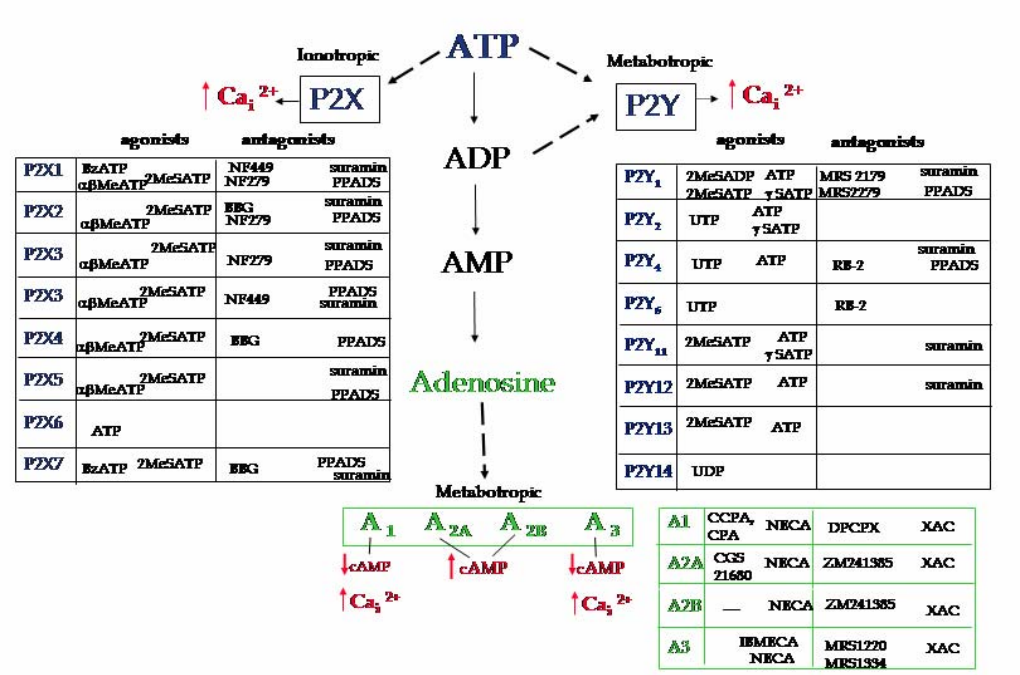
are expressed in developing pre-myelinating SCs, or which intracellular signaling pathways are activated in response to extracellular ATP.

Activation of different purinergic receptor subtypes could activate multiple intracellular second messengers and signal transduction pathways**.** Purinergic receptors could also be indirectly linked to other signaling systems, including growth factors, neurotransmitters, cell adhesion molecules, cytokines, hormones, and others, suggesting that they may be involved in many different cellular functions (Ralevic and Burnstock, 1999). Different receptors can have antagonistic actions on other purinergic receptors and intracellular signaling pathways in the same cell. In addition, the downstream pathway and biological effects appear to vary widely in different cell types, including different types of glial cells**.** Diversity of receptor expression and developmental and environmental influences likely contribute to these differences. For this reason, it will be important to characterize the specific signaling pathways linked to purinergic receptors that regulate SC development.

Figure 3. Families of ATP (P2) and Adenosine (P1) receptors.

P2 receptors divide into two families based on differences in molecular structure and signal transduction mechanisms. P2X are ligand-gated ion channels and P2Y are G-protein coupled receptors. Adenosine/P1 receptors are all coupled to G-proteins and can divided into A1, A2A, A2B, and A3 subtypes. Multiple subtypes of each family have been cloned and can be characterized with selective **(bold)** and non-selective receptor agonists and antagonists.

Figure 3.


ATP
Ionotropic
P2X
↑ Ca i 2+
agonists
antagonists
P2X1 BzATP αβMeATP 2MeSATP NF449 NF279 suramin PPADS
P2X2 2MeSATP αβMeATP BBG NF279 suramin PPADS
P2X3 2MeSATP αβMeATP NF279 suramin PPADS
P2X3 2MeSATP αβMeATP NF449 PPADS suramin
P2X4 2MeSATP αβMeATP BBG PPADS
P2X5 2MeSATP αβMeATP suramin PPADS
P2X6 ATP
P2X7 BzATP 2MeSATP BBG PPADS suramin
ADP
AMP
Adenosine
Metabotropic
A 1 A 2A A 2B A 3
↓cAMP ↑cAMP ↓cAMP
↑ Ca i 2+ ↑ Ca i 2+
Metabotropic
P2Y
↑ Ca i 2+
agonists
antagonists
P2Y1 2MeSADP 2MeSATP ATP γSATP MRS 2179 MRS2279 suramin PPADS
P2Y2 UTP ATP γSATP
P2Y4 UTP ATP RB-2 suramin PPADS
P2Y6 UTP RB-2
P2Y11 2MeSATP ATP γSATP suramin
P2Y12 2MeSATP ATP suramin
P2Y13 2MeSATP ATP
P2Y14 UDP
A1 CCPA, CPA NECA DPCPX XAC
A2A CGS 21680 NECA ZM241385 XAC
A2B — NECA ZM241385 XAC
A3 IBMECA NECA MRS1220 MRS1334 XAC

EXPERIMENTAL AIMS

This research will specifically test the hypotheses that pre-myelinating Schwann cells respond to neural impulse activity, and that activity-dependent signaling from axons regulates Schwann cell gene expression and function. Major objectives of these studies were to identify the activity-dependent signal(s) involved, the functional consequences which ensued, and to investigate the specific cellular and molecular mechanisms by which these signal(s) regulate Schwann cell function during development. To address these questions, a neuron/SC co-culture system equipped with stimulating electrodes was used to evoke action potentials in dorsal root ganglion neurons (DRGs), and study the ensuing effects in pre-myelinating SCs. This dissertation will examine the following questions:

1. Does activity-dependent communication regulate gene expression in pre-myelinated SCs? (Chapter 2)

a) Do Schwann cells detect action potential activity along pre-myelinated DRG axons?
b) What is the activity-dependent signal?
c) Does activity dependent signaling regulate gene expression in pre-myelinating Schwann cells?

2. What are the functional consequences of activity-dependent axon-Schwann cell communication? (Chapters 3 and 4)

a) Do action potentials and /or ATP regulate SC proliferation?

b) Does activity-dependent axon-SC communication and/or ATP regulate SC differentiation and/or myelination?

c) Does purinergic signaling regulate the cell cycle in SCs?

3) Which purinergic receptors regulate activity-dependent axon-SC communication? (Chapter 5)

a) Which specific purinergic receptors are expressed in developing SCs?

b) Which signal transduction pathway(s) are activated in response to activity-dependent release of ATP?

c) Independent roles of ATP and Adenosine in regulating MAPK pathways in developing SCs.

d) Which purinergic receptors mediate ATP-dependent inhibition of SC proliferation?

CHAPTER 2

INTRODUCTION

Electrical activity plays a fundamental role in modifying developing neural circuits, yet the role of neural impulse activity in regulating glial development has not been adequately explored. It is well established that myelinating glia are dependent on axonal signals during embryonic and early postnatal development (see review(s) by Mirsky and Jessen, 1996,1998), but the specific signals that regulate the timing of glial differentiation and myelination have remained elusive. The onset of high frequency action potential activity along developing peripheral nerves corresponds to the period when Schwann cells are exiting the cell cycle and initiating myelination, suggesting that neural impulse activity could serve as an important instructive signal regulating Schwann cell (SC) gene expression and development.

Calcium imaging studies in the central and peripheral nervous systems have been used to detect activity-dependent axon-glial communication in the postnatal nervous system. Repetitive firing of action potentials can trigger intracellular calcium increases in glia in the frog optic nerve, possibly through the release of neurotransmitters or co-transmitters (Kreigler and Chiu, 1993). The glial cell type has not been identified, but astrocytes, which express

metabotropic glutamate receptors, are a likely candidate. Astrocytes in culture respond to glutamate application (Cornell-Bell et al., 1990) and neuronal stimulation (Dani, Chernjavsky and Smith 1992) with a sustained increase in intracellular calcium.

In the peripheral nervous system (PNS), studies at the frog neuromuscular junction have demonstrated that repetitive stimulation causes an elevation of intracellular calcium in perisynaptic Schwann cells (Jahromi et al., 1992; Reist and Smith 1992), possibly mediated by the release of acetylcholine or ATP (Robitaille 1995) from the nerve terminal. Calcium responses were not detected in other Schwann cells along the myelinated axon (Jahromi et al., 1992; Reist and Smith, 1992), or in unmyelinated nerves (Wachteler et al.,1998), suggesting that this signaling may be limited to functions associated with synaptic transmission.

Activity-dependent calcium transients have also been detected in the paranodal loops of myelinated Schwann cells of the frog sciatic nerve in response to longer duration and higher frequency stimulation (Lev-Ram et al., 1995). The authors suggested that an increase in concentration of extracellular potassium at the node of Ranvier could depolarize voltage-sensitive calcium channels on the paranodal Schwann cells.

Glia along PNS and CNS axons express a variety of receptors and channels that could in theory mediate communication between axons and glia (Verkhratsky and Kettenmann, 1996; Chiu and Kreigler review, 1994). Optic

nerve and white matter glia respond to glutamate, adenosine, ATP, histamine, acetylcholine, serotonin, and potassium with increases in intracellular calcium (Bernstein et al., 1996; Kreigler and Chiu, 1993). Schwann cells *in vitro* and *in situ* are also responsive to many of these neuroactive substances. (Lyons et al., 1994;Yoder at al., 1992; Jahromi et al., 1992: Lev-Ram et al.,1992).

Possible activity-dependent communication between axons and pre-myelinating Schwann cells during development is less well studied. It is uncertain whether there is sufficient activity-dependent release of neurotransmitters from extrasynaptic regions of premyelinated axons to influence premyelinating Schwann cells, or whether extracellular potassium concentration would be increased as it is at the node of Ranvier, where potassium channels are concentrated in the paranodal region and where extracellular space is limiting to diffusion.

Various functions have been proposed for activity-dependent interactions between axons and glia in the nervous system (Smith 1998), including buffering extracellular potassium (Lev-Ram et al., 1995; Lieberman 1991), modulating neurotransmitter release at the synapse (Jahromi et al. 1992; Reist and Smith, 1992; Robitaille 1998), regulating synaptic structure (Trachenberg and Thompson 1997), and the excitability of neurons (Newman and Zahs, 1998). During development, activity-dependent axon-glial communication has been proposed to influence glial proliferation (Barres and Raff 1993), and myelination (Stevens et al., 1998; Demerens et al., 1996,

review by Zalc and Fields, 1999), but the activity-dependent signals involved have remained elusive. In premyelinated axons, which lack both nodes of Ranvier and synapses, action potential activity might influence Schwann cells to regulate survival, proliferation, or differentiation to meet functional requirements during development, but direct evidence is lacking.

The present study focuses on the following questions: (1) Can premyelinating Schwann cells detect action potentials from axons? (2) How is action potential activity transmitted to premyelinating Schwann cells? (3) What intracellular signaling molecules are involved? (4) Is gene transcription in Schwann cells influenced by action potential firing in premyelinated axons?

METHODS

ELECTRICAL STIMULATION.

Neurons were dissociated from the DRG of fetal mice and cultured for three weeks in multi-compartment chambers equipped for electrical stimulation (Fields et al., 1992). Axons grew into the central compartment by passing beneath high resistant barriers separating two side compartments. This allowed electrical stimulation of DRG neurons at various frequencies (1-10 Hz) and patterns (0.5 sec bursts at 10 Hz every 2 sec) with brief pulses (200 nsec, biphasic pulses at 5 V) from a custom made multi-channel stimulator. The stimulus was monitored continually by an oscilloscope and by light emitting diodes in series with stimulating electrodes in each dish. Up to 24 cultures could be stimulated simultaneously inside the incubator. Electrophysiological recording in DRG neurons labeled with DiI show that only those neurons with axons traversing the barrier are stimulated to fire action potentials (Li et al., 1996)

CELL CULTURE.

DRG neurons were obtained from embryonic day 13.5 mice and plated at a density of 0.35×10^6 cells/ml into the side compartments of multi-compartment chambers. DRG cultures were maintained in medium containing 50 ng/ml nerve growth factor (NGF). Schwann cells, obtained from the sciatic

nerve of postnatal mice (P2) and cultured and purified using the Brockes method (Kleitman et al., 1991) with the following modifications: Schwann cells were cultured in medium containing 5% horse serum, and purified after 1 week using anti-Thy1.1 and complement to lyse contaminating fibroblasts. Purified Schwann cells were maintained in 5% horse serum medium, without exogenous growth factors or mitogens until used in monoculture or co-cultured into the side compartments (30,000 cells / side) of chambers containing 3-week old DRG neurons. Schwann cells in co- and monoculture, were in culture 48-72 hours and incubated overnight in serum-free medium prior to all experiments.

CALCIUM IMAGING.

Intracellular Ca^{2+} was monitored in Schwann cells co-cultured for 48-72 hours with DRG neurons that were stimulated to fire action potentials in multi-compartment chamber preparations. Confocal microscopy (BioRad MRC 1024) with the calcium-sensitive indicator fluo-3 (Molecular Probes, Eugene, OR) was used to measure changes in fluorescence intensity ($\Delta F/O_f$) due to calcium transients in Schwann cells, DRG axons and cell bodies. Measurements were carried out at room temperature in a HEPES-buffered balanced salt solution, pH 7.2. A Nikon 40 X , 0.7 n.a. long-working distance lens was used for confocal imaging of cells grown on plastic dishes. A 40X Nikon, 1.3n.a. lens was used on cells cultured on 0.17mm thick glass

coverslips. Scanning argon ion or krypton-argon lasers emitting at 488 nM were used for excitation, and imaged with a pin-hole setting of 3.2-4.5mm. The optical sectioning by confocal microscopy allowed us to distinguish calcium responses in Schwann cells from responses in neurons and axons. Calcium transients were also measured in Schwann cells growing 24-72 hour in monoculture in response to candidate signaling molecules ATP (Mol. Probes), glutamate (Sigma), and potassium chloride (60mM).

ATP MEASUREMENTS.

ATP was measured in medium collected from DRG cultures using a luciferin/luciferase biolumunescence assay (Molecular Probes, Eugene, OR) and a luminometer (EG&G, Berthold, Germany). The growth medium was replaced with serum and NGF free medium 2 hours before collecting conditioned medium from the side compartments of control DRG cultures or cultures stimulated for 30 min at different frequencies (0.1-10 Hz). Culture medium was also collected from purified Schwann cells growing in the side compartments without neurons following 10 Hz stimulation. Experimental samples were run in duplicate and compared with an ATP standard curve (0.75-100nM).

IMMUNOCYTOCHEMISTRY.

Schwann cell/DRG co-cultures (as well Schwann cells in monoculture) were stimulated electrically or pharmacologically and fixed immediately (for P-CREB, P-MAPK) or after 1 hour (for c-Fos, Krox-24, Krox-20) with 1% paraformaldehyde. Cells were permeabilized with 0.2% Triton X-100 and non-specific peroxidases were blocked with 3% hydrogen peroxide, followed by a 3% normal goat serum block. Cultures were incubated with antibody against CREB phosphorylated at Ser-133 (1:800, New England Biolabs), phosphorylated MAPK (1:800, New England Biolabs), c-Fos (1:200, Calbiochem), Krox-20, and Krox-24 (1:200 – 1:1000, Santa Cruz) for 1 hour at room temperature. Cultures were then incubated with biotinylated goat anti-rabbit antibody (Vector Labs), and localized with the ABC method. The relative intensity of the stain was quantified by using image densitometry on a video microscope (Image-1, Universal Imaging). Immunocytochemical detection of S-100 (Sigma, 1:500) was performed according to the above protocol but was detected with fluorescein-conjugated goat F(AB')2 fragment to mouse IgG (Cappel, Aurora, OH) at a 1:200 dilution.

EXPERIMENTAL DESIGN AND DATA ANALYSIS.

The relative intensity of nuclear staining after immunocytochemistry was compared by imaging densitometry from multiple culture dishes representing controls and all relevant experimental treatments. Images were

acquired from at least 15 randomly chosen fields in each culture using a Nuvicon video camera and digitized on an 8-bit scale for storage and optical densitometry using Image-1 software (Universal Imaging, West Chester, PA). Statistical analysis was based on the mean staining intensity of nuclei in each dish, determined from measurements of all Schwann cell nuclei in each field (thus, n = number of cultures). All values were normalized to the mean nuclear staining intensity of control cultures in each experiment to allow pooling of replicate experiments [arbitrary O.D. units = (nuclear staining density/average staining density of control nuclei) X 100) – 100). This yields a scale of 0-55 O.D. units from gray (unstimulated control) to pure black (Fields et al., 1997). The results were presented as mean +- SEM, and statistical comparisons were evaluated by ANOVA or two-sample t-test using the statistical analysis software Minitab (State College, PA).

RESULTS

Confocal calcium imaging was used to determine whether neural impulse activity can be detected by premyelinating Schwann cells. Changes in intracellular calcium were measured in Schwann cells co-cultured with DRG neurons that were stimulated to fire action potentials in a multi-compartment chamber preparation equipped with stimulating electrodes (see fig. 4A). This culture preparation contained purified Schwann cells from the sciatic nerve of mice, co-cultured for 48-72 hours with a pure population of mouse DRG neurons that had been in culture for 3 weeks. Schwann cells displayed a spindle-shaped morphology, were S-100 positive and were just beginning to express the 04 antigen. DRG axons were stimulated at different frequencies (1-10 Hz) and changes in fluorescence intensity of the calcium indicator fluo-3 were measured. Optical sectioning by confocal microscopy permitted unambiguous distinction of Ca^{2+} responses in Schwann cells from those in neurons and axons.

Cytoplasmic Ca^{2+} levels increased sharply in DRG neurons and axons with the onset of 10 Hz stimulation, as would be expected from depolarization of the membrane by sodium-dependent action potentials and activation of voltage-sensitive calcium channels. Within 15 – 150 sec of stimulus onset, intracellular calcium levels in several Schwann cells in each microscopic field began to increase to high levels (Fig. 4B, b-d). The latency, amplitude and

concentration dynamics of the calcium response varied in different Schwann cells, from a sustained increase to a single or repetitive low frequency oscillation in calcium concentration. The oscillations outlasted the duration of the stimulus by several minutes in some cells. These responses were seen in response to a wide range of stimulus frequencies within the normal physiological range. From 1 Hz stimulation (the lowest frequency tested) to 10 Hz, the calcium response in Schwann cells varied proportionately with the stimulus frequency. The initial slope of the calcium increase in Schwann cells was steeper in response to higher frequency stimulation; more cells in the field showed responses; the peak amplitude of the calcium increase was higher, and the latency between the neuronal and SC response was shorter than for lower frequency stimulation (Fig. 4C, c vs. a,b). These results demonstrate that pre-myelinating Schwann cells are able to detect neural impulse activity well within the normal physiological range of firing, and Schwann cells are sensitive to different frequencies of action potentials.

The delay between the Schwann cell and neuronal response suggests involvement of a soluble signaling molecule released from axons in an activity-dependent manner to stimulate an increase in intracellular calcium in Schwann cells. Bath application of glutamate to purified Schwann cells failed to elicit a calcium response over a wide range of concentrations (10-600 μM), indicating that activity-dependent release of this neurotransmitter from DRG neurons is not likely responsible for the calcium responses in Schwann cells.

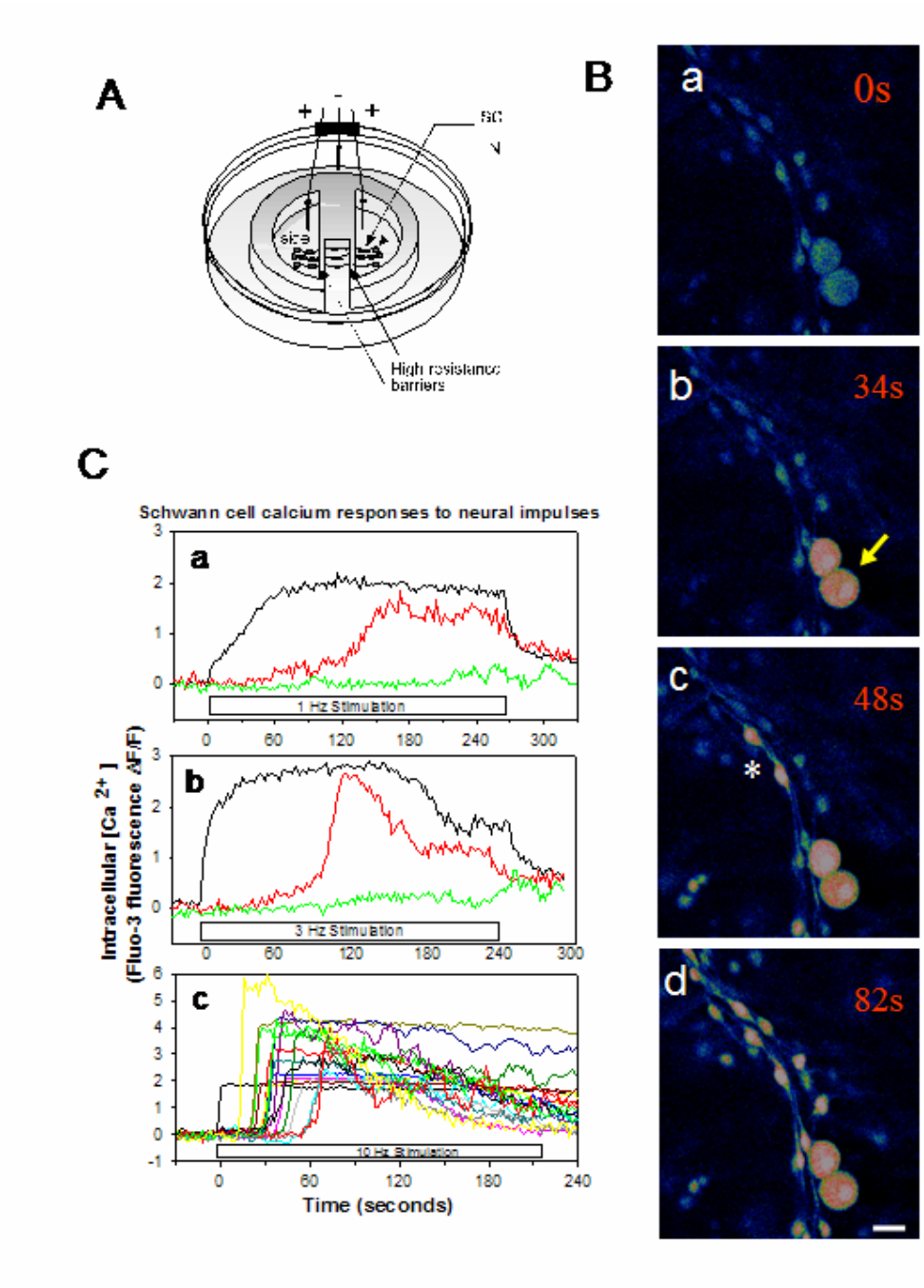
Figure 4. Activity-dependent communication between axons and pre-myelinating Schwann cells.

A. Mouse DRG axons growing beneath high resistance barriers of multicompartment cell culture chambers were stimulated by current pulses delivered through platinum electrodes. Schwann cells (SC) were plated on mouse DRG neurons (N) that were grown in culture for 3-weeks.

B. Action potentials in premyelinated axons increase intracellular Ca2+ in SCs. Scanning laser confocal microscopy was used to monitor changes in intracellular Ca2+ in SCs and DRG neurons in co-culture, using the fluorescent Ca2+ indicator fluo-3/AM. Action potentials induced an immediate influx of Ca2+ through voltage-sensitive Ca2+ channels in DRG neurons (arrow)(**34 s**). This was followed 15 to 150 s later by an increase in intracellular Ca2+ concentration in multiple SCs (*) (**48 and 82 s**).

C The Ca2+ responses for individual cells in (**B**) are plotted in (**C**) after stimulation at different frequencies (1-10 Hz). Note that Ca2+ increased in DRG neurons (**black traces**) immediately upon stimulation, but responses in SCs (**color traces**) were delayed and proportionate to the stimulus frequency between 1 Hz (**a**), 3 Hz (**b**) and 10 Hz (**c**) stimulus frequencies.

Figure 4.


A
B
C
High resistance barriers
0s
34s
48s
82s
Schwann cell calcium responses to neural impulses
1 Hz Stimulation
3 Hz Stimulation
10 Hz Stimulation
Intracellular [Ca 2+] (Fluo-3 fluorescence ΔF/F)
Time (seconds)

Depolarization with 60 mM KCl did increase intracellular calcium levels in a number of Schwann cells, consistent with the reports of voltage-sensitive calcium channels in these cells. (Ritchie et al., 1991). It is doubtful however, whether axonal stimulation at the low frequencies tested, for example 60 action potentials at 1 Hz, could produce sufficient increases in extracellular potassium concentration to depolarize Schwann cells and trigger the calcium response.

Schwann cells express P2-purinergic receptors, and ATP has been shown to elicit calcium responses in astrocytes, (Guthrie et al., 1999, Neary et al., 1991) oligodendrocytes, (Bernstein et al., 1996; Takeda et al., 1995) and myelinating and non-myelinating Schwann cells (Ansselin, et al, 1997;Green et al., 1997; Jahromi et al., 1992; Lyons et al. 1994, 1995; Mayer et al., 1997; Berti-Mattera et al., 1996; Verhraysky and Kettenman, 1996). Bath application of ATP (10 nM-1mM) to Schwann cells in monoculture induced a large increase in intracellular Ca2+ followed by sustained low frequency oscillations (see fig. 5A), which closely modeled the activity-induced response.

To test whether the activity-dependent calcium fluxes in Schwann cells were caused by ATP released from stimulated axons, Schwann cell calcium responses were measured in response to 10 Hz stimulation in co-culture with neurons in the presence of apyrase, an enzyme which degrades extracellular ATP to AMP (Guthrie et al., 1999). Before the application of apyrase, DRG neurons responded immediately to the 10 Hz stimulus and ~90 sec later,

intracellular calcium levels were elevated in a number of Schwann cells (see fig. 5Ba). Stimulation of the same field 45 minutes later in the presence of 30 U/ml apyrase completely blocked the calcium response in Schwann cells, but had no effect on the electrically-induced increase in intracellular calcium in DRG axons and cell bodies (Fig. 5Bb). Together, these results fail to support an alternative hypothesis that potassium or other diffusible molecules contributed to the activity-dependent calcium response measured in these Schwann cells, and implicate ATP as the intercellular signaling molecule.

To determine whether ATP is released extrasynaptically from DRG axons in an activity-dependent manner, conditioned medium collected from stimulated and control DRG cultures was assayed using the sensitive luciferase-bioluminescence ATP assay (Molecular Probes). Medium collected from DRG neurons stimulated at 10 Hz for 30 min showed a highly significant increase in ATP compared with unstimulated controls (n=69 experiments, $p<<0.004$) (Fig. 6A). A standard curve, calculated by linear regression of luciferase activity against a series of known ATP concentrations ($r^2 = 0.99$) indicates approximately 14.5 nM ATP in the stimulated cultures. This activity-dependent increase in ATP was completely blocked following 10 Hz stimulation in the presence of 1μM tetrodotoxin (TTX) which blocks sodium-dependent action potentials (see fig. 6A). Constant frequency stimulation was not required; a phasic pattern of stimulation (0.5 sec bursts @10 Hz every 2 sec) for 30 min also showed a significant increase in ATP levels compared

with unstimulated controls (n=57 experiments, $p<0.022$). Stimulation of DRG at lower frequencies (0.1 Hz, 30 minutes) failed to show this effect (ANOVA 0, 10 Hz, phasic, 10Hz + ttx, n = 102 experiments, $p<<0.003$ (Fig. 6A).

A timecourse experiment was performed to determine the kinetics of ATP release from DRG neurons. Electrical stimulation induced a rapid and substantial increase in ATP within the first 5 minutes electrical stimulation, which declined significantly by 30 minutes (Fig. 6B)**.** To test whether the source of ATP was neuronal, purified Schwann cells in monoculture were stimulated at 10 Hz for 30 minutes or depolarized with 60 mM KCl. There were no changes in ATP levels with either stimulus compared with controls (data not shown), indicating that Schwann cell are unable to release detectable levels of ATP in response to electrical depolarization, and they are not the source of ATP in co-cultures stimulated electrically. Furthermore, significant activity-dependent release of ATP was observed in the central axonal compartment of the chamber preparation, which lacked DRG cell bodies, suggesting that ATP was released from electrically active axons (see fig. 6C)**.**

Figure 5. Extracellular ATP mediates activity-dependent communication between neurons and SCs

A. Functional ATP receptors linked to Ca^{2+} signaling were detected by intracellular calcium imaging in premyelinating Schwann cells (SCs) monocultures in response to ATP (10μM).

B. In SCs the action potential-induced increase in intracellular Ca2+ (**color traces in a**) was blocked by electrical stimulation in the presence of 30 U/mL apyrase (**b**), but Ca2+ responses in DRG neurons (**black traces**) were not affected. Bar = 20 μM.

Figure 6. Extrasynaptic ATP release from electrically stimulated DRG neurons.

A. ATP, measured by luciferase activity assay, increased significantly in medium from DRG cultures stimulated at 10 Hz or in a phasic pattern (0.5 s bursts @ 10 Hz every 2 sec), but this was blocked by stimulation in the presence of the sodium channel blocker TTX.

B. Electrical stimulation (10 Hz) induced a rapid and substantial increase in extracellular ATP within the first 5 minutes stimulation, which declined significantly by 30 minutes.

C. Significant activity-dependent release of ATP was measured from culture medium collected from the side (cell bodies + axons) and central compartments (axons only).

Figure 5.

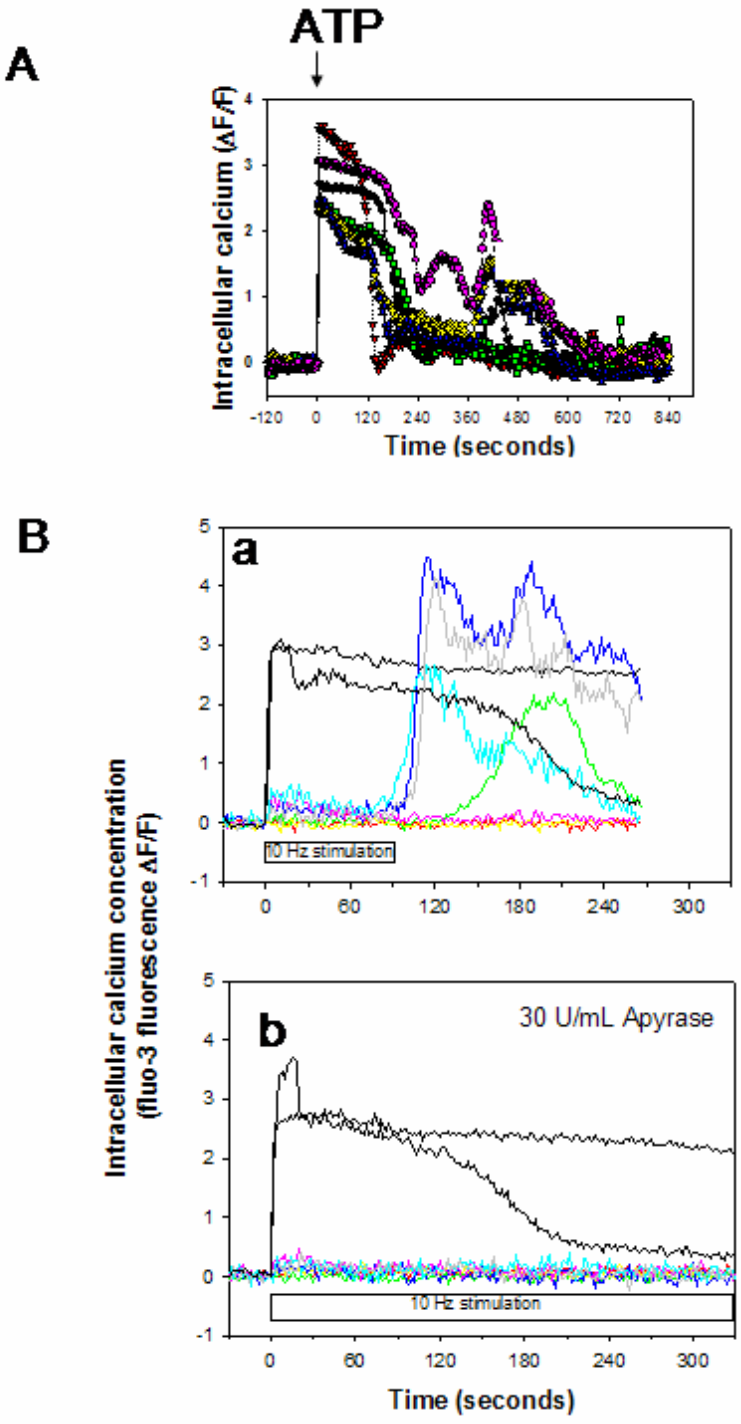
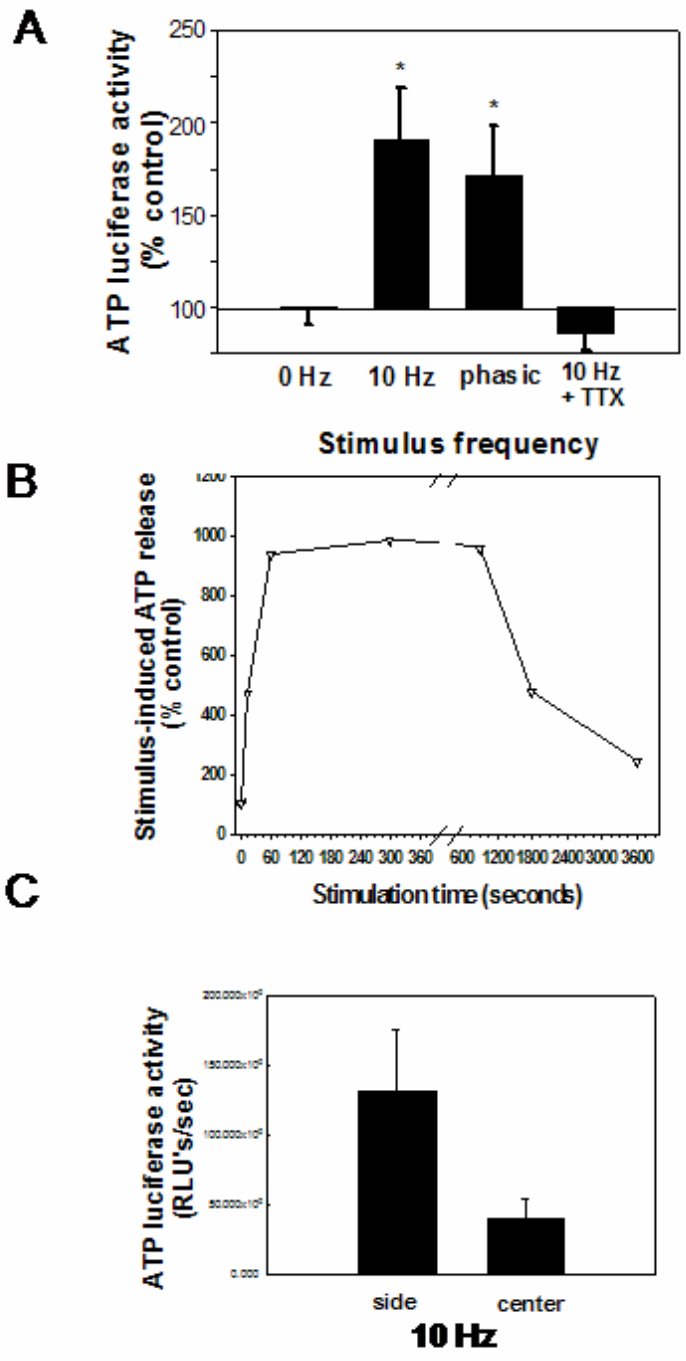

A
ATP
Intracellular calcium (ΔF/F)
Time (seconds)
B
a
10 Hz stimulation
b
30 U/mL Apyrase
10 Hz stimulation
Intracellular calcium concentration
(fluo-3 fluorescence ΔF/F)
Time (seconds)

Figure 6.


A
ATP luciferase activity
(% control)
250
200
150
100
*
*
0 Hz
10 Hz
phasic
10 Hz
+ TTX
Stimulus frequency
B
Stimulus-induced ATP release
(% control)
1000
800
600
400
200
0
0 60 120 180 240 300 360 600 1200 1800 2400 3000 3600
Stimulation time (seconds)
C
ATP luciferase activity
(RLU's/sec)
side
center
10 Hz

Calcium signals can induce long-term cellular responses by regulating the function of several transcription factors, thus leading to the induction of late-onset genes. cAMP response element binding protein (CREB) is an important transcription factor mediating calcium-dependent gene expression. Using a phospho-specific antibody, we observed that phosphorylation of CREB (P-CREB) at the regulatory site, Ser-133, was significantly increased in Schwann cells on axons stimulated at 10 Hz compared with unstimulated controls ($p<<0.001$, n=28 exps) (see fig. 7A, a and c). This effect was completely blocked by stimulation in the presence of 1μM TTX (Fig. 7B). Levels of P-CREB were elevated significantly by 30 min of stimulation, and returned to basal levels within 90 minutes after stopping stimulation ($p < 0.001$; n = 17 exps) (Fig. 7B). A phasic pattern of stimulation (0.5 sec bursts @ 10 Hz every 2 sec) delivered for 30 min also significantly increased P-CREB levels in Schwann cells (Fig 7B). At least two signaling pathways are involved in the phosphorylation of CREB in Schwann cells on electrically-stimulated axons. Electrical stimulation in the presence of the CaM kinase inhibitor KN62, or in the presence of the MEK-1 inhibitor PD098059, significantly inhibited CREB phosphorylation in Schwann cells relative to controls stimulated at 10 Hz ($p < 0.000$, n = 16 exp for KN62 and n = 14 experiments for PD098059) .

Several lines of evidence suggest that phosphorylation of CREB in Schwann cells in these experiments is the result of activity-dependent

release of ATP from axons. ATP of various concentrations (10nM-100μM) applied to purified Schwann cells in mono and DRG co-cultures caused a dose-dependent increase in CREB phosphorylation (ANOVA 0 μM, 10 μM, 100μM, n = 8, $p<0.023$) (see fig. 8A), and 10nM ATP, which we have shown to increase intracellular calcium levels in Schwann cell, significantly increased P-CREB levels compared with unstimulated controls (n=6, $p<0.024$). Electrical stimulation in co-cultures in the presence of apyrase, which degrades extracellular ATP (27 U/ml), blocked the increase in CREB phosphorylation in Schwann cells but not neurons, as did pre-incubation in 30 μM suramin, a purinergic antagonist, which blocks P2X and Y receptors (Fig. 8B).

Figure 7. Neural impulse activity phosphorylates CREB in associated pre-myelinating Schwann cells.

A. Electrical stimulation of DRG axons caused phosphorylation of CREB (c) compared with unstimulated co-cultures (a). Responses to forskolin (b) was used as a positive control. The staining intensity of Schwann cells was compared quantitatively by imaging densitometry.

B. CREB was phosphorylated to significantly higher levels in Schwann cells on axons stimulated at 10 Hz or in a phasic pattern (0.5 s pulses @ 10 Hz every 2 s), but this response was blocked by stimulation in the presence of TTX. Levels of CREB phosphorylation returned to baseline 60 min. after stopping axonal stimulation.

Figure 8. Activity-dependent CREB phosphorylation is mediated by extracellular ATP.

A. ATP of various concentrations (10nM-100μM) applied to purified Schwann cells in mono and DRG co-cultures caused a dose-dependent increase in CREB phosphorylation

B. CREB phosphorylation in Schwann cells in response to axonal stimulation at 10 Hz was blocked by stimulation in the presence of the P2Y receptor antagonist suramin (surm) or the enzyme apyrase (apyr), which degrades extracellular ATP.

Figure 7.

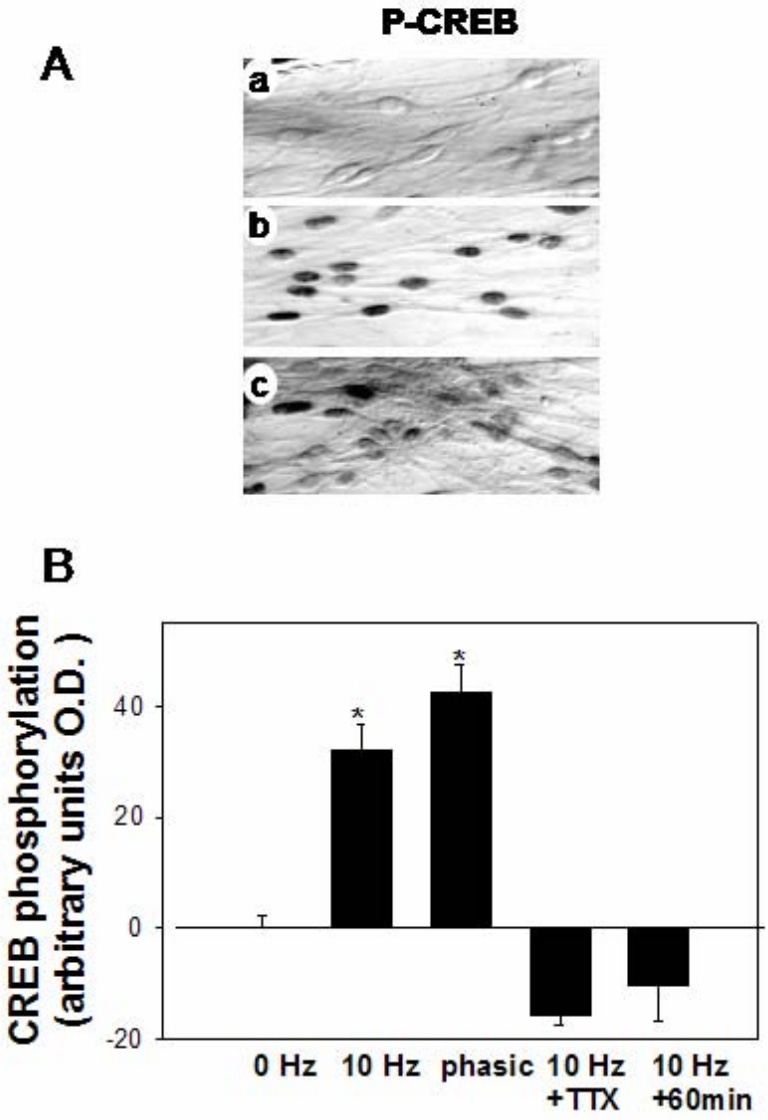
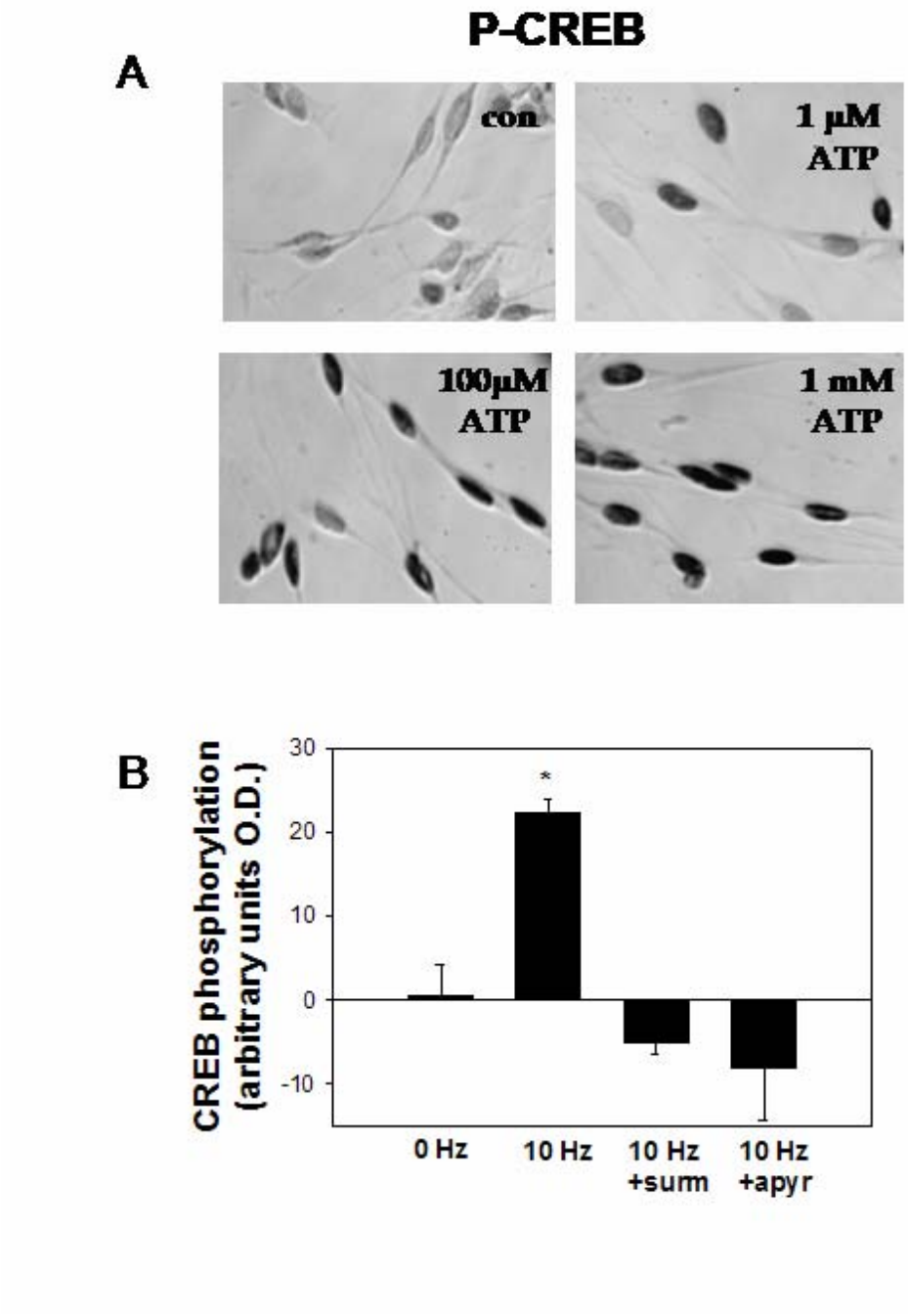

A
P-CREB
a
b
c
B
CREB phosphorylation
(arbitrary units O.D.)
40
20
0
-20
*
*
0 Hz
10 Hz
phasic
10 Hz
+TTX
10 Hz
+60min

Figure 8.


P-CREB
A
con
1 µM ATP
100µM ATP
1 mM ATP
B
CREB phosphorylation (arbitrary units O.D.)
30
20
10
0
-10
*
0 Hz
10 Hz
10 Hz +sum
10 Hz +apyr

Activity-dependent regulation of gene expression in Schwann cells could have important implications during development and regeneration, when impulse activity plays an important role. The promoter region of the immediate early gene c-*fos*, which has been implicated in adaptive responses of cells to extracellular stimulation, contains one CRE that binds CREB (Sheng et al., 1990). The c-*fos* gene codes a transcription factor that binds to AP-1 sites regulating transcription of several late onset genes. Acute application of ATP (30 min) significantly increased c-fos mRNA in purified SCs (see fig 9A). Using immunocytochemical staining, we found that c-Fos levels in nuclei of Schwann cells were increased significantly in neuronal cultures stimulated at 10 Hz for 30 min, and this effect was completely blocked when stimulation was performed in the presence of 27U/ml of apyrase (Fig. 9C) ($p < 0.000$; $n = 30$ exp). In addition, application of exogenous ATP (100μM) also induced c-Fos expression in purified Schwann cells (Fig. 9C).

The zinc-finger transcription factor *krox-24* is expressed in non-myelinating Schwann cells, where it has been implicated in the control of proliferation and differentiation of Schwann cells (Topilko et al., 1997). *Krox-24* has an AP-1 site in its promoter region, and experiments in striatal and neocortical neurons (Dragunow et al., 1994) report that expression of *krox-24* is blocked by antisense oligonucleotides against c-*fos*. Direct stimulation of purified SC monocultures with ATP (100μM, 30 min) resulted in a significant increase in *krox-24* mRNA, as determined by RT-PCR (see fig. 9A).

Importantly, we found that DRG axons firing at 10 Hz for 30 min significantly up-regulated nuclear Krox-24 protein levels in Schwann cells, and this activity-dependent increase was blocked with apyrase (Fig.9B, C). In addition, Schwann cells in monoculture stimulated with 100 μM ATP for 15 min significantly increased Krox-24 protein levels (Fig. 9C). Interestingly, another zinc-finger transcription factor, Krox-20, has been reported to have a reciprocal pattern of expression to Krox-24 during development of Schwann cells. Stimulation of DRG neurons at 10 Hz and with ATP increased expression of Krox-24, but it failed to induce expression of Krox-20 in Schwann cells (not shown).

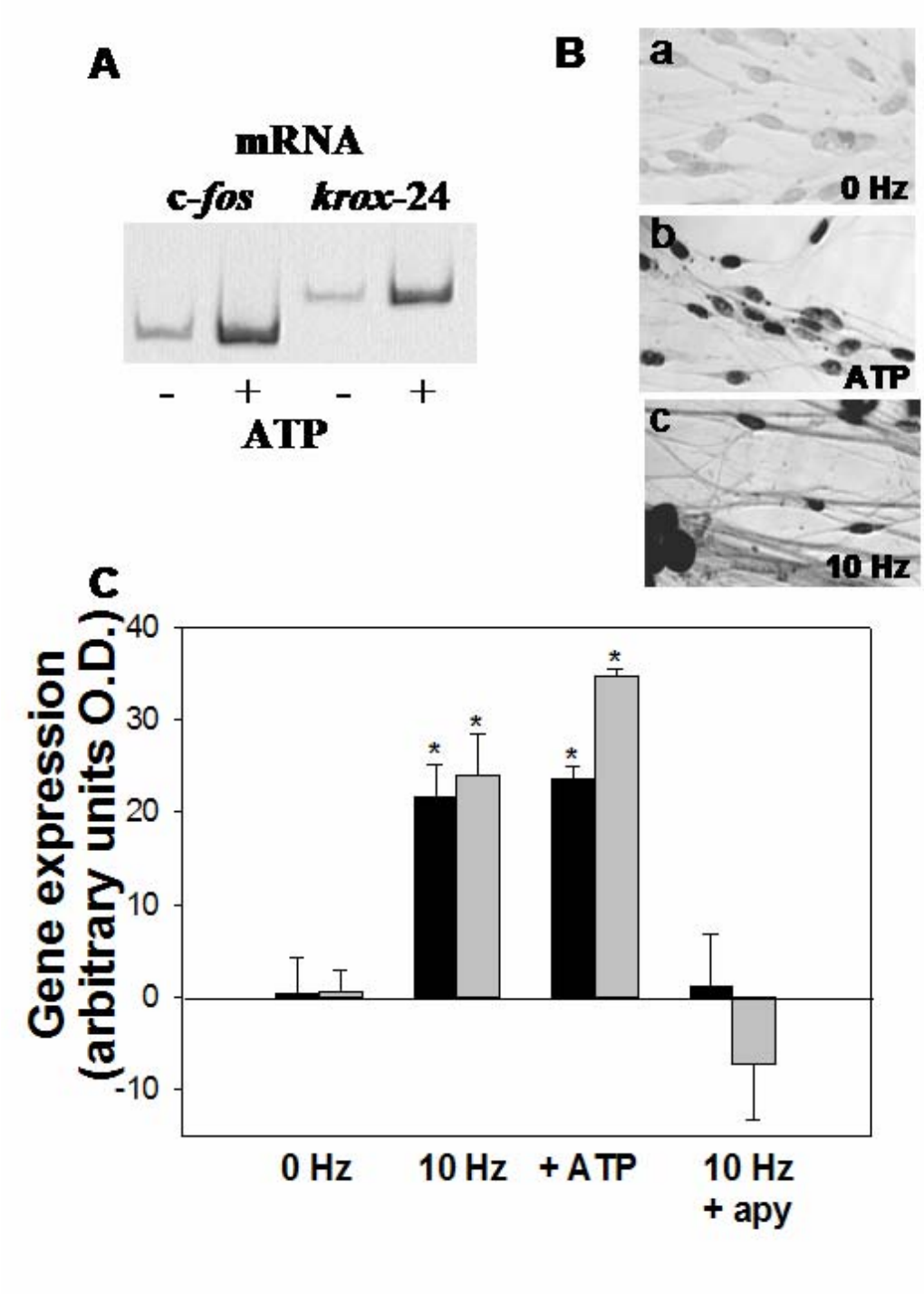
Figure 9. Action potentials induce gene expression in SCs by the release of ATP.

A. ATP (100μM, 15 minutes) stimulates expression of c-fos and krox-24. The ATP-induced increase in mRNA transcripts for both genes is shown by RT-PCR.

B Immunocytochemical staining for Krox 24 expression in DRG/Schwann cell co-cultures was increased significantly in Schwann cells in response to 100 μM ATP (b), or axonal stimulation (c).

C. Immunocytochemical staining for c-Fos (**black bars**) and Krox-24 (**grey bars**), showed an increase in nuclear expression of these genes in SCs on axons stimulated 30 min at 10 Hz (**10 Hz**) compared with unstimulated axons (**0 Hz**). Direct application of ATP (100 μM, 15 min) stimulates expression of both genes (**+ ATP**), and electrical stimulation in the presence of the ATP-degrading enzyme apyrase (27 U/mL) blocks the response (**10 Hz + apy**). No increase in a gene associated with myelinating phenotype, Krox-20, was detected in SCs in response to electrical stimulation or ATP application (data not shown). For Krox-24, $p = 0.000$, one-way ANOVA, $n = 28$; *$p < 0.005$ vs. control; for c-Fos $p < 0.006$; one-way ANOVA, $n = 19$; *$p < 0.05$ vs. control.

Figure 9.


A
mRNA
c-fos
krox-24
- + - +
ATP
B
a
0 Hz
b
ATP
c
10 Hz
c
Gene expression (arbitrary units O.D.)
40
30
20
10
0
-10
*
0 Hz
10 Hz
+ ATP
10 Hz + apy

DISCUSSION

The present observations indicate that ATP can act as an activity-dependent signal between premyelinated axons and Schwann cells. ATP is released non-synaptically from DRG neurons in a frequency-dependent manner, and it acts on Schwann cell purinergic receptors to increase intracellular calcium to regulate the transcription factor CREB and expression of c-*fos* and *Krox-24* genes in Schwann cells.

ATP AS AN AXON-SCHWANN CELL SIGNALING MOLECULE

Activity-dependent release of ATP has been detected from synaptic terminals (Wieraszko et al., 1989; Von Kugelgen et al., 1998), but ATP release from non-synaptic regions of myelinated, unmyelinated or premyelinated axons has not been shown previously. Release of the neurotransmitter glutamate from non-synaptic regions of axons has been detected in response to electrical stimulation of frog sciatic nerve (Weinreich and Hammerschlag, 1975;Wheeler et al., 1965) and optic nerve (Weinreich and Hammrschlag, 1975), and activity-dependent non-synaptic release of adenosine has also been shown in studies if the rabbit vagus nerve (Maire et al., 1984). At the NMJ, electrically-induced calcium responses in perisynaptic Schwann cells are inhibited by exogenous ATP or P2 receptor antagonists (Robitaille, 1995), consistent with the release of endogenous ATP from these synapses. In the

CNS, intense stimulation of brain slices has been reported to release ATP non-synaptically (Hamann and Atwell 1996), but this has been interpreted to be a result of neural injury, because the ATP release required intense stimulation (70- 100 V at 70 – 300 Hz) and release was not blocked by TTX. In the present experiments, action-potential-dependent ATP release was measured directly from DRG cultures that lack synapses. There is no evidence that stimulation of these cultures for up to 5 days causes neuronal injury or death (Itoh et al., 1995). In contrast to the hippocampal slice studies, minimal stimulation intensity (5V), frequency (1 Hz), and duration (1 min) induced the calcium response, and blocking sodium-dependent action potentials with TTX prevented the release of ATP and phosphorylation of CREB.

Schwann cells *in vitro* and *in vivo* express purinergic (P2) receptors which are linked to intracellular calcium stores and other signal transduction pathways (Ansselin et al., 1997; Berti-Mattera et al., 1996; Green et al., 1997; Lyons et al., 1995; Verkhratsky and Kettenman, 1996). Studies of biopsied human peripheral nerve (Wachteler, Mayer et al., 1996, Mayer, Quasthoff, Grafe 1998), and excised mammalian peripheral nerve indicate the presence of purinergic receptors on Schwann cells (Mayer, Wuasthoff, Grafe 1998; Mayer, Wachtler, Kamleiter, Grafe 1997); with the $P2Y_2$ subtype predominating on myelinating Schwann cells and the $P2Y_1$ subtype on non-myelinating Schwann cells (Mayer, Quasthoff, Grafe, 1998). The receptors are lost over several days in culture, but direct axon contact or stimulation with cAMP can prevent this

loss (Lyons, Morell, McCarthy 1994, 1995). In the present study, bath application of ATP to Schwann cell mono and co-cultures elicited a robust calcium response, indicating that the majority of Schwann cells in our system had not lost the ATP receptors. The presence of these receptors *in vivo* has also been shown by focal application of ATP, which stimulates calcium increases in paranodal Schwann cells of mammalian nerve (Wachteler et al., 1996; Lyons et al., 1995; Mayer et al., 1997), and perisynaptic Schwann cells at the amphibian neuromuscular junction (Jahromi, Robitaille, Charlton 1992; Robitaille 1995).

INTRACELLULAR SIGNALING FROM ACTION POTENTIALS

The results show that action potentials activate calcium-dependent intracellular signaling pathways leading to phosphorylation of the transcription factor CREB in Schwann cells. Although the multiple signaling pathways regulating CREB phosphorylation in neurons are understood in some detail, comparatively less is known of the mechanisms underlying CREB phosphorylation in glia. The MAPK/RSK pathway can mediate CREB phosphorylation in response to calcium, PKC, and growth factors in oligodendrocyte progenitor cells (Pende et al., 1996), and PKA activation in response to β-adrenergic stimulation can phosphorylate CREB in differentiated oligodendrocytes (Sato-Bigbee et al., 1998). In Schwann cells, axolemma fraction, acting through PKA and PKC pathways (Lee et al.,1999), or beta-

neuregulin acting through the MAPK pathway (Tabernerro et al., 1998), can phosphorylate CREB. The possible activity-dependent phosphorylation of CREB in glia or ATP-induced activation of this transcription factor in Schwann cells has not been investigated previously.

Three lines of evidence support the hypothesis that the activity-dependent release of ATP from peripheral axons as the mechanism stimulating CREB phosphorylation in premyelinated Schwann cells. First, the activity-induced CREB phosphorylation was blocked when purinergic receptors were blocked with suramin. Secondly, the activity-dependent effect was blocked when we included the ATP-degrading enzyme apyrase in the medium during electrical stimulation. Third, we have found that direct stimulation of Schwann cells with ATP, significantly increased P-CREB levels in a dose-dependent manner. The reduced CREB phosphorylation resulting from stimulation in the presence of the specific inhibitors of MEK or calmodulin-dependent kinases, suggest that both the CaM Kinase and MAPK pathways regulate CREB phosphorylation in Schwann cell following neural impulse activity.

REGULATION OF SCHWANN CELL GENE EXPRESSION BY NEURAL IMPULSES

The regulation of Schwann cell development is ultimately determined by signals from the axon, and identifying transcription factors which may be downstream targets of such signals is being actively pursued by a number of laboratories. An array of transcription factors including members of the Jun

family (c-Jun, Jun-B and D), CREB, POU domain proteins Oct-6 (SCIP), and the zinc-finger proteins Krox -20 and Krox24/Zif268/Egr-1 have been associated with Schwann cell proliferation and differentiation (see review by Mirsky and Jessen, 1999; Scherer , 1997). Each of these transcription factors has a unique expression pattern in Schwann cells during development, and may regulate Schwann cell phenotype through the activation of target genes controlling Schwann cell function. Neural impulse activity begins in DRG neurons at about E17 in the rat (Fitzgerald & Fulton, 1992). This corresponds to the time when pre-myelinated Schwann cells undergo a sharp decrease in proliferation and begin to differentiate into either myelinating or non-myelinating phenotypes (Stewart et al., 1993). The O4 antigen, which was just beginning to be expressed in these Schwann cell cultures, first appears at E16 in the rat. Activity-dependent regulation of glial gene expression has been detected in the optic nerve (Mack and Chiu, 1994), and in perisynaptic Schwann cells at the frog NMJ (Georgiou et al, 1994), but there is no evidence to date of activity-dependent regulation of genes in this critical developmental period of transition for pre-myelinating Schwann cells.

In this study we have shown that axonal stimulation at 10 Hz induces the expression of the immediate early gene c-*fos* in Schwann cells. Stimulation of co-cultures in the presence of apyrase (27 U/ml) blocked the activity-dependent induction of c-*fos*, and exogenously applied ATP (100uM, 15 min) directly stimulated c-*fos* expression in Schwann cells (Fig.9C). Taken together

these results suggest that Schwann cell gene expression can be regulated by ATP released from DRG axons in an activity-dependent manner. Although c-Fos may play a role during Wallerian degeneration (Liu et al, 1995), it does not yet have an associated function in developing Schwann cells. c-*fos* is a stress response gene which can respond rapidly to extracellular signals, such as action potential activity. c-Fos can dimerize with Jun proteins to bind to AP-1 sites of possible intermediate or target genes regulating Schwann cell development (proliferation, survival, differentiation, and myelination).

There is emerging evidence that both *krox-20* and *krox-24* are important regulators of Schwann cell development and differentiation, and c-*fos* may have a role in regulating these genes. c-Fos has been shown to suppress the zinc-family transcription factor, *krox-20*, which contains an AP-1 site in its promoter (Gius, D et al., 1990), and that c-*fos* may be necessary for induction of *krox-24* in some cells (Dragunow et al., 1994). We have shown that stimulation at 10 Hz and with exogenous ATP significantly increases the expression of Krox 24 in Schwann cells. Conversely, stimulation under the same conditions fails to increase Krox 20. *krox-24* has been implicated in cell proliferation (Topilko et al., 1994), and *krox-20* has a critical role in myelin formation. Knock-out of the *krox-20* gene leads to complete inhibition of compact myelin formation, and the subsequent expression of the myelin genes, Po and MBP (Topilko et al, 1994). Recent work by Zorick et al. has shown that SCIP, a POU domain transcription factor, which are expressed transiently

just prior to myelination, is significantly up-regulated in *krox-20* mutants. In these mice, Schwann cells are arrested in pro-myelinating stage, they continue to proliferate, and undergo increased apoptosis. It was concluded that Krox 20 controls SCIP expression, and without it, SCIP is incapable of self-down-regulation. Krox-24 expression is generally restricted to the non-myelinating, proliferating Schwann cells (Topilko et al., 1997). Interestingly, there is a narrow window at birth when the two genes are co-expressed in Schwann cells, and it has been suggested (Topilko et al., 1997) that this balance of expression between *krox-24* and *krox-20* may participate in the choice between myelinating and non-myelinating pathways. Neural impulse activity, which we have shown to up-regulate *krox-24* expression, and not *krox-20* in premyelinating Schwann cells in culture, may serve as one signal influencing this phenotypic decision. Similarly, the action potential-dependent release of ATP from premyelinated axons might contribute to regulation of Schwann cell proliferation or apoptosis during development. Future research on activity-dependent regulation of other genes implicated in phenotypic differentiation of Schwann cells will be helpful in providing a better understanding of the functional consequences of this phenomenon.

CONCLUSIONS

1. Pre-myelinating Schwann cells can detect action potential activity along sensory axons.

2. The activity-dependent signal between axons and pre-myelinating SCs was identified as extracellular ATP.

3. Activity-dependent release of ATP phosphorylates CREB, and activates the transcription factors, c-fos and krox-24 in premyelinating SCs.

CHAPTER 3

INTRODUCTION

Extracellular ATP was recently identified as a potent activity-dependent signal between axons and pre-myelinating Schwann cells (SCs) (Stevens and Fields, 2000). Axonal release of ATP induces calcium transients, and gene expression in associated SCs, and thus could serve as an instructive signal regulating SC development before the onset of myelination. One of the functional consequences of this activity-dependent axon-SC communication could be in regulating SC proliferation. The onset of high frequency stimulus-evoked firing along developing sensory axons corresponds to the pre-myelinating period (Fitzgerald, 1987), when embryonic SCs are undergoing dramatic changes in proliferation in response to axon-derived signals.

Earlier studies in the developing CNS suggest that electrical activity stimulates proliferation of myelinating glia (Barres and Raff, 1993). Blockade of axonal firing in developing optic nerve significantly inhibited oligodendrocyte progenitor cell (OPC) proliferation. The mechanism was thought to involve activity-dependent release of growth factors (PDGF) from neighboring astrocytes; however the activity-dependent axonal signal was not identified. The effects of neural impulse activity on SC proliferation and development are not known, but ATP has been implicated in regulating the

proliferation of other types of glial cells. Extracellular ATP can potently stimulate astrocyte proliferation (Neary et al., 1998; Franke et al., 1999; Neary et al., 1999b), both in culture (Rathbone et al., 1992; Abbracchio et al., 1994; Neary et al., 1994b) and *in vivo* (Franke et al., 1999; Franke et al., 2001). Activation of ATP/P2 receptors have also been shown to stimulate proliferation of Muller glia (Moll et al., 2002; Sanches et al., 2002; Milenkovic et al., 2003), as well as microglia, but it is not known whether ATP regulates SC proliferation.

REGULATION OF SCHWANN CELL PROLIFERATION

SCs continue to divide after migration into peripheral nerves. *In vivo* studies in the rat sciatic nerve demonstrated a rise in SC proliferation between E17-19 (Stewart et al., 1993). Direct contact of SCs with the axolemma, sustains SC proliferation until initiation of axon ensheathment, and a number of soluble mitogenic signals have been identified (Salzer et al., 1980; DeVries et al., 1982; Ratner et al., 1985). Release of ATP from electrically active axons might stimulate SC proliferation directly or indirectly by stimulating the secretion of mitogens.

While significant progress has been made in identifying factors that stimulate Schwann cells to divide, much less is known about the extracellular signals that inhibit SC proliferation *in vivo*, and *in vitro*. Just before birth, pre-myelinating SCs undergo a dramatic decline in their proliferation rate, which is

a requisite for induction of SC differentiation and myelination (Asbury, 1967; Stewart et al., 1993). Mechanisms that arrest SC proliferation could indirectly regulate myelination by enabling SCs to respond to myelin-inducing signals at the appropriate time. *In vivo* recordings from embryonic rat DRG neurons reveal that the onset of low frequency stimulus-evoked firing (10-20 Hz) occurs ~E17, and the frequency of firing, and the total number of impulses increase steadily between E17 and birth (Fitzgerald, 1987), when pre-myelinating exit the cell cycle and differentiate into myelinating or non-myelinating phenotypes. A specific aim of this research was to determine whether action potential activity can influence SC proliferation at the pre-myelination stage, and determine whether ATP release from electrically active axons could be involved.

REGULATION OF NUCLEAR CELL CYCLE PROTEINS IN GLIA

Major progress has been made in understanding the signals and proteins involved in the regulation of the cell cycle in oligodendrocyte progenitor cells (OPCs), astrocytes, and more recently, Schwann cells (Stevens and Fields, 2002). Cell proliferation is regulated by coordinated interactions between multiple proteins at distinct phases of the cell cycle. The three principal players are the cyclins, (D, E, A and B), cyclin-dependent kinases (Cdks), and cyclin dependent kinase inhibitors (CKIs) (see fig.10).

Growth factors and axonal mitogens stimulate quiescent glial cells to enter the G1 phase of the cell cycle by inducing expression of the D-type cyclins. These cyclins subsequently assemble with their catalytic partners, the cyclin-dependent kinases (cdk4 or cdk6). Cyclin D-cdk complexes are required for G1 progression, and serve to couple cell cycle machinery with extracellular signals. The sequential activation of different Cdks coordinates the orderly progression through the cell cycle. Negative regulation of the cell cycle is mediated by two families of cyclin-dependent kinase inhibitors (CKIs), the Cip/Kip family (p21, p27 p57) or Ink family (p15, p16, p18, p19) (Sherr and Roberts, 1999). All of these proteins bind and inhibit specific cyclin-Cdk complexes, giving an additional level of control of the cell cycle. Transition from G1 into S phase (DNA synthesis phase) is a critical cell cycle restriction point in glial cells. Progression to S phase in Schwann cells and OPCs requires formation and activation of cyclin-Cdk complexes (Tikoo et al., 2000; Ghiani and Gallo, 2001), and the subsequent phosphorylation (inactivation) of the retinoblastoma protein (pRb). CKIs can prevent entry into S phase, and induce cell cycle arrest or exit by binding to these G1 cyclin-Cdk complexes.

While there is growing evidence that purinergic signaling molecules regulate glial proliferation, it is not known whether activation of purinergic receptors regulates proteins that directly control cell cycle progression. The present study uses a DRG/SC co-culture system equipped with stimulating electrodes, to study the effects of action potential activity and ATP on

Schwann cell proliferation and, if apparent determine which aspects of cell cycle progression and regulation are affected.

Figure 10.

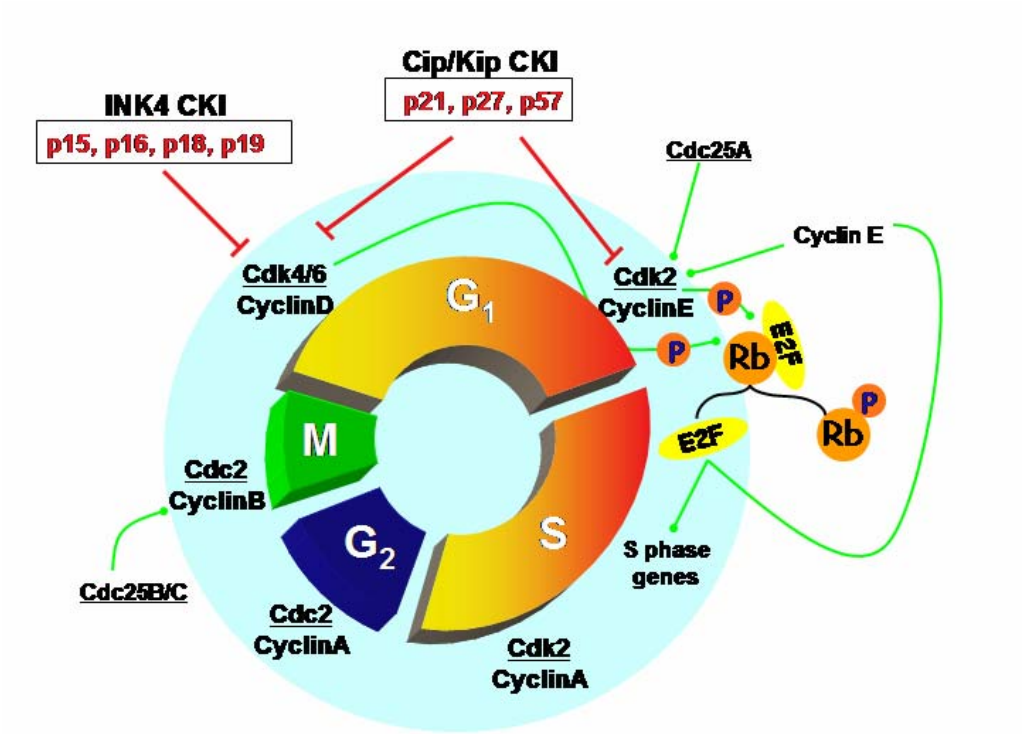


Figure 10. Glial cell proliferation is regulated by coordinated interactions between multiple proteins at distinct phases of the cell cycle.

The cyclins (D, E, A and B) assemble with their catalytic partners, the cyclin-dependent kinases (Cdk2, 4, 6 and cdc2), which are positively regulated by cyclin-cdk activating kinases (not shown), and via the serine/threonine phosphatase, cdc25. Negative regulation is mediated cyclin-dependent kinase inhibitors (CKIs), from the INK4 or Cip/Kip family, which bind to cyclin/cdk complexes. Progression to S phase requires the phosphorylation (inactivation) of retinoblastoma protein (pRb) by cyclinE-cdk2, which permits the E2F family of transcription factors (E2F) to regulate key S phase gene expression.

METHODS

CELL CULTURE.

DRG neurons were obtained from embryonic day 13.5 mice and plated at a density of $0.35x10^6$ cells/ml into the side compartments of multicompartment chambers. DRG cultures were maintained in medium containing 50ng/ml nerve growth factor (NGF). Schwann cells, obtained from the sciatic nerve of postnatal mice (P2) were cultured and purified using the Brockes method (Kleitman et al., 1991) with the following modifications: Schwann cells were cultured in medium containing 5% horse serum, and purified after 1 week using anti-Thy1.1 and complement to lyse contaminating fibroblasts. Purified Schwann cells were maintained in 5% horse serum medium, without exogenous growth factors or mitogens until used in monoculture or co-cultured into the side compartments (30,000 cells / side) of chambers containing 3-week old DRG neurons. Schwann cells in co- and monoculture, were in culture 48-72 hours and incubated overnight in serum-free medium prior to all experiments.

ELECTRICAL STIMULATION.

Neurons were dissociated from the DRG of fetal mice and cultured for three weeks in multicompartment chambers equipped for electrical stimulation

(Fields et al., 1992). Axons grew into the central compartment by passing beneath high resistant barriers separating two side compartments. This allowed electrical stimulation of DRG neurons at various frequencies (1-10 Hz) and patterns (0.5 sec bursts at 10 Hz every 2 sec) with brief pulses (200 nsec, biphasic pulses at 5 V) from a custom made multi-channel stimulator. The stimulus was monitored continually by an oscilliscope and by light emitting diodes in series with stimulating electrodes in each dish. Up to 24 cultures could be stimulated simultaneously inside the incubator. Electrophysiological recording in DRG neurons labeled with DiI show that only those neurons with axons traversing the barrier are stimulated to fire action potentials (Li et al., 1996).

PROLIFERATION ASSAY

SCs were co-cultured with DRG neurons, and axons were electrically stimulated at 10 Hz for 1-24 hrs. Purified SCs 48 hours in monoculture were serum starved for 18-24 hours before treatment with growth factors (10ng/mL PDGF or 250ng/mL B heregulin + 2μM forskolin) +/- ATP). Co and mono-cultures were pulsed with BrdU (Boehringer Mannheim) for 6 hrs, fixed and stained according to manufacturer instructions. Cultures were then counterstained with Hoechst nuclear stain (Molecular Probes) at a dilution of 1:2000 for 10 min. Proliferation rate was calculated as the ratio of BrdU/Hoechst positive SC nuclei/ microscope field. All SC nuclei stained

with BrdU and Hoechst were counted in each microscope field. 10-15 randomly chosen fields were sampled to obtain a mean for each culture well; n = number of culture wells.

TUNEL ASSAY

For the apoptosis assay, SCs were fixed with 4% paraformaldehyde for 1 hour following treatment. Cells were stained for TUNEL according to manufacturer's protocol (Roche), and the % of TUNEL-positive cells was determined from the ratio of TUNEL/Hoechst-positive cells/microscope field.

IMMUNOCYTOCHEMISTRY.

Schwann cell/DRG co-cultures (as well Schwann cells in monoculture) were stimulated electrically or pharmacologically and fixed immediately with 1% paraformaldehyde. Cells were permeabilized with 0.2% Triton X-100 and non-specific peroxidases were blocked with 3% hydrogen peroxide, followed by a 3% normal goat serum block. Cultures were incubated with antibody against cyclin D1, Cyclin E (1:100) p21 and p27 (Santa Cruz 1:500) for 1 hour at room temperature. Cultures were subsequently incubated with biotinylated goat anti-rabbit antibody (Vector Labs), and localized with the ABC method. The relative intensity of the stain was quantified by using image densitometry on a video microscope (Image-1, Universal Imaging). Immunocytochemical detection cyclin D was detected with fluorescein-conjugated goat F(ab')2 fragment to rabbit IgG (Jackson Immunoresearch. 1:100).

EXPERIMENTAL DESIGN AND DATA ANALYSIS.

The relative intensity of nuclear staining after immunocytochemistry was compared by imaging densitometry from multiple culture dishes representing controls and all relevant experimental treatments. Images were acquired from at least 15 randomly chosen fields in each culture using a Nuvicon video camera and digitized on an 8-bit scale for storage and optical densitometry using Image-1 software (Universal Imaging, West Chester, PA). Statistical analysis was based on the mean staining intensity of nuclei in each dish, determined from measurements of all Schwann cell nuclei in each field (thus, n = number of cultures). All values were normalized to the mean nuclear staining intensity of control cultures in each experiment to allow pooling of replicate experiments [arbitrary O.D. units = (nuclear staining density/average staining density of control nuclei) X 100) – 100). This yields a scale of 0-55 O.D. units from gray (unstimulated control) to pure black (Fields et al., 1997). The results were presented as mean +- SEM, and statistical comparisons were evaluated by ANOVA or two-sample t-test using the statistical analysis software Minitab (State College, PA).

RESULTS

ACTIVITY-DEPENDENT ATP RELEASE INHIBITS SCHWANN CELL PROLIFERATION

To determine the effects of impulse activity on SC proliferation, pre-myelinating SCs were co-cultured with DRG neurons for 2 days, and SC proliferation was determined following electrical stimulation at developmentally relevant patterns and durations using a BrdU incorporation assay (see fig. 11A)**.** As shown in Figure 11B, SC proliferation was significantly reduced following brief (1 hr) stimulation at all frequencies tested (0.1, 1, and 10 Hz). Chronic stimulation at 10 Hz also inhibited SC proliferation, but the inhibition was not significantly different from the 1 hour timepoint (ANOVA $p<0.000$, $n=66$)(Fig. 11B), indicating that only brief intervals of axonal firing are required to produce inhibitory effects on SC proliferation.

Moreover, direct application of ATP (300μM) for 24 hours, inhibited SC proliferation in SC-DRG co-cultures ($p<0.0009$, $n=76$) (Fig. 11C) and in purified SCs grown without axons ($p<0.009$, $n=22$). Importantly, electrical stimulation in the presence of apyrase (30U/ml), an enzyme that rapidly degrades extracellular ATP (Guthrie et al., 1999), completely inhibited the activity-dependent inhibition of SC proliferation (Fig. 11C). Taken together,

our results implicate ATP as the activity-dependent signal inhibiting SC proliferation in our system.

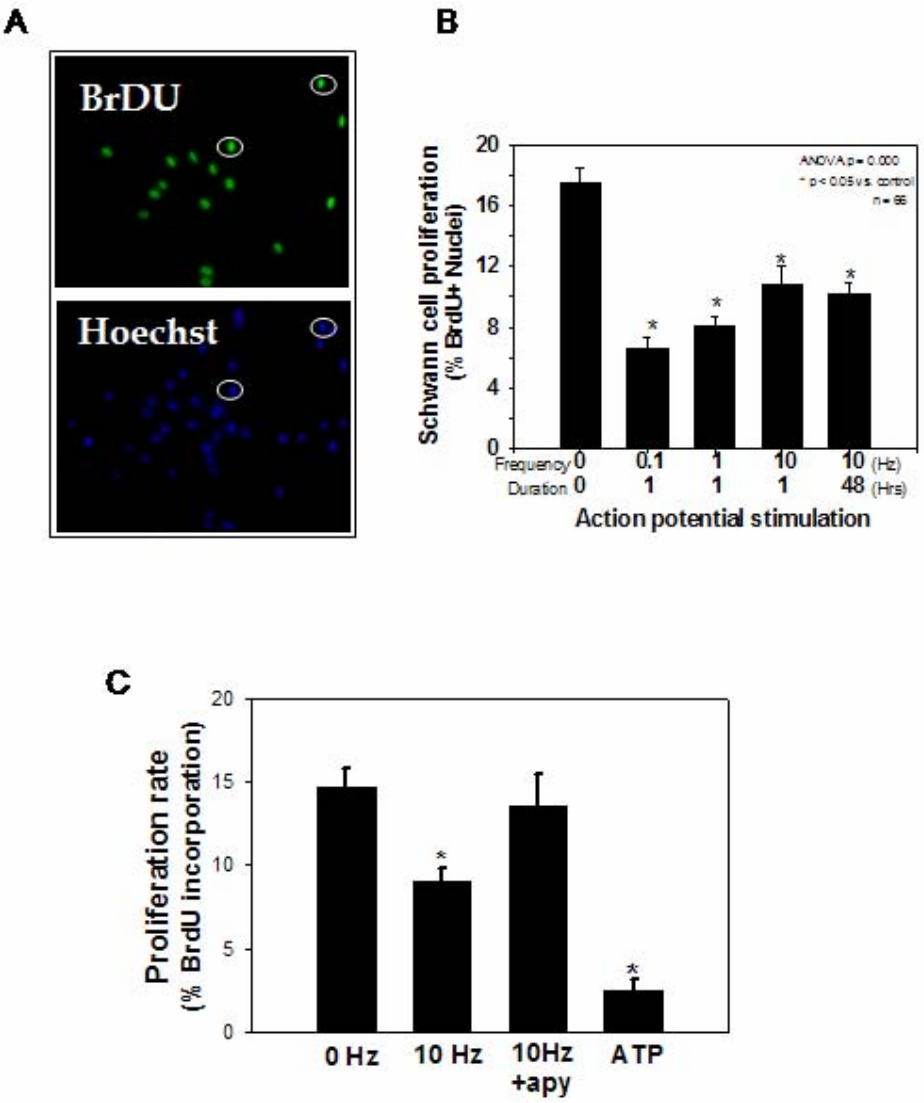
Figure 11. Inhibition of Schwann cell proliferation by activity-dependent release of ATP from DRG neurons

A. SC proliferation was measured using a BrdU assay. Proliferation rate was calculated as the ratio of BrdU /Hoechst positive SC nuclei/ microscope field. All SC nuclei stained with BrdU (green cells, top panel) and Hoechst (blue cells, bottom panel) were counted in each microscope field.

B. The proliferation rate of SCs was decreased on axons stimulated for 1 and 48 hrs at 10 Hz. Brief stimulation at lower frequencies (0.1 and 1 Hz for 1 hr) also inhibited SC proliferation.

C. The proliferation rate of SCs was decreased on axons stimulated 1 hr at 10 Hz (**10 Hz**) compared with SCs on unstimulated axons (**0 Hz**). Stimulation in the presence of 27U/ml apyrase (**10 Hz + apy**) prevented the reduction in proliferation rate, and direct application of 300 μM ATP for 24 hrs significantly inhibited SC proliferation on unstimulated axons. $p < 0.0001$, oneway ANOVA, $n = 126$; ***** $p < 0.005$ vs. control.

Figure 11.


A
BrDU
Hoechst
B
Schwann cell proliferation
(% BrdU+ Nuclei)
ANOVA p = 0.000
* p < 0.05 vs. control
n = 66
20
16
12
8
4
0
Frequency 0 0.1 1 10 10 (Hz)
Duration 0 1 1 1 48 (Hrs)
Action potential stimulation
C
Proliferation rate
(% BrdU incorporation)
20
15
10
5
0
0 Hz
10 Hz
10Hz
+apy
ATP

ATP INHIBITS CELL CYCLE PROGRESSION IN SCHWANN CELLS

Withdrawal from the cell cycle is a pre-requisite for myelin-related differentiation (Asbury, 1967; Morgan et al., 1991). ATP could inhibit proliferation by preventing entry into the G1 phase of the cell cycle upon stimulation with growth factor or axonal signals. Alternatively, ATP could induce cell cycle arrest in SCs, until the appropriate extracellular signals initiate differentiation. In order to distinguish between these two mechanisms, pre-myelinating SCs growing in monoculture were synchronized by serum starvation for 24-36 hr, which caused the majority cells to enter the quiescent, G0 phase. Cell cycle re-entry was then initiated by treatment of SCs with growth factors (10 ng/mL PDGF) in the presence and absence of ATP. Immunocytochemical analysis of G1-phase cyclin expression was measured using specific monoclonal antibodies against cyclin D1 and E at different timepoints after treatment.

Cyclin D is the first cyclin expressed early in G1 (1-3 hrs after addition of growth factors), and levels persists throughout the cell cycle. As shown in figure 12A, nuclear cyclin D1 levels were significantly increased 3-9 hours after the addition of growth factors both in control and ATP treated compared with serum deprived, control cultures (Fig 12A, b,c vs. a). These data suggest that ATP did not prevent SCs from entering G1 phase in response to growth factors. However, nuclear cyclin D levels were significantly reduced in SCs after 12 and 18 hrs of co-treatment of ATP and growth factors, compared to

controls (see fig. 12B). That ATP treatment inhibited growth factor-stimulation of cyclin D at later timepoints, suggests that ATP arrests SCs in mid-late G1 phase of the cell cycle.

In contrast to cyclin D, cyclin E is expressed briefly at the G1/S transition and levels decline precipitously in early S. As expected, cyclin E levels were low in Schwann cell nuclei in early G1 both in control and ATP-treated cultures (see fig 12C, a and b), but 18 hours after treatment, cyclin E was significantly lower in SCs co-treated with ATP (Fig 12C c and d; Fig 12D). Taken together, these data suggest that ATP could be arresting SC proliferation in late G1.

Figure 12. ATP treatment arrests Schwann cells in the G1 phase of the cell cycle.

A. Purified Schwann cells were immunolabeled with a monoclonal antibody against cyclin-D1, before (**a**) and after treatment with growth factors (**b**) and growth factors + 300μM ATP (**c**). Nuclear cyclin D levels were significantly increased 18 hours after the addition of growth factors in the presence and absence of ATP compared with serum starved, control cells (**b and c vs. a**).

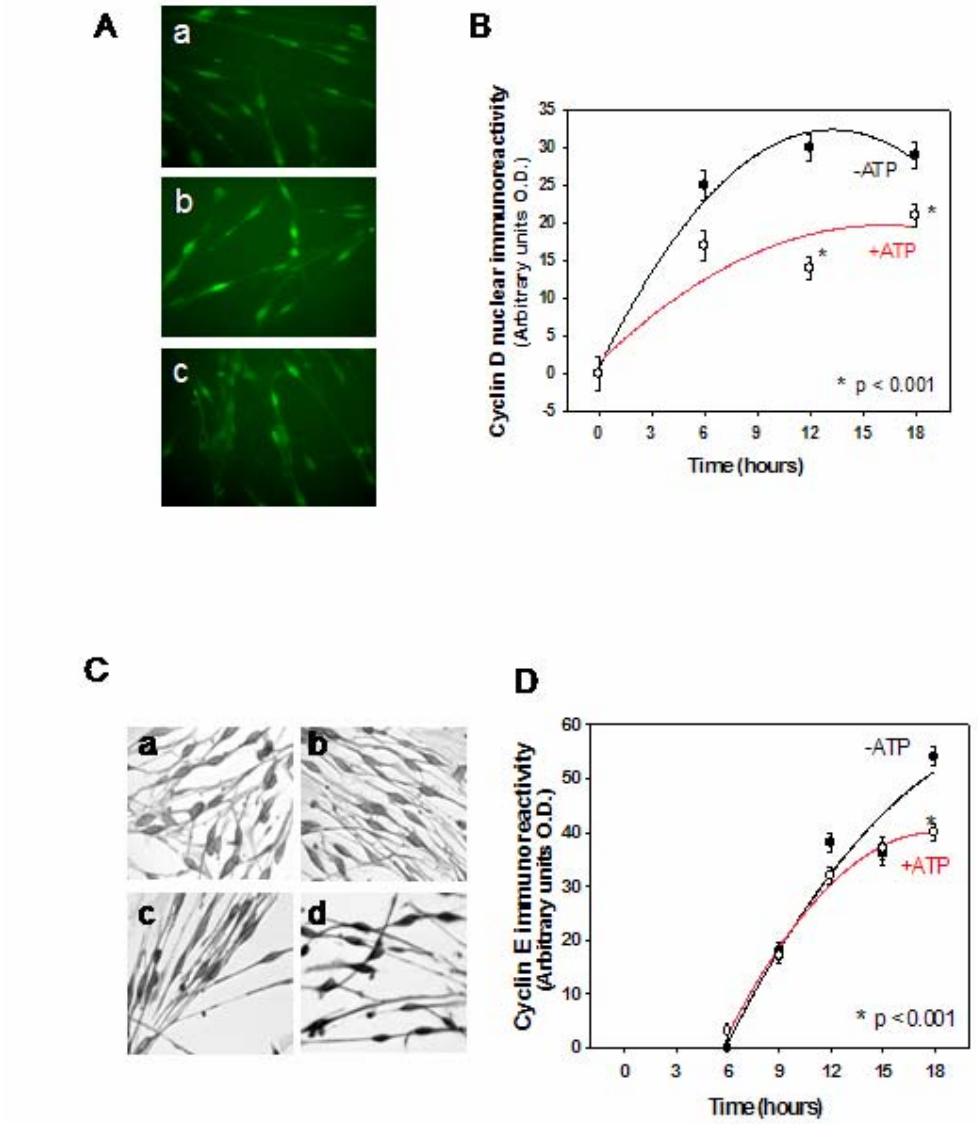
B. ATP inhibits the growth factor-mediated increase in cyclin D in the G1 phase of the cell cycle. Cyclin D levels sharply increased in Schwann cell nuclei 6-18 hours after addition of growth factors (**black curve**). ATP treatment inhibited this growth factor- stimulated cyclin D increase (**red curve**) at later timepoints. Cyclin D levels were significantly reduced at 12 and 18 hrs after treatment with growth factors + ATP compared with controls (GF only) (n= 923 cells).

C. Schwann cells were fixed and stained with anti-cyclin E every 3 hrs after the addition of growth factors +/- ATP. Cyclin E levels were low in Schwann cell nuclei in early G1 (6 hrs after addition of growth factors) both in control and ATP-treated cultures. (**a** and **b**), but nuclear cyclin E sharply increased in control cultures at 18 hours, and this increase was blocked with ATP treatment (**c vs. d**).

D. ATP inhibits growth factor-mediated increase in nuclear cyclin E in late G1. A timecourse of nuclear cyclin E expression showed no effect of ATP on the early increase in cyclin E between 9-15 hours in control (**black curve**) and ATP-treated cultures (**red curve**) (n = 1685 cells), but at 18 hours, nuclear cyclin E levels were

significantly lower in SC's treated with ATP compared with controls (n = 411 cells).

Figure 12.


A
a
b
c
B
Cyclin D nuclear immunoreactivity
(Arbitrary units O.D.)
-ATP
+ATP
* p < 0.001
Time (hours)
C
a
b
c
d
D
Cyclin E immunoreactivity
(Arbitrary units O.D.)
-ATP
+ATP
* p < 0.001
Time (hours)

Cyclin kinase inhibitors (CKIs) negatively regulate cell cycle progression by binding and inactivating specific cyclin-cyclin dependent kinase (cdk) complexes (Sherr and Roberts, 1999) (see fig. 10). CKI levels are high in quiescent cells, and during cell cycle arrest, but sharply decline in actively cycling cells in response to mitogenic signals. Since ATP appears to arrest SCs in the G1 phase, we focused on p21 and p27--two members of the CIP/Kip family that negatively regulate G1 progression.

To determine whether ATP regulates CDKI expression, SC monocultures were treated with PDGF with and without ATP (300μM), and p21 and p27 levels were determined immunocytochemically 24 and 48 hours later. Growth factor-stimulated entry into G1 resulted in the predicated down-regulation of nuclear p21 levels in SCs, but this growth factor-dependent decrease in p21 was prevented in the presence of ATP (24h). After 24 and 48 hours, nuclear p21 levels were significantly higher in cells treated with ATP compared with growth factor-treated controls (see fig 13 A). In contrast, there were no significant differences in p27 levels between growth factor and ATP-treated Schwann cells (Fig 13B). Collectively, our findings suggest that ATP may be arresting SC proliferation in late G1, by inhibiting G1 phase cyclins (D1 and E), and positively regulating the CKI, p21.

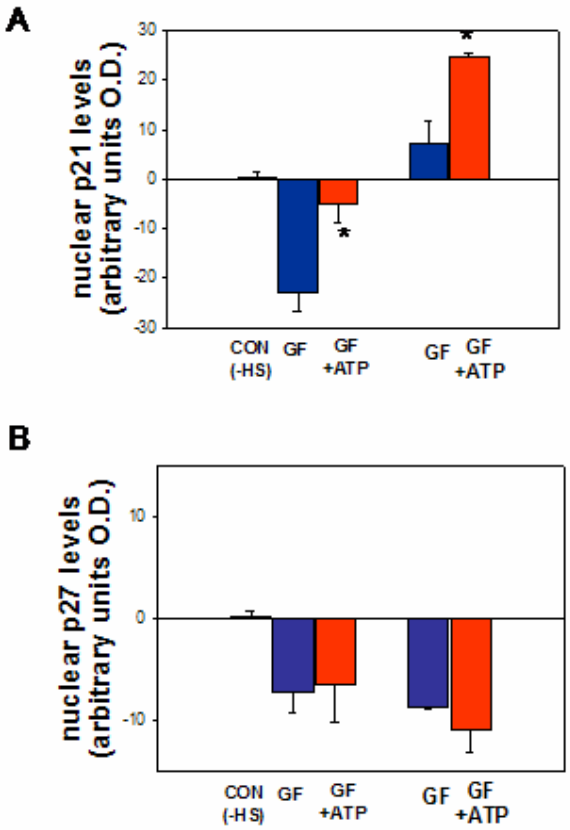
Figure 13. ATP prevents the growth factor-stimulated down-regulation of p21 in Schwann cells.

A. Growth factor-induced entry into the cell cycle resulted in a down-regulation of nuclear p21 levels in control cells (blue), but this decrease in p21 was blocked after 24 hours in the presence of ATP (red). After 24 and 48 hours, nuclear p21 levels were significantly higher in cells treated with ATP compared with growth factor-treated controls ($p < 0.0001$ one-way ANOVA, n=19 dishes; * $p<0.001$ vs. GF controls).

B. p27 levels were decreased after exposure to growth factors in the presence and absence of ATP, both at 24 and 48 hours compared with serum-starved control cultures (-**HS**).

Treatment with ATP did not affect this down- regulation, as there were no significant differences in p27 levels between growth factor (**GF**) and ATP-treated (**GF+ATP**) Schwann cells.

Figure 13.


A
nuclear p21 levels
(arbitrary units O.D.)
30
20
10
0
-10
-20
-30
*
*
CON
(-HS)
GF
GF
+ATP
GF
GF
+ATP
B
nuclear p27 levels
(arbitrary units O.D.)
10
0
-10
CON
(-HS)
GF
GF
+ATP
GF
GF
+ATP

DISCUSSION

ATP AS A CANDIDATE AXONAL SIGNAL INHIBITING SC PROLIFERATION DURING DEVELOPMENT

The present study identifies ATP as potent anti-mitotic signal that is released from sensory axons in an activity-dependent manner. ATP released from axons under physiological conditions significantly inhibited SC proliferation in DRG/SC co-cultures, despite the strong mitogenic influence of axons. Direct application of ATP also inhibited growth-factor induced proliferation in SC monocultures. Together these data suggest that extracellular ATP is sufficient to over-ride potent mitogenic signals and inhibit SC proliferation. In vivo, the abrupt decline in SC proliferation before the onset of myelination corresponds to the onset high frequency firing in DRG axons (Fitzgerald, 1987). We previously reported that ATP is released from cultured DRG neurons stimulated to fire action potentials through a TTX-dependent mechanism (Stevens and Fields, 2000). While ATP release has not been directly demonstrated *in vivo*, our present findings that apyrase blocked activity-dependent inhibition of SC proliferation, further implicate ATP as the primary signal inhibiting SC proliferation in response to axonal firing in our co-culture system.

Surprisingly little is known about the intracellular mechanisms that control growth arrest in Schwann cells. In general, cell differentiation occurs in two stages. The first involves inhibition of proliferation and arrest in the G1 phase of the cell cycle (ie, G1-S transition). The second involves changes in gene expression that direct a G1-arrested cell to the quiescent phase (i.e. G1-G0 transition). Our data suggest that ATP arrests SCs at the G1/S phase of the cell cycle through positively regulating the CDKI, p21. Inhibition of cyclin D1 and E expression in ATP-treated SCs, further imply a late G1 arrest.

Importantly, ATP-induced- G1 arrest did not lead to SC differentiation. Cell cycle arrest and withdrawal are associated with accumulation of cyclin dependent kinase inhibitors (CKIs), such as p21 and p27. Increased expression of CKIs in oligodendrocyte progenitors have been shown to induce cell cycle arrest, without leading to differentiation (Casaccia-Bonnefil et al., 1999). Consequently, independent signal(s) must initiate the second (G1-G0) phase of differentiation, which is likely the case in SCs treated with ATP. By holding SCs in this arrested, yet uncommitted stage, ATP could maintain a "plastic" pool of Schwann cells until secondary signals induce SC quiescence and myelin gene expression.

CONCLUSIONS

1. Activity-dependent release of ATP from axons significantly inhibits SC proliferation, and arrests SCs in the G1 phase of the cell cycle.

2. Cell cycle arrest occurs in late G1, and the present evidence indicates involvement of the cyclin-dependent kinase inhibitor (CKI), p21.

CHAPTER 4

INTRODUCTION

Schwann cell development is critically dependent on continuous signaling from peripheral axons. While major progress has been made in identifying the intracellular signals and transcription factors that regulate myelination, the molecular identity of the axonal signal(s) that control SC proliferation, differentiation, and myelination remain a mystery. Several *in vitro* studies have established that only post-mitotic Schwann cells (SCs) are responsive to myelin-inducing axon signals. During development SCs stop proliferating before differentiating into myelinating or non-myelinating phenotypes. The specific aim of these studies was to determine the consequences of activity-dependent release of ATP on SC differentiation following the arrest of proliferation that results from this axon-Schwann cell communication.

Growing evidence indicate that all types of glia (microglia, oligodendrocytes, astrocytes, and Schwann cells) have membrane receptors for extracellular ATP, and that many neurons can release ATP in an activity-dependent manner (Fields and Stevens, 2000). Consequently, extracellular ATP has been shown to regulate diverse glial functions in the CNS and PNS. For example, ATP plays an important role during injury, and in pathological conditions. ATP is released into the extracellular environment during

hyperexcitability and neurotrauma, where it can trigger astrocyte and microglial responses to injury and ischemia (Rathbone et al., 1998), as well as induce reactive astrogliosis *in vitro* and *in vivo* (Hindley et al., 1994; Bolego et al., 1997; Brambilla et al., 1999). Indeed, activation of purinergic receptors on astrocytes has been shown to regulate GFAP expression (Neary et al., 1994a), and induce morphological changes in cultured astrocytes (Guenard et al., 1996), both of which are indicators of astrogliosis.

ATP can also trigger microglia to synthesize and release various cytokines and chemokines involved in the immune response. Although the majority of studies carried out in astocytes and microglia have been placed in the context of neural injury, recent studies suggest that purinergic signaling could also regulate astrocyte differentiation during early postnatal development (Abe and Saito, 1999; Kukley et al., 2001; Zhu and Kimelberg, 2001).

The biological consequences of extrasynaptic ATP release on developing Schwann cells are unknown. During this period (~E16-birth in rats), SCs cells are highly plastic and exceptionally sensitive to instructive signals from axons. Embryonic Schwann cells undergo dramatic changes in morphology, and gene expression as they cease proliferation and differentiate into either mature myelinating or non-myelinating cells (Jessen and Mirsky, 1999a; Mirsky and Jessen, 1999). The initiation of SC differentiation *in vitro* can be controlled by the addition of ascorbic acid to post mitotic SCs associated with DRG neurons. This results in formation of basal lamina, and

marked changes in SC morphology, and gene expression associated with myelination (Eldridge et al., 1987; Wood et al., 1990a).

The developmental role of impulse activity in regulating glial responses, such as myelination, is controversial. Some studies suggest that impulse activity inhibits myelination (Stevens et al., 1998), and others indicate that impulse activity promotes myelination (Demerens et al., 1996), or has no effect (Shrager and Novakovic, 1995). Activity-dependent release of ATP from pre-myelinated axons could influence maturation of SCs and differentiation into either myelinating or non-myelinating phenotypes.

METHODS

CELL CULTURE.

DRG neurons were obtained from embryonic day 13.5 mice and plated at a density of 0.35×10^6 cells/ml into the side compartments of multicompartment chambers. DRG cultures were maintained in medium containing 50 ng/ml nerve growth factor (NGF). Schwann cells were obtained from the sciatic nerve of postnatal mice (P2) and cultured and purified using the Brockes method (Kleitman et al., 1991) with the following modifications: Schwann cells were cultured in medium containing 5% horse serum, and purified after 1 week using anti-Thy1.1 and complement to lyse contaminating

fibroblasts. Purified Schwann cells were maintained in 5% horse serum medium, without exogenous growth factors or mitogens until used in monoculture or co-cultured into the side compartments (30,000 cells / side) of chambers containing 3-week old DRG neurons. Schwann cells in co- and monoculture, were in culture 48-72 hours and incubated overnight in serum-free medium prior to all experiments.

ELECTRICAL STIMULATION.

Neurons were dissociated from the DRG of fetal mice and cultured for three weeks in multicompartment chambers equipped for electrical stimulation (Fields et al., 1992). Axons grew into the central compartment by passing beneath high resistant barriers separating two side compartments. This allowed electrical stimulation of DRG neurons at various frequencies (1-10 Hz) and patterns (0.5 sec bursts at 10 Hz every 2 sec) with brief pulses (200 nsec, biphasic pulses at 5 V) from a custom made multi-channel stimulator. The stimulus was monitored continually by an oscilliscope and by light emitting diodes in series with stimulating electrodes in each dish. Up to 24 cultures could be stimulated simultaneously inside the incubator. Electrophysiological recording in DRG neurons labeled with DiI show that only those neurons with axons traversing the barrier are stimulated to fire action potentials (Li et al., 1996).

SC DIFFERENTIATION AND MYELINATION

Differentiation was induced in one-week old DRG/SC co-cultures, by the addition of ascorbic acid (50ug/mL) in medium containing 5-10% horse serum +/- ATP (1-500□M). Morphology changes were observed 3-4 days later, and SCs were stained with antibodies against the O4 antigen. Live cultures were incubated with monoclonal O4 antibody (1:10 for 1 hr), and antigens were detected using a fluorescein-conjugated goat anti-mouse IgM antibody (Jackson Immunoresearch). The 04 antibody was generously provided by Dr. Vittorio Gallo. Myelin profiles were evident by phase contrast microscopy and MBP staining within 10-14 days of the medium shift. Cultures received half changes of medium daily with or without 300 μM ATP.

TUNEL ASSAY

For the apoptosis assay, SCs were fixed with 4% paraformaldehyde following treatment. Cells were stained for TUNEL according to manufacturer's protocol (Roche), and the % of TUNEL-positive cells was determined from the ratio of TUNEL/Hoechst-positive cells/microscope field.

IMMUNOCYTOCHEMISTRY

Co-cultures were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with monoclonal antibodies against myelin basic protein and Gal-C (Boehringer-Mannheim) at a dilution of 1:500 (MBP)

and 1:50 (Gal-C) for 1 hr at RT. Primary antibodies were detected with fluorescein-conjugated goat F(ab')2 fragment to mouse IgG (Organon Teknika) for MBP and Rhodamine-conjugated Fc-specific goat anti-mouse (Jackson Immunoresearch) for Gal-C. Immunocytochemical detection of S-100 (Sigma, 1:500) was detected with fluorescein-conjugated goat F(ab')2 fragment to mouse IgG (Cappel).

RESULTS

ACTIVITY-DEPENDENT ATP RELEASE ARRESTS SC DIFFERENTIATION

To determine whether ATP can influence SC differentiation, SC/DRG co-cultures (1 week *in vitro*) were treated with 50 μg/ml ascorbic acid + 5% horse serum with and without ATP (300 μM), as described (Stevens et al., 1998). Surprisingly, the normal morphological changes associated with maturation of SCs were completely prevented by ATP treatment over a 4 day period. Under control conditions, SC displayed a rounded, flattened morphology three to four days after the switch into differentiating media, as expected. In complete contrast, SCs treated with ATP (300μM), remained spindle shaped, and failed to closely align with DRG axons (see fig. 14A, a vs. c).

In addition, expression of the 04 antigen, a marker of SC lineage progression, was strongly inhibited in SCs treated with ATP (Fig. 14A b vs. d). Chronic treatment of co-cultures with 300 μM ATP resulted in a ~50- fold reduction in the number of SCs expression 04 antigen compared to controls, while adenosine treatment had no significant effect. The ATP-dependent inhibition of 04 was mimicked by the non-hydrolysable ATP receptor agonist, 2MeSATP ($p < 0.0001$, n=15 cultures) (Fig. 14 B), implicating involvement of P2 purinergic receptors. Lower concentrations of ATP were also effective in

inhibiting 04 expression in SCs. The proportion of 04-positive SCs was measured by computerized optometry, and showed a dose-dependent relation to ATP concentration (1-100μM; $p<0.001$ linear regression against the log of ATP concentration; n=8) (Fig.15A a,b).

Importantly, the inhibitory effects of ATP on SC differentiation were mimicked by neural impulse activity. The number of 04-positive SCs was reduced to 33.5% of controls in cultures stimulated 24-48 hours after co-culture in a phasic pattern for 7-10 days without ascorbic acid ($p<0.03$; n=21) (see fig. 15B). The total number of SCs was not different at this timepoint (104 +/- 7.2 versus 108+/- 13 per field), and there was no evidence of apoptosis in these co-cultures as determined by cell counts or TUNEL assay after 1 hr or 7-days ATP treatment. In the rat sciatic nerve, 04 expression begins prior to differentiation into myelinating or nonmyelinating phenotype and continues to be expressed in both phenotypes into adulthood (Mirsky et al., 1990; Jessen and Mirsky, 1992). These findings suggest that impulse activity could prevent or delay SC differentiation at the pre-myelinating stage of development, before differentiation into either myelinating or non-myelinating phenotypes.

Figure 14. ATP, but not adenosine, arrests maturation and differentiation of SCs.

A. Chronic treatment of SCs with 300 μM ATP in co-culture with DRG neurons for 4 days, prevented the normal development from spindle shaped to rounded, flattened morphology (**a vs. c**). ATP prevented expression of the 04 antigen (**b vs. d**), a marker of SC lineage progression from an immature stage, just prior to differentiation into premyelinated or promyelinated phenotypes

B. ATP (300μM) and the P2 receptor agonist, 2MeSATP (100μM) significantly inhibited 04 expression in SCs (18.2+/- 5.7 vs. 0.03+/-0.04, 04 positive cell/field con vs. ATP, n = 24, $p<0.004$, t-test). In contrast, treatment of SCs with adenosine (300μM) had no significant effect.

Figure 15. Neural impulse activity inhibits SC differentiation.

A. The proportion of 04-positive SCs was measured by computerized morphometry, and showed a dose-response relation to ATP concentration (1-100μM; $p<0.0001$ linear regression against the log of ATP concentration; n=8).

B. The number of 04-positive SCs was also significantly reduced in co-culture with DRG axons following 7-10 days of phasic electrical stimulation without ascorbic acid.

Figure 14.

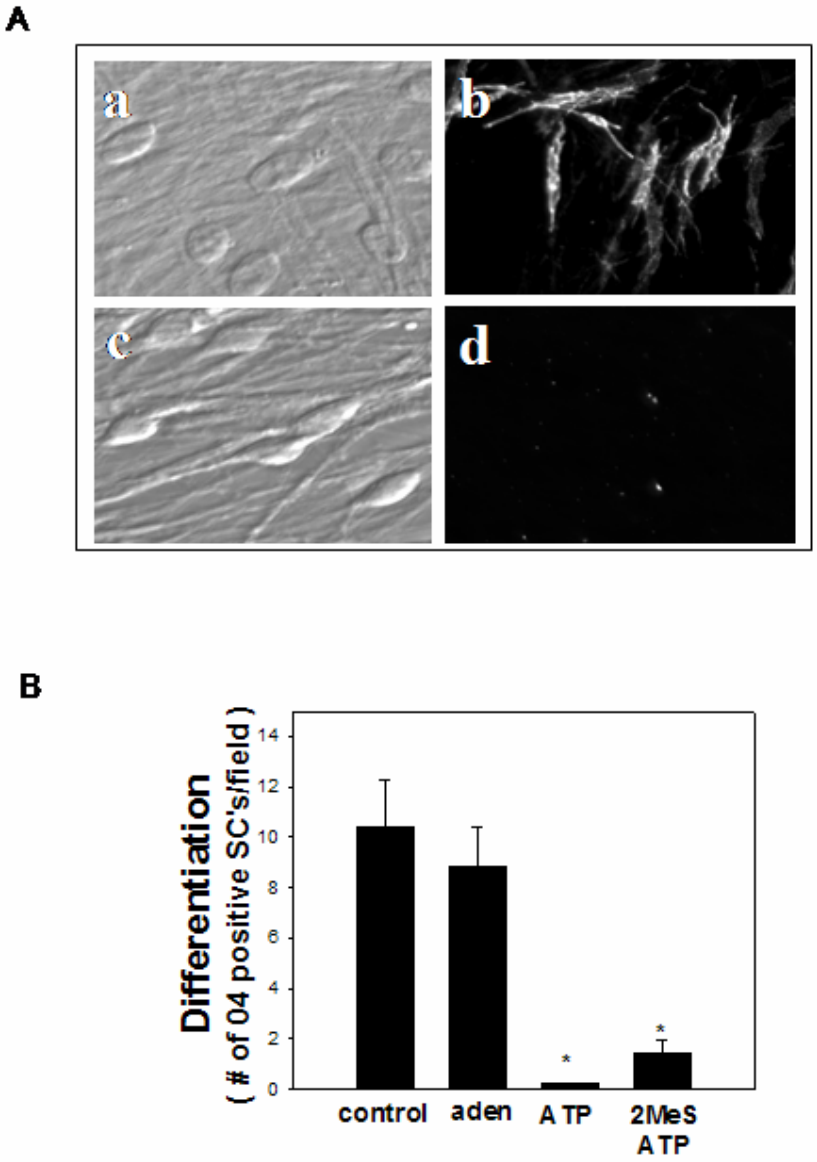
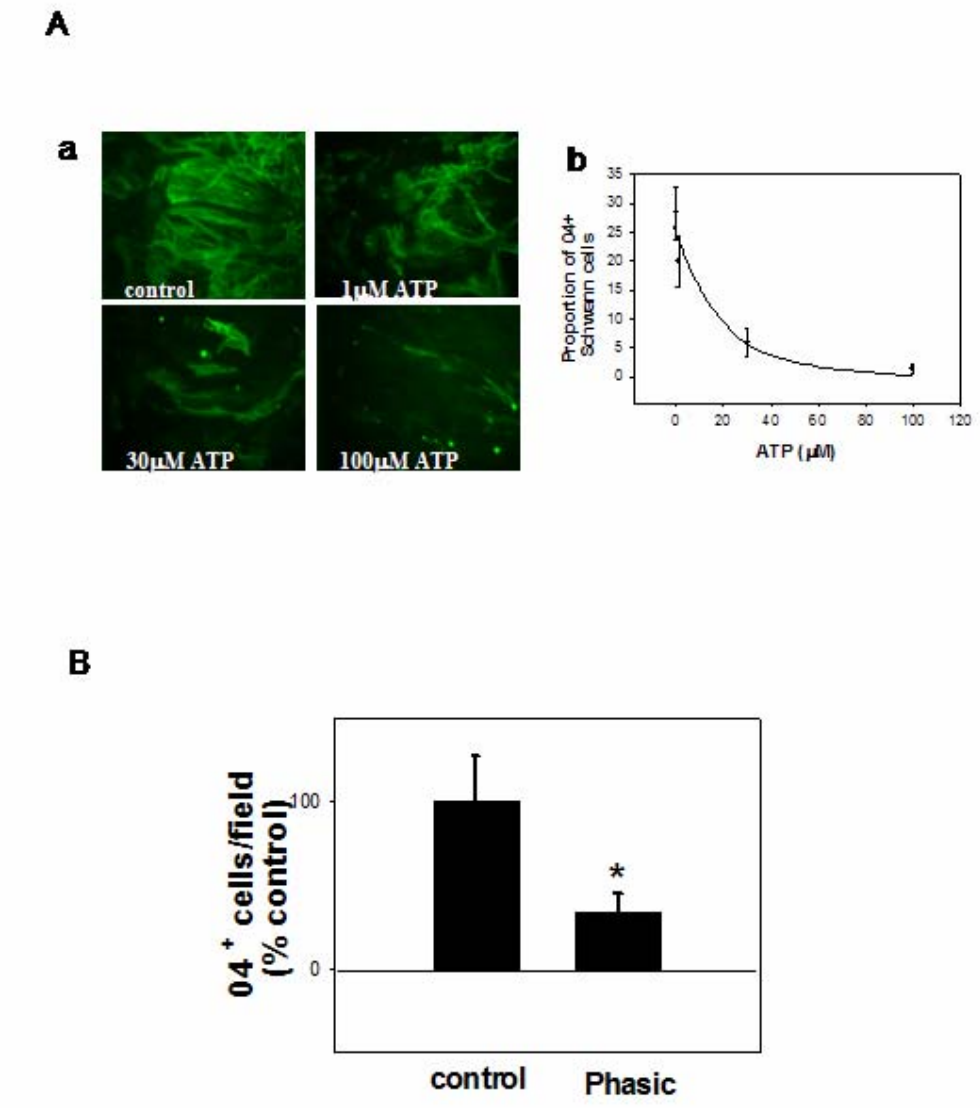

A
a
b
c
d
B
Differentiation
(# of 04 positive SC's/field)
0
2
4
6
8
10
12
14
*
*
control
aden
ATP
2MeS
ATP

Figure 15.


A
a
control
1μM ATP
30μM ATP
100μM ATP
b
Proportion of O4+ Schwann cells
35
30
25
20
15
10
5
0
0
20
40
60
80
100
120
ATP (μM)
B
O4+ cells/field (% control)
100
0
*
control
Phasic

ATP INHIBITS MYELIN GENE EXPRESSION AND FORMATION OF MYELIN

We next looked at several myelin-related proteins to determine whether impulse activity could influence myelination by inhibiting the maturation and differentiation of SCs. In the sciatic nerve, the myelin glycolipid Galactocerebrocide (Gal C) appears in cells of the myelin pathway before the myelin proteins are expressed, and in maturing non-myelinating SCs (Jessen 1985). In the present study, the majority of pre-myelinating SCs express Gal-C in serum/ascorbate-free conditions in DRG/SC co-cultures, which is consistent with published reports (Seilheimer et al., 198; (Wood et al., 1990b). Within several days of the addition of ascorbic acid, Gal-C was dramatically down-regulated in the majority of SCs, and expression was restricted to SCs forming myelin sheaths. Under control conditions, nearly all non-myelinating SCs became Gal-C negative at this timepoint, indicating that SCs have progressed to the pro-myelinating stage in co-cultures. When long term co-cultures were treated with ATP and ascorbic acid, the down-regulation of Gal-C in SCs was prevented compared to controls, indicating failure to SCs to differentiate beyond the immature, pre-myelinating stage (7.4 +/1 0.5 0.5+/- 0.28 Gal-C positive SCs/field in ATP vs. control, n = 8 cultures, $p<0.0001$ t-test) (see fig. 16A).

The zinc family transcription factor, krox-20 is expressed in embryonic SCs before the onset of myelination, but its expression is later restricted to

myelinating SCs. Consistent with these findings, Krox 20 levels are not expressed in SCs that differentiate into non-myelinating phenotypes in the adult animal (Topilko et al., 1997). We observed moderate Krox-20 expression in SCs in short term co-culture with DRG neurons, which is consistent with the observation that Krox 20 can be induced in cultured SCs 24-48 hours after direct contact with axons (Murphy et al., 1996). Krox-20 levels were significantly reduced in they majority of SCs in long term DRG/SC cocultures treated with ascorbic acid. This ascorbic acid-dependent down-regulation of krox -20 was prevented by ATP (89.7% vs. 50% Krox 20 + cells, ATP vs. control, p= 0.000,χ^2 test, n = 1691 cells) (Fig. 16B). Interestingly, the majority of differentiating, 04-positive SCs in control cultures did not co-express krox-20 (Fig. 16C), suggesting that in the absence of ATP, these SCs may be differentiating into the non-myelinating phenotype.

SCs in the pro-myelinating stage establish a 1:1 association with an axon, and initiate ensheathment. In the presence of ATP, SCs remained spindle-shaped and were not aligned with axons even after two weeks co-treatment with ascorbic acid and ATP (Fig.17 a and c). Moreover, compact myelin was not observed in ATP treated cultures, and myelin basic protein (MBP), a component of compact myelin, could not be detected by immunocytochemistry (Fig. 17 b,d). Collectively, the biochemical and

morphological evidence suggests that ATP arrests SC maturation prior to differentiation into either the myelinating or nonmyelinating phenotypes.

Figure 16. ATP prevented the down-regulation of GalC and Krox 20 expression in differentiating, non-myelinating SCs.

A. ATP prevented the ascorbate-induced down-regulation of Gal-C in non-myelinating SCs compared to controls (7.4 +/1 0.5 0.5+/- 0.28 Gal-C positive SCs/field in ATP vs. control, n = 8 cultures, $p<0.0001$ t-test).

B. ATP also prevented the ascorbic acid-dependent down-regulation of krox -20 (89.7% vs. 50% Krox 20 + cells, ATP vs. control, $p = 0.000$, χ^2 test, n = 1691 cells).

C. Differentiating SCs in co-culture double labeled with antibodies specific for 04 (a) and Krox-20 (b). The majority of differentiating, 04-positive SCs (white arrow, a vs. b) in control cultures did not co-express krox-20.

Figure 17. Chronic ATP treatment prevented the formation of myelin.

ATP treatment for 10 days prevented the close association of SC with axons (**a vs. c**) and the formation of compact myelin and expression of myelin basic protein (MBP) (**b vs. d**). (The areas shown contain no neurons).

Figure 16.

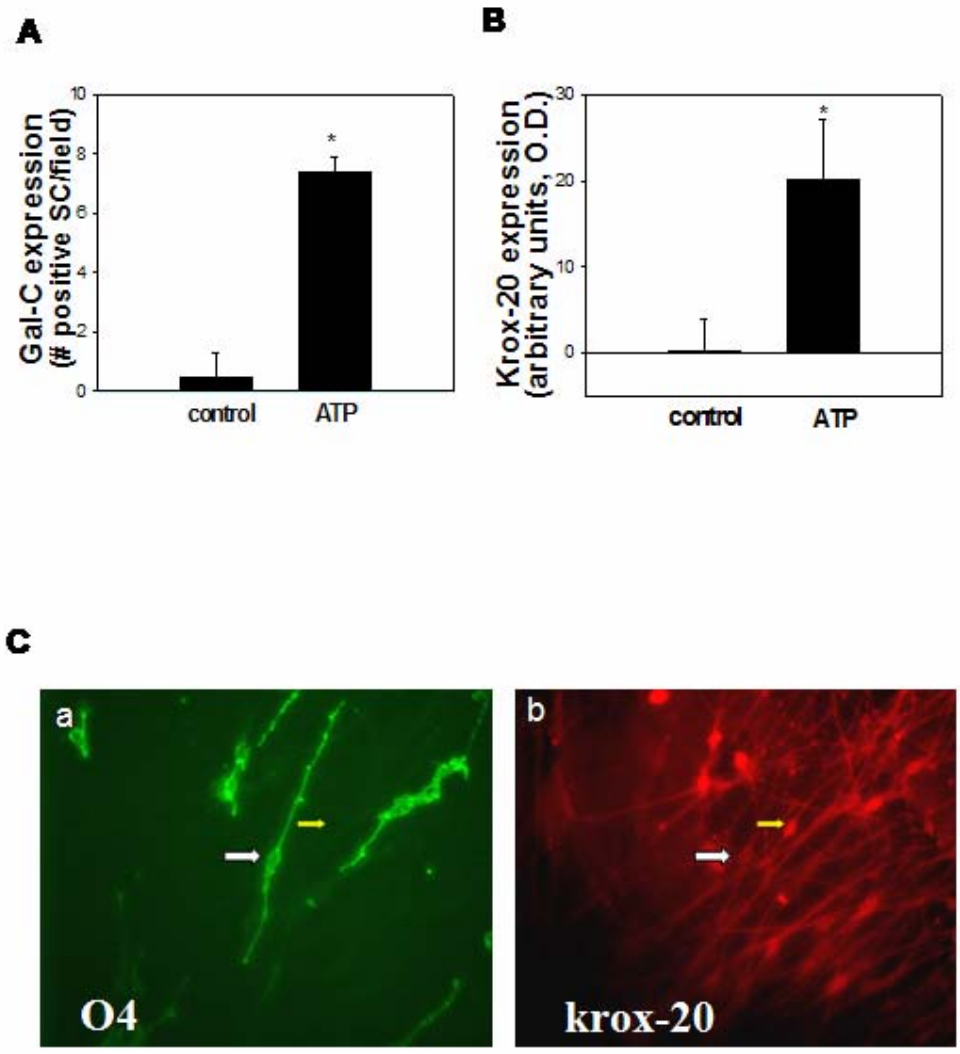
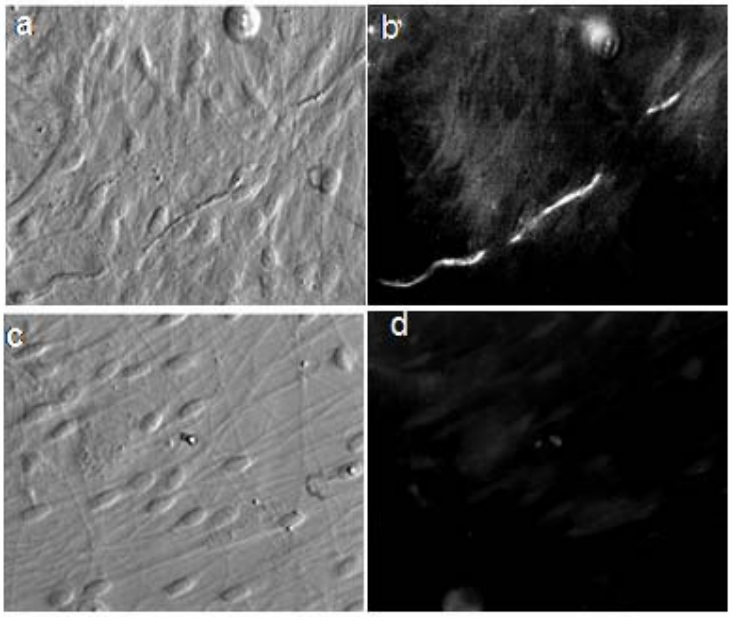

A
B
Gal-C expression (# positive SC/field)
10
8
6
4
2
0
*
control
ATP
Krox-20 expression (arbitrary units, O.D.)
30
20
10
0
*
control
ATP
C
a
O4
b
krox-20

Figure 17.



DISCUSSION

The maturation of a myelinating SC involves migration, recognition, proliferation, ensheathment, and the establishment of an intimate 1:1 relationship between a SC and a peripheral axon. The timing of each stage of SC development is tightly controlled by multiple extrinsic and axon- derived factors to ensure that normal myelination begins around the time of birth (Jessen and Mirsky, 1999a). The present study identifies extracellular ATP as an important activity-dependent signal that arrests SC development at a highly dynamic stage, prior to the onset of myelination. Through this mechanism, neural impulse activity could delay terminal SC differentiation until the appropriate axon signal(s) are in place to initiate the myelination program.

ATP MAY CONTROL TIMING OF SC DIFFERENTATION AND MYELINATION

This study specifically focuses on the effects of ATP on pre-myelinating SCs, which are bipolar, dividing cells that have not yet committed to either the myelinating or nonmyelinating fate. We provide several lines if evidence that extracellular ATP arrests SCs in the pre-myelinating stage of development.

We found that ATP prevents axon-induced induction of 04 in SCs growing in differentiating medium. 04 is a surface glycoprotein that served as

an early differentiation marker. 04 appears as early as ~E16/17 in the rat sciatic nerve, and it continues to be expressed in both myelinating and nonmyelinating lineages into adulthood (Morgan et al., 1991). Axon contact, and elevated cAMP have been shown to induce surface expression of 04 in the early pre-myelination period in vitro (Mirsky et al., 1990). We observed a dose –dependent inhibition of 04 by ATP, indicating that physiological levels of ATP are sufficient to inhibit 04 expression. This is further supported by our findings that chronic electrical stimulation of DRG/SC co-cultures mimics the effects of ATP in inhibiting 04 expression. In addition, SCs treated with ATP were morphologically immature compared to control conditions, SCs remained spindle shaped and randomly associated with axons in the presence of ATP, even after a week in ascorbate medium.

Not surprisingly, the ATP-induced SC arrest at the pre-myelinating stage, completely prevented subsequent stages of differentiation, including myelination. The onset of spontaneous activity along rat DRG axons *in vivo* precisely corresponds to the appearance of 04 in SCs in the developing rat sciatic nerve (Fitzgerald, 1987; Morgan et al., 1991). Although it is difficult to directly compare expression of SC markers *in vivo* and *in vitro*, our results obtained in DRG/SC co-cultures appear to closely parallel the in vivo environment.

In contrast to other myelin –related proteins, 04 expression can be induced equally in dividing and non-dividing SCs (Morgan et al., 1991). Since

ATP both prevented expression of 04 in the early pre-myelinating stage, and induced a G1-cell cycle arrest, it is tempting to speculate that the two effects could be linked. An ATP-induced G1 growth arrest could prevent differentiation to the 04-positive stage. This could maintain a synchronized pool of early premyelinating cells so that induction of 04 and later differentiation markers are timed according to the functional requirements of axons. Through this mechanism, ATP could prevent premature differentiation into the non-myelinating phenotype, and indirectly promote myelination.

That SCs remained morphologically immature even after 2 weeks in differentiation medium, suggest that the inhibitory effects of ATP were sufficient to override the myelin promoting effects of ascorbic acid, which promotes basal lamina formation and stimulate myelination *in vitro*. Initiation of myelination would thus require that the inhibitory effects of ATP be overridden. Several mechanisms could overcome the inhibitory effects of ATP. ATP could be chronically released from axons, since neural impulse activity continues at higher frequencies in the adult. In this scenario, ATP may only be inhibitory during a narrow window before the appropriate myelin inducing signals are in place. A second possibility is that ATP is only released during a narrow window of development, before the onset of myelination. The mechanism(s) regulating activity-dependent ATP release from axons are still not well understood, but it remains possible that ATP release is developmentally regulated. SCs may also control their own receptiveness to

ATP via developmental regulation of purinergic receptor expression, or other receptor systems that could interact with purinergic receptors to inhibit SC development. Future studies addressing these issues are required before the mechanisms underlying ATP-dependent regulation of myelination are understood *in vivo*.

CONCLUSIONS

1. Chronic ATP treatment of SC/DRG co-cultures prevents morphological changes associated with SC differentiation, and potently inhibits expression of the early differentiation marker, 04.

2. Chronic electrical stimulation of DRG/SC co-cultures mimic the effects of ATP in inhibiting SC differentiation.

3. This ATP-induced developmental arrest ultimately prevented the expression of myelin basic protein and formation of compact myelin *in vitro*.

CHAPTER 5

INTRODUCTION

Extracellular ATP released from pre-myelinated axons was recently identified as an important activity-dependent transmitter inhibiting proliferation and differentiation of Schwann cells (SCs), the myelinating glia in the PNS (Stevens and Fields, 2000). Activity-dependent ATP release from cultured DRG neurons induces calcium transients and gene expression in associated SCs (Stevens and Fields, 2000), but the specific types of purinergic receptor(s), and intracellular signaling pathways involved in this form of neuron-glia communication are not known.

There are multiple types of ligand-gated ionotropic (P2X) and G-protein coupled metabotropic (P2Y) ATP receptors which could interact to regulate Schwann cell function in response to extracellular ATP (Ralevic and Burnstock, 1998). In addition, hydrolysis of extracellular ATP by ectonucleotidaes can generate adenosine (Zimmermann, 2000), which can activate several families of metabotropic adenosine (P1) receptors (Ralevic and Burnstock, 1998; Klotz, 2000). Emerging evidence indicate that CNS glia express a complex array of purinergic receptors. Astrocyes, for example, express multiple adenosine and ATP receptor subtypes, often in the same cell (King et al., 1996; Neary et al., 1998). A similar picture is beginning to

emerge for cells of the oligodendrocyte lineage (Stevens et al., 2002a). It is not yet known whether pre-myelinating SCs express functional adenosine receptors, or which types of ATP receptors are expressed in developing pre-myelinating SCs that could be activated in response to extracellular ATP.

Simultaneous activation of different purinergic receptors on the same cell can activate multiple intracellular signal transduction pathways (Ralevic and Burnstock, 1998; Burnstock, 1999). In addition, purinergic receptors can be indirectly linked to other signaling systems, including growth factors, neurotransmitters, cytokines, and others (Neary et al., 1996; Rathbone et al., 1998; Neary et al., 1999a; D'Ambrosi et al., 2001). Consequently, the biological outcome of a particular purinergic agonist has been shown to vary widely in different cell types. Activation of ATP receptor stimulates proliferation in many cells, including astrocytes (Neary et al., 1998; Neary et al., 1999b), whereas ATP inhibits Schwann cell proliferation and differentiation (Stevens and Fields, 2000). A more detailed characterization of the specific purinergic receptor subtypes and key intracellular signaling pathways involved may help explain the disparate functional effects in response to purinergic signaling molecules in different cellular systems.

We recently reported involvement of the ERK/MAPK pathway in activity-dependent axon-SC communication, since inhibition of MEK blocked action-potential induced phosphorylation of CREB in SCs (Stevens and Fields, 2000), but direct activation of ERK/MAPK by ATP receptor agonists has not

been directly demonstrated. All three members of the MAP family kinases, ERK1/2 MAPK, p38MAPK, and JNK-MAPK, can independently and cooperatively communicate signals from the membrane to the nucleus to regulate gene expression involved in cell cycle and differentiation (Johnson and Lapadat, 2002). The ultimate biological effect of such signaling is dependent upon several factors, including the duration and level of kinase activation, and the degree of communication between MAPK families and other signaling systems, such as purinergic receptors. Consistent with this notion, new research is demonstrating considerable crosstalk between purinergic receptors and MAPK signaling pathways in several cells, including Muller glia (Milenkovic et al., 2003) and astrocytes (Neary et al., 1998; Lenz et al., 2001; Milenkovic et al., 2003; Schulte and Fredholm, 2003).

New research also indicates an important role for trophic factors in modulating purinoreceptor-mediated ERK/MAPK activity. Adenosine receptor agonists can either enhance or inhibit ERK/MAPK activation, depending on the presence of growth factors in the extracellular environment (Arslan et al., 1997; Arslan and Fredholm, 2000). Axonal signals and growth factors such as PDGF and neuregulins are critical regulators of SC survival, proliferation and development (Stewart et al., 1991; Jessen and Mirsky, 1998; Mirsky and Jessen, 1999). Both growth factors activate the ERK/MAPK pathway in cultured SCs (Pleasure et al., 1985; Eccleston et al., 1987; Kim et al., 1997a; Maurel and Salzer, 2000), and are potent SC mitogens (Pleasure et

al., 1985; Eccleston et al., 1987; Pleasure et al., 1985;Eccleston et al., 1987), either alone or in combination with factors which increase intracellular cAMP (Eccleston et al., 1990; Stewart et al., 1991; Kim et al., 1997a). Since growth factors are present at different levels during periods of SC plasticity, i.e. when SC are exiting the cell cycle and initiating myelination, it will be important to determine whether growth factors influence the functional responses of extracellular ATP.

The present study demonstrates that electrical stimulation of DRG neurons directly activated the ERK/MAPK pathway in associated pre-myelinating SCs in DRG/SC cocultures, as predicted from our previous work (Stevens and Fields, 2000). Surprisingly however, the activity dependent signal was identified as adenosine, acting through A2 receptors on premyelinating SCs, and not ATP. In contrast, we show that ATP can inhibit ERK1/2 MAPK in SCs by activating P2Y1 receptors and downstream P38 MAPK. Further, we provide evidence that growth factors cooperate with the purinergic signaling system to regulate SC proliferation and ERK/MAPK. Both ATP and adenosine inhibited growth factor-dependent proliferation and growth factor induction of ERK1/2 in SCs. Collectively, our findings suggest that axonal ATP and adenosine released from electrically active axons, activate a complex intracellular signaling network, in which ATP and adenosine act together, and with growth factors to regulate ERK/MAPK and proliferation in premyelinating SCs during development.

METHODS

CELL CULTURE.

DRG neurons were obtained from embryonic day 13.5 mice and plated at a density of $0.35x10^6$ cells/ml into the side compartments of multicompartment chambers. DRG cultures were maintained in medium containing 5% horse serum supplemented with 50 ng/ml nerve growth factor (NGF) according to previously published methods (Fields et al., 1990). Schwann cells, obtained from the sciatic nerve of postnatal mice (P2) and cultured and purified using the Brockes method (Kleitman et al., 1991) with the following modifications: Schwann cells were cultured on to poly-L-Lysine coated dishes in medium containing 5% horse serum. The following day, cultures were treated with 10^{-5} M cytosine arabinoside (AraC) to prevent proliferation of non-neuronal cells. Contaminating fibroblasts were eliminated by complement-mediated lysis during passaging with antibody to Thy1.1 one week later, and purified Schwann cells were used in experiments within 1 week. Purified Schwann cells were maintained in 5% horse serum medium, without exogenous growth factors or mitogens until passaged and replated on to collagen coated dishes/coverslips, or co-cultured with DRG neurons. Pre-myelinated SCs in co- and monoculture, were in culture 48-72 hours and incubated overnight in serum-free medium prior to all experiments.

CHEMICALS

ATP, 2-(methylthio)-adenosine diphosphate (2MeSADP), 2-(methylthio)-adenosine triphosphate (2MeSATP), αβ-methylene adenosine 5'-triphosphate (αβ-meATP), 2'-3'-o-(4-benzoyybenzoyl)-adenosine 5'-triphosphate (BzATP), uridine-5' triphosphate (UTP) were purchased from Sigma. CGS221680, CCPA, IBMECA, ZM241385, MRS2179 were purchased from Tocris Cookson Inc.

ELECTRICAL STIMULATION.

Neurons were dissociated from the DRG of fetal mice and cultured for three weeks in multicompartment chambers equipped for electrical stimulation (Fields and Nelson, 1992). Axons grew into the central compartment by passing beneath high resistant barriers separating two side compartments. This allowed electrical stimulation of DRG neurons (10 Hz) from a custom made multi-channel stimulator. The stimulus was monitored continually by an oscilliscope and by light emitting diodes in series with stimulating electrodes in each dish. Up to 24 cultures could be stimulated simultaneously inside the incubator. Electrophysiological recording in DRG neurons labeled with DiI

show that only those neurons with axons traversing the barrier are stimulated to fire action potentials (Li et al., 1996).

CALCIUM IMAGING.

Intracellular Ca^{2+} was monitored in Sacs cultured 48-72 hrs on Poly-L Lysine + collagen-coated glass cover slips. Co focal microscopy (Byroad MRC 1024) with the calcium-sensitive indicator fluo-3 (Molecular Probes, Eugene, OR) was used to measure changes in fluorescence intensity ($\Delta F/F0$) due to calcium transients in Schwann cells in response to purinergic agonists. Solutions were applied locally through a multi-barrel pipette using electronically controlled valves (Harvard Apparatus). Measurements were carried out at room temperature in a HEPES-buffered balanced salt solution, pH 7.2. A Nikon 40 X , 0.7 n.a. long-working distance lens was used for confocal imaging of cells grown on plastic dishes. A 40X Nikon, 1.3n.a. lens was used on cells cultured on 0.17nm thick glass coverslips. Scanning argon ion or krypton-argon lasers emitting at 488 nM were used for excitation, and imaged with a pin-hole setting of 3.2-4.5mm. The optical sectioning by confocal microscopy allowed us to distinguish calcium responses in Schwann cells from responses in neurons and axons.

IMMUNOCYTOCHEMISTRY.

Schwann cell/DRG co-cultures (as well Schwann cells in monoculture) were stimulated electrically (10 Hz, 30 min), or pharmacologically and fixed immediately with 1-4% paraformaldehyde. Cells were permeabilized with 0.2% Triton X-100 and non-specific peroxidases were blocked with 3% hydrogen peroxide, followed by a 5% normal goat serum block. Cultures were incubated with antibody against CREB phosphorylated at Ser-133 (1:800, Cell Signaling), phosphorylated MAPK (1:500, Cell Signaling), phospho-p38MAPK (1:250, Cell Signaling) in 3% BSA/PBS overnight at 4 degrees. Cultures were then incubated with biotinylated goat anti-rabbit antibody (Vector Labs), and localized with the ABC method. The relative intensity of the stain was quantified by using image densitometry on a video microscope (Image-1, Universal Imaging).

EXPERIMENTAL DESIGN AND DATA ANALYSIS.

The relative intensity of nuclear staining after immunocytochemistry was compared by imaging densitometry from multiple culture dishes representing controls and all relevant experimental treatments. Images were acquired from at least 15 randomly chosen fields in each culture using a Nuvicon video camera and digitized on an 8-bit scale for storage and optical densitometry using Image-1 software (Universal Imaging, West Chester, PA).

Statistical analysis was based on the mean staining intensity of SC cytoplasm and/or nucleus in each dish, determined from measurements of all SCs in each field (thus, n = number of cultures). All values were normalized to the mean nuclear staining intensity of control cultures in each experiment to allow pooling of replicate experiments [arbitrary O.D. units = (nuclear staining density/average staining density of control nuclei) X 100) – 100). This yields a scale of 0-55 O.D. units from gray (unstimulated control) to pure black (Fields et al., 1997). The results were presented as mean +- SEM, and statistical comparisons were evaluated by ANOVA or two-sample t-test using the statistical analysis software Minitab (State College, PA).

RT-PCR

RNA was isolated using TRIzol (Invitrogen), from purifed SC monocultures, and RT-PCR was performed using 1 µg RNA in a Retroscript kit (Ambion), and 5 µl of RT product was amplified using SuperTaq (Ambion) in 30 cycles of 94^{o} C (1 min)/60^{o} C (1 min), with 72^{o} C (7 min) after the final cycle. Primers for mouse: A2a: 5’ CTC ACG CAG AGT TCC ATC TTC 3’ & 5’ GAA GCA GTT GAT GAT GTG CAG 3’ (500bp); A2b: 5’ CAG ACC CCC ACC AAC TAC TTT 3’& 5’ TGT CAG AGG ACA GCA GCT TTT 3’ (396 bp). Products were resolved by electrophoresis on a 2% agarose gel.

PROLIFERATION ASSAY

SCs 48 hours in culture were serum starved for 18-24 hours before treatment with growth factors (10ng/mL PDGF or 250ng/mL B heregulin + 2μM forskolin) +/- selective purinergic agonists. Cultures were pulsed with BrdU (Boehringer Mannheim) for 6 hrs, fixed and stained according to manufacturer instructions. Cultures were then counterstained with Hoechst nuclear stain (Molecular Probes) at a dilution of 1:2000 for 10 min. Proliferation rate was calculated as the ratio of BrdU/Hoechst positive SC nuclei/ microscope field. All SC nuclei stained with BrdU and Hoechst were counted in each microscope field. 10-15 randomly chosen fields were sampled to obtain a mean for each culture well; n = number of culture wells.

.

RESULTS

ACTION POTENTIAL-DEPENDENT ACTIVATION OF ERK1/2 MAPK IS NOT MEDIATED BY EXTRACELLULAR ATP

Activity-dependent release of extracellular ATP from DRG neurons could activate multiple purinergic receptors and intracellular signaling pathways to phosphorylate the transcription factor CREB in pre-myelinating SCs (see fig. 18). We previously reported that action-potential dependent-CREB phosphorylation was blocked when co-cultures were pre-incubated with the MEK1 inhibitor PD098059**,** suggesting that ATP released from stimulated axons may signal through the ERK1/2 MAPK pathway to regulate gene expression and SC function during development (Stevens and Fields, 2000).

To test this hypothesis, DRG neurons were electrically stimulated (10 Hz, 30 min), and active ERK1/2 MAPK levels were determined immunocytochemically in SCs co-cultured with DRGs in a multicompartment chamber equipped with stimulating electrodes (Stevens et al., 1998; Stevens and Fields, 2000) (Fig 19A)**.** Electrical stimulation of DRG neurons significantly increased phospho- ERK1/2 MAPK levels in pre-myelinated SCs associated with electrically stimulated DRG axons compared with unstimulated controls ($p<0.0001$; $n=21$, t-test) (Fig. 19 B). Only DRG neurons that extend axons under the high resistant barriers into the central

compartment are electrically stimulated (Li et al., 1996), therefore imaging densitometry was performed in 10-15 random fields along the central barrier region of each side compartment (see fig. 19A). This quantification method takes into account the heterogeneity of MAPK staining in our co-cultures, since not all SCs are necessarily associated with electrically stimulated axons.

Since we previously established that ATP is released from electrically stimulated DRG neurons (Stevens and Fields, 2000), we next tested whether ATP directly activated ERK/MAPK in SCs. Surprisingly, ATP and non-hydrolysable ATP agonist, γSATP failed to activate ERK/MAPK in SCs over a wide range of concentrations. As shown in Figure 19C, agonists selective for P2Y (UTP [P2Y2,4,6]) and P2X subtypes (αBMeATP [P2X]) were also ineffective in activating ERK/MAPK in SCs. In fact, treatment of SC with the potent P2 receptor agonist, 2MeSATP, significantly inhibited ERK1/2 levels in SCs compared with unstimulated controls ($p<0.001$, n=31 exps) (Fig. 19C).

Figure 18.

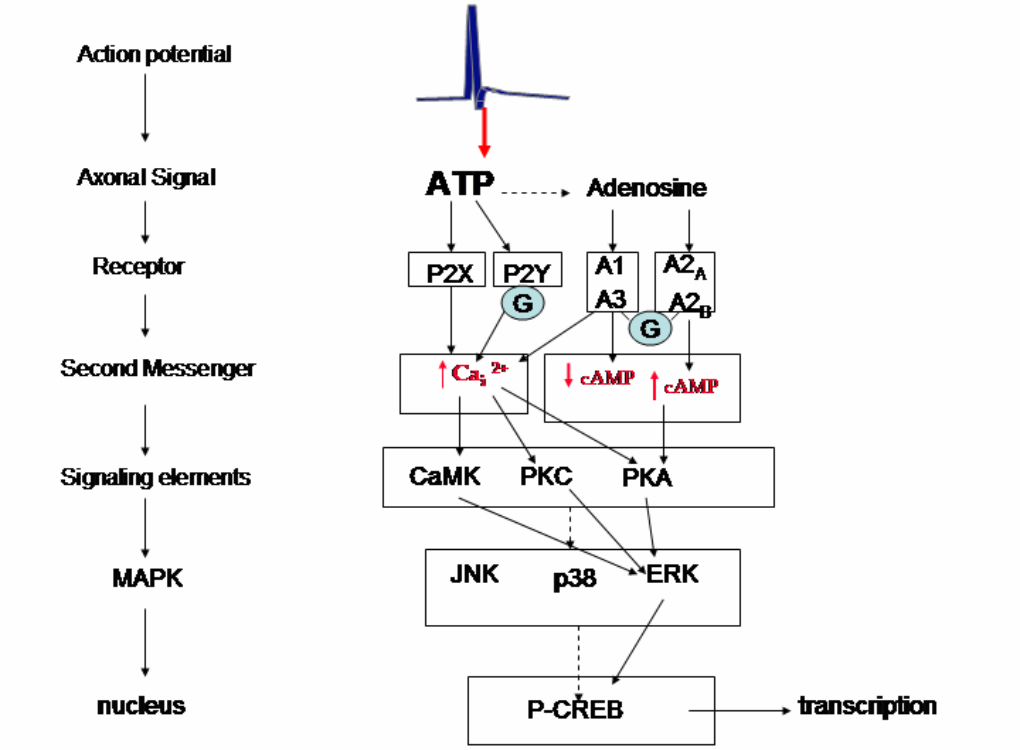


Figure 18. Possible intracellular signaling pathways activated in Schwann cells by neural impulse activity.

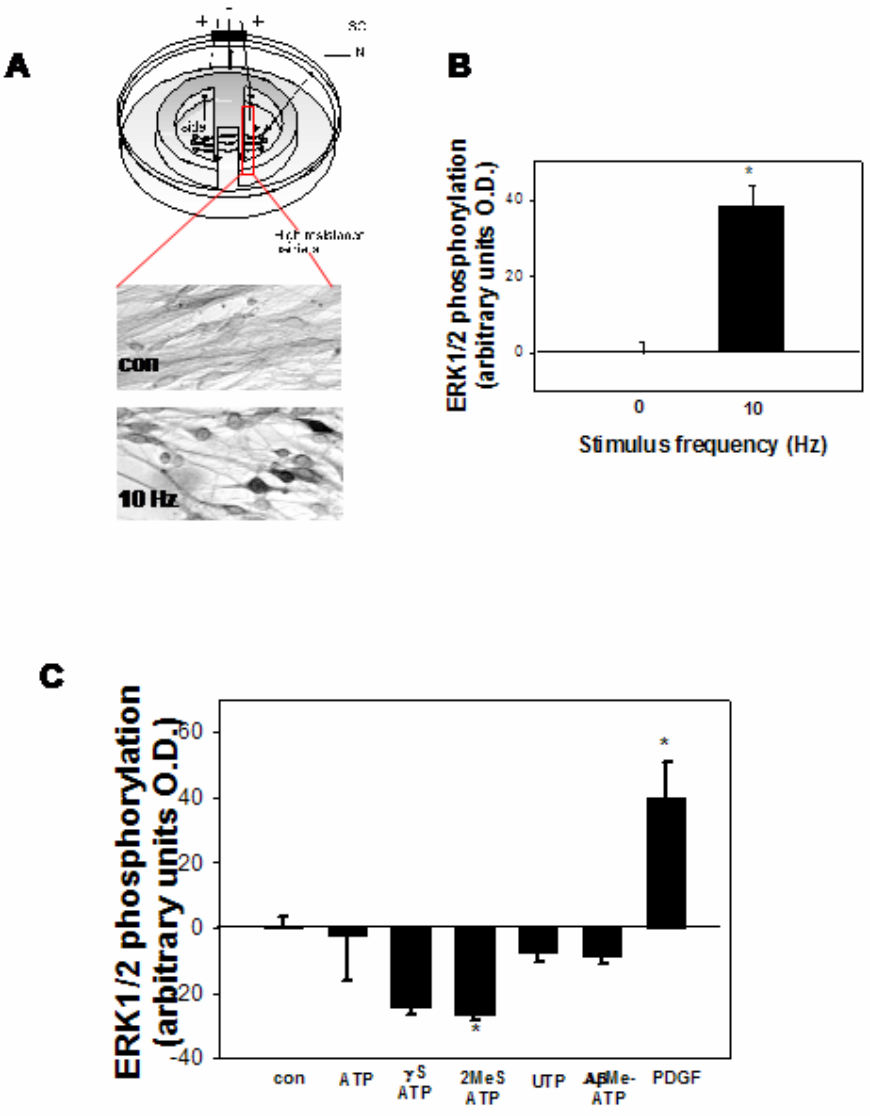
Figure 19. Action potential-dependent activation of ERK1/2 MAPK in Schwann cells is *not* mediated by extracellular ATP.

A. DRG neurons were electrically stimulated (10 Hz, 30 min), and active ERK1/2 MAPK levels were determined immunocytochemically in SCs co-cultured with DRGs in a multicompartment chamber equipped with stimulating electrodes. Electrical stimulation of DRG axons caused phosphorylation of ERK/MAPK **(10 Hz)** compared with unstimulated co-cultures **(con).** The staining intensity of Schwann cells was compared quantitatively by imaging densitometry. Only DRG neurons that extend axons under the high resistant barriers into the central compartment are electrically stimulated, therefore imaging densitometry was performed in 10-15 random fields along the central barrier region of each side compartment.

B, Electrical stimulation of DRG neurons significantly increased phospho-ERK1/2 MAPK levels in pre-myelinated SCs associated with electrically stimulated DRG axons compared with unstimulated controls ($p<0.0001$; $n=21$, t-test)

C. ATP and non-hydrolysable ATP agonist, γSATP failed to activate ERK/MAPK in SCs over a wide range of concentrations. Agonists selective for P2Y (UTP [P2Y2,4,6]) and P2X subtypes (αBMeATP [P2X]) were also ineffective in activating ERK/MAPK in SCs. Treatment of SC with the potent P2 receptor agonist, 2MeSATP, significantly inhibited ERK1/2 levels in SCs compared with unstimulated controls ($p<0.001$, $n=31$ exps).

Figure 19.


A
con
10 Hz
B
ERK1/2 phosphorylation (arbitrary units O.D.)
40
20
0
0
10
Stimulus frequency (Hz)
C
ERK1/2 phosphorylation (arbitrary units O.D.)
60
40
20
0
-20
-40
con
ATP
γS ATP
2MeS ATP
UTP
PDGF

P2Y1 RECEPTOR ACTIVATION INHIBITS ERK1/2 MAPK IN SCHWANN CELLS

There are several subtypes of ionotropic (P2X) and metabotropic (P2Y) ATP receptors which could mediate ATP-dependent inhibition of ERK1/2 MAPK in SCs (see Chapter 1, Fig 3)**.** Non-myelinating, adult Schwann cells express P2Y1 receptors both *in situ*, while myelinating Schwann cells primarily express P2Y2 receptors (Mayer et al., 1998), but the P2 receptor subtypes expressed in pre-differentiated SCs are not known. Time lapse confocal microscopy was used to monitor changes in intracellular calcium (Ca^{2+}_i) in SCs in response to selective ATP (P2) agonists. Our results indicate that pre-myelinating SCs express several subtypes of functional P2Y and P2X receptors, often in the same cell. Cultured SC responded robustly to P2Y1-receptor agonists, 2MeSADP and 2MeSATP, as well as the P2Y2/4 agonist, UTP (see fig. 20A). Dose-dependent calcium responses were also observed for the P2X agonists, αβMe-ATP (general) and BzATP (P2X7) (Fig. 20B). Since neither P2Y2/4 (UTP) or P2X (αB–MeATP) receptor agonists inhibited ERK1/2 MAPK in Schwann cells (Fig. 19C), we postulated that the 2MeSATP-dependent inhibition of ERK MAPK may be mediated by P2Y1 receptors.

2MeSATP activates P2Y1 receptors with high affinity, but can also bind to other P2 receptors when applied at higher doses (Mayer et al., 1998; Ralevic and Burnstock, 1998). Brief application of 2MeSATP (30 minutes) to

SCs grown in monoculture inhibited ERK1/2 MAPK activity in a dose-dependent manner. Lower doses (1μM) of 2MeSATP, significantly inhibited ERK1/2 immunostaining, while higher doses (30μM-300μM) were less effective (data not shown). RT-PCR using primers selective for mouse P2Y1 receptors confirmed that pre-myelinating SCs express the P2Y1 receptor mRNA (see fig. 21A). Consistent with these data, pre-treatment of SC cultures with the highly selective P2Y1 receptor antagonist, MRS 2179 (10μM) prevented the 2MeSATP-dependent inhibition of ERK1/2 MAPK compared to controls ($p<0.001$, n=24 exps) (Fig. 21B). Inhibition of ERK was also observed when SCS were treated with the selective P2Y1 receptor agonist, 2MeSADP, and this inhibition was also blocked by MRS 2179 (Fig. 21B). Collectively, these data support the hypothesis that P2Y1 receptor activation negatively regulates ERK1/2 MAPK in SCs.

Figure 20. Pre-myelinating SCs express functional P2Y and P2X ATP receptors

A. Time lapse confocal microscopy was used to monitor changes in intracellular calcium (Ca^{2+}_i) in SCs in response to P2Y receptor agonists (10μM). Cultured SC responded robustly to P2Y1-receptor agonists, 2MeSADP and 2MeSATP, as well as the P2Y2/4 agonist, UTP **.**

B. Dose-dependent calcium responses were also observed for the P2X agonists, αβMe-ATP (general) and BzATP (P2X7).

Figure 21 P2Y1 receptor activation inhibits ERK1/2 MAPK in Schwann cells.

A. RT-PCR using primers selective for mouse P2Y1 receptors confirmed that pre-myelinating SCs express mRNA transcripts for the P2Y1 receptor.

B. Pre-treatment of SC cultures with the highly selective P2Y1 receptor antagonist, MRS 2179 (10 μM) prevented the 2MeSATP-dependent inhibition of ERK1/2 MAPK compared to controls ($p<0.001$, n=24 exps)**.** Inhibition of ERK was also observed when SCs were treated with the selective P2Y1 receptor agonist, 2MeSADP, and this inhibition was also blocked by MRS 2179 at 10μM.

Figure 20.

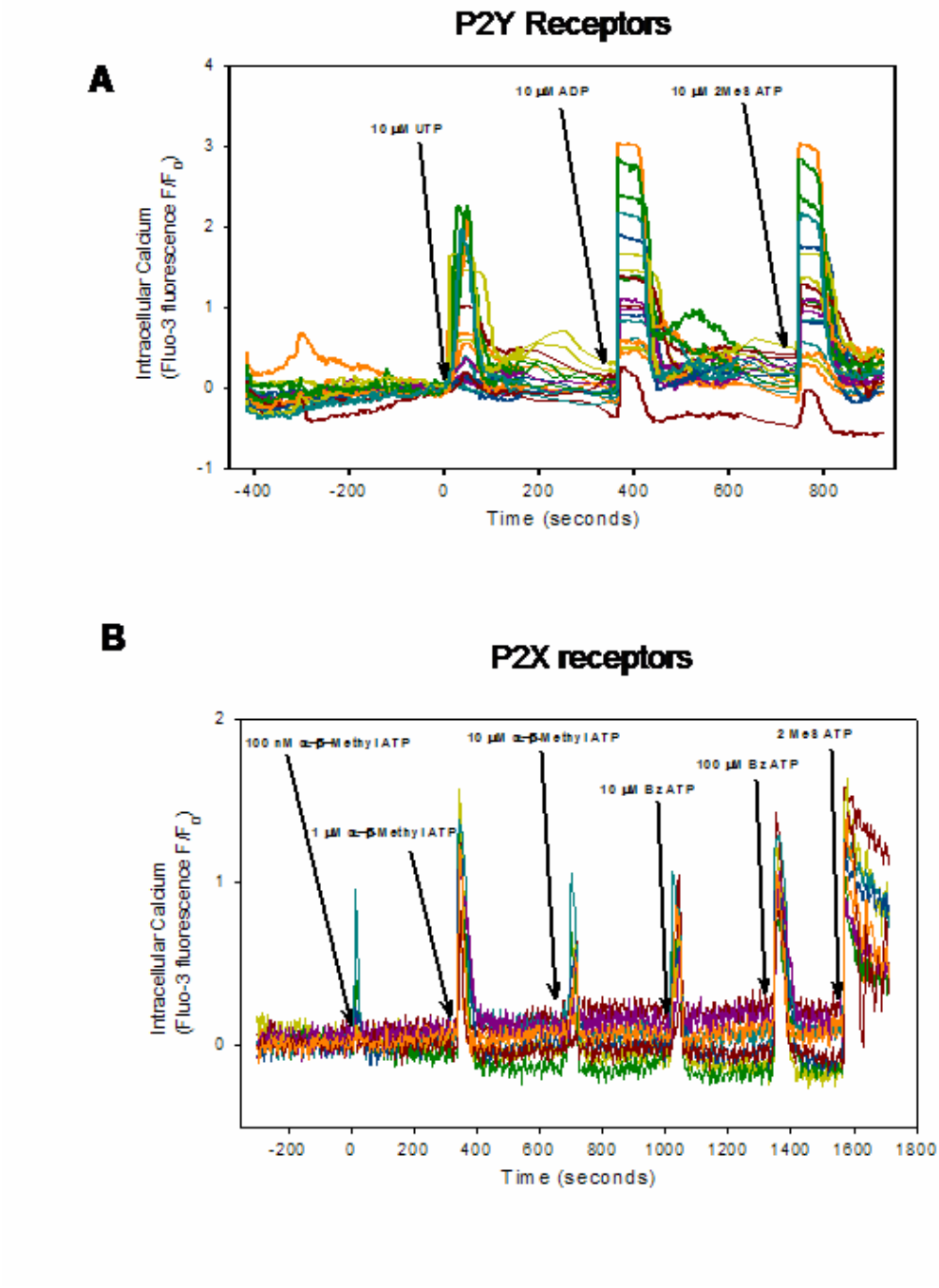
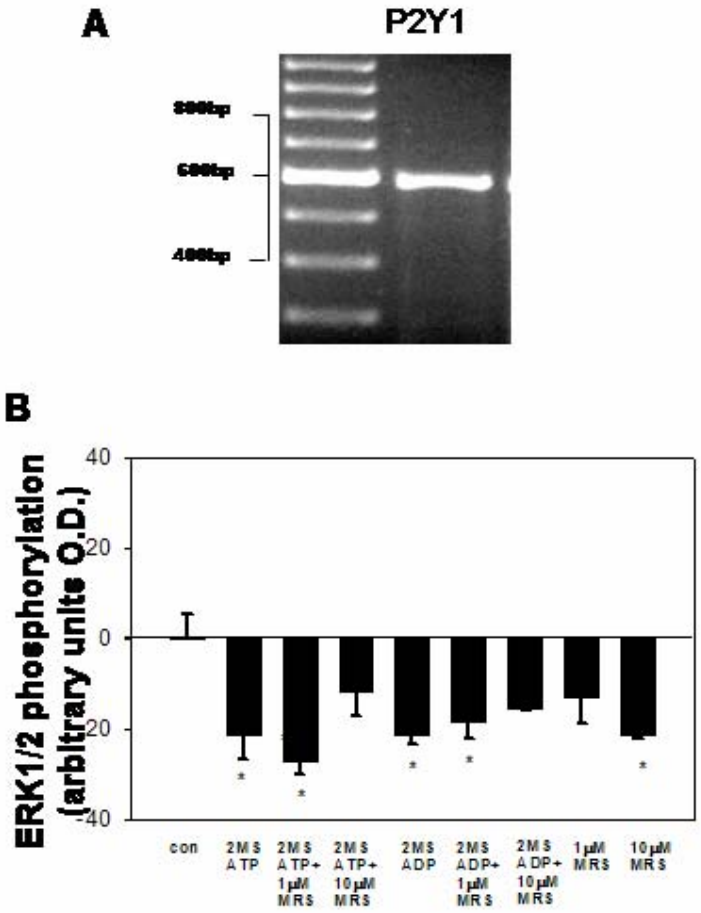

P2Y Receptors
A
10 µM UTP
10 µM ADP
10 µM 2MeS ATP
Intracellular Calcium
(Fluo-3 fluorescence F/F0)
Time (seconds)
B
P2X receptors
100 nM α,β-Methyl ATP
1 µM α,β-Methyl ATP
10 µM α,β-Methyl ATP
10 µM BzATP
100 µM BzATP
2 MeS ATP
Intracellular Calcium
(Fluo-3 fluorescence F/F0)
Time (seconds)

Figure 21.


A
P2Y1
B
ERK1/2 phosphorylation (arbitrary units O.D.)
40
20
0
-20
-40
con
2MS ATP
2MS ATP+ 1μM MRS
2MS ATP+ 10μM MRS
2MS ADP
2MS ADP+ 1μM MRS
2MS ADP+ 10μM MRS
1μM MRS
10μM MRS

ATP NEGATIVELY REGULATES ERK1/2 VIA ACTIVATION OF THE P38 MAPK PATHWAY

ATP receptor activation could activate multiple downstream kinase pathways, including PKA, PKC, CaMK, and p38-MAPK (Zimmermann, 1994; Fredholm, 1995; Neary, 1996), to phosphorylate CREB and regulate gene expression in SCs (see Fig 18). In order to determine which of these pathways is activated by extracellular ATP, we stimulated SC monocultures with ATP (100-300μM for 15- 30 minutes) in the presence of specific inhibitors to each of the kinases mentioned above and measured CREB phosphorylation immunocytochemically. Selective inhibition of the p38 MAPK pathway by SB203580 (20μM) completely prevented ATP-stimulated CREB phosphorylation. Pre-incubation of PKC inhibitor, GF109203X (1μm) also blocked the ATP-dependent CREB phosphorylation in SCs, but inhibition of PKA (2μM KT5720), CAMK (30μM KN62) were without effect ($p<0.001$, n=34 exps) (see fig. 22A). Consistent with the inability of ATP to activate ERK/MAPK, the MEK-1 inhibitor, PD098059 failed to block the ATP-dependent increase in P-CREB, suggesting that activity-dependent release of ATP does not signal though the ERK/MAPK to activate CREB as previously thought. Taken together our results suggest that ATP may signal through the p38 MAPK and PKC pathways to regulate CREB in SCs.

The function of p38 MAPK signaling in SCs is largely unexplored. While ATP has been shown to activate p38 MAPK in glia involved in inflammatory responses, regulation of p38 MAPK by purinergic signaling molecules has not been demonstrated in CNS or PNS myelinating glia. Using a phospho-specific antibody, we observed a rapid increase in phospho-p38 MAPK in SCs following direct application 2MeSATP compared to untreated control cultures (Fig. 22Ba). Maximal increases in phospho-p38 levels were observed between 5-10 minutes of 2MeSATP application, and returned to control levels after 30 minutes (Fig. 22Bb). In addition, the P2Y1 receptor agonist, 2MeSADP also activated p38 MAPK with similar kinetics (Fig. 22Bc). Interestingly, the concentrations of 2MeSATP and 2MeSADP that activated p38 MAPK in (1μM) also significantly inhibited ERK/MAPK, suggesting a possible link in these two signaling pathways.

Indeed, activation of p38 MAPK has been shown to inhibit ERK 1/2 MAPK in several cell types (Ding and Adrian, 2001; Zhang et al., 2001; Lee et al., 2002; Rice et al., 2002), but antagonistic interactions between p38 and ERK/MAPK pathways has not been demonstrated in glia. To determine whether the 2MeSATP-mediated inhibition of ERK1/2 MAPK is dependent on the p38 MAPK pathway, we stimulated SCs with 2MeSATP in the presence of the selective p38 MAPK inhibitor, SB203580. Pre-incubation of SC cultures with SB203580 completely blocked the 2MeSATP-dependent inhibition of ERK1/2 ($p<0.001$, n=10 exps) (see fig. 22C), and treatment of SCs with

SB203580 alone was without effect. Collectively, our results suggest that P2Y1 receptor activation by ATP inhibited ERK1/2 MAPK in SCs via a p38MAPK-dependent mechanism. These finding are the first to demonstrate possible antagonistic interactions between the p38MAPK and ERK/MAPK in glial cells, and identifies a novel signaling pathway by which ATP could communicate signals to the nucleus to regulate gene expression in Schwann cells.

Figure 22. P2Y1- receptor mediated activation of p38 MAPK in SCs

A. SCs were stimulated with 300μM ATP for 15 minutes following pre-incubation (1 hour) with inhibitors of MEK, CAM kinase, PKA, PKC, and p38 MAPK pathways. The ATP-mediated increase in P-CREB was significantly blocked by the PKC inhibitor (1μM GF109203X), and completely blocked by the p38 MAPK inhibitor (20μM SB203580) ($p<<0.001$ ANOVA; n=34 cultures). Inhibitors of MEK (50μM PD98059), PKA (2μM KT5720), and CaMK (30μM KN62) had no effect.

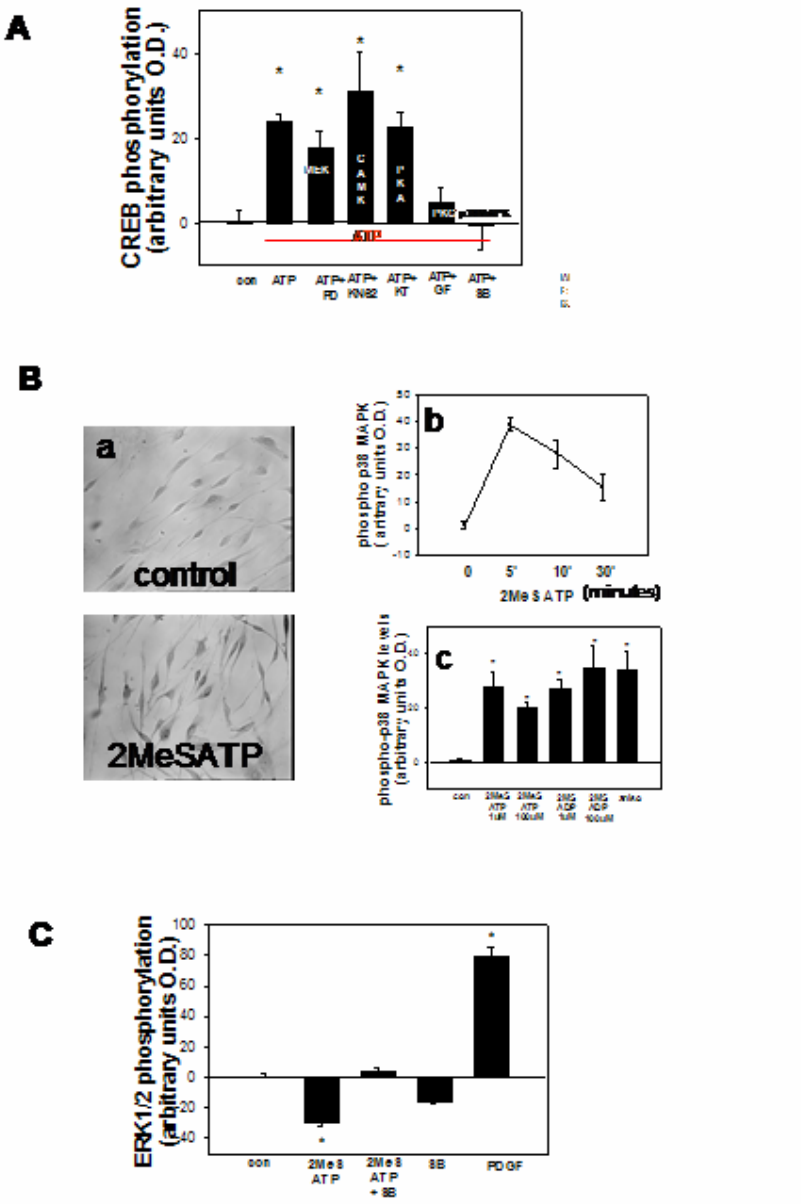
B. (a) Immunocytochemical detection of phosphorylated p38 MAPK indicate a rapid increase in phospho-p38 MAPK in SCs following direct application 2MeSATP compared to untreated control cultures.

(b) Maximal increases in phospho-p38 levels were observed between 5-10 minutes of 2MeSATP application, and returned to control levels after 30 minutes.

(c) 2MeSATP, as well as the P2Y1 receptor agonist, 2MeSADP activated p38 MAPK with similar kinetics.

C. Pre-incubation of SC cultures with SB203580 completely blocked the 2MeSATP-dependent inhibition of ERK1/2 ($p<0.001$, n=10 exps)**,** and treatment of SCs with SB203580 alone was without effect.

Figure 22.


A
CREB phosphorylation (arbitrary units O.D.)
40
20
0
MEK
CAMK
PKA
PKC
AKT
con
ATP
ATP+ PD
ATP+ KN62
ATP+ KT
ATP+ GF
ATP+ 8B
B
a
control
2MeSATP
b
phospho p38 MAPK (arbitrary units O.D.)
0
5'
10'
30'
2MeS ATP (minutes)
c
phospho-p38 MAPK levels (arbitrary units O.D.)
con
C
ERK1/2 phosphorylation (arbitrary units O.D.)
100
80
60
40
20
0
-20
-40
con
2MeS ATP
2MeS ATP + 8B
8B
PDGF

PRE-MYELINATING SCHWANN CELLS EXPRESS FUNCTIONAL ADENOSINE RECEPTORS

If not ATP, than what is the activity-dependent signal activating ERK1/2 MAPK Schwann cells in response to axonal firing? Since extracellular ATP is hydrolyzed to adenosine by ectonucleases (Zimmermann and Braun, 1996), we considered adenosine as a candidate axonal signal. Adenosine was recently identified as an important activity-dependent signal regulating oligodendrocyte development (Stevens et al., 2002a), but it is not known whether pre-myelinating SCs express functional adenosine receptors.

Time lapse confocal calcium imaging was used to determine whether pre-myelinating SCs in monoculture responded to adenosine receptor agonists. Consistent with published reports, adenosine (300μM) and selective adenosine receptor agonists (CCPA [A1], IBMECA [A3], and NECA (A1, A2 and A3]) (100 μM) failed to elicit calcium responses in SCs (data not shown). The A2a family of adenosine receptors, signal through cAMP, and are not coupled to PLC/IP3, thus A2a receptors would not have been detected in the calcium experiments described above. A2b receptors are also positively coupled to cAMP, and can mediate PLC/IPC-dependent Ca_i^{2+} increases in some cells types (Yakel et al., 1993; Feoktistov and Biaggioni, 1995). RT-PCR using specific primers for mouse A2a and A2b adenosine receptors detected mRNA transcripts for both subtypes of A2 receptors in SCs (see fig. 23A). These data

are the first show that SCs express adenosine receptors *in vitro*, and implicate adenosine as a possible activity-dependent signal between neurons and pre-myelinating SCs in our system.

ACTIVITY-DEPENDENT ACTIVATION OF A2 ADENOSINE RECEPTOR ACTIVATES ERK1/2 MAPK IN SCHWANN CELLS

To determine whether adenosine activates ERK1/2 MAPK in SCs, we applied selective agonists for A1 (CCPA), A2 (CGS21680) and A3 (IBMECA) receptors to SC monocultures, and measured ERK1/2 MAPK levels immunocytochemically as described previously. The selective A2 receptor agonist, CGS21680 (10μM) significantly increased phospho-ERK1/2 in SCs. In contrast, A1 and A3 selective receptor agonists (10μM CPA[A1]; 10μM IBMECA [A3]), were without significant effect ($p<0.005$, n=19 exps) (Fig. 23B). The general adenosine receptor agonist, NECA activated ERK/MAPK in SCs in a dose-dependent manner, with maximal activation occurring at lower doses (1 μM). Higher concentrations of NECA (300μM) had no significant effect on ERK/MAPK (Fig. 23C). In contrast, a dose –dependent increase in ERK activation was observed with the selective A2a receptor agonist, CGS21680 (100nM-30μM), with maximal activation occurring at higher doses ($p<0.003$, n=9 exps). Since NECA can activate both $A2_A$ and receptors, the $A2_A$-selective antagonist, ZM241385 was used to discriminate between these two receptor subtypes. Pre-incubation with (1μM) ZM241385

completely blocked the NECA-stimulated increase in P-ERK1/2 in SCs, and there were no significant effects on P-ERK1/2 MAPK levels in SCs following treatment with ZM241385 alone ($p<0.0001$, n=16 exps) (Fig. 23D). We were unable to directly test involvement of $A2_B$ receptors, since selective $A2_B$ agonists and antagonists are not available; however, ZM241385 was used at doses that should preclude $A2_B$ receptors.

A number of soluble signaling molecules could be released from DRG axons in an activity-dependent manner to activate ERK/MAPK in SCs. To determine whether adenosine mediates activity-dependent SC-axon communication in our system, DRG/SC co-cultures were pre-incubated with the A2 receptor antagonist ZM241385, and then stimulated at 10 Hz for 30 minutes. As shown in figure 23E, electrical stimulation in the presence of ZM231385 significantly inhibited the activity-dependent increase in phospho-ERK1/2 immunostaining following electrical stimulation of DRG neurons ($p<0.004$; n=13 exps) (Figure 23E). Taken together, our results implicate extracellular adenosine as the primary activity-dependent signal activating the ERK/MAPK pathways in Schwann cells acting through SC A2 adenosine receptors.

Figure 23. Activity-dependent release of adenosine activates ERK/MAPK in SCs

A. mRNA for the cAMP-dependent A2A and A2B receptors was detected by RT-PCR using specific adenosine receptor primers in SCs after 2 days in monoculture.

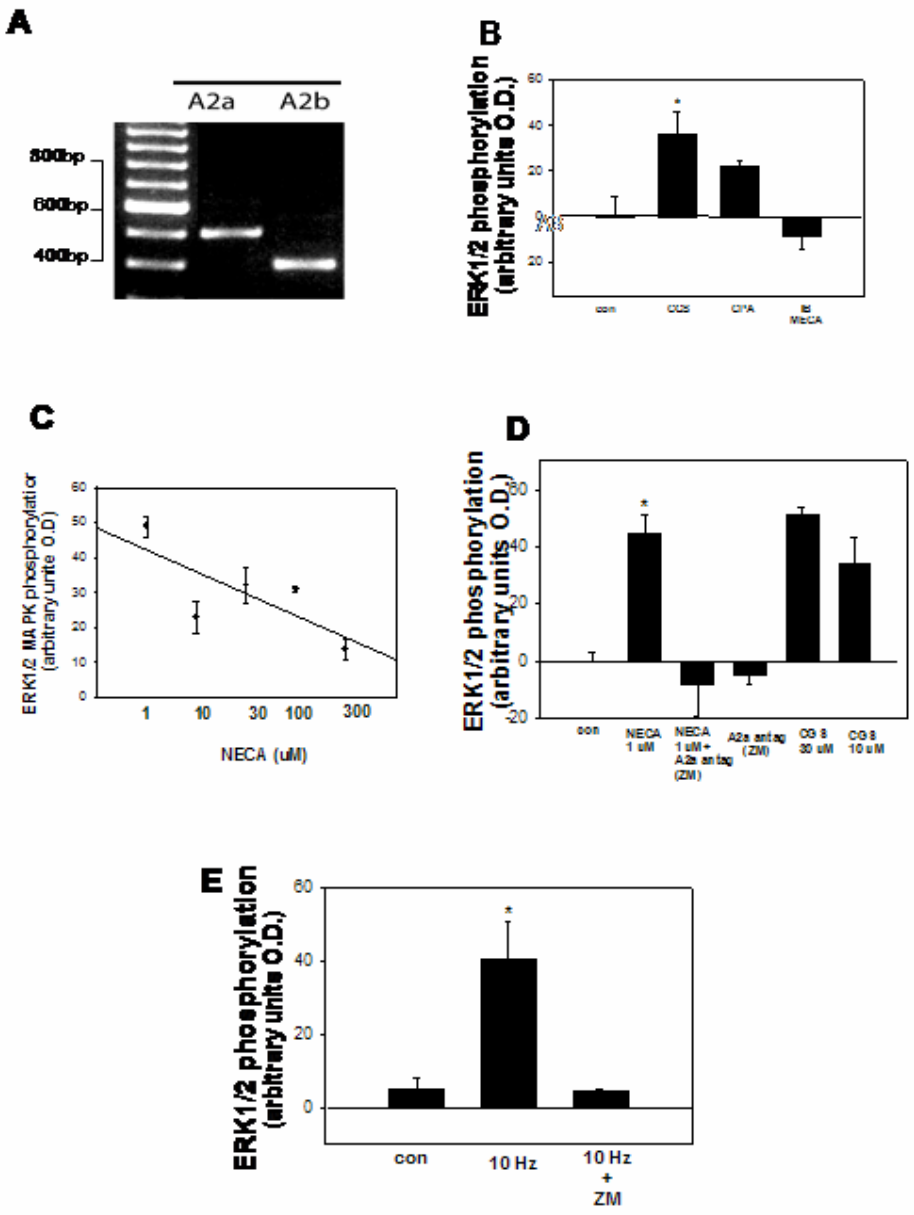
B. The selective A2 receptor agonist, CGS21680 (10μM) significantly increased phospho-ERK1/2 in SCs. In contrast, A1 and A3 selective receptor agonists (10μM CPA[A1]; 10μM IBMECA [A3]), were without significant effect ($p<0.005$, n=19 exps).

C. The general adenosine receptor agonist, NECA activated ERK/MAPK in SCs in a dose-dependent manner, with maximal activation occurring at lower doses (1 μM). Higher concentrations of NECA (300μM) had no significant effect on ERK/MAPK.

D. Pre-incubation with (1μM) ZM241385 completely blocked the NECA-stimulated increase in P-ERK1/2 in SCs, and there were no significant effects on P-ERK1/2 MAPK levels in SCs following treatment with ZM241385 alone ($p<0.0001$, n=16 exps).

E. DRG/SC co-cultures were pre-incubated with the A2 receptor antagonist ZM241385, and then stimulated at 10 Hz for 30 minutes. Electrical stimulation in the presence of ZM231385 significantly inhibited the activity-dependent increase in phospho- ERK1/2 immunostaining following electrical stimulation of DRG neurons ($p<0.004$; n=13 exps).

Figure 23.


A
A2a
A2b
800bp
600bp
400bp
B
ERK1/2 phosphorylation (arbitrary units O.D.)
con
CGS
CHA
IB MECA
C
ERK1/2 MAPK phosphorylation (arbitrary units O.D.)
NECA (uM)
D
ERK1/2 phosphorylation (arbitrary units O.D.)
con
NECA 1 uM
NECA 1 uM + A2a antag (ZM)
A2a antag (ZM)
CGS 30 uM
CGS 10 uM
E
ERK1/2 phosphorylation (arbitrary units O.D.)
con
10 Hz
10 Hz + ZM

BOTH ATP AND ADENOSINE INHIBIT GROWTH FACTOR-INDUCED SCHWANN CELL PROLIFERATION

The ERK/MAPK pathway plays a central role in regulating growth factor- dependent proliferation and differentiation of most mammalian cells, including developing SCs (Maurel and Salzer, 2000; Meintanis et al., 2001). Growth factors, such as PDGF and neuregulins signal through the Ras/Raf/MAPK pathway to regulate cell cycle progression (Kim et al., 1997a; Maurel and Salzer, 2000; Kim et al., 2001b). Cross talk between growth factors and purinergic receptors regulate ERK/MAPK signaling in astrocytes (Munsch et al., 1998; Neary et al., 1999b; Lenz et al., 2000; Panenka et al., 2001; Neary et al., 2003), but interactions between growth factors and purinergic receptors has not been explored in myelinating glia. Since growth factors are critical regulators of SC growth and development, we were interested in determining whether purinergic signaling molecules could regulate growth factor-dependent stimulation of SC proliferation.

We previously reported that ATP released from axons in response to axonal firing significantly inhibits proliferation of cultured SCs (Stevens and Fields, 2000), but the specific type(s) of purinergic receptors or signaling pathways involved were not identified. In order to determine which receptors mediate inhibition of SC proliferation by ATP, we applied selective ATP and adenosine receptor agonists over a wide range of concentrations to SC monocultures which had been serum starved for 24 hours. Growth factors

(PDGF, 10 ng/mL) were applied 15 minutes after the addition of purinergic agonists in order to stimulate entry in to the cell cycle. SC proliferation rates were then determined 24 hours later using a BrdU incorporation assay after a 6 hour pulse of BrdU at the end of the 24 hour period.

As shown in Figure 24, both ATP and adenosine receptor agonists inhibited growth factor-mediated SC proliferation. 2 MeSATP significantly inhibited SC proliferation by ~1.5 fold at all concentrations tested (1, 100 and 300 μM), and this inhibitory effect was mimicked by the P2Y1 receptor agonist, 2MeSADP ($p<0.004$, 18.6+/- 2.1[con] vs. 6.2 +/- 1.0 [ADP]; t-test, n=11 exps). In contrast, agonists selective for P2X receptors (alpha-beta methyl ATP and Bz ATP), and P2Y2/4 receptors (UTP) failed to inhibit SC proliferation (100 μM) (Figure 24A). These data implicate involvement of P2Y1 receptors in the ATP-dependent inhibition of SC proliferation.

Treatment of SCs with adenosine receptor agonists resulted in a more pronounced inhibition of proliferation compared to ATP receptor agonists. Adenosine at 100 μM inhibited SC proliferation by ~ 10 fold (21.6%+/- 2.8[con] vs. 2%+/- 0.65[Aden];t-test, n=6). A similar inhibition was observed with NECA (100 μM), and with A2a-selective agonist, CGS21680 at 100nM (Figure 24B). We found no evidence for cell death following adenosine or ATP receptor agonist treatment at the concentrations which inhibited SC proliferation, since the total number of SCs following the 24 hour period did not fall below unstimulated controls cultures. Taken together, our results

indicate that both ATP and adenosine inhibit growth factor-induction of SC proliferation through activation of distinct receptor subtypes (P2Y1, and A2), despite their opposing effects on ERK/MAPK, a critical regulator of cell growth. These data imply possible crosstalk between purinergic receptors and growth factor signaling pathways in controlling SC proliferation, and further suggest that growth factors and purinergic receptors could also cooperatively regulate ERK/MAPK.

ATP (P2) AND ADENOSINE (A2) RECEPTOR ACTIVATION INHIBITS GROWTH FACTOR-DEPENDENT ACTIVATION OF ERK1/2 IN SCS

Growth factors such as PDGF, and glial growth factor (GGF) are potent SC mitogens via activation of tyrosine kinase receptors and downstream Ras/Raf ERK1/2 MAPK pathway (Kim et al., 1997a; Maurel and Salzer, 2000; Kim et al., 2001a; Parkinson et al., 2002). Since adenosine receptor agonists have recently been shown to have opposing effects of ERK/MAPK in the presence and absence growth factors in other PC12 cells (Arslan et al., 1997; Arslan and Fredholm, 2000), we next investigated possible cross talk between adenosine (A2) and ATP (P2Y) receptors and growth factors upstream of ERK/MAPK. Since the growth factor activation of the ERK/MAPK pathway is necessary for G1 progression into the S phase in SCs (Kim et al., 2001a), we postulated that the same P1 and P2 receptor agonists

which inhibited growth-factor induction of proliferation may do so by interfering with growth-factor induction of the ERK1/2 MAPK cascade in SCs.

To determine the effects of ATP and adenosine on growth factor activation of ERK/MAPK, SCs were pre-treated with selective P1 and P2 purinergic agonists 15 minutes prior to application of PDGF, and ERK/MAPK was measured in SCS 30 minutes later. Consistent with our hypothesis, there was a dose-dependent correlation between the specific purinoreceptor agonists that inhibited both PDGF-dependent activation of ERK and PDGF-stimulation of SC proliferation (see fig. 25 A vs. B). Pre-incubation of SCs with the A2a agonist (30nM-1μM) CGS21680 significantly inhibited ERK1/2 MAPK levels in a dose –dependent manner, compared to SCs treated with PDGF alone ($p<0.006$;n=9 exps)(Fig. 25B). NECA (1-300μM) also significantly reduced PDGF-induced ERK/MAPK at the same concentrations which inhibited PDGF- stimulated SC proliferation (1-300μM) ($p<0.0001$, n=11 exps). In contrast, adenosine failed to inhibit PDGF-induced ERK MAPK in SCs, despite its potent inhibitory effect of SC proliferation (at 100 and 300μM), Since adenosine is hydrolyzed into AMP and inosine by ectoenzymes (Zimmermann, 2000), it is possible that extracellular adenosine inhibition of proliferation is indirect, or that anti-proliferative effects mediated by adenosine are independent of the ERK/MAPK pathway.

As demonstrated in Fig 25, 2MeSATP and P2Y1-selective agonist, 2MeSADP significantly inhibited PDGF-dependent activation of ERK/MAPK

when applied at the doses that also inhibited growth factor-dependent proliferation($p<0.005$, $n=10$ exps for 2MesATP; $p<0.005$, $n=9$ exps for 2MeSADP). These findings suggest a mechanism by which increased neural impulse activity, through release of purinergic signaling molecules, may over-ride the mitogenic axonal signals to inhibit SC proliferation before the onset of myelination.

Figure 24. Both ATP and adenosine receptor agonists inhibited growth factor-mediated SC proliferation.

A. 2 MeSATP significantly inhibited SC proliferation ,and this inhibitory effect was mimicked by the P2Y1 receptor agonist, 2MeSADP ($p<0.004$, 18.6+/- 2.1[con] vs. 6.2 +/- 1.0 [ADP]; t-test, n=11 exps). In contrast, agonists selective for P2X receptors (alpha-beta methyl ATP and Bz ATP), and P2Y2/4 receptors (UTP) failed to inhibit SC proliferation (100 μM).

B. Adenosine at 100 μM inhibited SC proliferation by ~ 10 fold. A similar inhibition was observed with NECA (100 μM), and with A2a-selective agonist, CGS21680 at 100nM.

Figure 24.

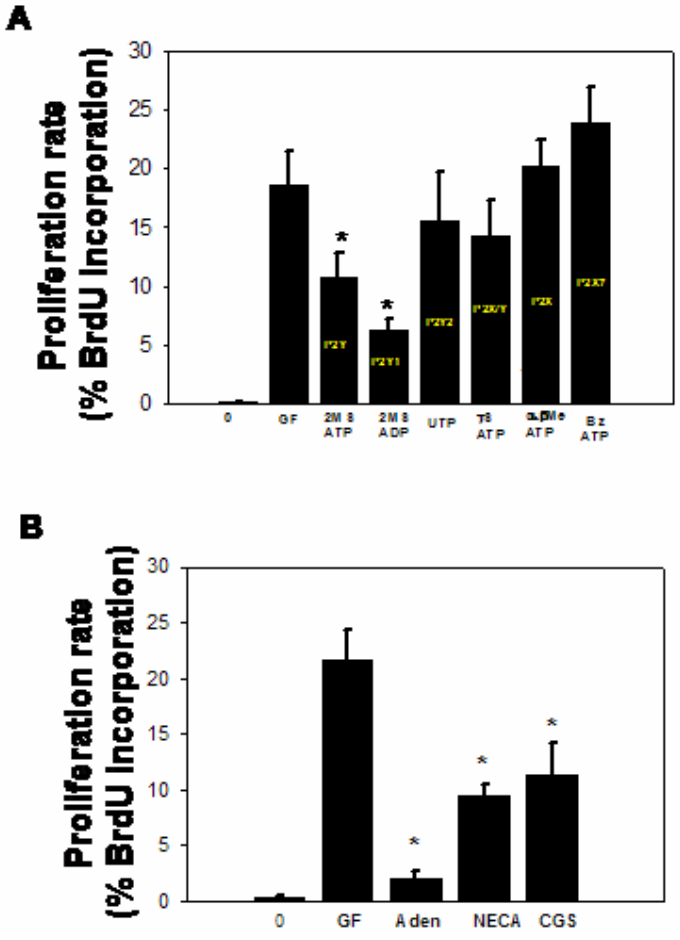

A
Proliferation rate
(% BrdU Incorporation)
30
25
20
15
10
5
0
*
*
P2Y
P2Y1
P2Y2
P2X/Y
P2X
P2X7
0
GF
2M S ATP
2M S ADP
UTP
Bz ATP
B
Proliferation rate
(% BrdU Incorporation)
30
25
20
15
10
5
0
*
*
*
0
GF
Aden
NECA
CGS

Figure 25. Selective ATP and Adenosine receptor agonists prevented growth-factor induction of ERK and growth factor stimulation of SC proliferation.

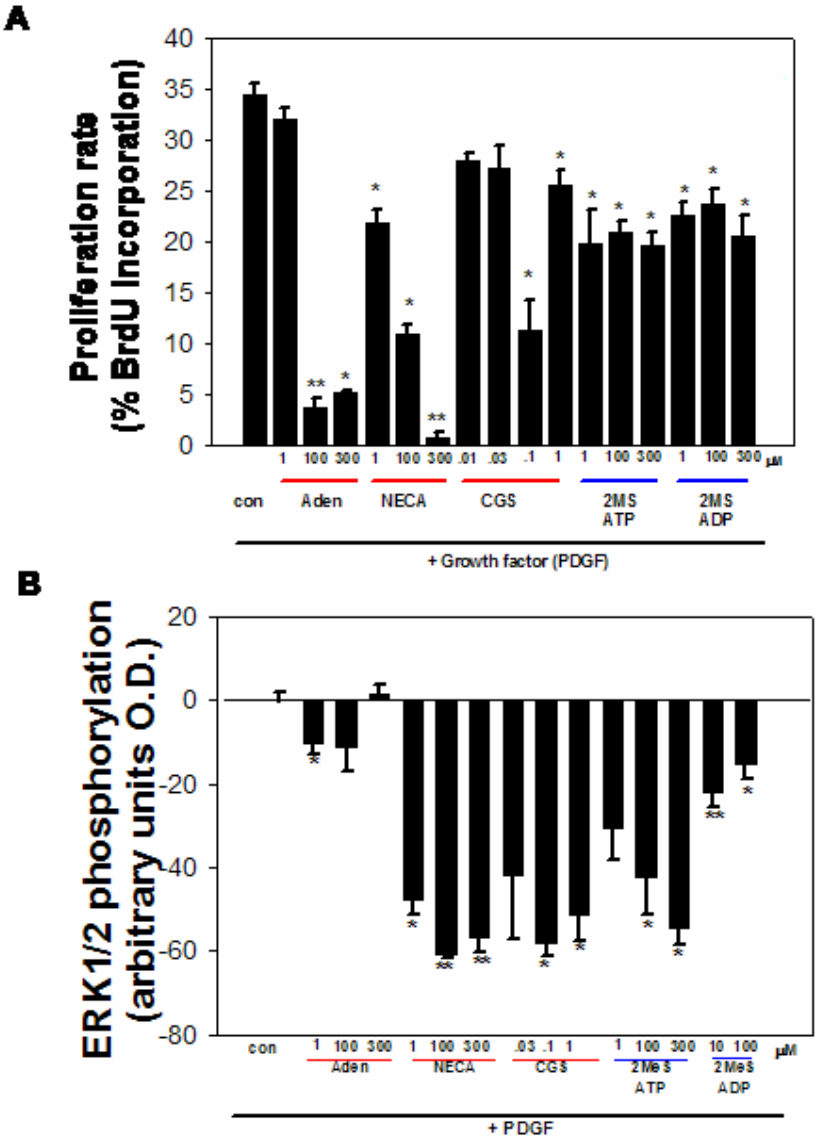
A. Pre-incubation of SCs with the adenosine (100, 300μM), A2a agonist (30nM-1μM) CGS21680, and the general adenosine receptor agonist NECA (1-300μM), significantly inhibited growth-factor stimulation of SC proliferation **(red lines).**

2MeSATP and P2Y1-selective agonist, 2MeSADP **(blue lines)** also inhibited growth factor-dependent proliferation

B. Pre-incubation of SCs with the A2a agonist (30nM-1μM) CGS21680, and the general adenosine receptor agonist NECA (1-300μM) , significantly inhibited growth factor activation of ERK1/2 MAPK levels in a dose – dependent manner. In contrast, adenosine failed to inhibit PDGF-induced ERK MAPK in SCs despite its potent inhibitory effect of SC proliferation (at 100 and 300μM) as shown above in A.

2MeSATP and P2Y1-selective agonist, 2MeSADP **(blue lines)** significantly inhibited PDGF-dependent activation of ERK/MAPK when applied at the doses that also inhibited growth factor-dependent proliferation.

Figure 25.


A
Proliferation rate
(% BrdU Incorporation)
40
35
30
25
20
15
10
5
0
1 100 300 1 100 300 .01 .03 .1 1 1 100 300 1 100 300 μM
con
Aden
NECA
CGS
2MS ATP
2MS ADP
+ Growth factor (PDGF)
B
ERK1/2 phosphorylation
(arbitrary units O.D.)
20
0
-20
-40
-60
-80
con
1 100 300 1 100 300 .03 .1 1 1 100 300 10 100 μM
Aden
NECA
CGS
2MeS ATP
2MeS ADP
+ PDGF

DISCUSSION

The present work identifies adenosine as novel axonal signal mediating activity-dependent communication between neurons and pre-myelinating Schwann cells. Using a combination of pharmacological and molecular approaches, we found that cultured mouse SCs express functional adenosine receptors, in addition to multiple subtypes of ATP receptors (P2X and P2Y) -- a far more complex array of purinergic receptors than previously thought. While both adenosine and ATP receptors are activated in SCs in response to neural impulse activity, these two families of purinergic receptors can have opposing effects on ERK/MAPK activity. Adenosine activated ERK/MAPK through stimulation of A2 adenosine receptors. In contrast, extracellular ATP inhibited ERK/MAPK activity, through activation of P2Y1-receptors coupled to the p38MAPK pathway. These findings are summarized in Figure 26. We further demonstrated that the presence of growth factors in the extracellular environment can alter the effects of ATP and adenosine on ERK/MAPK. These results are considered further below.

Figure 26.

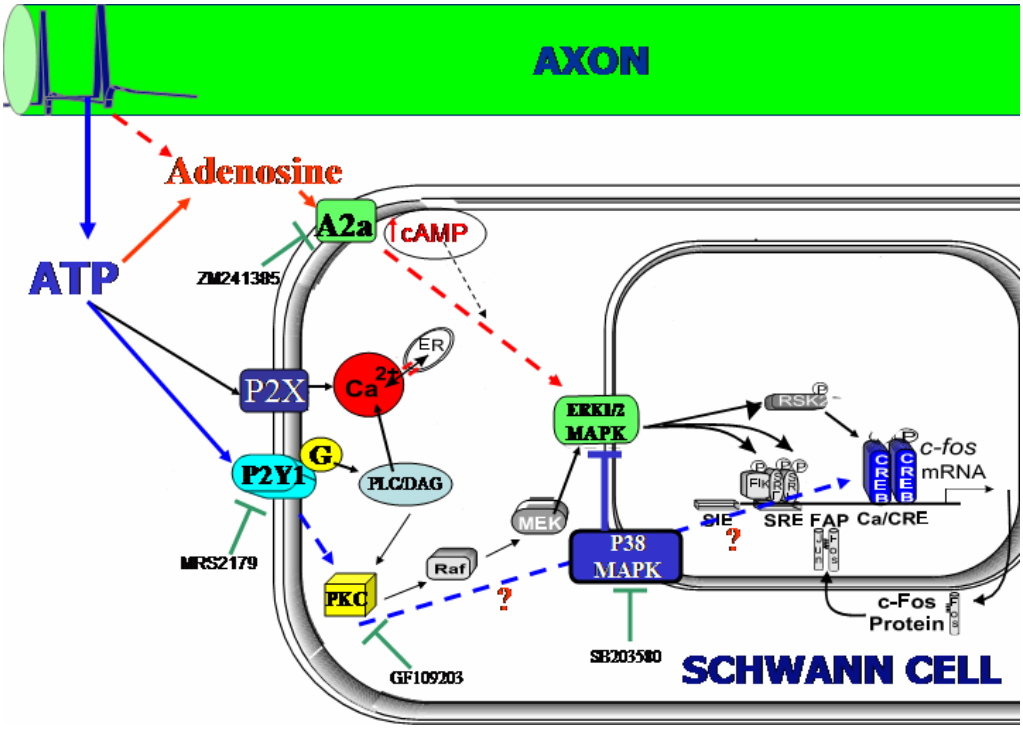


Figure 26. Antagonistic roles of ATP and adenosine on ERK/MAPK signaling in Schwann cells: a working model

PRE-MYELINATING SCHWANN CELLS EXPRESS FUNCTIONAL ADENOSINE AND ATP RECEPTORS

A major finding in this study is that pre-myelinating SCs express functional adenosine receptors. Recent reports have demonstrated expression of all four subtypes of adenosine receptors in cultured and acutely isolated oligodendrocyte progenitor cells (Stevens et al., 2002), and astrocyes (Pilitsis and Kimelberg, 1998), however adenosine receptors in PNS glia have not been previously characterized. Calcium transients have been observed in perisynaptic Schwann cells surrounding the neuromuscular junction, but these specialized PNS glia are structurally and functionally distinct from SCs associated with extrasynaptic myelinated and non-myelinated axons. In fact, several reports have demonstrated that adenosine failed to elicit calcium responses in SCs, both in vitro and in situ (Lyons et al., 1994; Mayer et al., 1997; Mayer et al., 1998). Using RT-PCR and selective adenosine receptor agonists and antagonists, we identified functional A2-type adenosine receptor subtypes in cultured, pre-myelinated SCs. Since this class adenosine receptors are positively coupled to cAMP, and not Ca_i^{2+}, it is possible that these receptor subtypes were present in studies in which adenosine failed to elicit calcium responses in SCs.

We also identified both ionotropic (P2X), and distinct subtypes of metabotropic ATP (P2Y), in pre-myelinating SCs. Our previous work implicated involvement of P2Y receptors in axon-SC communication, since

ATP-evoked calcium transients were observed in SC in the absence of extracellular calcium. In addition, activity and ATP-dependent gene expression in SCs was blocked by the general P2 receptor antagonist, suramin (Stevens and Fields, 2000), but these findings did not preclude expression of P2X receptors. This study found that while pre-myelinating SCs expressed P2X receptors, they do not appear to regulate ERK/MAPK or proliferation in SCs. In addition, pre-myelinating SCs co-express of P2Y1 and P2Y2 (and possibly P2Y4) receptors, but here we have shown that inhibition of ERK/MAPK and SC proliferation was selectively mediated by P2Y1 receptors. Further molecular and functional characterization of the specific subtypes of P2X and Y receptors expressed in pre-myelinated and differentiated SCs may identify other classes of P2 receptors which could further regulate SC gene expression and function.

ADENOSINE AND ATP CAN HAVE OPPOSING EFFECTS ON THE ERK/MAPK PATHWAY IN SCHWANN CELLS

Activation of ERK/MAPK underlies the mitogenic effects of many growth factors, and it is now widely accepted that regulation of ERK/ MAPK is far more complex than a linear pathway from membrane receptor to kinase. ERK/MAPK may represent a critical point of convergence from multiple intracellular signaling cascades coupled to diverse membrane receptors. ERK/MAPK appears to integrate these complex signals and communicate this

information to the nucleus to regulate genes involved in cell cycle and differentiation (Bhalla and Iyengar, 2001; Bhalla et al., 2002). Consequently, the degree and kinetics of ERK activation appear to be far more important in determining the biological outcome to an extracellular signal than simply activation itself. This hypothesis may explain why the same signal can have opposing effects in different cell types, or why the effects of a particular signal on ERK/MAPK varies in the same cell, depending on the biological context. Our results suggest that the net effect on ERK/MAPK in Schwann cells is likely dependent on several factors including, purinergic receptors expression, extracellular concentration and rate of ATP hydrolysis, and importantly, the degree of cross talk among purinergic receptor subtypes and other signaling systems.

Our findings suggest that activation of specific purinergic receptors can differentially regulate ERK/MAPK in SCs. This is consistent with a recent study by Lenz et al (2000), which showed distinct P2Y subtypes can recruit different MEK activators in cultured astrocytes (Lenz et al., 2000; Lenz et al., 2001). Our results indicate that different types of ATP and adenosine receptors can have can have opposing effects on ERK/MAPK in SCs. Acute application of ATP, and its non-hydrolysable analog, γSATP, had no effect on ERK/MAPK activity in SC monocultures. In contrast, activation of A2 adenosine receptors significantly increased ERK/MAPK levels, where as P2Y1 receptors inhibited basal ERK/MAPK activity in SCs. A2 adenosine receptors

are positively coupled to adenylate cyclase via G_s proteins, and increase intracellular cAMP levels (Seidel et al., 1999; Schulte and Fredholm, 2000; Klinger et al., 2002; Schulte and Fredholm, 2003). In SCs, $A2_A$ may activate ERK/MAPK via the cAMP-dependent PKA pathway, as has been demonstrated in several cell types levels (Seidel et al., 1999; Schulte and Fredholm, 2000; Klinger et al., 2002; Schulte and Fredholm, 2003). In contrast, ATP/P2Y receptors appear to signal through the p38MAPK pathway, and not ERK/MAPK. Our findings that ATP- mediated phosphorylation of CREB is dependent on both PKC and p38MAPK, and independent of PKA and MEK/ERK is consistent with this notion. Although this study did not extensively characterize the upstream elements involved these two signaling cascades, our observed antagonistic effects of ATP and adenosine on ERK/MAPK indicate that different families/subtypes of purinergic receptor expressed in pre-myelinating SCs may cooperatively modulate ERK/MAPK activity in SCs.

Importantly, this study provided evidence that action-potential dependent release of ATP from sensory axons activates ERK/MAPK in associated SCs. While pharmacological stimulation of specific receptor subtypes allowed us to characterize the receptors that regulate ERK/MAPK in SCs, it is unlikely that a single receptor subtype would be activated under physiological conditions, especially given our findings that SCs express multiple purinoreceptor subtypes on their surface. Our co-culture system more

closely models the in vivo environment by allowing pre-myelinating SCs to naturally associate with axons--which provide a host of survival and growth signals absent in SC monocultures. In addition, our system enabled electrical stimulation of axons at frequencies representative of developing axons before the onset of myelination (Fitzgerald, 1987).

Several lines of evidence implicate adenosine as the primary activity-dependent signal mediating ERK activation in SCs. Activity-dependent activation of ERK/MAPK was blocked by the selective A2 receptor antagonists ZM241385. Consistent with these data, the selective A2 receptor agonist, CGS 21680 activated ERK in a dose dependent manner, as did NECA at nM concentrations. That ATP failed to activate ERK under the conditions of our study ERK/MAPK, further excluded involvement of ATP/P2 receptors in activity-dependent activation of ERK/MAPK.

We previously reported that ATP is released extrasynaptically from DRG neurons in an activity-dependent manner (Stevens and Fields, 2000). Extracellular ATP is hydrolyzed to ADP, AMP and ultimately to adenosine, which can modulate cell function via its own receptors. The rate and degree of ATP hydrolysis, is tightly regulated by several complex families of ectonucleases, which act together to terminate nucleotide signaling at their respective receptors (Zimmermann et al., 1998). Our findings suggest that ATP is sufficiently hydrolyzed to adenosine during the 30 minute stimulation period in DRG/SC co-cultures to exert functional effects in SCs.

Ectonuceotidases may be absent in SCs grown without axons, since acute stimulation (30 min) of ATP to SCs monocultures failed to activate ERK, where as activity-dependent generation of ATP activated ERK in SCs in DRG/SC co-cultures. Consistent with this notion, agents that increase intracellular cAMP levels (thus mimic axonal signals) have been recently shown to significantly up-regulate an ATPase in cultured Schwann cells (Bermingham et al., 2001). We have not ruled out the alternative possibility that adenosine is directly released from DRG neurons in an activity –dependent manner. Future studies will be necessary to determine whether ectonucleotidases are involved in regulating activity-dependent axon-SC communication.

ATP ACTIVATES P38 MAPK IN SCHWANN CELLS AND INHIBITS ERK/MAPK

Our results are the first to demonstrate activation of p38 MAPK by purinoreceptors in myelinating glia. In contrast to ERK and JNK family-MAPKs, the role of p38 MAPK in the nervous system is poorly understood. As its name implies, the stress activated protein kinase (SAPK), p38 MAPK is preferentially activated by environmental stress such as UV radiation, heat and osmotic shock, and by inflammatory signals such as TNF, IL-1, and TGF-β (Koistinaho and Koistinaho, 2002). Activation of the p38 MAPK pathway also regulates cytokine synthesis and secretion in a number of cells (Da Silva

et al., 1997; Ridley et al., 1997; Ridley et al., 1998; Chae et al., 2001). In the CNS, p38 MAPK is activated in microglia (Bhat et al., 1998; Pocock and Liddle, 2001; Shigemoto-Mogami et al., 2001) and astrocytes (Bhat et al., 1998; Panenka et al., 2001; Schinelli et al., 2001) in response to hypoxia, and injury-related signals including ATP, which is consistent with the recognized role of these glial cells in acute brain injury and neurodegenerative diseases. Much less is known about the function of p38MAPK in myelinating glia in the central and peripheral nervous system.

Here we presented the first evidence of ATP-dependent activation of p38 MAPK in Schwann cells. Direct activation of ATP/P2Y receptors at low concentrations, induced rapid and transient activation p38 MAPK in pre-myelinated SCs. Both 2MeSADP and 2Me SATP activated p38MAPK at the same concentrations and kinetics, suggesting involvement of P2Y1 receptors. In addition, ATP-dependent CREB phosphorylation was blocked by p38 MAPK inhibitor, SB203580, further implicating involvement of this MAPK. Although the specific regulators of p38 MAPK cascade are poorly defined, CREB has been reported as a downstream target of p38 MAPK in a number of cell types (Xing et al., 1998; Hansen et al., 1999; Pugazhenthi et al., 1999; Schinelli et al., 2001), and PKC has been reported as an upstream activator of p38 MAPK (Loubani and Galinanes, 2002). Here we showed that selective inhibition of PKC by GF109203X and p38 MAPK by SB203580 both inhibited ATP-dependent CREB phosphorylation in SCs. Whether PKC acts

upstream of p38MAPK in SCs has not been determined. PKC and p38 MAPK could alternately activate CREB through parallel pathways, as has been reported (Fiebich et al., 2001).

Interestingly, our observation that P2Y1-receptor mediated inhibition of ERK/MAPK in SCs was dependent on p38 MAPK is in line with several recent reports demonstrating negative regulation of the ERK1/2 pathway by p38 MAPK (Singh et al.,1999; Hall et al., 2002; Lee et al., 2002; Ahuirre-Ghiso et al., 2001; Ding et al., 2001). p38 MAPK has been shown to inhibit ERK by two different mechanisms: via activation of protein phosphatases, which inhibit MEK/ERK activation (Westernmarck et al. 2001), and via direct physical interactions between p38α and ERK1/2, in which p38 appears to sequester ERK1/2 and sterically block their phosphorylation by MEK1 (Zhang et al 2000).

In addition to regulating stress-related functions, the p38 MAPK pathway also plays a critical role in regulating cell cycle, growth and differentiation (Bogre et al., 2000; Ono and Han, 2000). Activation of p38MAPK was recently shown to induce cell cycle arrest via inhibition of the Raf/ERK pathway during muscle differentiation (Lee et al., 2002). p38 MAPK was also shown to negatively regulate PDGF-mediated proliferation of myofibroblasts by directly interacting with ERK1/2 and suppressing its phosphorylation (Rice et al., 2002). In addition, there is growing evidence that p38 MAPK inhibits cell cycle machinery independently of ERK. Activation of

p38 MAPK has been shown to negatively regulate the transcription if Cyclin D1, and induce cyclin-dependent kinase inhibitors (CDKIs) (Page and Hershenson, 2001; Lavoie et al., 1996).

The mechanisms by which p38 negatively regulates ERK1/2 in SCs remains to be determined, but it is tempting to speculate that ATP-dependent inhibition of ERK by p38 MAPK via one of above mechanisms could underlie ATP/P2Y receptor mediated inhibition of SC proliferation. Unpublished observations from our laboratory indicate that ATP receptor activation in SCs induced G1 arrest, inhibited cyclin D1, and prevented the down-regulation of the CDKI, p21 in the presence of growth factors (see Chapter 3; Figs 12,13). Future studies using dominant negative approaches may be useful in directly testing this hypothesis, since pharmacological perturbation of p38 and ERK kinases can inhibit SC proliferation, and interfere with proliferation experiments.

CROSS TALK BETWEEN PURINERGIC AND GROWTH FACTOR RECEPTORS IN REGULATION OF ERK/ MAPK AND PROLIFERATION IN SCS

Schwann cell proliferation is regulated by direct interactions with axons during embryonic and early postnatal development (Salzer and Bunge, 1980; Wood and Bunge, 1975), or in response to peripheral nerve injury (Bradley and Ashbury, 1970; Salzer et al.,1980). Growth factors, including PDGF and

neuregulins are potent SC mitogens to cultured SCs when applied in combination with agents that increase cAMP (Stewart et al., 1991). It has been proposed that increased levels of intracellular cAMP, induced by unknown axonal factors, act as a primary signal that synergizes with growth factors to activate ERK/MAPK, and stimulate cell cycle progression (Kim et al., 1997a; Kim et al., 2001a; Stevens and Fields, 2002). At the same time, cAMP elevation in post-mitotic SCs promotes differentiation and myelination (Sobue and Pleasure, 1984; Sobue et al., 1986; Morgan et al., 1991), indicating that cAMP can exert opposing functional effects (proliferation vs. differentiation) in the same cell type under different biological contexts.

Recent studies in SCs (Kim et al. 1997), and a variety of other cell types indicate that cAMP/PKA plays a crucial and generalized role in transducing growth factor signals to the nucleus (Stork and Schmitt, 2002). In Schwann cells, the ERK/MAPK cascade represents a point of cross-talk between growth factors and cAMP signaling pathways, whereby cAMP can either enhance or inhibit growth-factor mediated ERK signaling (and cell cycle progression) by regulating kinases upstream of ERK, such as Ras/Raf-1 or Rap-1/BRaf (Kim et al., 1997a; Kim et al., 1997b; Kim et al., 2001a). For example, pretreatment of SCs with forskolin, significantly reduced neuregulin-induced ERK/MAPK in SCs (Kim et al., 1997a). In addition, the intracellular concentration of cAMP is also important in determining the level and duration of ERK/MAPK activity and biological outcome in SCs. Mutoh et al., (1998)

demonstrated that low concentration of cAMP (0.1mM) activated S6 kinase in SCs (an immediate target of activated ERK), resulting in transient, and high levels of ERK1/2--conditions that stimulate SCs proliferation. In contrast, high concentrations of cAMP (>1mM), induced a smaller activation of S6 kinase activation, and smaller but prolonged ERK/MAPK--conditions that induce SC differentiation (Sobue et al., 1986) (Mutoh et al., 1998). A similar paradigm has been shown in PC12 cells (Marshall, 1995). Thus, the ability of cAMP to both promote proliferation and differentiation in SCs appear dependent on relative concentrations of cAMP and the kinetics of ERK1/2 activation, rather that simply activation or inhibition.

The identity of the axon signal(s) which increase cAMP to differentially regulate SC proliferation and differentiation during development are not known, but our results suggest adenosine as a possible candidate. Our in vitro findings suggest that adenosine may be released from electrically active axons (either directly or via breakdown of ATP) during the time when SCs are exiting from the cell cycle, and differentiating into myelinating or nonmyelinating phenotypes. G-protein coupled -A2 adenosine receptors are positively coupled to adenosine and increase intracellular cAMP, and cAMP-dependent activation of ERK1/2 by Gs-coupled A2a and b receptors have been shown in several cell types (Schulte and Fredholm, 2003).

Adenosine can exert an ERK1/2- dependent mitogenic effects in some cells, while also exerting inhibitory effects on growth-factor-induced ERK

phosphorylation. Arslan and Fredholm (2000) reported that acute treatment with adenosine receptor agonists alone activated ERK/MAPK in PC12 cells, even at low doses. However, when adenosine was acutely applied to cells in the presence of NGF, adenosine inhibited NGF-induced ERK. This was attributed to an inhibition of Raf1in PC12 cells by high levels of cAMP, which consequently interfered with NGF-mediated effects (Arslan and Fredholm, 2000). These results are strikingly similar to our present findings in SCs. While acute activation of A2 adenosine receptors activated ERK/MAPK in SCs, adenosine blocked the PDGF-induced activation ERK, and proliferation in the presence of growth factors. Interestingly, $A2_A$ receptor expression is dramatically down regulated in differentiated cells, compared to pre-differentiated PC12 cells, which highly express $A2_A$ receptors (Arslan et al., 1997). Therefore adenosine can exert different functional effects in pre-differentiated vs. post differentiated PC12 cells, in response to the same purinergic signal. This is in line with the longstanding observations that cAMP can opposing effects on ERK and SC function in differentiated vs. pre-differentiated stage in development.

The onset of high frequency neural impulse activity, and possible ATP release from sensory axons, corresponds to a critical period in development, when SCs stop proliferating, and differentiate into mature myelinating or non-myelinating phenotypes (Fitzgerald, 1987; Fields and Stevens, 2000). Our results suggest that extracellular ATP and adenosine may act together, and

with trophic factors to balance MAPK signaling in SCs to meet functional requirements during development, regeneration, and nervous system plasticity.

CONCLUSIONS

1. Electrical stimulation of DRG neurons directly activates the ERK/MAPK pathway in associated pre-myelinating SCs in DRG/SC co-cultures.

2. The activity-dependent signal was identified as adenosine, acting through A2 receptors on premyelinating SCs, and not ATP.

3. ATP can inhibit ERK1/2 MAPK in SCs by activating P2Y1 receptors and downstream P38 MAPK.

4. Both ATP and adenosine inhibited growth factor-dependent proliferation and growth factor induction of ERK1/2 in SCs.

CHAPTER 6

DISCUSSION

Electrical activity plays a crucial role in modifying developing neural circuits in the perinatal period. Neural impulse activity regulates neuronal gene expression, neurite outgrowth, neuronal morphology, and the stabilization of synaptic connections (Buonanno and Fields, 1999; Penn and Shatz, 1999; Fields et al., 2001), yet much less is known about the role of neural activity in controlling glial development. The present findings are the first to demonstrate activity-dependent communication between axons and pre-myelinating Schwann cells, and identify extracellular ATP as a potent and dynamic axonal signal regulating Schwann cell gene expression, proliferation, differentiation, and myelination in response to axonal firing.

Here these data will be put into a larger developmental context, and the implications of these findings will be further discussed with respect to the specific aims outlined in the introduction. Additionally, the strengths and limitations of the methodologies used in these studies will be highlighted, with particular emphasis on the requirements necessary for implicating ATP as an axonal signal regulating Schwann cell development *in vivo*. Future directions and open questions relating to this research will also be discussed.

ACTIVITY-DEPENDENT COMMUNICATION BETWEEN AXONS AND DEVELOPING SCHWANN CELLS

The first fundamental question that was addressed in this research was whether immature, pre-myelinating Schwann cells could respond to neural impulse activity. While there is evidence to support activity-dependent neuron-Schwann cell communication at the synapse, as well as the paranode of mature myelinated axons, the present findings are the first to identify this form of communication between axons and developing, pre-myelinated SCs. A DRG/SC co-culture system equipped with stimulating electrodes was developed to electrically stimulate DRG neurons, and study the ensuing effects in pre-myelinating SCs. Confocal calcium imaging experiments revealed that electrical stimulation of DRG neurons elicited robust calcium responses in associated SCs. These calcium transients were observed several seconds up to minutes after the onset of neural impulse activity, which strongly suggested involvement of a soluble axonal signal. As will be discussed below, the activity-dependent signal between axons and pre-myelinating SCs was identified as extracellular ATP. It was further found that activity-dependent release of ATP activated multiple intracellular signaling pathways in SCs, phosphorylated CREB, and increased levels of the transcription factors, c-fos and krox-24 in SCs. Together these findings address each of the questions posed in the first experimental aim (Chapter 2)(Stevens and Fields, 2000), and

will be further discussed and contrasted with related experiments in the adult nervous system.

This research identified ATP as the activity-dependent signal mediating calcium signaling and gene expression in pre-myelinating SCs. Although SCs are responsive to multiple neuroactive signals that could be released from DRG axons in an activity-dependent manner, this research provides several lines of evidence that ATP is the primary activity-dependent axonal signal in our system. First, pre-myelinating SCs express functional ATP receptors. Using a combination of imaging, pharmacological and molecular approaches, several types of ATP receptors were identified in pre-myelinating SCs (P2Y and P2X). Second, selective ATP receptor agonists mimicked the electrically induced calcium responses in SCs. Third, activity-dependent calcium transients were completely blocked in Schwann cells in the presence of apyrase, an enzyme that rapidly degrades extracellular ATP. Activity-dependent increases in PCREB, c-fos and krox-24 were also prevented when cultures were stimulated in the presence of apyrase, and/or the general P2 receptor antagonist, suramin.

Fourth, extracellular ATP was released from DRG axons in response to sodium-dependent action potential activity. Medium collected from pure DRG cultures stimulated at 10 Hz showed a highly significant increase in ATP compared with unstimulated controls. Electrically induced ATP release occurred with rapid kinetics (within the first several minutes of electrical

stimulation), and was completely prevented when sodium-dependent action potentials were blocked with TTX. Furthermore, increased levels of ATP were observed in the central axonal compartment of our chamber preparation that lacked DRG cell bodies, suggesting that ATP was released from electrically active axons. The mechanisms of extrasynaptic ATP release are currently under active investigation. Together these findings strongly implicate extracellular ATP as the activity-dependent signal regulating calcium signaling and gene expression in pre-myelinating SCs.

While there are general limitations associated with an *in vitro* system, our co-culture model has several unique advantages for the study of activity-dependent communication between neurons and developing SCs. First, only neurons with axons traversing the central barriers are stimulated to fire action potentials, thus SCs are not directly stimulated. Second, DRG neurons are not spontaneously active, nor do they form synapses in vitro (Li et al., 1996). These features allowed electrical stimulation of DRG neurons at developmentally relevant frequencies (1, 3, 10 Hz), and the study of SC responses in an extrasynaptic environment. Finally, the developmental stage of SCs cultured from rodent sciatic can be tightly controlled and characterized at the morphological and biochemical levels. Since SC lineage progression in DRG/SC co-cultures closely parallels that of the intact sciatic nerve, SCs were co-cultured at the immature, pre-myelinated stage, in order to study the effects on neural impulse activity on SC gene expression and development before the

onset of differentiation and myelination. Using a combination of imaging, biochemical, and pharmacological approaches, this model system enabled us to identify ATP as an activity-dependent signal between neurons and pre-myelinating SCs. These studies lay the foundation for designing specific experiments to address similar questions *in vivo*. As will be discussed below, several *in situ* studies provide evidence for activity-dependent communication between neurons and Schwann cells in the adult animal.

Purinergic signaling molecules have also been shown to mediate activity-dependent neuron-SC communication at the adult neuromuscular synapse (Castonguay and Robitaille, 2001). Terminal Schwann cells surrounding synaptic terminals at the neuromuscular synapse express functional ATP (P2Y and X), and synaptic release of ATP has been shown to induce calcium transients in terminal SCs at the frog neuromuscular junction (NMJ) (Robitaille, 1995). Although certain parallels exist between synaptic and the extrasynaptic neuron-SC signaling presented here, there are several significant difference between studies at the adult synapse, and the present research.

First, terminal Schwann cells are specialized cells, which more closely resemble perisynaptic CNS astrocytes that myelinating or cultured SCs. Aside for the obvious functional differences, terminal Schwann cell differ structurally and biochemically from SCs associated with extrasynaptic regions of axons (Son et al., 1996; Castonguay and Robitaille, 2002; Auld and Robitaille, 2003).

Although they express several myelin-related proteins, (such as P0, MBP, and GalC), terminal SCs do not form myelin.

A second major distinction is that ATP does not appear to mediate activity dependent SC-axon communication at the frog NMJ (Rochon et al., 2001), as it has been reported in the frog (Robitaille, 1998). Curiously, both rodent and frog terminal SCs express functional ATP receptors, but P2 receptor antagonists failed to reduce activity-dependent calcium transients in rodent terminal SCs, suggesting functional differences in purinergic signaling exist in these two species. This discrepancy may reflect evolutionary modifications in the way that terminal SCs respond to synaptic activity (Auld and Robitaille, 2003). Alternatively, these difference could be due to technical issues such as species –specific selectivity of purinergic antagonists used in these studies (Fredholm, 1995) .

In addition, ATP mediated activity-dependent gene expression has not been demonstrated in terminal SCs in either the rodent or frog NMJ. Although synaptic activity has been shown to down-regulate GFAP expression in terminal SCs at the frog NMJ, the activity dependent neuron-SC signal was identified as acetylcholine (acting on muscarinic AchRs), and not ATP or adenosine (Georgiou et al., 1994). In addition, ATP and/or activity-dependent regulation of SC proliferation or differentiation has not been adequately explored in terminal SCs surrounding the synapse.

Activity-dependent axon-SC signaling has also been studied to some extent in myelinating SCs at the amphibian Node of Ranvier (Lev-Ram and Ellisman, 1995), however the involvement of purinergic signaling molecules at the adult node in unclear. Paranodal SCs responded to high frequency axonal stimulation *in situ*, however the activity-dependent signal(s) were not characterized. It was hypothesized that potassium efflux from axons following activity induced depolarization on the paranodal loops, resulting in calcium influx from external sources (possibly voltage-gated calcium channels). It remains possible that ATP may have contributed to the activity-dependent calcium fluxes in myelinating SCs, but this was not directly investigated. In fact, *in situ* studies of adult, rodent peripheral nerves demonstrated that paranodal SCs express functional metabotropic (P2Y2) and ionotropic (P2X7) ATP receptors (Mayer et al., 1997; Grafe et al., 1999), suggesting that adult myelinating SCs are indeed responsive to extracellular ATP.

In contrast, electrical stimulation of adult frog NMJ failed to elicit calcium transients in the proximal myelinated SCs in another study (Reist and Smith, 1992), but calcium transients were observed in the terminal SCs. Electrical stimulation of unmyelinated adult rat vagus nerve also failed to elicit calcium responses in associated SCs. As discussed briefly in chapter 2, these discrepancies may reflect different stimulation paradigms used in each study. Given that adult myelinating and non-myelinating SCs can respond to ATP agonists *in situ* (Wachtler et al., 1998; Grafe et al., 1999), but not necessarily to

electrical stimulation (Reist and Smith, 1992; Mayer et al., 1998; Wachtler et al., 1998), suggests that ATP may not be released extrasynaptically in the adult nervous system, or perhaps it may be released with different kinetics than what we have observed *in vitro*. Indeed, extrasynaptic release of ATP has been reported from large diameter sensory afferents in the spinal cord (Sawynok et al., 1993; Sawynok and Liu, 2003). Various technical issues related to *in situ* calcium imaging (i.e. difficulties in loading calcium indicators into myelinated segments of nerves) may also contribute to the lack of calcium responses in myelinating SCs proximal to the nerve terminal. Future experiments investigating purinergic receptor expression, as well as ATP release in adult vs. embryonic myelinated and non-myelinated nerves, may help resolve these discrepancies, as well as provide important insight into possible developmental regulation of purinergic signaling between axons and SCs *in vivo*.

CORRELATIONS BETWEEN ACTIVITY PATTERNS AND KEY SC DEVELOPMENTAL STAGES IN EMBRYONIC PERIPHERAL NERVES IN VIVO AND IN VITRO

Comparisons of the electrophysiological properties of embryonic sensory neurons *in vivo* with Schwann cell lineage studies in the developing rodent sciatic nerve, reveal striking parallels between the timecourse and firing patterns of embryonic DRG axons and key transitions in Schwann cell

development before the onset of myelination (Fitzgerald, 1987; Jessen and Mirsky, 1991, 2002) (see fig 27)**.**

In the rat, axons project out into the hind limb between E13 and E14. At this stage, SC precursor cells are associated with axons that are vigorously growing toward their target tissue. Recordings from single embryonic lumbar DRG neurons reveal significant spontaneous activity (0.5 Hz- 2 Hz) beginning at ~E16, and peaking at E 18-19. The onset of spontaneous activity corresponds with the transition of SC precursors to embryonic, premyelinating SCs. This is the stage at which SCs first express the S100 antigen, initiate autocrine survival loops, and begin proliferation in response to β neuregulin and axon-derived mitogens. The onset of low frequency stimulus-evoked firing (10-20 Hz) occurs ~ E17, which corresponds precisely to the first appearance of the 04 antigen on the surface of pre-myelinating SCs in vivo. This is also the stage at which DRG afferents innervate central targets in spinal cord grey matter. The frequency of firing and the total number of impulses increase steadily between E17 and birth, when pre-myelinating SC envelope bundles of axons, form a basal lamina, and begin differentiation in to myelinating or non-myelinating phenotyes. Spontaneous activity completely disappears by birth, and is absent in the postnatal period, when myelination begins.

While it is not yet known whether ATP is released from developing DRG axons in response to action potentials *in vivo*, the links between the time

course and pattern of impulse activity with key stages in SC development suggest that activity-dependent release could have significant effects on SC development *in vivo*. The present research demonstrated that ATP is released from DRG axons following electrical stimulation at frequencies comparable to the firing patterns observed in rat DRG neurons *in vivo* during the premyelination period (10 Hz). In addition, calcium imaging experiments indicate that SCs respond to axonal activity in a frequency-dependent manner (1 and 3 Hz vs. 10 Hz) (Chapter 2, Fig 4C). We also have preliminary evidence suggesting that ATP is released from DRG axons in response to different patterns of impulse activity.

Our DRG/SC co-culture model closely models the time course of SC developmental stages described in vivo. Robust proliferation of S100-positive SC occurs during the first several days in co-culture, which correspond to the pre-myelinating stage. Expression of the 04 marker is observed after ~1 week, and increases slowly with days in co-culture. Addition of ascorbic acid induces basal lamina formation, and strongly up regulates the expression of 04, and other differentiation markers. The first MBP-positive myelin profile is generally observed about a week after addition of ascorbic acid (Stevens et al., 1998).

Since the onset of neural impulse activity corresponds to the pre-myelinating period of SC development, we hypothesized that ATP release from electrically active DRG neurons could regulate SC proliferation,

differentiation, and possibly myelination (Experimental Aim 2). Indeed, SC differentiation and proliferation were inhibited by action potential activity in our DRG/SC co-culture model. Chronic electrical stimulation of post-mitotic DRG/SC co-cultures significantly inhibited the expression of 04 in pre-myelinating SCs. This period in culture may correspond to the onset of stimulus-evoked firing (10-20 Hz) at embryonic day 17. In addition, high frequency stimulation of DRG/SC co-cultures significantly inhibited axon-induced SC proliferation, which was blocked by apyrase, implicating extracellular ATP. These findings correlate with the dramatic inhibition of proliferation that occurs *in vivo* before the onset of myelination, when DRG axons are firing at higher frequencies. The specific mechanisms mediating activity-dependent regulation of Schwann cell function will be further discussed in the next passage.

ACTIVITY-DEPENDENT RELEASE OF PURINERGIC SIGALING MOLECULES REGULATES SCHWANN CELL FUNTION DURING DEVELOPMENT

We previously reported that chronic, low frequency electrical stimulation of DRG/SC co-cultures inhibits PNS myelination (Stevens et al., 1998). This inhibition only occurred following electrical stimulation at the firing pattern and duration (0.1 Hz, 5 days) that specifically down-regulated the cell adhesion molecule, L1 on DRG axons (Itoh et al., 1995). Homophilic

interactions between L1 expressed on axons and SCs is important for the initial stages of ensheathment that precedes myelination (Seilheimer and Schachner, 1988; Wood et al., 1990b). We concluded that electrical activity disrupted L1-mediated axon-SC interactions, and inhibited SC myelination via regulating molecules expressed on the axon (Stevens et al., 1998).

The present research provides evidence for a second mechanism by which neural impulse activity inhibits SC myelination. This study was the first to demonstrate a direct effect of action potential activity on Schwann cell development through the release of a soluble signaling molecule from axons. As described in chapter 3, extracellular ATP had profound effects on Schwann cell development. Activity-dependent release of ATP significantly inhibited SC proliferation, and arrested SC differentiation at an immature, pre-myelinating stage prior to onset of 04 expression. Chronic treatment of SC/DRG co-cultures with ATP maintained SCs in a morphologically immature stage, and completely prevented the formation of myelin (Stevens and Fields, 2000).

Importantly, chronic stimulation mimicked the effects of ATP in inhibiting SC differentiation and 04 expression, suggesting that ATP can arrest differentiation under physiological conditions. It is important to note that the experimental design and stimulation conditions were dramatically different from the conditions that inhibited L1-mediated myelination described above (Stevens et al., 1998). In the present study, electrical stimulation began 1 week

after DRG/SC co-cultures were established. Thus, at the time of stimulation, most SCs were associated with axons, and were post-mitotic. Co-cultures were subsequently stimulated for up to one week in the absence of ascorbic acid, at a high frequency, phasic pattern (5 pulses at 10 Hz at 2 sec intervals). We have established that this pattern of firing significantly increases ATP release (Chapter 2, Fig 6A) (Stevens and Fields, 2000).

In contrast, in the L1 myelin study, low frequency electrical stimulation (0.1 Hz for 5 days) was initiated 1 day after SCs were co-cultured with neurons (Stevens et al., 1998). In this paradigm, electrical stimulation was carried out during the initial stages of SC adhesion, and association with axons when homophilic L1-L1 interactions play a critical role. In these experiments, co-cultures were switched into differentiating medium five days after co-culture, and myelination was significantly reduced 10 days later following 0.1 Hz, but not higher frequency conditions (1 and 3 Hz). We have preliminary data indicating ATP release is frequency-dependent, and calcium imaging experiments indicate that fewer SCs responded, albeit robustly, with increased latency following electrical stimulation at lower frequencies (1 and 3 Hz vs. 10 Hz) (Chapter 2, Fig 4C). Thus, it is possible that ATP may not affect the initial stages of SC-axon association, suggesting the timing of ATP treatment may be important.

PURINERGIC SIGNALING IN PRE-MYELIMNATING SCHWANN CELLS

The unexpected finding that pre-myelinating SCs express adenosine receptors, suggested that the functional effects described thus far could be mediated by ATP, adenosine, or both. While this discovery complicates the story considerably, it highlights the complexity of purinergic signaling, and suggests that ATP, and its metabolites (ADP, AMP, adenosine), could modulate Schwann cell gene expression and function through multiple mechanisms. Indeed, this has been demonstrated in many cell types, including astrocytes. Different families of purinergic receptors (i.e. ATP [P2] vs. Adenosine [P1]) can activate different second messengers and consequently distinct intracellular signaling pathways. This is also the case for different subtypes of purinergic receptors within the same family (i.e. P2Y1 vs. P2Y2). For example, distinct P2Y subtypes can recruit different MEK activators in cultured astrocytes (Lenz et al., 2000; Lenz et al., 2001).

Since extracellular ATP can activate multiple types of purinergic receptors (see chapter 1, Fig 3), we explored the specific purinergic receptors and signaling pathways that could regulate ATP-mediated functional effects in SCs (Experimental Aim 3). Chapter 4 presented pharmacological, molecular, and functional evidence that pre-myelinating SCs express a far more complex array of ATP (P2X and P2Y) receptors than previously thought. Surprisingly, it was found that pre-myelinating SCs express functional A2 adenosine

receptors (A2a and A2b). These findings are the first to demonstrate functional adenosine receptors in SCs, with the exception of specialized terminal SCs at the neuromuscular junction, which express calcium-dependent A1 adenosine receptors (Robitaille, 1995). These recent findings lead us to re-examine some of our previous experiments involving chronic treatment of SCs with extracellular ATP. Since ATP is rapidly degraded to adenosine by ectonucleotidases, adenosine may have contributed to the ATP-mediated inhibition of SC proliferation and /or differentiation.

REGULATION OF SC PROLIFERATION AND DIFFERENTIATION BY ATP AND ADENOSINE

Using more selective adenosine and ATP receptors agonists, it was determined that both ATP and adenosine inhibited SC proliferation. Chronic treatment with adenosine, the general agonist NECA, as well as the A2a receptor agonist, CGS21680, significantly inhibited growth factor-stimulated SC proliferation compared to controls (Chapter 5). In addition, P2Y receptor agonists also significantly inhibited SC proliferation, while general P2X agonists were without significant effect. That 2MeSATP and 2MeSADP both inhibited SC proliferation, suggested that the ATP-mediated responses were likely mediated by P2Y1 receptors. While the specific pathways mediating the anti-proliferative effects involving A2a and P2Y receptors were not directly

determined, these findings provided important insights as to how these receptors differentially regulated MAPK signaling in premyelinating SCs.

It is widely appreciated that ERK/MAPK represents a point of convergence from multiple intracellular signaling cascades coupled to diverse membrane receptors. ERK/MAPK appears to integrate and communicate this information to the nucleus to regulate genes involved in cell cycle and differentiation (Bhalla and Iyengar, 2001; Bhalla et al., 2002). In Schwann cells, the ERK/MAPK cascade represents a point of cross-talk between growth factors and cAMP signaling pathways, whereby cAMP can either enhance or inhibit growth-factor mediated ERK signaling, and proliferation. For example, cAMP can synergize with growth factors to activate ERK/MAPK and stimulate cell cycle progression (Kim et al., 1997a; Kim et al., 2001a; Stevens and Fields, 2002), or it can significantly reduce growth factor-induced ERK/MAPK in SCs (Kim et al., 1997a) and induce SC differentiation. Growing evidence suggest that the intracellular concentration of cAMP is important in determining the level and duration of ERK/MAPK activity, and the ultimate biological outcome in SCs. For example, low concentration of cAMP (0.1mM) can cause high, but transient levels of ERK1/2 in SCs--conditions that stimulate SCs proliferation. In contrast, high concentrations of cAMP (>1mM), produce smaller but prolonged ERK/MAPK--conditions that induce SC differentiation (Sobue et al., 1986) (Mutoh et al., 1998).

The identity of the axon signal(s) which increase intracellular cAMP in SCs to differentially during development are not known, but our findings suggest adenosine as a possible candidate. Adenosine could be released from electrically active axons during the time when SCs are exiting from the cell cycle, and differentiating into myelinating or nonmyelinating phenotypes. Chapter 5 provided evidence that activity-dependent release of adenosine activates ERK/MAPK in SCs. While adenosine was applied with growth factors, adenosine inhibited growth-factor stimulation of ERK and SC proliferation. Although intracellular cAMP in SCs in response to adenosine or A2 receptor agonists were not directly determined in this study, G-protein coupled -A2 adenosine receptors are positively coupled to adenosine and increase intracellular cAMP. In addition, cAMP-dependent activation of ERK1/2 by Gs-coupled A2a and b receptors have been shown in several cell types (Schulte and Fredholm, 2003). Together these data suggest that adenosine interacts with growth factors to modulate ERK/MAPK in SCs. This may be a mechanism by which adenosine could either over-ride or synergize with mitogenic signals to regulate SC proliferation. These findings are in line with the longstanding observations that cAMP can have opposing effects on ERK and SC function in differentiated vs. pre-differentiated stage in development.

Activation of P2Y/ATP receptors also inhibited SC proliferation. In contrast to adenosine, ATP can inhibit ERK/MAPK in SCs. Our results

suggest that ATP-dependent inhibition of ERK is dependent on P2Y1-receptor activation of p38 MAPK in SCs. Negative regulation of ERK by p38MAPK has been recently demonstrated in several reports, and the p38 MAPK pathway has been shown to inhibit proliferation and induce cell cycle arrest in a many cell types. Although the mechanisms by which p38 negatively regulates ERK1/2 in SCs remains to be determined, it is tempting to speculate that ATP-dependent inhibition of ERK by p38 MAPK could underlie ATP/P2Y receptor mediated inhibition of SC proliferation. This hypothesis is in line with our findings that ATP receptor activation in SCs inhibited cyclin D1, positively regulated p21, and induced G1 arrest in the presence of growth factors (Chapter 3). Purinergic signaling in SCs is exceedingly complex, the present research has opened more questions than it has answered. However, our results thus far suggest that ATP and adenosine may act together, in cooperation with growth factors to modulate MAPK signaling in SCs. Since the levels and types of growth factor likely change with development, purinergic signaling molecules could have diverse biological outcomes in SCs.

Interestingly, in contrast to ATP, chronic treatment of DRG/SC co-cultures with adenosine (300 μM) does not appear to influence SC differentiation (Chapter 4, Fig. 14). Since this experiment was performed before A2 adenosine receptors were identified in SCs, it will be important to re-evaluate possible roles of adenosine in regulating SC differentiation and myelination, especially in light of the established role of cAMP in regulating

myelin gene expression. These signaling studies have highlighted the importance of determining a dose response with selective agonists and antagonists. Future studies should include more selective adenosine receptor agonists and antagonists at a wide range of concentrations. The identification of the specific subtypes of ATP (P2) involved in arresting SC development is also currently being investigated. As described in Chapter 4, 2MeSATP mimicked ATP in inhibiting 04 expression, suggesting involvement of P2Y receptors. Since 2MeSATP can activate multiple subtypes of P2 receptors, including P2X, it will be important to look more closely with selective P2X and Y receptor agonists and antagonists. Identification of the specific P2 receptor(s) involved, could provide important insights into possible downstream signaling pathways involved in regulating PNS myelination.

DIFFERENTIAL EFFECTS OF ATP AND ADENOSINE ON DEVELOPMENT OF CENTRAL AND PERIPHERAL GLIA

Growing evidence indicate that astrocytes, oligodendrocytes, and Schwann cells each express functional ATP and adenosine receptors (Fields and Stevens, 2000). Despite this apparent correspondence, recent research has demonstrated that the same extracellular signal can result in opposing biological outcomes in CNS and PNS glia. An interesting story is beginning to emerge regarding activity-dependent regulation of glial differentiation and myelination in the CNS and PNS by purinergic signaling molecules. As

summarized in Figure 28, ATP appears to be the dominant purinergic signal inhibiting SC differentiation and myelination, while adenosine has no apparent effect (Stevens and Fields, 2000). In the CNS, adenosine is the primary activity-dependent signal promoting oligodendrocytes differentiation and myelination, while ATP has no apparent effect (Stevens et al., 2002b). Functional differences also exist between Schwann cells and astrocytes (Neary et al., 1998; Franke et al., 2001). Difference is the number and types of purinergic receptors in these cells likely contribute to some of these functional differences. In addition, interactions between purinergic receptors and other signaling systems in the extracellular environment may also underlie diverse biological outcomes in CNS and PNS glia.

Figure 27.

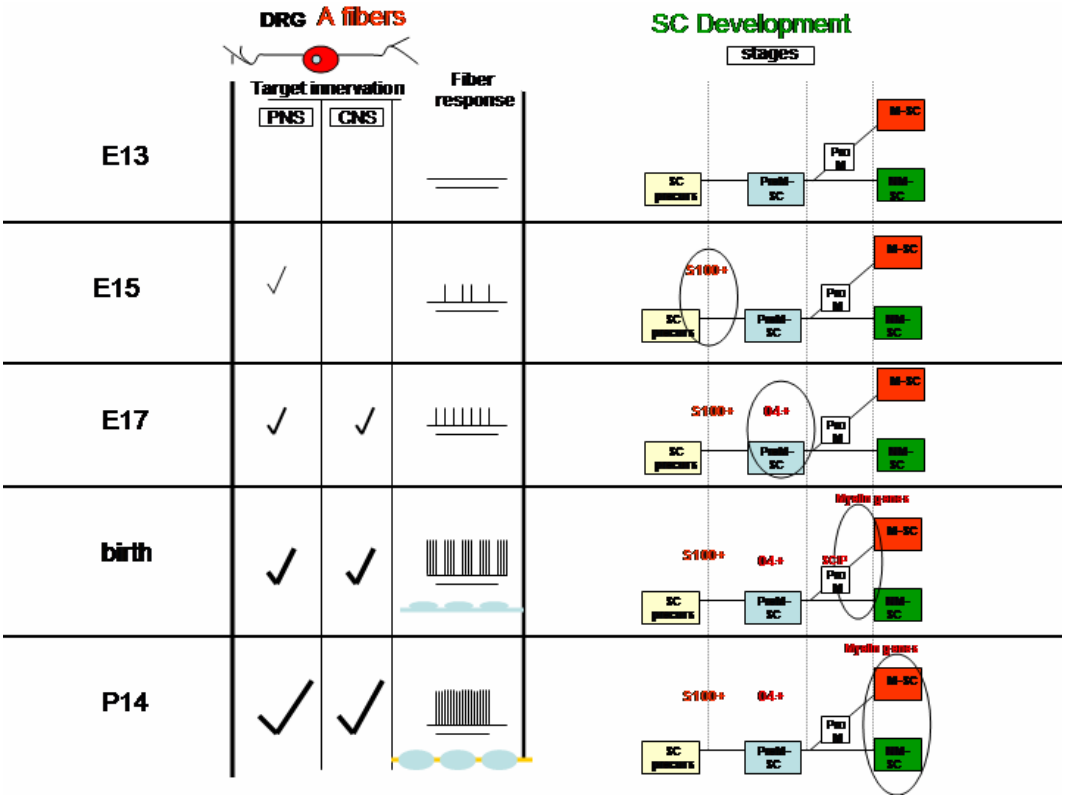


Figure 27. Correlations between activity patterns in sensory axons and key developmental stages in Schwann cells *in vivo*. (Embryonic day 13 (E13)- postnatal day 14 (P14). See text p. 174 for details).

CONCLUSION

The major goal of this research was to determine whether developing Schwann cells respond to neuronal activity. While this goal has been largely achieved, the findings have opened more questions than they have answered. Purinergic signaling is an important new signaling system between axons and Schwann cells, with significant effects on development and myelination. Yet to be determined are a complete characterization of these receptors on SCs, how the expression changes during development and in response to injury, and how this purinegic signaling system may affect activity -dependent signaling with other glial cells.

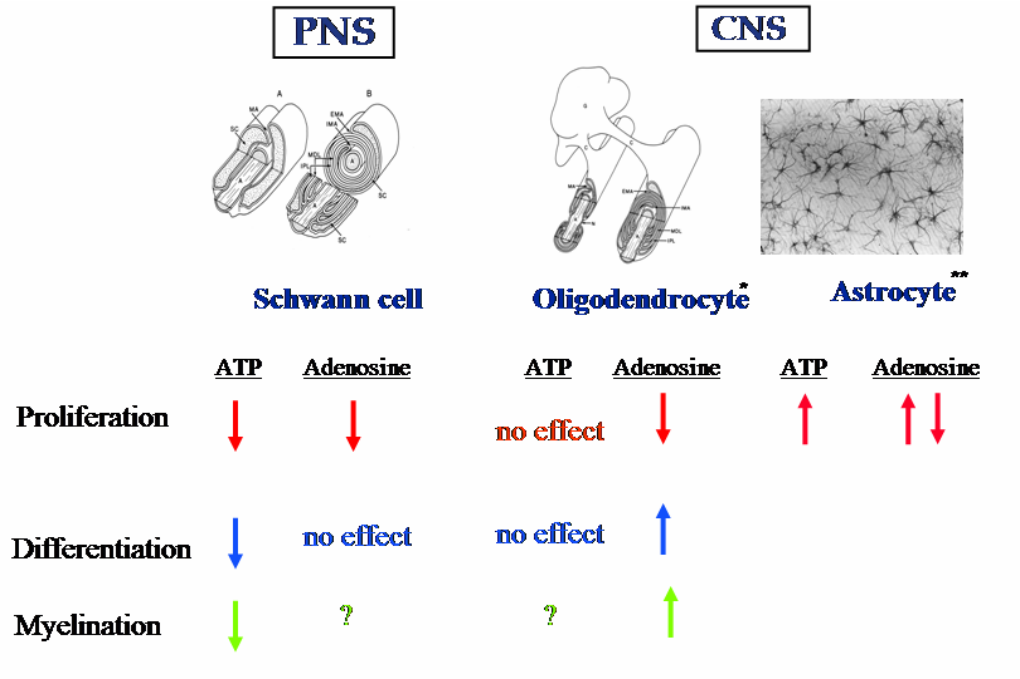


Figure 28. Differential effects of ATP and adenosine on myelinating glia in the CNS and PNS. Results in the PNS represent a summary of the present studies (Stevens and Fields, 2000). Results in the CNS are based on the following recent studies: *(Stevens et al., 2002b) ; ** (Neary et al., 1998).

REFERENCES

Abbracchio MP, Saffrey MJ, Hopker V, Burnstock G (1994) Modulation of astroglial cell proliferation by analogues of adenosine and ATP in primary cultures of rat striatum. Neuroscience 59:67-76.

Abe K, Saito H (1999) Effect of ATP on astrocyte stellation is switched from suppressive to stimulatory during development. Brain Res 850:150-157.

Araque A, Parpura V, Sanzgiri RP, Haydon PG (1999) Tripartite synapses: glia, the unacknowledged partner. Trends Neurosci 22:208-215.

Arslan G, Fredholm BB (2000) Stimulatory and inhibitory effects of adenosine A(2A) receptors on nerve growth factor-induced phosphorylation of extracellular regulated kinases 1/2 in PC12 cells. Neurosci Lett 292:183-186.

Arslan G, Kontny E, Fredholm BB (1997) Down-regulation of adenosine A2A receptors upon NGF-induced differentiation of PC12 cells. Neuropharmacology 36:1319-1326.

Asbury AK (1967) Schwann cell proliferation in developing mouse sciatic nerve. A radioautographic study. J Cell Biol 34:735-743.

Auld DS, Robitaille R (2003) Perisynaptic Schwann cells at the neuromuscular junction: nerve- and activity-dependent contributions to synaptic efficacy, plasticity, and reinnervation. Neuroscientist 9:144-157.

Barres BA, Raff MC (1993) Proliferation of oligodendrocyte precursor cells depends on electrical activity in axons. Nature 361:258-260.

Barres BA, Chun LL, Corey DP (1990) Ion channels in vertebrate glia. Annu Rev Neurosci 13:441-474.

Bermingham JR, Jr., Shumas S, Whisenhunt T, Rosenfeld MG, Scherer SS (2001) Modification of representational difference analysis applied to the isolation of forskolin-regulated genes from Schwann cells. J Neurosci Res 63:516-524.

Berti-Mattera LN, Wilkins PL, Madhun Z, Suchovsky D (1996) P2-purigenic receptors regulate phospholipase C and adenylate cyclase activities in immortalized Schwann cells. Biochem J 314 (Pt 2):555-561.

Berti-Mattera LN, Harwalkar S, Hughes B, Wilkins PL, Almhanna K (2001) Proliferative and morphological effects of endothelins in Schwann cells: roles of p38 mitogen-activated protein kinase and Ca(2+)-independent phospholipase A2. J Neurochem 79:1136-1148.

Bhalla US, Iyengar R (2001) Functional modules in biological signalling networks. Novartis Found Symp 239:4-13; discussion 13-15, 45-51.

Bhalla US, Ram PT, Iyengar R (2002) MAP kinase phosphatase as a locus of flexibility in a mitogen-activated protein kinase signaling network. Science 297:1018-1023.

Bhat NR, Zhang P, Lee JC, Hogan EL (1998) Extracellular signal-regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate

inducible nitric oxide synthase and tumor necrosis factor-alpha gene expression in endotoxin-stimulated primary glial cultures. J Neurosci 18:1633-1641.

Bogre L, Meskiene I, Heberle-Bors E, Hirt H (2000) Stressing the role of MAP kinases in mitogenic stimulation. Plant Mol Biol 43:705-718.

Bolego C, Ceruti S, Brambilla R, Puglisi L, Cattabeni F, Burnstock G, Abbracchio MP (1997) Characterization of the signalling pathways involved in ATP and basic fibroblast growth factor-induced astrogliosis. Br J Pharmacol 121:1692-1699.

Brambilla R, Burnstock G, Bonazzi A, Ceruti S, Cattabeni F, Abbracchio MP (1999) Cyclo-oxygenase-2 mediates P2Y receptor-induced reactive astrogliosis. Br J Pharmacol 126:563-567.

Buonanno A, Fields RD (1999) Gene regulation by patterned electrical activity during neural and skeletal muscle development. Curr Opin Neurobiol 9:110-120.

Burnstock G (1999) Purinergic cotransmission. Brain Res Bull 50:355-357.

Casaccia-Bonnefil P, Hardy RJ, Teng KK, Levine JM, Koff A, Chao MV (1999) Loss of p27Kip1 function results in increased proliferative capacity of oligodendrocyte progenitors but unaltered timing of differentiation. Development 126:4027-4037.

Castonguay A, Robitaille R (2001) Differential regulation of transmitter release by presynaptic and glial Ca2+ internal stores at the neuromuscular synapse. J Neurosci 21:1911-1922.

Castonguay A, Robitaille R (2002) Xestospongin C is a potent inhibitor of SERCA at a vertebrate synapse. Cell Calcium 32:39-47.

Castonguay A, Levesque S, Robitaille R (2001) Glial cells as active partners in synaptic functions. Prog Brain Res 132:227-240.

Chae H, Lee J, Byun J, Jung W, Kwak Y, Chae S, Kim H (2001) Blockade of p38 mitogen-activated protein kinase pathway inhibits interleukin-6 release and expression in primary neonatal cardiomyocytes. Res Commun Mol Pathol Pharmacol 110:209-228.

Cotrina ML, Lin JH, Alves-Rodrigues A, Liu S, Li J, Azmi-Ghadimi H, Kang J, Naus CC, Nedergaard M (1998) Connexins regulate calcium signaling by controlling ATP release. Proc Natl Acad Sci U S A 95:15735-15740.

Da Silva J, Pierrat B, Mary JL, Lesslauer W (1997) Blockade of p38 mitogen-activated protein kinase pathway inhibits inducible nitric-oxide synthase expression in mouse astrocytes. J Biol Chem 272:28373-28380.

D'Ambrosi N, Murra B, Cavaliere F, Amadio S, Bernardi G, Burnstock G, Volonte C (2001) Interaction between ATP and nerve growth factor

signalling in the survival and neuritic outgrowth from PC12 cells. Neuroscience 108:527-534.

Davis JB, Stroobant P (1990) Platelet-derived growth factors and fibroblast growth factors are mitogens for rat Schwann cells. J Cell Biol 110:1353-1360.

Deitmer JW, Verkhratsky AJ, Lohr C (1998) Calcium signalling in glial cells. Cell Calcium 24:405-416.

Demerens C, Stankoff B, Logak M, Anglade P, Allinquant B, Couraud F, Zalc B, Lubetzki C (1996) Induction of myelination in the central nervous system by electrical activity. Proc Natl Acad Sci U S A 93:9887-9892.

DeVries GH, Salzer JL, Bunge RP (1982) Axolemma-enriched fractions isolated from PNS and CNS are mitogenic for cultured Schwann cells. Brain Res 255:295-299.

Ding XZ, Adrian TE (2001) MEK/ERK-mediated proliferation is negatively regulated by P38 map kinase in the human pancreatic cancer cell line, PANC-1. Biochem Biophys Res Commun 282:447-453.

Dong Z, Brennan A, Liu N, Yarden Y, Lefkowitz G, Mirsky R, Jessen KR (1995) Neu differentiation factor is a neuron-glia signal and regulates survival, proliferation, and maturation of rat Schwann cell precursors. Neuron 15:585-596.

Eccleston PA, Jessen KR, Mirsky R (1987) Control of peripheral glial cell proliferation: a comparison of the division rates of enteric glia and Schwann cells and their response to mitogens. Dev Biol 124:409-417.

Eccleston PA, Collarini EJ, Jessen KR, Mirsky R, Richardson WD (1990) Schwann Cells Secrete a PDGF-like Factor: Evidence for an Autocrine Growth Mechanism involving PDGF. Eur J Neurosci 2:985-992.

Eldridge CF, Bunge MB, Bunge RP, Wood PM (1987) Differentiation of axon-related Schwann cells in vitro. I. Ascorbic acid regulates basal lamina assembly and myelin formation. J Cell Biol 105:1023-1034.

Fam SR, Gallagher CJ, Salter MW (2000) P2Y(1) purinoceptor-mediated Ca(2+) signaling and Ca(2+) wave propagation in dorsal spinal cord astrocytes. J Neurosci 20:2800-2808.

Feoktistov I, Biaggioni I (1995) Adenosine A2b receptors evoke interleukin-8 secretion in human mast cells. An enprofylline-sensitive mechanism with implications for asthma. J Clin Invest 96:1979-1986.

Fiebich BL, Schleicher S, Spleiss O, Czygan M, Hull M (2001) Mechanisms of prostaglandin E2-induced interleukin-6 release in astrocytes: possible involvement of EP4-like receptors, p38 mitogen-activated protein kinase and protein kinase C. J Neurochem 79:950-958.

Fields RD, Nelson PG (1992) Activity-dependent development of the vertebrate nervous system. Int Rev Neurobiol 34:133-214.

Fields RD, Stevens B (2000) ATP: an extracellular signaling molecule between neurons and glia. Trends Neurosci 23:625-633.

Fields RD, Stevens-Graham B (2002) New insights into neuron-glia communication. Science 298:556-562.

Fields RD, Neale EA, Nelson PG (1990) Effects of patterned electrical activity on neurite outgrowth from mouse sensory neurons. J Neurosci 10:2950-2964.

Fields RD, Eshete F, Stevens B, Itoh K (1997) Action potential-dependent regulation of gene expression: temporal specificity in ca2+, cAMP-responsive element binding proteins, and mitogen-activated protein kinase signaling. J Neurosci 17:7252-7266.

Fields RD, Eshete F, Dudek S, Ozsarac N, Stevens B (2001) Regulation of gene expression by action potentials: dependence on complexity in cellular information processing. Novartis Found Symp 239:160-172; discussion 172-166, 234-140.

Fitzgerald M (1987) Spontaneous and evoked activity of fetal primary afferents in vivo. Nature 326:603-605.

Franke H, Krugel U, Illes P (1999) P2 receptor-mediated proliferative effects on astrocytes in vivo. Glia 28:190-200.

Franke H, Krugel U, Schmidt R, Grosche J, Reichenbach A, Illes P (2001) P2 receptor-types involved in astrogliosis in vivo. Br J Pharmacol 134:1180-1189.

Fredholm BB (1995) Purinoceptors in the nervous system. Pharmacol Toxicol 76:228-239.

Garratt AN, Voiculescu O, Topilko P, Charnay P, Birchmeier C (2000) A dual role of erbB2 in myelination and in expansion of the schwann cell precursor pool. J Cell Biol 148:1035-1046.

Georgiou J, Robitaille R, Charlton MP (1999) Muscarinic control of cytoskeleton in perisynaptic glia. J Neurosci 19:3836-3846.

Georgiou J, Robitaille R, Trimble WS, Charlton MP (1994) Synaptic regulation of glial protein expression in vivo. Neuron 12:443-455.

Ghiani C, Gallo V (2001) Inhibition of cyclin E-cyclin-dependent kinase 2 complex formation and activity is associated with cell cycle arrest and withdrawal in oligodendrocyte progenitor cells. J Neurosci 21:1274-1282.

Grafe P, Mayer C, Takigawa T, Kamleiter M, Sanchez-Brandelik R (1999) Confocal calcium imaging reveals an ionotropic P2 nucleotide receptor in the paranodal membrane of rat Schwann cells. J Physiol 515 (Pt 2):377-383.

Guenard V, Frisch G, Wood PM (1996) Effects of axonal injury on astrocyte proliferation and morphology in vitro: implications for astrogliosis. Exp Neurol 137:175-190.

Guenard V, Rosenbaum T, Gwynn LA, Doetschman T, Ratner N, Wood PM (1995) Effect of transforming growth factor-beta 1 and -beta 2 on Schwann cell proliferation on neurites. Glia 13:309-318.

Guthrie PB, Knappenberger J, Segal M, Bennett MV, Charles AC, Kater SB (1999) ATP released from astrocytes mediates glial calcium waves. J Neurosci 19:520-528.

Hansen TV, Rehfeld JF, Nielsen FC (1999) Mitogen-activated protein kinase and protein kinase A signaling pathways stimulate cholecystokinin transcription via activation of cyclic adenosine 3',5'-monophosphate response element-binding protein. Mol Endocrinol 13:466-475.

Haydon PG (2001) GLIA: listening and talking to the synapse. Nat Rev Neurosci 2:185-193.

Hindley S, Herman MA, Rathbone MP (1994) Stimulation of reactive astrogliosis in vivo by extracellular adenosine diphosphate or an adenosine A2 receptor agonist. J Neurosci Res 38:399-406.

Hutchinson DO, Walls TJ, Nakano S, Camp S, Taylor P, Harper CM, Groover RV, Peterson HA, Jamieson DG, Engel AG (1993) Congenital endplate acetylcholinesterase deficiency. Brain 116 (Pt 3):633-653.

Itoh K, Stevens B, Schachner M, Fields RD (1995) Regulated expression of the neural cell adhesion molecule L1 by specific patterns of neural impulses. Science 270:1369-1372.

Jessen KR, Mirsky R (1991) Schwann cell precursors and their development. Glia 4:185-194.

Jessen KR, Mirsky R (1992) Schwann cells: early lineage, regulation of proliferation and control of myelin formation. Curr Opin Neurobiol 2:575-581.

Jessen KR, Mirsky R (1997) Embryonic Schwann cell development: the biology of Schwann cell precursors and early Schwann cells. J Anat 191 (Pt 4):501-505.

Jessen KR, Mirsky R (1998) Origin and early development of Schwann cells. Microsc Res Tech 41:393-402.

Jessen KR, Mirsky R (1999a) Developmental regulation in the Schwann cell lineage. Adv Exp Med Biol 468:3-12.

Jessen KR, Mirsky R (1999b) Schwann cells and their precursors emerge as major regulators of nerve development. Trends Neurosci 22:402-410.

Jessen KR, Mirsky R (2002) Signals that determine Schwann cell identity. J Anat 200:367-376.

Johnson GL, Lapadat R (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 298:1911-1912.

Katz LC, Shatz CJ (1996) Synaptic activity and the construction of cortical circuits. Science 274:1133-1138.

Kim HA, DeClue JE, Ratner N (1997a) cAMP-dependent protein kinase A is required for Schwann cell growth: interactions between the cAMP and neuregulin/tyrosine kinase pathways. J Neurosci Res 49:236-247.

Kim HA, Ling B, Ratner N (1997b) Nf1-deficient mouse Schwann cells are angiogenic and invasive and can be induced to hyperproliferate: reversion of some phenotypes by an inhibitor of farnesyl protein transferase. Mol Cell Biol 17:862-872.

Kim HA, Ratner N, Roberts TM, Stiles CD (2001a) Schwann cell proliferative responses to cAMP and Nf1 are mediated by cyclin D1. J Neurosci 21:1110-1116.

Kim J, Yoon MY, Choi SL, Kang I, Kim SS, Kim YS, Choi YK, Ha J (2001b) Effects of stimulation of AMP-activated protein kinase on insulin-like growth factor 1- and epidermal growth factor-dependent extracellular signal-regulated kinase pathway. J Biol Chem 276:19102-19110.

King BF, Neary JT, Zhu Q, Wang S, Norenberg MD, Burnstock G (1996) P2 purinoceptors in rat cortical astrocytes: expression, calcium-imaging and signalling studies. Neuroscience 74:1187-1196.

Klinger M, Kuhn M, Just H, Stefan E, Palmer T, Freissmuth M, Nanoff C (2002) Removal of the carboxy terminus of the A2A-adenosine receptor blunts constitutive activity: differential effect on cAMP accumulation and MAP kinase stimulation. Naunyn Schmiedebergs Arch Pharmacol 366:287-298.

Klotz KN (2000) Adenosine receptors and their ligands. Naunyn Schmiedebergs Arch Pharmacol 362:382-391.

Koistinaho M, Koistinaho J (2002) Role of p38 and p44/42 mitogen-activated protein kinases in microglia. Glia 40:175-183.

Kriegler S, Chiu SY (1993) Calcium signaling of glial cells along mammalian axons. J Neurosci 13:4229-4245.

Kukley M, Barden JA, Steinhauser C, Jabs R (2001) Distribution of P2X receptors on astrocytes in juvenile rat hippocampus. Glia 36:11-21.

Lee J, Hong F, Kwon S, Kim SS, Kim DO, Kang HS, Lee SJ, Ha J (2002) Activation of p38 MAPK induces cell cycle arrest via inhibition of Raf/ERK pathway during muscle differentiation. Biochem Biophys Res Commun 298:765-771.

Lenz G, Goncalves D, Luo Z, Avruch J, Rodnight R, Neary JT (2001) Extracellular ATP stimulates an inhibitory pathway towards growth factor-induced cRaf-1 and MEKK activation in astrocyte cultures. J Neurochem 77:1001-1009.

Lenz G, Gottfried C, Luo Z, Avruch J, Rodnight R, Nie WJ, Kang Y, Neary JT (2000) P(2Y) purinoceptor subtypes recruit different mek activators in astrocytes. Br J Pharmacol 129:927-936.

Lev-Ram V, Ellisman MH (1995) Axonal activation-induced calcium transients in myelinating Schwann cells, sources, and mechanisms. J Neurosci 15:2628-2637.

Li M, Jia M, Fields RD, Nelson PG (1996) Modulation of calcium currents by electrical activity. J Neurophysiol 76:2595-2607.

Loubani M, Galinanes M (2002) Pharmacological and ischemic preconditioning of the human myocardium: mitoKATP channels are upstream and p38MAPK is downstream of PKC. BMC Physiol 2:10.

Lyons SA, Morell P, McCarthy KD (1994) Schwann cells exhibit P2Y purinergic receptors that regulate intracellular calcium and are up-regulated by cyclic AMP analogues. J Neurochem 63:552-560.

Lyons SA, Morell P, McCarthy KD (1995) Schwann cell ATP-mediated calcium increases in vitro and in situ are dependent on contact with neurons. Glia 13:27-38.

Marshall CJ (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80:179-185.

Maurel P, Salzer JL (2000) Axonal regulation of Schwann cell proliferation and survival and the initial events of myelination requires PI 3-kinase activity. J Neurosci 20:4635-4645.

Mayer C, Quasthoff S, Grafe P (1998) Differences in the sensitivity to purinergic stimulation of myelinating and non-myelinating Schwann cells in peripheral human and rat nerve. Glia 23:374-382.

Mayer C, Wachtler J, Kamleiter M, Grafe P (1997) Intracellular calcium transients mediated by P2 receptors in the paranodal Schwann cell region of myelinated rat spinal root axons. Neurosci Lett 224:49-52.

Meintanis S, Thomaidou D, Jessen KR, Mirsky R, Matsas R (2001) The neuron-glia signal beta-neuregulin promotes Schwann cell motility via the MAPK pathway. Glia 34:39-51.

Milenkovic I, Weick M, Wiedemann P, Reichenbach A, Bringmann A (2003) P2Y receptor-mediated stimulation of Muller glial cell DNA synthesis: dependence on EGF and PDGF receptor transactivation. Invest Ophthalmol Vis Sci 44:1211-1220.

Mirsky R, Jessen KR (1996) Schwann cell development, differentiation and myelination. Curr Opin Neurobiol 6:89-96.

Mirsky R, Jessen KR (1999) The neurobiology of Schwann cells. Brain Pathol 9:293-311.

Mirsky R, Dubois C, Morgan L, Jessen KR (1990) 04 and A007-sulfatide antibodies bind to embryonic Schwann cells prior to the appearance of galactocerebroside; regulation of the antigen by axon-Schwann cell signals and cyclic AMP. Development 109:105-116.

Moll V, Weick M, Milenkovic I, Kodal H, Reichenbach A, Bringmann A (2002) P2Y receptor-mediated stimulation of Muller glial DNA synthesis. Invest Ophthalmol Vis Sci 43:766-773.

Morgan L, Jessen KR, Mirsky R (1991) The effects of cAMP on differentiation of cultured Schwann cells: progression from an early phenotype (04+) to a myelin phenotype (P0+, GFAP-, N-CAM-, NGF-receptor-) depends on growth inhibition. J Cell Biol 112:457-467.

Morrissey TK, Levi AD, Nuijens A, Sliwkowski MX, Bunge RP (1995) Axon-induced mitogenesis of human Schwann cells involves heregulin and p185erbB2. Proc Natl Acad Sci U S A 92:1431-1435.

Munsch N, Gavaret JM, Pierre M (1998) Ca2+ dependent purinergic regulation of p42 and p44 MAP kinases in astroglial cultured cells. Biomed Pharmacother 52:180-186.

Murphy P, Topilko P, Schneider-Maunoury S, Seitanidou T, Baron-Van Evercooren A, Charnay P (1996) The regulation of Krox-20 expression reveals important steps in the control of peripheral glial cell development. Development 122:2847-2857.

Mutoh T, Li M, Yamamoto M, Mitsuma T, Sobue G (1998) Differential signaling cascade of MAP kinase and S6 kinase depends on 3',5'-monophosphate concentration in schwann cells: correlation to cellular differentiation and proliferation. Brain Res 810:274-278.

Neary JT (1996) Trophic actions of extracellular ATP on astrocytes, synergistic interactions with fibroblast growth factors and underlying signal transduction mechanisms. Ciba Found Symp 198:130-139; discussion 139-141.

Neary JT, Whittemore SR, Zhu Q, Norenberg MD (1994a) Destabilization of glial fibrillary acidic protein mRNA in astrocytes by ammonia and protection by extracellular ATP. J Neurochem 63:2021-2027.

Neary JT, Baker L, Jorgensen SL, Norenberg MD (1994b) Extracellular ATP induces stellation and increases glial fibrillary acidic protein content and DNA synthesis in primary astrocyte cultures. Acta Neuropathol (Berl) 87:8-13.

Neary JT, McCarthy M, Kang Y, Zuniga S (1998) Mitogenic signaling from P1 and P2 purinergic receptors to mitogen-activated protein kinase in human fetal astrocyte cultures. Neurosci Lett 242:159-162.

Neary JT, McCarthy M, Cornell-Bell A, Kang Y (1999a) Trophic signaling pathways activated by purinergic receptors in rat and human astroglia. Prog Brain Res 120:323-332.

Neary JT, Kang Y, Willoughby KA, Ellis EF (2003) Activation of extracellular signal-regulated kinase by stretch-induced injury in astrocytes involves extracellular ATP and P2 purinergic receptors. J Neurosci 23:2348-2356.

Neary JT, Rathbone MP, Cattabeni F, Abbracchio MP, Burnstock G (1996) Trophic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. Trends Neurosci 19:13-18.

Neary JT, Kang Y, Bu Y, Yu E, Akong K, Peters CM (1999b) Mitogenic signaling by ATP/P2Y purinergic receptors in astrocytes: involvement

of a calcium-independent protein kinase C, extracellular signal-regulated protein kinase pathway distinct from the phosphatidylinositol-specific phospholipase C/calcium pathway. J Neurosci 19:4211-4220.

Ono K, Han J (2000) The p38 signal transduction pathway: activation and function. Cell Signal 12:1-13.

Panenka W, Jijon H, Herx LM, Armstrong JN, Feighan D, Wei T, Yong VW, Ransohoff RM, MacVicar BA (2001) P2X7-like receptor activation in astrocytes increases chemokine monocyte chemoattractant protein-1 expression via mitogen-activated protein kinase. J Neurosci 21:7135-7142.

Parkinson DB, Langner K, Namini SS, Jessen KR, Mirsky R (2002) beta-Neuregulin and autocrine mediated survival of Schwann cells requires activity of Ets family transcription factors. Mol Cell Neurosci 20:154-167.

Parpura V, Liu F, Jeftinija KV, Haydon PG, Jeftinija SD (1995) Neuroligand-evoked calcium-dependent release of excitatory amino acids from Schwann cells. J Neurosci 15:5831-5839.

Peles E, Salzer JL (2000) Molecular domains of myelinated axons. Curr Opin Neurobiol 10:558-565.

Penn AA, Shatz CJ (1999) Brain waves and brain wiring: the role of endogenous and sensory-driven neural activity in development. Pediatr Res 45:447-458.

Pilitsis JG, Kimelberg HK (1998) Adenosine receptor mediated stimulation of intracellular calcium in acutely isolated astrocytes. Brain Res 798:294-303.

Pleasure D, Kreider B, Shuman S, Sobue G (1985) Tissue culture studies of Schwann cell proliferation and differentiation. Dev Neurosci 7:364-373.

Pocock JM, Liddle AC (2001) Microglial signalling cascades in neurodegenerative disease. Prog Brain Res 132:555-565.

Pugazhenthi S, Boras T, O'Connor D, Meintzer MK, Heidenreich KA, Reusch JE (1999) Insulin-like growth factor I-mediated activation of the transcription factor cAMP response element-binding protein in PC12 cells. Involvement of p38 mitogen-activated protein kinase-mediated pathway. J Biol Chem 274:2829-2837.

Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. Pharmacol Rev 50:413-492.

Rathbone MP, Middlemiss PJ, Kim JK, Gysbers JW, DeForge SP, Smith RW, Hughes DW (1992) Adenosine and its nucleotides stimulate proliferation of chick astrocytes and human astrocytoma cells. Neurosci Res 13:1-17.

Rathbone MP, Middlemiss P, Andrew C, Caciagli F, Ciccarelli R, Di Iorio P, Huang R (1998) The trophic effects of purines and purinergic signaling in pathologic reactions of astrocytes. Alzheimer Dis Assoc Disord 12 Suppl 2:S36-45.

Ratner N, Bunge RP, Glaser L (1985) A neuronal cell surface heparan sulfate proteoglycan is required for dorsal root ganglion neuron stimulation of Schwann cell proliferation. J Cell Biol 101:744-754.

Reist NE, Smith SJ (1992) Neurally evoked calcium transients in terminal Schwann cells at the neuromuscular junction. Proc Natl Acad Sci U S A 89:7625-7629.

Rice AB, Ingram JL, Bonner JC (2002) p38 mitogen-activated protein kinase regulates growth factor-induced mitogenesis of rat pulmonary myofibroblasts. Am J Respir Cell Mol Biol 27:759-765.

Ridley SH, Dean JL, Sarsfield SJ, Brook M, Clark AR, Saklatvala J (1998) A p38 MAP kinase inhibitor regulates stability of interleukin-1-induced cyclooxygenase-2 mRNA. FEBS Lett 439:75-80.

Ridley SH, Sarsfield SJ, Lee JC, Bigg HF, Cawston TE, Taylor DJ, DeWitt DL, Saklatvala J (1997) Actions of IL-1 are selectively controlled by p38 mitogen-activated protein kinase: regulation of prostaglandin H synthase-2, metalloproteinases, and IL-6 at different levels. J Immunol 158:3165-3173.

Robitaille R (1995) Purinergic receptors and their activation by endogenous purines at perisynaptic glial cells of the frog neuromuscular junction. J Neurosci 15:7121-7131.

Robitaille R (1998) Modulation of synaptic efficacy and synaptic depression by glial cells at the frog neuromuscular junction. Neuron 21:847-855.

Rochon D, Rousse I, Robitaille R (2001) Synapse-glia interactions at the mammalian neuromuscular junction. J Neurosci 21:3819-3829.

Salzer JL, Bunge RP, Glaser L (1980) Studies of Schwann cell proliferation. III. Evidence for the surface localization of the neurite mitogen. J Cell Biol 84:767-778.

Sanches G, de Alencar LS, Ventura AL (2002) ATP induces proliferation of retinal cells in culture via activation of PKC and extracellular signal-regulated kinase cascade. Int J Dev Neurosci 20:21-27.

Sawynok J, Liu XJ (2003) Adenosine in the spinal cord and periphery: release and regulation of pain. Prog Neurobiol 69:313-340.

Sawynok J, Downie JW, Reid AR, Cahill CM, White TD (1993) ATP release from dorsal spinal cord synaptosomes: characterization and neuronal origin. Brain Res 610:32-38.

Scherer SS (1997) The biology and pathobiology of Schwann cells. Curr Opin Neurol 10:386-397.

Schinelli S, Zanassi P, Paolillo M, Wang H, Feliciello A, Gallo V (2001) Stimulation of endothelin B receptors in astrocytes induces cAMP

response element-binding protein phosphorylation and c-fos expression via multiple mitogen-activated protein kinase signaling pathways. J Neurosci 21:8842-8853.

Schulte G, Fredholm BB (2000) Human adenosine A(1), A(2A), A(2B), and A(3) receptors expressed in Chinese hamster ovary cells all mediate the phosphorylation of extracellular-regulated kinase 1/2. Mol Pharmacol 58:477-482.

Schulte G, Fredholm BB (2003) Signalling from adenosine receptors to mitogen-activated protein kinases. Cell Signal 15:813-827.

Schumacher M, Jung-Testas I, Robel P, Baulieu EE (1993) Insulin-like growth factor I: a mitogen for rat Schwann cells in the presence of elevated levels of cyclic AMP. Glia 8:232-240.

Seidel MG, Klinger M, Freissmuth M, Holler C (1999) Activation of mitogen-activated protein kinase by the A(2A)-adenosine receptor via a rap1-dependent and via a p21(ras)-dependent pathway. J Biol Chem 274:25833-25841.

Seilheimer B, Schachner M (1988) Studies of adhesion molecules mediating interactions between cells of peripheral nervous system indicate a major role for L1 in mediating sensory neuron growth on Schwann cells in culture. J Cell Biol 107:341-351.

Shah NM, Groves AK, Anderson DJ (1996) Alternative neural crest cell fates are instructively promoted by TGFbeta superfamily members. Cell 85:331-343.

Sherr CJ, Roberts JM (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 13:1501-1512.

Shigemoto-Mogami Y, Koizumi S, Tsuda M, Ohsawa K, Kohsaka S, Inoue K (2001) Mechanisms underlying extracellular ATP-evoked interleukin-6 release in mouse microglial cell line, MG-5. J Neurochem 78:1339-1349.

Shrager P, Novakovic SD (1995) Control of myelination, axonal growth, and synapse formation in spinal cord explants by ion channels and electrical activity. Brain Res Dev Brain Res 88:68-78.

Sobue G, Pleasure D (1984) Schwann cell galactocerebroside induced by derivatives of adenosine 3',5'-monophosphate. Science 224:72-74.

Sobue G, Shuman S, Pleasure D (1986) Schwann cell responses to cyclic AMP: proliferation, change in shape, and appearance of surface galactocerebroside. Brain Res 362:23-32.

Son YJ, Trachtenberg JT, Thompson WJ (1996) Schwann cells induce and guide sprouting and reinnervation of neuromuscular junctions. Trends Neurosci 19:280-285.

Stevens B, Fields RD (2000) Response of Schwann cells to action potentials in development. Science 287:2267-2271.

Stevens B, Fields RD (2002) Regulation of the cell cycle in normal and pathological glia. Neuroscientist 8:93-97.

Stevens B, Tanner S, Fields RD (1998) Control of myelination by specific patterns of neural impulses. J Neurosci 18:9303-9311.

Stevens B, Porta S, Haak LL, Gallo V, Fields RD (2002a) Adenosine. A neuron-glial transmitter promoting myelination in the CNS in response to action potentials. Neuron 36:855-868.

Stevens B, Porta S, Haak LL, Gallo V, Fields RD (2002b) Adenosine: a neuron-glial transmitter promoting myelination in the CNS in response to action potentials. Neuron 36:855-868.

Stewart HJ, Eccleston PA, Jessen KR, Mirsky R (1991) Interaction between cAMP elevation, identified growth factors, and serum components in regulating Schwann cell growth. J Neurosci Res 30:346-352.

Stewart HJ, Morgan L, Jessen KR, Mirsky R (1993) Changes in DNA synthesis rate in the Schwann cell lineage in vivo are correlated with the precursor--Schwann cell transition and myelination. Eur J Neurosci 5:1136-1144.

Stork PJ, Schmitt JM (2002) Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol 12:258-266.

Svenningsen AF, Kanje M (1996) Insulin and the insulin-like growth factors I and II are mitogenic to cultured rat sciatic nerve segments and stimulate

[3H]thymidine incorporation through their respective receptors. Glia 18:68-72.

Tikoo R, Zanazzi G, Shiffman D, Salzer J, Chao MV (2000) Cell cycle control of Schwann cell proliferation: role of cyclin-dependent kinase-2. J Neurosci 20:4627-4634.

Topilko P, Murphy P, Charnay P (1996) Embryonic development of Schwann cells: multiple roles for neuregulins along the pathway. Mol Cell Neurosci 8:71-75.

Topilko P, Levi G, Merlo G, Mantero S, Desmarquet C, Mancardi G, Charnay P (1997) Differential regulation of the zinc finger genes Krox-20 and Krox-24 (Egr-1) suggests antagonistic roles in Schwann cells. J Neurosci Res 50:702-712.

Topilko P, Schneider-Maunoury S, Levi G, Baron-Van Evercooren A, Chennoufi AB, Seitanidou T, Babinet C, Charnay P (1994) Krox-20 controls myelination in the peripheral nervous system. Nature 371:796-799.

Verkhratsky A, Steinhauser C (2000) Ion channels in glial cells. Brain Res Brain Res Rev 32:380-412.

Wachtler J, Mayer C, Grafe P (1998) Activity-dependent intracellular Ca2+ transients in unmyelinated nerve fibres of the isolated adult rat vagus nerve. Pflugers Arch 435:678-686.

Wood P, Moya F, Eldridge C, Owens G, Ranscht B, Schachner M, Bunge M, Bunge R (1990a) Studies of the initiation of myelination by Schwann cells. Ann N Y Acad Sci 605:1-14.

Wood PM, Schachner M, Bunge RP (1990b) Inhibition of Schwann cell myelination in vitro by antibody to the L1 adhesion molecule. J Neurosci 10:3635-3645.

Wu R, Jurek M, Sundarababu S, Weinstein DE (2001) The POU gene Brn-5 is induced by neuregulin and is restricted to myelinating Schwann cells. Mol Cell Neurosci 17:683-695.

Xing J, Kornhauser JM, Xia Z, Thiele EA, Greenberg ME (1998) Nerve growth factor activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation. Mol Cell Biol 18:1946-1955.

Yakel JL, Warren RA, Reppert SM, North RA (1993) Functional expression of adenosine A2b receptor in Xenopus oocytes. Mol Pharmacol 43:277-280.

Zanazzi G, Einheber S, Westreich R, Hannocks MJ, Bedell-Hogan D, Marchionni MA, Salzer JL (2001) Glial growth factor/neuregulin inhibits Schwann cell myelination and induces demyelination. J Cell Biol 152:1289-1299.

Zhang H, Shi X, Hampong M, Blanis L, Pelech S (2001) Stress-induced inhibition of ERK1 and ERK2 by direct interaction with p38 MAP kinase. J Biol Chem 276:6905-6908.

Zhang LI, Poo MM (2001) Electrical activity and development of neural circuits. Nat Neurosci 4 Suppl:1207-1214.

Zhu Y, Kimelberg HK (2001) Developmental expression of metabotropic P2Y(1) and P2Y(2) receptors in freshly isolated astrocytes from rat hippocampus. J Neurochem 77:530-541.

Zimmermann H (1994) Signalling via ATP in the nervous system. Trends Neurosci 17:420-426.

Zimmermann H (2000) Extracellular metabolism of ATP and other nucleotides. Naunyn Schmiedebergs Arch Pharmacol 362:299-309.

Zimmermann H, Braun N (1996) Extracellular metabolism of nucleotides in the nervous system. J Auton Pharmacol 16:397-400.

Zimmermann H, Braun N, Kegel B, Heine P (1998) New insights into molecular structure and function of ectonucleotidases in the nervous system. Neurochem Int 32:421-425.

Zorick TS, Lemke G (1996) Schwann cell differentiation. Curr Opin Cell Biol 8:870-876.

Zorick TS, Syroid DE, Arroyo E, Scherer SS, Lemke G (1996) The transcription factors SCIP and Krox-20 mark distinct stages and cell fates in Schwann cell differentiation. Mol Cell Neurosci 8:129-145.