Université de Montréal

Molecules and Mechanisms of Glial and Synaptic Plasticity

by
Keith Jeffrey Todd

Département de physiologie
Faculté de médecine

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Molecules and Mechanisms of Glial and Synaptic Plasticity

Présenté par:
Keith Jeffrey Todd

a été évaluée par un jury composé des personnes suivantes :

Vincent Castellucci (président-rapporteur)

Richard Robitaille (directeur de recherche)

Adriana Di Polo (membre du jury)

Rita Balice-Gordon (examinateur externe)

John Kalaska (représentant du doyen de la FES)

RÉSUMÉ ET LES MOTS CLÉS FRANÇAIS

La reconnaissance des cellules gliales comme partenaires dynamiques de la fonction synaptique au sein du système nerveux est grandissante. Cette évolution de pensée, vers un rôle actif des cellules gliales, a débuté dans les années 1990 avec des travaux pionniers démontrant que la transmission synaptique induit des élévations calciques gliales. De plus, nous savons maintenant que les cellules gliales ont la capacité de moduler les fonctions neuronales et synaptiques sur des échelles de temps variables. Cette influence des cellules gliales sur les synapses contribue à l'une des propriétés synaptiques fondamentales, soit leur habilité à être modifiée ou leur plasticité. Les mécanismes gliaux influençant la direction de la plasticité de la synapse vers des états potentialisés ou déprimés ont récemment gagnés en compréhension. Toutefois, les mécanismes impliqués dans la plasticité des cellules gliales elles-mêmes demeurent inconnus.

Les neurotrophines font partie d'un groupe de facteurs contribuant aux mécanismes de plasticité dans le système nerveux et sont impliquées dans la fonction des cellules gliales. Ces observations suggèrent donc que les neurotrophines puissent contribuer à la plasticité des cellules gliales. Ainsi, je propose dans cette thèse :

1) D'étudier l'un des aspects de base des interactions neurone-glie, soit, de déterminer comment s'explique la génération d'une action positive versus négative des cellules gliales sur la synapse.

2) Déterminer le rôle des neurotrophines sur la plasticité des cellules gliales.

Dans la première section, nous avons démontré que les réponses calciques gliales sont différentes selon les paramètres de stimulation nerveuse et que ces dernières exercent à leur tour un effet rétroactif différentiel sur les synapses. Cette étude démontre donc que les patrons d'activité neuronale dictent les patrons d'activité gliale menant ultimement à

des différences dans la contribution des cellules gliales à la plasticité synaptique.

Dans la deuxième section, l'implication des neurotrophines dans la plasticité gliale a été établie. En effet, l'application de différentes neurotrophines, au cours de l'activité synaptique évoquée, a permis de démontrer que ces facteurs altèrent les réponses calciques gliales. Ainsi, ces résultats démontrent que les élévations calciques gliales sont plastiques et modifiables par les neurotrophines.

Les résultats présentés dans cette thèse démontrent l'importance des cellules gliales dans la régulation de la direction de la plasticité synaptique. D'autre part, ces études démontrent l'importance des neurotrophines dans la communication neurone-glie. En effet, en considérant l'importance des différences dans les réponses calciques gliales sur la plasticité synaptique, la capacité des neurotrophines à les moduler démontre bien leur rôle clé dans la fonction synaptique. Ces études révèlent finalement l'importance des changements fins du signal calcique glial sur la fonction des synapses ainsi que l'importance des neurotrophines dans leur régulation.

Mots clés- interactions neurone-glie; neurotrophines;.

ABSTRACT AND ENGLISH KEY WORDS

In recent years, understanding of glial cell function has evolved from a merely supportive role to one of primary function in synaptic transmission. This shift in thinking began around 1990 with pioneering work demonstrating that glial cells *in situ* could actively respond to synaptic transmission with elevations of intracellular calcium. We now know that glial cells also provide feedback to neurons and synapses to modulate short-, and long-term function. This influence of glial cells on synapses contributes to a fundamental property of synapses, their plasticity. However, little is known about what determines whether glial cells influence synaptic plasticity in positive versus negative directions. Furthermore, although we have gained more information regarding the role of glial cells in synaptic plasticity, little is known about the plasticity of glial cells themselves. One group of factors that is implicated in a wide variety of plasticity mechanisms throughout the nervous system is the neurotrophins. We also know that these factors are involved in glial cell function, and this knowledge led me to become interested in their potential contribution to glial cell plasticity. This thesis addresses the fundamental questions of both glial contribution to synaptic plasticity and the plasticity of glial cells themselves. It will address the following specific questions: 1) what underlies the generation of positive, in comparison to negative feedback, to synapses by glial cells; and 2) the role of neurotrophins on glial cell plasticity. These studies will provide information on mechanisms of neuron-glial interactions as well as some molecules that could be important for these interactions.

In the first study I found that modulation of nerve activity induced distinct glial calcium responses. These different glial calcium elevations were shown to be involved in providing either positive or negative feedback to synapses. This study demonstrated that the pattern of neuronal activity dictates the pattern of glial activation, which ultimately leads to glial-mediated differences in synaptic plasticity.

Secondly, I investigated neurotrophin signalling to glial cells and the roles these factors have in glial cell plasticity itself. Application of different neurotrophins during nerve-evoked activity altered glial calcium responses. I found that glial cell calcium elevations are plastic and can be changed by neurotrophins.

The results presented in this thesis demonstrate the crucial role of glial cells in regulating the direction of synaptic plasticity. Furthermore, I reveal the highly sensitive nature of glial cell calcium elevations, where small changes in glial calcium signals dramatically alter synaptic function. In addition I demonstrate that glial cells are themselves plastic. When considering the observed importance of differences in glial calcium elevations on synaptic plasticity, neurotrophins may be vital for directing the outcome of neuron-glial communication.

Keywords – neuron-glial interactions; neurotrophin;

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List of abbreviations

ACh – acetylcholine

ATP – adenosine triphosphate

BDNF – brain derived neurotrophic factor

CAPS – calcium activated protein for secretion

CNS – central nervous system

EDL – extensor digitorum longus

mAChR – muscarinic acetylcholine receptor

nAChR – nicotinic acetylcholine receptor

NGF – nerve growth factor

NMJ – neuromuscular junction

NO – nitric oxide

NOS – nitric oxide synthase

NT-3 – neurotrophin 3

NT-4 – neurotrophin 4

PNS – peripheral nervous system

PSC – perisynaptic Schwann cell

PTP – post-tetanic potentiation

ROS – reactive oxygen species

SNARE – soluble N-ethylmaleimide-sensitive-factor attachment protein receptor

Sol – soleus

TrkB – tropomyosin-related kinase receptor B

TrkC – tropomyosin-related kinase receptor C

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1. INTRODUCTION

1.1. PREAMBLE

The classical view of the synapse includes the presynaptic terminal and the postsynaptic neuron (Fig. 1). However, over the past two decades glial cells have emerged as a third cellular component important for synaptic function. This development has lead to the use of the term "tripartite synapse" when referring to synapses to better reflect our new understanding of synapses as structures that not only include the pre- and postsynaptic neurons, but also the surrounding glia (Fig. 2).

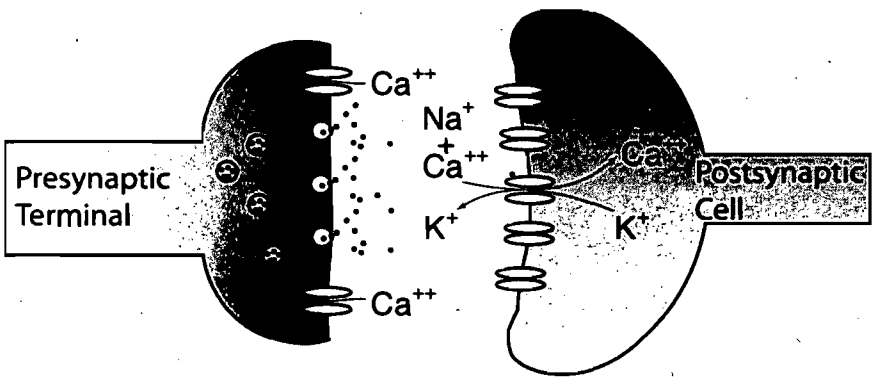

Figure 1. Classic view of the synapse.
Communication between two neurons involves release of neurotransmitter from the presynaptic terminal that binds to receptors on the postsynaptic cell.

Synapses are plastic, and therefore can be strengthened or weakened depending on the changing demands of the nervous system. Classically, plasticity was thought to involve the pre- and postsynaptic neurons of the synapse. However, glial cells surrounding the synapses have now been implicated in these phenomena throughout the nervous system (Allen and Barres, 2005; Todd et al., 2006).

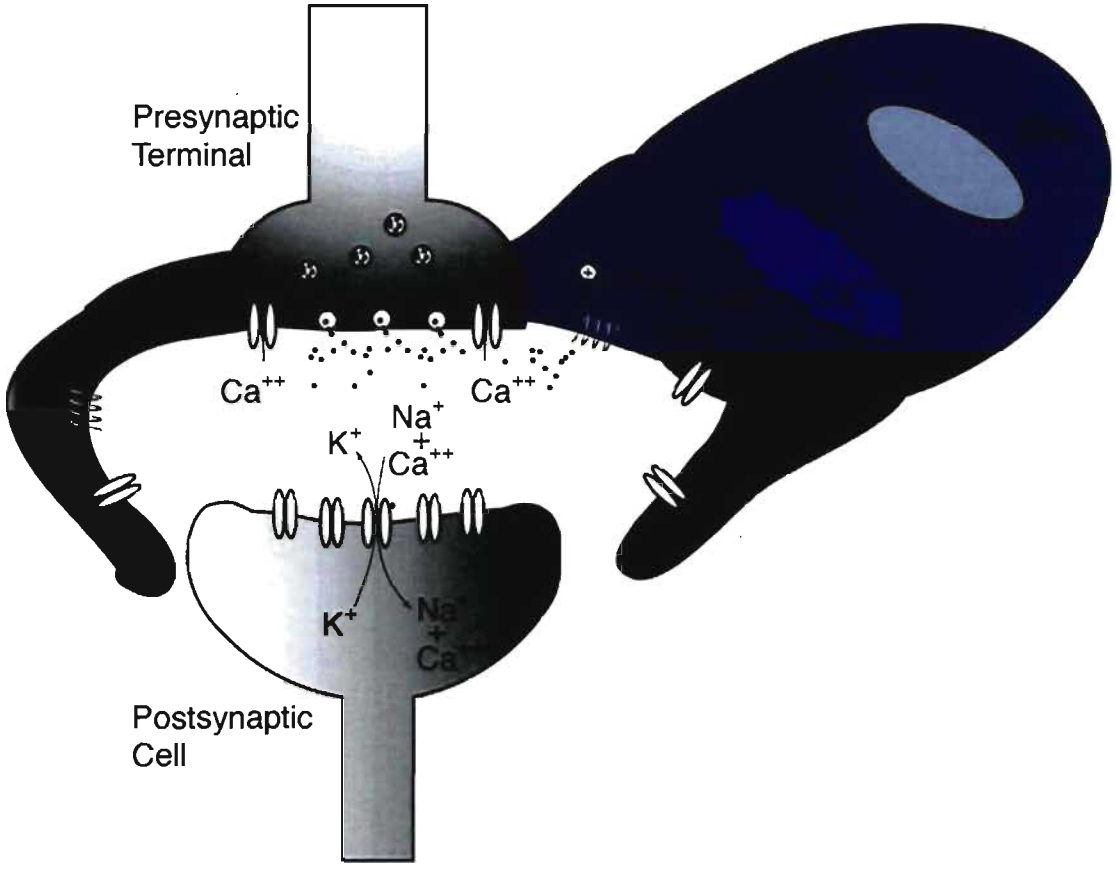

Figure 2. The tripartite synapse.
The glial component is added in to the classical view of the synapse and is an active partner in synaptic transmission both receiving input and providing feedback to neurons.

The study of communication between neurons and glia (ie neuron-glial interactions) began with the observation that glia could respond to neuronal activity with elevations of intracellular calcium (Dani et al., 1992; Jahromi et al., 1992; Reist and Smith, 1992). Since then, it has been demonstrated that glial cell responses to nerve activity are not simply on or off, but are graded and dependent on the frequency of stimulation (Pasti et al., 1997), and are also input specific (Perea and Araque, 2005). This acute variability in glial calcium responses illustrates the dynamic nature of their interactions at the synapse. However, the contributions of distinct glial calcium signals on synaptic transmission remain unknown. One wonders if

glial calcium responses, as with synapses, are modifiable and plastic in a manner that would allow them to undergo lasting changes (minutes to hours) to their responsiveness.

Following the demonstration of glial cell responsiveness to synaptic activity, a number of laboratories investigated the capacity of glial cells to modify neuronal and synaptic function (Kang et al., 1998; Newman and Zahs, 1998; Robitaille, 1998) through the release of transmitter substances from glial cells termed gliotransmitters. Since these first discoveries, a number of different examples have been published using different preparations and approaches to demonstrate the involvement of glial cells in synaptic plasticity (Castonguay and Robitaille, 2001; Ullian et al., 2001; Fellin et al., 2004; Fiacco and McCarthy, 2004; Hama et al., 2004; Christopherson et al., 2005; Pascual et al., 2005; Ge et al., 2006; Panatier et al., 2006; Serrano et al., 2006; Stellwagen and Malenka, 2006; Perea and Araque, 2007). Surprisingly, however, no one has yet demonstrated the ability of the same glial cell to induce both increases and decreases in synaptic function in the same neuron. Since synapses need to be able to be modified both positively and negatively, it seems likely that glial cells could be involved in both processes. Furthermore, if glial cells are to change their responsiveness under different synaptic situations, they too must be plastic.

In this thesis I have provided information regarding the role of glial cells in different forms of plasticity and in glial plasticity itself. To investigate these unanswered problems, I studied the involvement of glial cells in the generation of distinct forms of plasticity, specifically, their ability to detect different patterns of neuronal activity, and the resulting differences in their feedback to synapses. In addition, I have investigated the contribution of the neurotrophins, on the modulation of glial cell plasticity and neuron-glial interactions.

First, I demonstrate that glia from the same synapse could detect differences in the pattern of endogenous neuronal activity, which is reflected in their calcium elevations. Second, activating the glial cells with distinct patterns of nerve activity resulted in feedback that was dependent on the initial pattern of activity. Finally, in Chapter 3 I demonstrate that glial cell calcium elevations are indeed plastic and that the neurotrophins are one group of molecules that are implicated in this plasticity.

The work contained in this thesis provides important insight into the mechanisms and some of the molecules involved in neuron-glial interactions. These finding take steps towards explain the complex interactions between neurons and glia throughout the nervous system.

1.2. The Synapse

Synapses are connections between two cells that allow the transfer and modulation of neuronal information. Most commonly, these are connections between two neurons where one neuron, the presynaptic cell, releases a transmitter substance that will bind to receptors on the postsynaptic cell. The binding of neurotransmitter to its receptors causes signalling to occur in the postsynaptic neuron either through the flux of ions through pore-forming receptors or through complex cascades of intracellular messengers.

Transmitter release is a tightly regulated process for reliable communication in the nervous system. It occurs at active zones, specialized regions of the presynaptic terminal where vesicle fusion occurs (Fig. 3) and it is regulated by many intracellular proteins as well as some transmembrane proteins that span the synapse to physically join the pre-postsynaptic cells. Some of the most studied presynaptic proteins that are involved in vesicle trafficking and release make up a group known as the SNARE proteins. The SNARE proteins link together the nerve terminal

membrane, vesicles and calcium channels to tightly regulate vesicular release. They consist of SNAP-25, synaptobrevin, syntaxin and the associated calcium-sensitive synaptotagmin (Bennett et al., 1992; Geppert et al., 1994; Nishiki and Augustine, 2004). Calcium channels bind to the SNARE proteins SNAP-25 and syntaxin (Leveque et al., 1994; Sheng et al., 1994; Martin-Moutot et al., 1996; Jarvis et al., 2002; Keith et al., 2007). Association with these presynaptic scaffolding proteins keeps calcium channels in close proximity to active zones, an organization that has been repeatedly suggested for over 30 years (Heuser et al., 1974; Robitaille et al., 1990). This close proximity between calcium channels and active zones (probably less than 50 nm) leads to high fidelity of transmitter release. In fact, following an action potential it takes approximately 0.2 ms for vesicle fusion to occur. Presynaptic active zones allows for rapid and reliable vesicle release following arrival of an action potential.

Postsynaptically, neurotransmitter binds to receptors to induce a response. Receptors are located across from active zones and held in place by their interactions with scaffolding proteins that combine to make up the postsynaptic density (Fig. 3). The postsynaptic density contains receptors, scaffolding proteins and signalling proteins involved in the generation of the postsynaptic response.

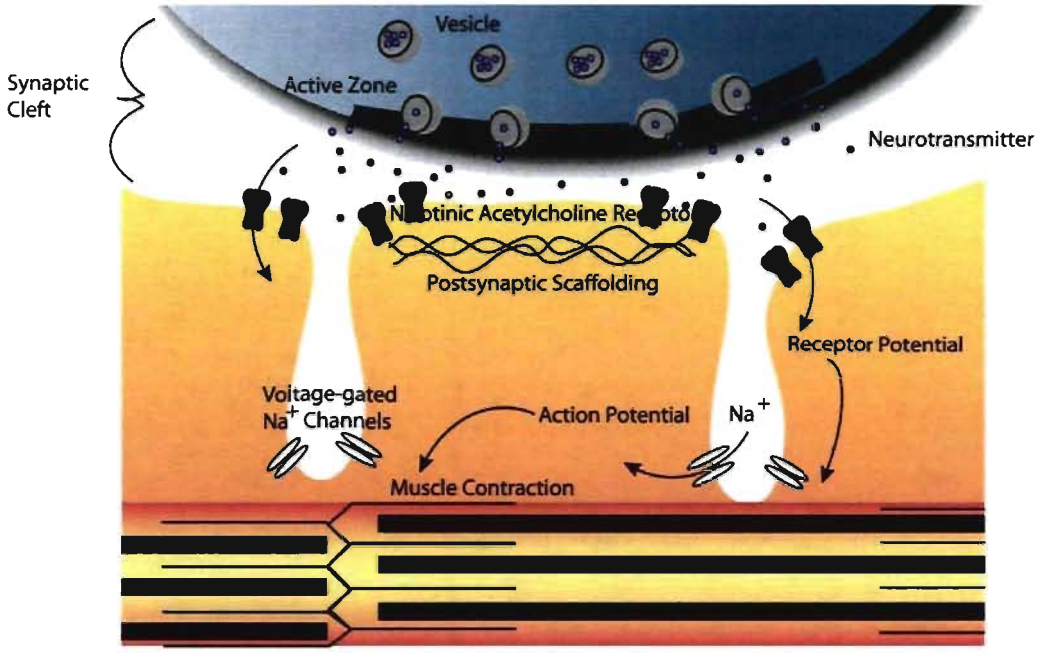

Figure 3. Presynaptic and Postsynaptic Machinery.
At the presynaptic terminal vesicles fuse with the presynaptic membrane at active zones, regions specialized for the fusion of vesicles and the release of neurotransmitter upon arrival of action potentials. Once neurotransmitter is released into the synaptic cleft is crosses to the postsynaptic cell where it binds to postsynaptic receptors. In muscle, the postsynaptic membrane is composed of folds and valleys with nAChRs positioned at the tops of the folds and voltage-gated sodium channels at the bottoms of the valleys. Activation of the nAChRs by neurotransmitter binding results in a receptor potential that propagates passively down the membrane until it reaches the voltage-gated sodium channels where action potential initiation and ultimately muscle contraction occur.

Synaptic structures are modifiable both in morphology and in terms of the strength of the connection. Morphological adjustments can occur, for example, through actual changes in the size of the cellular elements or through their proximity to each other. Having said this, synapses are relatively stable structures once formed (Grutzendler et al., 2002). Aside from morphological alterations, synaptic strength can also be changed. Changes in the strength of synapses can be both increasing and

decreasing. These changes are generally referred to as synaptic plasticity. The mechanisms and processes involved will be discussed in more detail later. However, at a basic level these plasticity events can be the result of changes to the presynaptic terminal, the postsynaptic cell as well as neighbouring glia.

This section has introduced general aspects of the chemical synapse. Synapses include the presynaptic terminal, postsynaptic cell and surrounding glia. Synaptic transmission occurs through release of vesicles from the active zone, which binds to postsynaptic receptors. Most commonly we think of neuron-to-neuron synapses, however, one of the best-understood synapses in the nervous system is the neuromuscular junction (NMJ). The NMJ is the equivalent of neuron-to-neuron synapses, but is specialized to allow for synaptic transmission between neurons and muscles. Like central nervous system synapses the NMJ is surrounded by glia that participate in its function (Auld et al., 2003). These properties make the NMJ an excellent model for studying neuron-glial interactions. In the sections to follow properties of the NMJ will be discussed in more detail.

1.3. Neuromuscular Junction

The neuromuscular junction has been studied for the longest amount of time of any synapse in the nervous system. Chemical synaptic transmission has been best described in this system beginning in the 1950s with the work of Bernard Katz and colleagues (Del Castillo and Katz, 1954). Since then the NMJ has continued to be a useful model for understanding mechanisms of release (Poage and Meriney, 2002; Rizzoli et al., 2003), postsynaptic mechanisms (Sanes and Lichtman, 2001) and more recently, for studying the tripartite synapse (Auld and Robitaille, 2003a).

1.3.1. Organization and Function of the NMJ

The NMJ, like other synapses, is composed of a presynaptic nerve terminal, a postsynaptic cell (muscle fibre) and surrounding glial cells (perisynaptic Schwann cells; PSCs). This is a relatively simple organization with only one innervating nerve terminal (at mature synapses), one postsynaptic cell, and about 3-4 PSCs, with all cellular compartments being visually identifiable.

1.3.1.1. Presynaptic Terminal of the NMJ

The presynaptic terminal of the mammalian NMJ is an elaborate structure that roughly takes on a pretzel-like shape. Release at the neuromuscular junction occurs upon the arrival of an action potential and results in the liberations of tens to hundreds of vesicles. Calcium and presynaptic proteins associated with vesicular release play similar roles at the NMJ as previously mentioned for synapses in general.

Presynaptic terminals functionally match the properties of their associated muscle. For example the presynaptic terminal must be able to induce the form of contraction required by a given muscle, and some muscles are required to be active for brief periods of intense activity whereas others require more prolonged periods of activation. This is achieved through a variety of presynaptic terminal properties such as the number of vesicles available and released (Reid et al., 1999). These differences in properties give some terminals the ability to be active for longer periods of time, while others will fatigue more easily. Synapses that initially release fewer vesicles but can remain active for longer periods of time are called weak and fatigue resistant. Strong synapses fatigue more easily as they are normally only active for shorter periods of

time(Wood and Slater, 1997; Reid et al., 1999). Specific properties will be discussed in more detail in the following section.

1.3.1.2. Properties of Presynaptic Release

Although the structure of NMJs can vary widely amongst different species, the functional aspects seem to be well conserved. For instance, frog NMJs, which can be hundreds of micrometers long, also release hundreds of vesicles for each action potential (Heuser et al., 1979; Katz and Miledi, 1979). However, rat terminals, which are around 50 micrometers in length also release around 50 vesicles per action potential (Wood and Slater, 1997). This suggests that a size/release relationship exists that is conserved at least amongst vertebrates. However, there are observable differences between neuromuscular synapses. For instance, much interest has been focussed on the adaptive mechanisms that are associated with differences in weak and strong neuromuscular synapses. In the mammalian system, one commonly used comparison for weak and strong synapses is that of *soleus* (Sol; weak) and *extensor digitorum longus* (EDL; strong) muscles. At the weak Sol synapses, less fatigue is observed and release can be maintained for longer periods of time at lower frequencies (~20 Hz). However, the EDL displays rapid fatigue and is only active for short bursts of activity at high frequencies (~80 Hz) (Hennig and Lomo, 1985). Corresponding to the functional differences between these synapses are morphological ones, with the Sol occupying larger synaptic areas than EDL NMJs (Waerhaug, 1992; Wood and Slater, 1997). In conjunction with occupancy of large synaptic areas, Sol nerve terminals also have a larger synaptic vesicle pool in comparison to those of EDL NMJs (Reid et al., 1999). These terminals also display differences in initial quantal release (Gertler and Robbins,

1978; Wood and Slater, 1997; Reid et al., 1999). However, the time required for vesicle recycling appears to be quite similar for both terminals even across species, ~115 sec (Betz and Bewick, 1992, 1993; Ryan et al., 1993; Reid et al., 1999). Since recycling time is similar, this suggests smaller release per stimulus and unit area along with the larger vesicle pool size contribute to the greater degree of fatigue resistance at Sol nerve terminals. Although there are some subtle differences in release properties and vesicle number, the same basic mechanisms apply to all NMJs.

1.3.1.3. Synaptic Plasticity

The amount of transmitter release that occurs is variable and changes in the amount of release are referred to as synaptic plasticity. Synaptic plasticity can occur over different time frames from milliseconds to days and can be broadly broken up into short-, and long-term plasticity.

1.3.1.3.1 Potentiation

Enhancement of synaptic transmission can be divided temporally into facilitation, lasting tens of milliseconds, augmentation, lasting seconds, post-tetanic potentiation (PTP), lasting 10s of seconds and finally short-term plasticity, which lasts for minutes (Zucker and Regehr, 2002). Calcium plays important roles in all forms of synaptic enhancement although the mechanisms are different. Facilitation is the result of residual calcium in the presynaptic terminal when a second impulse triggers release. This causes greater fusion of synaptic vesicles and a larger postsynaptic response. Katz and Miledi first suggested the involvement of calcium in facilitation using the NMJ preparation (Katz and Miledi, 1968). More recent work suggests that facilitation is defined

merely by the rate with which calcium diffuses away from active zones (Cooper et al., 1996). In comparison, augmentation, which is longer lasting, is mediated by the rate of calcium removal. It has been suggested for a number of years that the processes regulating facilitation and augmentation are different (Landau et al., 1973; Magleby and Zengel, 1982). There is evidence suggesting that calcium pumps and exchangers can be involved in regulating the duration of augmentation and PTP (Parnas et al., 1982; Wojtowicz and Atwood, 1985). This probably occurs through a build up of both sodium and calcium presynaptically. This build up then results in decreased removal by exchangers due to the increased intracellular sodium concentration (Birks and Cohen, 1968b, 1968a; Mulkey and Zucker, 1992). Another factor that can influences PTP is mitochondrial calcium loading. This also occurs during periods of prolonged activity where mitochondria are loaded with calcium that is slowly released following cessation of activity (Tang and Zucker, 1997; David et al., 1998; David and Barrett, 2000). All of these different mechanisms lead to different forms of synaptic enhancement, largely delineated by their duration.

1.3.1.3.2 Depression

Aside from synaptic potentiation, depression also occurs. At the NMJ there are two main phases of depression that are often described. The first and shorter phenomenon occurs over seconds during prolonged activity, while the other is a modification of longer duration. One of the main causes suggested to underlie the shorter depression mentioned above is depletion of the vesicle pool (Betz, 1970). This occurs during prolonged periods of activity, where vesicle release outpaces recovery and recycling, such that fewer readily releasable vesicles are available. Once presynaptic activity stops, vesicle recycling

can catch up and return the vesicle pool to normal, and thus, no depression is apparent.

Other mechanisms of depression that involve longer processes include modulation of presynaptic function by neuromodulators and postsynaptic desensitization (Zucker and Regehr, 2002). Some of these mechanisms have been described in our laboratory and include neuromodulators such as nitric oxide (NO) and glutamate that can induce long lasting depression of neuromuscular transmission (Thomas and Robitaille, 2001; Pinard et al., 2003). Interestingly, the depression induced by these two transmitters appear to be quite similar. Recently, it has been demonstrated that in fact glutamate and NO are linked and cooperate to induce depression at the NMJ (Pinard and Robitaille, in press). This appears to be through presynaptic release of glutamate that binds to postsynaptic metabotropic glutamate receptors, which activate NO synthase to produce NO. The NO is then able to diffuse back to the presynapse to cause depression.

The processes leading to synaptic plasticity are fundamental for proper synaptic functioning. Many of the early discoveries surrounding plasticity were made using NMJ preparations, discoveries that have proven to hold true throughout the nervous system.

1.3.1.4. Postsynaptic Muscle Fibres

The postsynaptic cell associated with NMJs is the muscle fibre. There is only one presynaptic cell for each postsynaptic cell, unlike the organization of the CNS, however, during development muscle fibres are innervated by multiple presynaptic terminals (Balice-Gordon and Lichtman, 1993).

Strong and weak synapses innervate different muscle fibre types. The differences in muscle fibres have been categorized a number of different ways based on myofibrillar ATPase isoform, twitch kinetics,

myosin heavy chain (MHC) isoform, and metabolic enzymes and processes used. This results in Type I fibres corresponding to slow twitch, and red fibres expressing MHCIβ and being innervated by weak presynaptic terminals. These fibres are high in mitochondria, undergo oxidative metabolism and are fatigue resistant (Peter et al., 1972; Pette and Staron, 2000). They also have a smaller diameter than other fibre types. Type IIB fibres are larger in diameter, are one type of fast twitch, white fibre, that are low in mitochondria, run on glycolysis and express MHCIIb (Peter et al., 1972; Pette and Staron, 2000). These properties make white fibres more prone to fatigue and are innervated by the strong presynaptic terrninals. The other pure fast twitch fibres include type IIA and IID. Finally there are mixed fibres that display a mixture of the above-mentioned properties(Pette and Staron, 2000). Most mammalian muscles are composed of a mixture of fibre types rather than being purely one type or another.

1.3.1.5. The End-Plate and Receptors

Synaptic transmission occurs through the release of acetylcholine and activation of postsynaptic nicotinic acetylcholine receptors (nAChRs). These receptors are pentameric in structure and are permeable to monovalent and divalent cations (Villarroel and Sakmann, 1996). As with all postsynaptic cells, densities of receptor clusters and postsynaptic scaffolding proteins are present on muscle fibres in the end-plate region. The clusters of nAChRs are located at the peaks of a number of folds in the postsynaptic membrane that make up the end-plate and are located directly across from active zones. In the troughs of these folds are located the voltage-gated sodium channels responsible for generating action potentials.

The NMJ has been an important model for studies of pre and postsynaptic development, for example receptor clustering. Early studies observed some spontaneous clustering of nAChRs (Vogel et al., 1972; Fischbach and Cohen, 1973), however, synapse formation and clustering associated with mature synapses requires activity (Anderson and Cohen, 1977; Frank and Fischbach, 1979), something that was also confirmed *in vivo* (Liu and Westerfield, 1992). Along with receptor clustering, receptor synthesis is also increased with activity (Anderson and Cohen, 1977; Jessell et al., 1979; Role et al., 1985). Two factors released in an activity-dependent manner that are important for these processes are neuregulin (Usdin and Fischbach, 1986; Falls et al., 1993; Jo et al., 1995), and agrin (Godfrey et al., 1984; Meier et al., 1997). Recent *in vivo* work has found that spontaneous nAChR clusters are actually the site of presynaptic contact and that these clusters can be incorporated into maturing end-plates (Flanagan-Steet et al., 2005; Panzer et al., 2005).

1.3.1.6. Perisynaptic Schwann Cells

Perisynaptic Schwann cells (PSCs) are geno- and phenotypically distinct from myelinating Schwann cells. Whereas myelinating Schwann cells express high levels of proteins such as P0 and myelin basic protein, PSCs express low levels of these proteins and high levels of proteins such as NCAM and S100 (Mirsky and Jessen, 1996). In terms of phenotype, PSCs do not wrap axons, but rather surround nerve terminals and postsynaptic densities. In mammals PSCs arrive at NMJs starting in early postnatal life and increase in numbers to about three or four in adults (Love and Thompson, 1998). During development, Schwann cells migrate along axons rather than the opposite as previously thought (Gilmour et al., 2002).

PSCs are found surrounding the synaptic cleft and can, in fact, participate in synaptic transmission. They express receptors for the neurotransmitters of the NMJ and also transporters that could participate in regulating plasticity events (Pinard et al., 2003). This will be discussed further in the following section (1.4.4). Aside from developmental and synaptic functions of PSCs, they are also important for synapse stability in mature animals (Reddy et al., 2003). PSCs are an integral part of neuromuscular synapses, participating in development, maturation, stability and function.

1.4. Neuron-glial Interactions in Synaptic Plasticity

Glial cells are the other major cell type in the nervous system aside from neurons. Like neurons they are heterogeneous and fall into a number of large categories; 1) microglia, the immune cells of the brain, 2) myelinating glia, oligodendrocytes of the CNS and Schwann cells of the PNS, and 3) astroglia, which are the non-myelinating synaptic glial cells that are represented by PSCs at the NMJ.

Beginning in 1992 glial cells *in situ* were shown to actively respond to synaptic transmission (Dani et al., 1992; Jahromi et al., 1992; Reist and Smith, 1992). It was another six years before it was demonstrated that glial cells could also provide modulation of synaptic function through feedback (Kang et al., 1998; Newman and Zahs, 1998; Robitaille, 1998). Now, it is widely accepted that glia are active partners in synaptic function that play a number of different roles in synaptic transmission. This evidence has been derived from work at the NMJ and with CNS preparations.

1.4.1. A Brief History of Synapse-Glia Interactions

Although it was not until relatively recently that glial cells were demonstrated to actively participate in synaptic function, observations made by anatomists around the early 1900's suggested a variety of such functions (Somjen, 1988). For instance, Lugaro (Somjen, 1988) suggested that astrocytes might be involved in neurotransmitter removal to regulate transmission, something that is now known to be true (Rothstein et al., 1996; Bergles and Jahr, 1997, 1998; Huang and Bordey, 2004).

The first description of a dynamic glial response to synaptic transmission came from the work of Stephen Kuffler in the 60's. He and colleagues showed that activity in neighbouring nerves could induce depolarization of glial cells that was dependent on extracellular potassium (Kuffler et al., 1966; Orkand et al., 1966). From these first observations was derived the

idea that glial cells buffered the extracellular milieu, however, a major advancement in our ability to study glial cells came with the finding that they responded dynamically with calcium elevations. Since then a number of different studies using isolated cell cultures, and a variety of semi-intact, and more recently, even *in vivo* studies, have revealed a vast number of functions for glial cells in brain physiology (Volterra and Meldolesi, 2005; Haydon and Carmignoto, 2006).

In terms of chemical activation of glial cells, the first studies to show this were done using cultured astrocytes and bulk application of transmitter substances (Cornell-Bell et al., 1990). Following this, a number of studies used more intact preparations to demonstrate that this was not simply an artefact of culture (Dani et al., 1992; Jahromi et al., 1992; Reist and Smith, 1992). Two of these studies, from Stephen Smith's laboratory, used different preparations. One was the hippocampal slice, and the other the NMJ preparation and PSCs. These two studies arrived at the same basic conclusion, that glial cells respond with calcium elevations to nerve activity.

Following the establishment of glia as active responders to synaptic transmission, the question turned to their involvement in providing modulatory feedback to synapses. This could occur in a number of ways: regulated clearance of neurotransmitter, morphological changes affecting transmission, and release of chemical gliotransmitters. As already mentioned, a major breakthrough occurred in 1998 when a number of papers from independent laboratories published results showing that glial cells from both the CNS and PNS could indeed modulate neuronal function (Kang et al., 1998; Newman and Zahs, 1998; Robitaille, 1998). Since then, work has provided information on many of the finer points of neuron-glial communication and the many and varied roles that glial cells play in synaptic function.

1.4.2. Calcium: A Breakthrough in the Study of Glial Cell Function

The major acute response detectable in glial cells is the calcium response. This dynamic form of cellular response was discovered in the 1980's and has since been influential in studies of glial cell function and neuron-glial interactions (for example see Sugino et al., 1984).

The intracellular signalling cascades responsible for generating glial calcium responses are the inositol 1,4,5-triphosphate (IP_3) and ryanodine receptor pathways (Haak et al., 2001; Holtzclaw et al., 2002). However, calcium responses can also be generated through influx of extracellular calcium (Verkhratsky and Kettenmann, 1996). The IP_3 pathway is initiated through G-protein-mediated activation of phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) to form diacylglycerol and IP_3. The newly formed IP_3 then activates IP_3 receptors to release intracellular stores of calcium (Fig. 2).

Calcium elevations in glia can be generated spontaneously and through agonist-mediated activation. In terms of synaptic function, agonist-mediated activation of glial cells can be critically important for subsequent modifications to that function. For instance, in the hippocampus, calcium signalling in astrocytes can be initiated by $GABA_B$ receptors that lead to potentiation of release by inhibitory interneurons (Kang et al., 1998). This initially occurs through the presynaptic release of a neurotransmitter that then binds to receptors on the perisynaptic glial cell. This leads to a calcium elevation in the glial cell and subsequent release of gliotransmitter that acts through feedback on the presynaptic terminal or onto the postsynaptic cell. Interestingly, glial calcium responses are not all or none events. They are modifiable and dependent on the input used to activate them and the intensity of stimulus (Pasti et al., 1997; Perea and Araque, 2005). Glial cells, like neurons, express a number of different receptors and therefore, can be activated in a number of different ways. In neurons, different frequencies

and patterns of activity lead to different changes in synaptic plasticity. Although glial cells respond with different calcium elevations to different frequency input, it is unknown if this impacts any ongoing process. Furthermore, nothing is known regarding the influence of different patterns of stimulation on glial activity. It is likely that differences in glial activation lead to differences in feedback to neurons, although this has never specifically been tested.

1.4.3. CNS Synaptic Function and the Implication of Glia

Due to issues with complexity and accessibility much of the early CNS glial work was performed in culture. However, it was soon realized that observations made in culture were not always representative of those occurring in more intact preparations (Kimelberg et al., 1997). Now slice preparations are commonly used and even some *in vivo* studies are emerging (Wang et al., 2006). Despite early problems with accessibility, *in situ* CNS-glial preparations have provided original pieces of information, and continue to improve our understanding of neuron-glial interactions.

1.4.3.1. Gliotransmission and Glial-to-Neuron Signalling

1.4.3.1.1 Chemical Transmitter Release

Gliotransmission (the release of chemical transmitters from glia) is one discovery made through studies of CNS preparations. In fact, the release of gliotransmitters in response to activation has only definitively been demonstrated in the CNS. There is also evidence for astrocytic vesicles (Bezzi et al., 2004), and vesicular release machinery (Zhang et al., 2004). Furthermore, release of glutamate has been recorded in culture (Parpura et al., 1994), and suggested, somewhat controversially, to occur in slice preparations (Angulo et al., 2004; Fellin et al., 2004; Fiacco and McCarthy, 2004). Although glutamate release by astrocytes leading to modulation of

CNS synapses is an attractive hypothesis, the evidence is currently under debate (Angulo et al., 2004; Fellin et al., 2004; Fiacco et al., 2007).

Another transmitter known to be released by glia is ATP (Fields and Burnstock, 2006; Zhang et al., 2007). The release of ATP by glia is one of the major mechanisms used to propagate signals across glial networks (Newman and Zahs, 1997; Haas et al., 2006). Another role for astrocyte purine signalling has recently emerged from two laboratories using pharmacological and molecular approaches. These reports demonstrated the impact of such signalling in hippocampal plasticity where glial signalling and ATP release is fundamental for the generation of heterosynaptic plasticity (Pascual et al., 2005; Serrano et al., 2006). Similarly, ATP release by astrocytes was earlier found to be involved in heterosynaptic suppression, which occurs on a faster time scale (Zhang et al., 2003). Purine release in neuron-glial interactions is a major factor in both the CNS and PNS. The roles that purines play in neuron-glial interactions at the NMJ will be discussed in the following section.

1.4.3.1.2 GLIOTRANSMISSION AND SYNAPSE DEVELOPMENT

Other important actions of factors released by glial cells have been shown in the context of development. For instance early studies in culture revealed the importance of glia for synapse development and synaptic transmission (Pfrieger and Barres, 1997; Ullian et al., 2001). A number of different factors have been shown to be important for these developmental changes such as cholesterol (Mauch et al., 2001), thrombospondins (Christopherson et al., 2005), and TNFα (Beattie et al., 2002; Stellwagen and Malenka, 2006).

These studies often focus on excitatory synaptic transmission, however, roles for glia in inhibitory synapse formation have also recently come to light. For instance, Balice-Gordon and colleagues demonstrated that

astrocyte conditioned medium could enhance both inhibitory synaptic transmission and postsynaptic $GABA_A$ receptor density (Elmariah et al., 2005b). This appears to occur through two separate mechanisms that rely on the release of BDNF from neighbouring astrocytes. An interesting link with the previous discussion of purinergic signalling comes from work in the PNS showing that axonal release of ATP could initiate the release of BDNF from Schwann cells in a regulated manner (Verderio et al., 2006). This study was performed in the context of regeneration; however, similar mechanisms may influence developmental progressions as well. In addition to releasing neurotrophins, glial cells can also respond, to at least BDNF, with intracellular calcium elevations (Rose et al., 2003). This indicates that the neurotrophins are likely important molecules for neuron-to-glial communication and glial-to-neuron signalling.

1.4.3.1.3 Physical Glial-to-Neuron Signalling

Aside from factors that are released, glial cells can communicate with neurons through a wide variety of mechanisms. For example, one mechanism of communication is the direct interaction of neuronal and glial cells, since glia enwrap synapses (Ventura and Harris, 1999; Murai et al., 2003). These interactions are known to influence dendritic morphological plasticity (Murai et al., 2003), and possibly synaptic development (Hama et al., 2004). Interestingly, astocytes occupy distinct domains, do not overlap, but do contact synapses on multiple neurons (Bushong et al., 2002; Halassa et al., 2007). This provides the potential for inter-neuronal interactions outside the normal axon pathways.

Aside from release and direct cell-to-cell contact, the morphological arrangement of a synapse can also affect its function. As mentioned, a phenomenon such as this was described in the hippocampus where glial contact increased dendritic stability (Murai et al., 2003). Another example of

the role that morphology could have on synaptic function has emerged from studies in the hypothalamus where lactation causes a retraction of glial processes from around synapses (Oliet et al., 2001) and ultimately changes the plasticity expressed by these synapses (Panatier et al., 2006). It is quite possible that differences in the anatomical arrangement of different synapses in other regions of the brain can have important effects on synaptic function, similar to what has been reported in the hypothalamus.

1.4.3.2. Waves of Activation

Glial calcium waves have been observed in a number of brain regions including the cortex (Haas et al., 2006) and retina (Newman and Zahs, 1997). Glial calcium waves are proposed to have a number of roles in CNS function. For instance, they appear to be linked to metabolic functions to regulate glucose uptake over a larger area (Bernardinelli et al., 2004). Furthermore, these waves are likely involved in regulating neuro-vascular coupling (Metea and Newman, 2006; Takano et al., 2006), since calcium signals in glial endfeet can induce changes in vascular dilation (Zonta et al., 2003; Mulligan and MacVicar, 2004). In terms of direct synaptic functioning, glial calcium waves may also be involved in long-distance communication and possibly even synchronization of multiple neurons through coherent release of gliotransmitter (Angulo et al., 2004; Fellin et al., 2004).

There are many molecules and mechanisms that are implicated in the birdirectional communication between neurons and glial cells. It is becoming clear that the heterogeneity of glial cells is leading to an almost endless number of possibilities for the way they interact with neurons.

1.4.4. NMJ Modulation and the Involvement of PSCs

The neuromuscular junction has proven to be a valuable model for investigating synapse-glia interactions. In fact, some of the pioneering

studies were performed on this preparation (Jahromi et al., 1992; Reist and Smith, 1992; Robitaille, 1998). In the following sections neuron-glial intractions involved in NMJ plasticity will be discussed.

1.4.4.1. Acute Actions of PSCs

Perisynaptic Schwann cells of the NMJ can be thought of as the peripheral nervous system equivalent to astrocytes of the CNS. They are non-myelinating, occupy distinct domains and surround the synapse. As mentioned, nerve activity induces PSC calcium responses through activation of a variety of receptors. PSCs are also involved in modulating synaptic function, through regulation of transmitter in the synapse (Smit et al., 2001), signalling to the presynaptic terminal (Robitaille, 1998; Castonguay and Robitaille, 2001) and providing overall stability to the synapse (Reddy et al., 2003). These aspects of the NMJ-PSC preparation allow for its utility as a model for studies of neuron-glial interactions.

As with CNS astrocytes, PSCs are able to respond to a number of different neurotransmitters. The most predominant are acetylcholine (ACh), which is released from the nerve terminal, and the purines, which will be discussed in the review to follow. ACh is the primary neurotransmitter at the neuromuscular junction. In addition to activating the postsynaptic muscle fibre, it can also induce responses in PSCs through metabotropic muscarinic acetylcholine receptors (mAChRs). At frog NMJs, mAChRs are suggested to be of an atypical form due to their unique pharmacological profile (Robitaille et al., 1997).

The primary known and acute result of PSC activation is synaptic modulation. This can occur in the form of either potentiation (Castonguay and Robitaille, 2001) or depression (Robitaille, 1998). The mechanisms of this modulation are presently unknown, however, the outcome of glial

modulation appears to be dependent on the intracellular signalling cascade that is recruited following nerve activity.

1.4.4.2. Long-term Actions of PSCs

Some of the most well described PSC functions are those occurring over longer periods of time. Interestingly, results from the laboratory of Milton Charlton have suggested that mAChRs might be involved in regulating morphology of PSCs through control of cytoskeletal elements. This was suggested through investigations showing that blockade of nerve activity resulted in the rapid upregulation of glial fibrillary acidic protein (GFAP) in PSCs (Georgiou et al., 1994). This group went on to show that the receptors involved were mAChRs (Georgiou et al., 1999). These results suggest that not only does neurotransmission have acute affects on PSCs, but that it likely also influences glial and synaptic stability. The reciprocal of this is true as well, where removal of PSCs from the synapse results in synaptic disassembly (Reddy et al., 2003), suggesting that PSCs also provide stability signals to presynaptic terminals at NMJs.

1.4.4.2.1 PSCs and Synapse Development

Intestingly, PSCs, like astrocytes, also seem to release substances that encourage synaptic development. In culture, the addition of neurotrophic factors maintains the survival of neurons, but prevents synapse formation. On the other hand, when Schwann cell conditioned media is added, synaptogenesis can occur (Peng et al., 2003). Furthermore, evidence has recently emerged suggesting that factors released by glial cells can modulate synaptic transmission during development, possibly to facilitate this process (Cao and Ko, 2007). This illustrates the similarity in function between PSCs and astrocytes of the CNS.

Neurotrophins are also known to be important for Schwann cell development and myelination. For instance, NT-3 can induce migration of Schwann cells prior to myelination (Cosgaya et al., 2002; Yamauchi et al., 2003). Following migration, a developmental switch in neurotrophin release induces myelination (Chan et al., 2001). Recently, an investigation of NT-3 expression and its effect on PSCs indicated that overexpression of NT-3 results in an increase in the number of PSCs at NMJs (Hess et al., 2007). In adults, however, TrkC expression is reduced, suggesting a primarily developmental role for NT-3-TrkC signalling in PSCs. Other more long-term roles for neurotrophins may also exist as it is known that disruption of TrkB signalling results in disassembly of postsynaptic receptor clusters (Gonzalez et al., 1999). This postsynaptic stabilizing influence of TrkB may be carried out by other neurotrophins or other factors acting at nerve terminals and PSCs.

One such molecule, which is important for NMJ development, and negatively affects PSC stability, is neuregulin. Neuregulin expression can be regulated by BDNF, GDNF, and NT-3 (Loeb et al., 2002). Neuregulin is a potent factor for NMJ development and PSC survival during development (Trachtenberg and Thompson, 1996). It also known that following denervation PSCs send processes to neighbouring junctions that guide reinnervation (Son and Thompson, 1995b, 1995a). The elaboration of PSC sprouts is partially mimicked by induction of neuregulin signalling in PSCs (Hayworth et al., 2006), indicating its importance in a variety of processes.

The studies mentioned here demonstrate the complexity of interactions between axons, muscle fibres and PSCs. A number of different factors are implicated in any given process. However, the neurotrophins alone appear to have the potential for both direct and indirect actions on PSCs in development and pathology.

One of the last major groups of molecules that I will discuss in the context of NMJ and PSC function are the purines. They are important for

both modulation of synaptic activity and neuron-glial interactions and are discussed in the review to follow.

In the following pages readers will find the review article:

Todd KJ, Robitaille R (2006) Purinergic modulation of synaptic signalling at the neuromuscular junction. Pflugers Arch 452:608-614.

1.4.4.3. Review Article

Purinergic Signalling at the Neuromuscular Junction

Keith J. Todd and Richard Robitaille
Département de physiologie
and
Centre de recherche en sciences neurologiques
Université de Montréal
Montréal, QC
Canada, H7G 1T7

Address correspondence to: Richard Robitaille
Département de physiologie
Université de Montréal
P.O. box 6128, Station Centre-Ville
Montréal, Québec
Canada, H3C 3J7
e-mail [redacted]

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INTRODUCTION

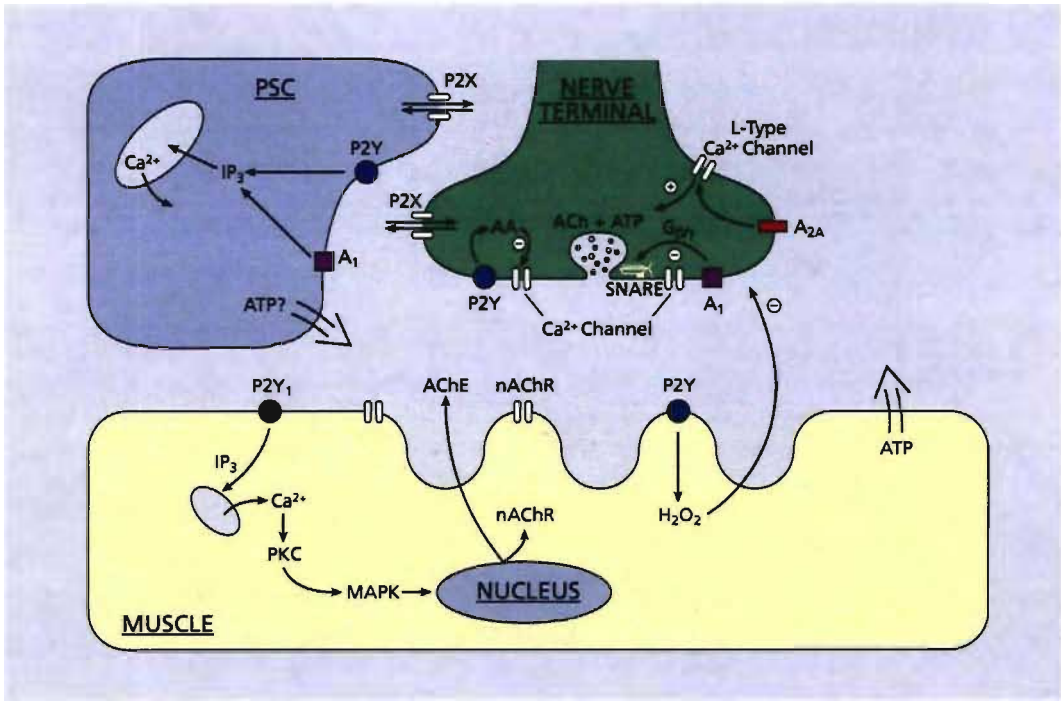
Adenosine triphosphate (ATP) was found to be co-released with acetylcholine (ACh) at neuromuscular junctions (NMJs) over 30 years ago (Silinsky, 1975). Interestingly, purines can also be released from muscle fibres (Smith, 1991; Santos et al., 2003) and Schwann cells (Liu et al., 2005). Since then, the functions of purines at NMJs have been examined in a number of different preparations, revealing diverse functions. A number of different receptors are present on all three elements of the NMJ: the presynaptic nerve terminal, postsynaptic muscle and perisynaptic Schwann cells (PSCs). These receptors include P1 adenosine receptors on presynaptic terminals (Correia-de-Sa et al., 1996; Silinsky, 2004; Baxter et al., 2005) and PSCs (Robitaille, 1995; Rochon et al., 2001) and P2 ATP receptors on PSCs (Robitaille, 1995; Rochon et al., 2001), nerve terminals (Grishin et al., 2005; Moores et al., 2005) and muscle fibres (Collet et al., 2002; Choi et al., 2003; Santos et al., 2003). Purinergic function in the peripheral nervous system and at the NMJ indicates roles during development (Fu and Poo, 1991), at mature NMJs (Redman and Silinsky, 1994; Correia-de-Sa et al., 1996), in PNS myelination (Stevens and Fields, 2000) and neuron-glial interactions (Robitaille, 1995; Rochon et al., 2001). This review will discuss the role of purines in modulation of NMJ function giving special focus to the involvement of purines in neuron-glia interactions. Some elements of this review have been published as part of a Novartis Foundation Symposium (Todd and Robitaille, 2006).

PURINE RECEPTORS AND PRESYNAPTIC MODULATION

Purinergic modulation of NMJ function can occur in a bidirectional manner, as either potentiation or depression of transmitter release (Correia-de-Sa et al., 1996). The outcome of the

purinergic modulation depends on a number of factors. For instance, the function of purinergic signalling changes with the developmental stage of the NMJ, where, during development of the tadpole NMJ, purines seem to potentiate neurotransmitter release (Fu and Poo, 1991) while in the adult frog the effect is opposite (Giniatullin and Sokolova, 1998). Dual effects of purines have also been reported at adult NMJs. For instance, using a mammalian preparation, Correia-de-sá and colleagues (Correia-de-Sa et al., 1996) demonstrated purine-mediated potentiation and depression of transmitter release depending on the frequency and pattern of stimulation. In this study, activation of P1 adenosine receptors of the A_1 or A_{2A} subtypes were responsible for inducing the depressive and potentiating effects respectively. This model suggests that the A_1 receptor effect dominates during low-frequency activity, while higher levels of synaptic adenosine are required for activation of A_{2A} receptors. These higher levels of adenosine are achieved when short-duration bursts of activity that cause the release of larger amounts of ATP that is then converted into adenosine by ectonucleotidases (Zimmermann et al., 1998). However, it is suggested that during prolonged high-frequency activation, release of ATP leads to inhibition of ectonucleotidases such that the production of adenosine is reduced (Cunha, 2001). Correia-de- sá and colleagues suggested that during high-frequency bursts of nerve activity the accumulation of synaptic adenosine is greater and activation of adenosine receptors shifts such that transmitter release is potentiated through activation of A_{2A} receptors (Correia-de-Sa et al., 1996; Oliveira et al., 2004). These phenomena are thought to function to preserve transmitter during times of prolonged release and to potentiate release during times where run-down has already occurred so as to ensure muscle activation.

Other mechanisms of modulation can occur through direct activation of ATP receptors (Giniatullin and Sokolova, 1998; Giniatullin et al., 2005; Grishin et al., 2005; Moores et al., 2005). These actions have also been shown to be potentiating or depressing depending on the receptors involved. When metabotropic P2 receptors are activated, they have an inhibitory effect similar to that of adenosine (Giniatullin and Sokolova, 1998). However, Morres et al. (Moores et al., 2005) found evidence for presynaptic ionotropic $P2X_7$ receptors that potentiate transmitter release due to an increase in non-selective cation conductance. Therefore, it appears that multiple types of purinergic receptors may have similar actions and can operate in parallel to modulate transmitter release at the NMJ (Fig. 1).



Review Figure 1. Schematic representation of the purinergic modulation of synaptic functions at the NMJ.

Diagram depicting the presynaptic nerve terminal (green), perisynaptic Schwann cell (PSC, blue) and postsynaptic muscle fibre (yellow). Sources of purines include presynaptic co-release with ACh, release from muscle fibres and possibly from PSCs. A first site of purines modulation is the presynaptic terminal where they regulate transmitter release. Both A_1 and P2Y receptors decrease transmitter release where P2Y receptors are thought to inhibit calcium channels through production of arachidonic acid (AA) (Grishin et al., 2005). In the mouse A_1 receptors were shown to cause inhibition of calcium channels through interaction of $G_{\beta/\gamma}$ with the SNARE complex protein, syntaxin, which also interacts with the calcium channels (Silinsky, 2005). Transmitter release can also be up-regulated through activation of P2X and A_{2A} receptors. P2X, ionotropic receptors increase non-specific cation conductance leading to Ca^{2+} entry while A_{2A} receptors are involved in activation of L-type calcium channels (Oliveira et al., 2004). Further inhibitory action can be derived from production of reactive oxygen species such as H_2O_2. The location for production of reactive oxygen species is not isolated to any specific compartments, however, muscle fibres are a likely source. Their production is linked to activation of P2Y receptors (Giniatullin et al., 2005). Other short-term modulation of the NMJ comes from purine-mediated signalling with PSCs. Purines are at least partially involved in feedback modulation of transmitter release by PSCs (Robitaille, 1998; Castonguay and Robitaille, 2001). Purines are also involved in long-term regulation of the NMJ, in part

through P2Y1-mediated activation of transcription of nicotinic ACh receptor (nAChR) subunits and acetylcholinesterase (AChE) (Choi et al., 2001; Choi et al., 2003).

MECHANISMS OF PRESYNAPTIC MODULATION

The mechanisms of purinergic modulation vary depending on the receptors involved and, to some extent, on the species being investigated. Purinergic depression of transmitter release in amphibians seems to modulate the mechanisms of transmitter release without altering presynaptic calcium (Redman and Silinsky, 1994; Robitaille et al., 1999; Huang et al., 2002). However, recently, Grishin and colleagues (Grishin et al., 2005) demonstrated a decrease in presynaptic calcium currents at the frog NMJ mediated by P2Y activation. This effect was mimicked with the addition of arachidonic acid (AA), suggesting its involvement as a second messenger. It is unclear why the difference in results found in this new study occurred. However, Grishin investigated P2Y receptors while Robitaille (Robitaille et al., 1999) investigated actions of adenosine as did Huang et al. (Huang et al., 2002) and Redman and Silinsky (Redman and Silinsky, 1994). Interestingly, it has been observed that presynaptic calcium currents may be altered by purinergic signalling in other NMJs. This type of modulation has been well established at mammalian NMJ (Hamilton and Smith, 1991; Silinsky, 2004) where modulation of calcium currents is thought to occur through hydrolysis of ATP to adenosine, which then activates adenosine receptors (Hamilton and Smith, 1991). Once activated, adenosine A1 receptors seem to inhibit P/Q-type calcium channels (Fig. 1) that, in mammals, are responsible to calcium entry during transmitter release (Silinsky, 2004). Interestingly, the inhibition of calcium currents is not the only site of purinergic modulation. Recently, Silinsky demonstrated that disruption of the

presynaptic release machinery, specifically the SNARE complex, decreased the effect of adenosine signalling on calcium currents (Silinsky, 2005). This occurs through interactions with the synaptic protein syntaxin, which has binding domains for P/Q calcium channels and the $G_{\beta/\gamma}$ subunit. The $G_{\beta/\gamma}$ interaction with syntaxin decreases P/Q channel activity and ultimately inhibits transmitter release (Fig.1)(Silinsky, 2005). These results are a clear demonstration of G-protein modulation of presynaptic release machinery. It would be interesting to know whether similar interactions between purinergic receptors and presynaptic machinery also exist in amphibian NMJs.

Another effect of purinergic signalling on calcium channels was recently described where this group investigated a potentiating mechanism mediated by A_{2A} receptors (Oliveira et al., 2004). In this model, during high-frequency continuous nerve activity, the inhibitory A_1 effects were dominant. However, during high-frequency bursts of activity, levels of ACh release are increased through activation of A_{2A} receptors, which leads to recruitment of L-type calcium channels (Fig. 1). They suggested that this modulation may be an endogenous mechanism for ensuring release during repetitive burst activity.

The purinergic modulation described above implies a direct regulation mediated by presynaptic auto-receptors for ATP and adenosine. However, there is evidence that ATP may act also via a diffusible retrograde messenger. Indeed, Giniatullin and colleagues demonstrated the involvement of reactive oxygen species (ROS) in purine-mediated inhibition. The production of ROS, such as H_2O_2, seemed to be dependent on ATP signalling, but not adenosine (Fig. 1). Fluorescent staining for ROS with carboxy-2',7'-dichlorodihydrofluorescein revealed ATP-mediated production of

ROS in muscle fibres, however, general staining was observed in other synaptic components. The authors suggested that PSCs could be involved in production of ROS although it was not tested directly. Regardless of the source, production of ROS results in feedback modulation of the NMJ such that ACh release is decreased.

Purinergic signalling can alter calcium currents, calcium-independent processes and recruit other signalling cascades. Overall, this leads to a fine balance of NMJ signalling such that sufficient presynaptic release can be maintained at all times. The exact mechanisms for many of these processes have yet to be resolved; however, it appears that redundant mechanisms exist for both potentiation and inhibition of release.

PURINERGIC SIGNALLING TO SCHWANN CELLS

Purines appear to be important factors for signalling to glia in both the CNS and PNS. We will only discuss purine signalling to PNS glia (Schwann cells) in this review and the reader is referred to Haydon and colleagues (this issue) for a review of the role of purines in synapse-glia interactions in the CNS. In the PNS there are three types of Schwann cells: the myelinating and non-myelinating Schwann cells associated with axons and the perisynaptic Schwann cells (PSCs) located at NMJs. PSCs perform similar functions to CNS astrocytes, where they participate in synapse-glia signalling, modulate synaptic function and are in all senses of the term active synaptic partners (Auld and Robitaille, 2003a). Purinergic signalling to Schwann cells has been investigated in culture and in situ revealing roles for purine signalling in both axonal Schwann cells and PSCs.

Myelinating Schwann cells have been shown to express both ionotropic (Grafe et al., 1999; Colomar and Amedee, 2001) and

metabotropic (Lyons et al., 1994; Ansselin et al., 1997) ATP receptors. Expression of these receptors appears to be dependent on neuronal interaction (Lyons et al., 1995) demonstrating the synergistic relationship between neurons and glia in roles such as structural support, maintenance and stability, differentiation and development. Purinergic signalling between neurons and Schwann cells was also shown to be important for regulating the developmental progression of myelination (Stevens and Fields, 2000; Stevens et al., 2004). These data demonstrated that signalling through P2 receptors could arrest developing Schwann cells in a pre-myelinating state through activation of mitogen-activated protein kinase (MAPK) (Stevens and Fields, 2000). Following this, it was shown that ATP and adenosine could have different effects on Schwann cell development (Stevens et al., 2004). Activation of A_{2A} receptors resulted in the inhibition of Schwann cell proliferation through activation of the extracellular signal-regulated kinase (ERK)/MAPK pathway. It appears that this cascade is regulated separately from the P2 initiated cascade, which leads to inhibition of differentiation rather than proliferation (Stevens et al., 2004). These results demonstrate the complex nature of purinergic signalling where different outcomes can be mediated through similar cascades. Additionally, it indicates a role of purinergic signalling in long-term regulation of Schwann cells. Interestingly, Schwann cells were also shown to release ATP through mechanisms requiring activation of $P2Y_2$ receptors (Liu et al., 2005). Release of ATP from Schwann cells is suggested to provide excitatory feedback to axons, however, this has yet to be confirmed in situ. These studies indicate the importance of purinergic signalling for Schwann cell function and proper myelination during development.

Purinergic signalling for PSCs has also revealed multiple receptor pathways and signalling cascades (Robitaille, 1995; Rochon et al., 2001). Purinergic signalling to PSCs was first described in 1992 and was observed in acutely isolated muscles using calcium imaging (Jahromi et al., 1992). Since the initial description of ATP signalling to PSCs, information has been provided regarding similar signalling pathways in amphibian (Robitaille, 1995) and mouse PSCs (Rochon et al., 2001). Importantly, it has been shown that multiple receptors are expressed at amphibian and mammalian NMJs, leading to the possibility of complex regulation of PSC function and neuron-glia interactions.

At the frog NMJ, PSCs express both ATP and adenosine receptors. The expression of ATP receptors appears to be heterogeneous with expression of both P2X and P2Y receptors (Robitaille, 1995). The presence of both ionotropic and metabotropic receptors likely allows for differential signalling through calcium entry. Calcium responses to purines are efficacious with almost 100% of PSCs responding to exogenous local application. This is also true for the mammalian NMJ (Rochon et al., 2001). In the mouse, calcium rises can also be elicited following application of ATP or adenosine. Here also, it appears that both P2X and P2Y receptors are present. The exact sub-type of these receptors have not been clearly identified although the pharmacology suggests that they might be of the P2X1 or P2X5 group while the P2Y receptors are probably of group 1. Activation of P2X receptors trigger the entry of Ca^{2+} from the external milieu while P2Y receptors elicit intracellular rises in calcium, most likely through IP_3-mediated stores. This is supported by recent work in our laboratory at the mouse NMJ. This was shown through inhibition of ATP signalling in the presence of the PLC inhibitor U73122, a step upstream of IP_3

production (Todd and Robitaille, unpublished results). Regarding the adenosine receptors, they appear to be of the A_1 type while there is no evidence for the presence of A_{2A} receptors (Robitaille, 1995). Although these results confirm the presence and action of these receptors, they do not address the involvement of purines in PSC signalling during evoked activity.

At the frog NMJ, Robitaille determined the contribution of purines to PSC calcium responses (Robitaille, 1995). This was done through nerve stimulation in the presence and absence of the general P2 receptor antagonist suramin. In the presence of suramin, PSC calcium responses were reduced by about 50 per cent. This suggests that, at this synapse, purines provide about half of the stimulus for the observed response. The other part of the response is due to activation of muscarinic receptors that are also expressed on the PSCs (Robitaille et al., 1997). This suggests parallel pathways for PSC activation that may have different roles in short- or long-term signalling. In his study, Robitaille did not find any evidence for a direct activation of PSCs by adenosine during sustained synaptic activity. The contribution of purines to PSC responses observed during nerve activity at the mouse NMJ is not as clear. Also, it seems that the purinergic activation may not be driven by P2 receptors but rather by adenosine P1 receptors (Rochon et al. 2001). At the mouse NMJ, however, muscarinic signalling to PSCs also appears to be important for activation of PSCs.

The presence of purinergic and cholinergic signalling systems on PSCs suggests the ability to differentially modulate PSC function. Although the contribution of each receptor family to PSC responses during nerve activity has not been investigated in the mouse, it is thought to be similar to the amphibian PSCs.

Interestingly, purinergic and cholinergic signalling systems have been shown to interact at the mammalian NMJ nerve terminal (Oliveira et al., 2002) where adenosine, acting through A_1 and A_{2A} receptors, can decrease presynaptic M1 and M2 receptor activity. This has been addressed in both the frog and mouse NMJs, but no evidence for interaction between purinergic and muscarinic systems has been observed in PSCs (Rochon et al., 2001). Although these two pathways do not seem to interact, other mechanisms for modulation of PSC signalling do exits.

MODULATION OF PSC SIGNALLING

Since purinergic signalling is clearly the most efficacious calcium-dependent pathway on PSCs, it stands to reason that purinergic modulatory pathways would also exist. In particular, modulation by peptides and diffusible transmitters appears important (Bourque and Robitaille, 1998; Thomas and Robitaille, 2001).

Exogenous application of substance P at the frog NMJ induced calcium rises in glial cells (Bourque and Robitaille, 1998). Induction of PSC calcium responses by SP occurred through activation of NK-1 receptors. Interestingly, this signalling appears to be involved in modulation of PSC responses during nerve activity. During repetitive evoked activity, PSC responses are gradually reduced. However, when Bourque and Robitaille blocked NK-1 activation, the rundown of PSC responses was prevented (Bourque and Robitaille, 1998). This reveals a peptidergic modulation of PSC signalling. This endogenous modulation was in part due to SP effects on the purinergic signalling as suggested by the observation that PSC responses to exogenously applied ATP were reduced by half in the presence of bath applied SP. This is one pathway

regulating purinergic signalling in PSCs and ultimately of neuron-glial communication.

Another pathway involved in modulating purinergic signalling to PSCs is the production of nitric oxide (NO). Synthesis of NO was observed in both PSCs and muscle fibres at the frog NMJ (Descarries et al., 1998). This was determined through localization of NO synthase to these compartments. Production of NO occurs both tonically, providing a basal level of NO, and in response to activity where it is involved in mediating synaptic depression at the NMJ (Thomas and Robitaille, 2001). Interestingly, during high levels of nerve activity NO may signal to PSCs, resulting in a reduction of purinergic calcium responses as suggested by the observation that exogenous application of NO donors resulted in a reduction of ATP-induced calcium responses (Descarries et al., 1998).

Another important effect of NO at the NMJ is its ability to modulate adenosine-mediated depression of synaptic release since NO chelation prior to adenosine-mediated depression, attenuated the adenosine effect (Thomas and Robitaille, 2001). The role of NO in modulation of NMJ activity could occur either directly or indirectly. Indeed, it is possible that NO modulates presynaptic activity through interactions with presynaptic adenosine receptors. However, it is also possible that these effects are occurring indirectly through modulation of PSC signalling that in turn differentially signal to the presynaptic terminal.

Changes in purinergic signalling could also be altered during development or denervation (Son and Thompson, 1995a; Robitaille et al., 1997). It is possible that purinergic signalling could alter expression of a number of genes in PSCs as seen in myelinating Schwann cells (Stevens and Fields, 2000). Additionally, similar long-term roles for purines have been described in muscle

fibres where purinergic signalling is involved in regulating acetylcholinesterase and acetylcholine receptor expression (Fig. 1) (Choi et al., 2003; Tung et al., 2004). However, purines were found to have no effect on the inhibition of the gene expression and protein synthesis of glial fibrillary acidic protein (GFAP) in PSCs (Georgiou et al., 1994). Indeed, this inhibition is due to cholinergic signalling to PSCs via muscarinic receptors (Georgiou et al., 1999). The expression of cytoskeletal components such as GFAP is required for the elaboration of new PSC processes that are formed during synaptic weakening or denervation. Therefore, decreased cholinergic signalling to PSCs should facilitate this process.

Furthermore, the close interaction between the three NMJ elements is important for signalling the overall health and state of the NMJ. In the event of perturbation such as denervation, PSC receptor expression changes indicating the sensitivity of PSCs to NMJ health (Robitaille et al., 1997). Interestingly, changes in receptor expression at PSCs of the frog NMJ were observed in the cholinergic pathway (Robitaille et al., 1997), but there is no evidence for changes in purinergic signalling to PSCs (R. Robitaille unpublished observation). This could provide some mechanism by which signalling at NMJs can be maintained to facilitate re-innervation. Indeed, the maintenance of purinergic signalling on PSCs makes intuitive sense. ATP released from muscle fibres and PSCs (Smith, 1991; Santos et al., 2003) may allow for the maintenance of communication through purinergic signalling between PSCs and muscles of the NMJ during denervation. This may contribute to the proper environment for re-innervation by presynaptic terminals.

Purines have multiple pathways on PSCs to induce diverse signals. To date it is known that these pathways are

modulated by both NO and SP. Although only short-term roles in NMJ function have been described for purines, it remains possible that they mediate changes in gene expression that have not been discovered. Additionally, it is hypothesized that purines are crucial for maintenance of signalling between denervated muscle and PSCs that may in part facilitate re-innervation.

PURINE-MEDIATED SYNAPSE-GLIA INTERACTIONS

To this point, solely the mechanisms for purinergic signalling and modulation at the NMJ have been discussed. An additional aspect of purinergic signalling, however, is their involvement in synaptic function, in particular their role in PSC-mediated synaptic modulation. This information is derived from experiments employing specific perturbation of PSC function.

The involvement of purine receptors was investigated through the manipulation of the various G-proteins recruited by cholinergic and purinergic signalling (Robitaille, 1995; Robitaille et al., 1997). These data indicate that muscarinic receptors on PSCs signal through G-proteins that are insensitive to pertussis toxin (PTX), likely of the $G\alpha_{i/o}$ type (Robitaille et al., 1997). On the other hand, purinergic receptors on PSCs are probably linked to $G\alpha_q$ type G-proteins, which are sensitive to PTX (Robitaille, 1995). The downstream signalling cascades recruited would involve purine-mediated production of IP_3, while muscarinic signalling probably regulates levels of cAMP. The involvement of these two pathways was investigated in terms of PSC-mediated synaptic depression. Robitaille (Robitaille, 1998) found that both PTX-sensitive and insensitive G-proteins were involved in PSC-mediated modulation of synaptic transmission. Further work on this topic has revealed a specific role for IP_3 production in PSCs (Castonguay and Robitaille,

2001). These results revealed that injection of IP_3 into PSCs potentiated synaptic transmission. These observations suggest the involvement of PSC purinergic signalling, based on the inferred pathway recruited by these receptors.

Importantly, the combined results, where PSCs were specifically perturbed, provide evidence for bidirectional modulation of synaptic transmission by PSCs. As shown by Robitaille (Robitaille, 1998), PSCs can decrease high-frequency synaptic transmission. This seems to occur partially through muscarinic signalling since a PTX-insensitive pathway is involved. The follow-up study indicates PSC-mediated potentiation of transmitter release through the IP_3 pathway (Castonguay and Robitaille, 2001). PSCs, therefore, can dynamically respond to the ongoing changes in NMJ activity and respond appropriately with reciprocal signals that alter transmitter release. The existence of different signalling cascades recruited to mediate facilitation and depression is consistent with the synaptic properties of the NMJ since synaptic depression and potentiation both occur at this synapse and can be recruited at different times. Furthermore, this probably allows PSCs to adapt to the synaptic environment by favouring either depression or potentiation according to the state and health of the synapse.

CONCLUSION

Purinergic signalling at the NMJ has definite roles in synaptic modulation. In developmental models, ATP appears to potentiate synaptic transmission (Fu and Poo, 1991), and at mature synapses both potentiation (Correia-de-Sa et al., 1996) and depression (Giniatullin and Sokolova, 1998) have been described, the decisive factor being the type of receptor activated. Furthermore, the modulatory effects of purines can alter both quantal and non-

quantal release (Galkin et al., 2001). Not only does the release of purines provide auto-feedback to the presynaptic terminal, they also maintain communication with PSCs by inducing Ca^{2+} elevation in these cells (Jahromi et al., 1992; Robitaille, 1995). Purines also signal to muscle fibres through P2 receptors to induce short-term modulation (Giniatullin et al., 2005) and long-term changes (Choi et al., 2001; Tung et al., 2004). Activation of purine receptors can have bidirectional effects on synaptic release and are quite important for neuron-glial communication and synaptic modulation. Finally, these factors could play a pivotal role during development and following injury to maintain signalling between muscle and glial components.

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1.4.4.4. Conclusions and Perspectives

Glial cells at NMJs perform similar functions to astrocytes of the CNS. They respond to synaptic activity, provide modulatory feedback to synapses and are involved in long-term regulation of synaptic stability. These functions arrive through signalling via purines, ACh, neurotrophins and other yet unknown factors. Further study into the functions of PSCs in synaptogenesis at the NMJ as well as in adult synaptic function should provide insight into broader functions of glial cells throughout the nervous system.

1.5. NEUROTROPHINS

The neurotrophins are a family of molecules largely known for their role in neuronal survival. Since their discovery, they have been implicated in many processes throughout the nervous system aside from survival, such as development and plasticity. The discovery of neurotrophins began with nerve growth factor (NGF), discovered by Levi-Montalcini, and later purified by Cohen (Levi-Montalcini and Hamburger, 1951; Cohen, 1960). Since this discovery, the family of neurotrophins has grown to include three other members (BDNF, NT-3 and NT-4) and four receptor proteins (Pitts et al., 2006). Receptor binding and subsequent signal cascades vary widely.

Neurotrophins are important for a variety of functions including NMJ development, synaptic plasticity and long-term NMJ stability. It was also recently demonstrated that neurotrophins could have rapid signalling effects on glial cells in the cerebellum (Rose et al., 2003). It is known that Schwann cells of the peripheral nervous system and at the NMJ express a variety of neurotrophin receptors (Pitts et al., 2006) suggesting a potential role for these molecules in neuron-glial interactions in the PNS.

1.5.1. NEUROTROPHIN PROTEINS

Neurotrophins exist as homodimers of two polypeptides held together by non-covalent bonds. The dimeric interaction is quite stable and is supported by three disulfide bridges. This form was determined by the crystal structure of NGF (McDonald et al., 1991).

The neurotrophins are synthesized in a precursor, immature form and are later cleaved by convertases to produce the mature forms (Roux and Barker, 2002). Originally, the precursor form was thought to be inactive, however, evidence now exists that pro forms of neurotrophins can be involved in cell signalling via p75 receptors (Woo et al., 2005).

In the central nervous system neurotrophins are synthesized in both neurons and glia. In the peripheral nervous system this is also true (Bandtlow et al., 1987; Acheson et al., 1991), suggesting that perisynaptic Schwann cells may release neurotrophins at the neuromuscular junction. This has in fact been suggested through *in vitro* methods (Alderson et al., 2000).

1.5.2. Regulation of Expression

1.5.2.1. Activity-Dependence of Expression

Expression of both NT-3 and NT-4 can occur in an activity-dependent manner at the neuromuscular junction (Funakoshi et al., 1995; Xie et al., 1997). In rat skeletal muscle, NT-4 expression is dependent on AChR activation while no change in NT-3 or BDNF was observed (Funakoshi et al., 1995). Interestingly, only NT-3 mRNA was reported to change in an activity-dependent manner in Xenopus nerve-muscle cocultures (Xie et al., 1997). These differences may simply reflect species differences or perhaps culture artefact.

1.5.2.2. Expression During Development and Injury

Expression of the neurotrophins changes over the course of development. In the rat, muscle fibre expression of NT-3 and NT-4 is highest at the embryonic and neonatal stages and decreases into adulthood (Griesbeck et al., 1995; Hess et al., 2007). BDNF, on the other hand, is expressed at relatively low levels at embryonic stages and not at all after birth (Griesbeck et al., 1995).

This pattern of expression is not recapitulated following nerve injury, where NT-3 remains low, NT-4 decreases and BDNF increases (Funakoshi et al., 1993; Griesbeck et al., 1995; Hess et al., 2007). The increase in BDNF appears to occur in Schwann cells rather than muscle (Griesbeck et

al., 1995). The broad expression of neurotrophins throughout the nervous system suggests broad functions. At the neuromuscular junction, this expression seems to be important for development and repair following injury.

1.5.3. Neurotrophin Release

The release of neurotrophins remains somewhat of a mystery. However, recent progress has revealed some of the mechanism and proteins involved.

1.5.3.1. The Release Process

There has been much debate over how neurotrophins may be released and many questions remain unanswered. However, to date it has been suggested that NT-4 (Hibbert et al., 2003), NT-3 and NGF are released constitutively (Mowla et al., 1999; Farhadi et al., 2000) or by regulated secretion (Blochl and Thoenen, 1996; Wu et al., 2004). It does seem, however, that BDNF is primarily released by regulated processes (Haubensak et al., 1998). In response to this controversy the four neurotrophins were labelled with GFP and monitored in hippocampal cultures (Brigadski et al., 2005). They found that while BDNF and NT-3 were primarily associated with release granules while less than half of the cells had NGF or NT-4 containing granules. However, these cells without NGF or NT-4 granules were still capable of undergoing constitutive release (Brigadski et al., 2005). It, therefore, seems that all of the neurotrophins are released through a combination of regulated and constitutive mechanisms. However, the relative proportion of each mechanism used appears to be dependent on the neurotrophin in question and likely the cells involved.

1.5.3.2. Mechanisms and Effectors of Release

Although it is now better understood that both activity-dependent and constitutive release can both be responsible for neurotrophin release, the mechanisms and release properties are only beginning to be understood. Through the use of GFP tagged neurotrophins it has been possible to dynamically monitor release (Haubensak et al., 1998; Hartmann et al., 2001; Brigadski et al., 2005). This regulated release appears to be quite slow, about 10 times, in comparison to conventional, SNARE-dependent synaptic release (Brigadski et al., 2005). Also through the use of GFP-tagged neurotrophins, and other methods, it was determined that regulated release of neurotrophins is calcium-dependent (Canossa et al., 2001; Balkowiec and Katz, 2002; Wang et al., 2002b). This dependency seems to work through a PLC-IP_3 pathway that ultimately results in fusion of neurotrophin containing granules. Recently a potential calcium sensor protein was reported to be associated with neurotrophin containing granules in the cerebellum (Sadakata et al., 2004). Activity of the Calcium-Activated Protein for Secretion (CAPS) was correlated with increased neurotrophin release in this study. CAPS was first described in 1992 in adrenal cells and was associated with dense core vesicles (Walent et al., 1992). It is intimately linked to cell membrane, however, it seems to be associated only with dense core vesicles as it is not reported to link to small, clear synaptic vesicles (Berwin et al., 1998). This suggests that CAPS is related to secretion of peptides, but not neurotransmitters such as glutamate. This, in fact, seems to hold true for Drosophila neuromuscular synapses, which are glutamatergic, but also release peptide transmitters from dense core vesicles (Renden et al., 2001).

Regulated release of neurotrophins, therefore, seems to occur through liberation of dense core vesicles and may rely on CAPS to sense the calcium trigger. These advances in our understanding of neurotrophin

release will help to further elucidate the conditions for release and subsequent actions of neurotrophins.

1.5.4. Neurotrophin Receptors

Neurotrophin receptors are most commonly thought to be the receptor tyrosine kinase or tropomyosin related kinase (Trk) type. However, all of the neurotrophins also bind to the p75 neurotrophin receptor that is part of the tumour necrosis factor receptor family. In fact it was the p75 receptor that was first reported to bind to NGF (Johnson et al., 1986).

The first Trk receptor to be described was actually TrkB a receptor that was found throughout the nervous system (Klein et al., 1989; Klein et al., 1990). However, its ligand was not discovered until 1991 (Squinto et al., 1991). TrkA was later described as the receptor for NGF (Kaplan et al., 1991) and then finally TrkC as the NT-3 receptor (Lamballe et al., 1991). Trk receptors are transmembrane proteins with particular extracellular domains that contain an immunoglobulin-like region important for ligand binding (Fig. 4). Above the Ig region are two cysteine-rich domains on either side of a series of three leucine-rich domains. Although the greatest contribution to ligand binding comes from the Ig domain, both the cysteine and leucine regions can affect binding (MacDonald and Meakin, 1996; Arevalo et al., 2001). Further modulation of ligand binding comes from heteromeric interactions between p75 and Trk receptors. It appears that direct binding by neurotrophins to p75 receptors is not necessary and p75 has more of a conformational effect on the Trk receptors (Esposito et al., 2001). However, crystallography shows that direct interactions between p75 and neurotrophins can occur (He and Garcia, 2004; Gong et al., 2008). Furthermore, p75 interactions with Trk receptors leads to increased ligand specificity for example only NGF binding to TrkA rather than NT-3 and NGF (Mischel et al., 2001).

Trk receptors have a number of different splice variants. Truncated receptor forms lack intracellular tyrosine kinase domains and seem to lack binding domains. However, these receptors can function in a dominant negative manner to block neurotrophin signalling (Gonzalez et al., 1999) and may be involved in uptake of released neurotrophins (Alderson et al., 2000). There have also been some reports of direct functions for truncated receptors themselves (Yacoubian and Lo, 2000; Rose et al., 2003), although they may also be functional through interactions with other proteins (Kryl and Barker, 2000).

Trk receptors form dimers in response to ligand binding, and subsequently autophosphorylate, which enhances their kinase activity. Phosphorylation also allows for interaction with down-stream signalling molecules.

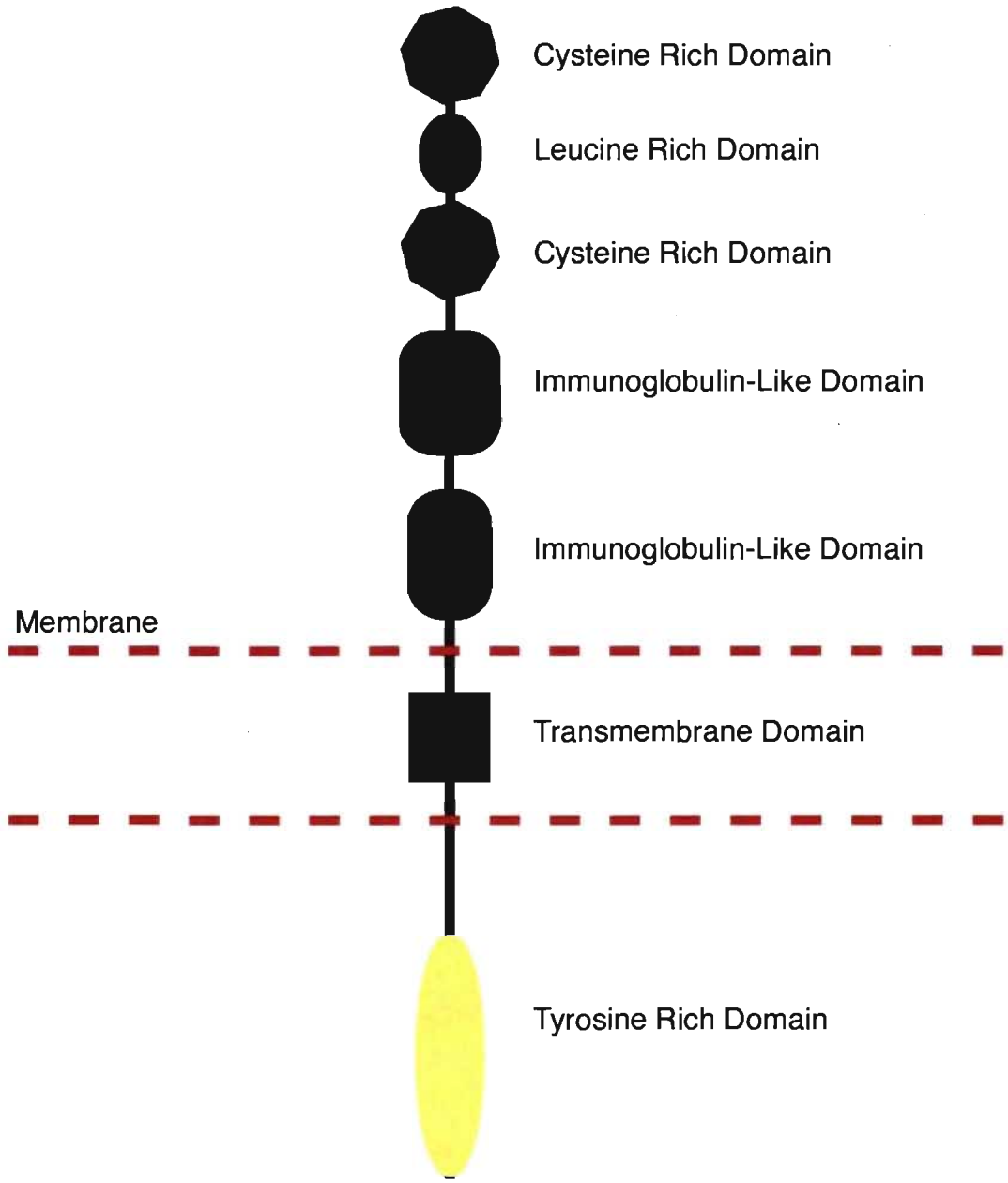

Figure 4. Trk Receptor Domains.

Trk receptors are all similar with 5 extracellular domains, 2 cysteine rich domains flanking a leucine rich region and two immunoglobulin-like domains important for ligand binding. Intracellularly is found the tyrosine rich domain. When receptors dimerize they autophosphorylate this region, which increases their own phosphorylating activity.

1.5.5. Neurotrophin-Mediated Signalling

Trk receptors interact with a plethora of intracellular signalling molecules. These molecules lead to down-stream modulation of gene expression, ion channel function and cellular processes such as calcium dynamics (Huang and Reichardt, 2003). Many of these receptor-effector interactions are modulated by phosphorylation of the Trk receptors following ligand binding. However, other signalling cascades can also require internalization and retrograde transport of the receptors to the nucleus.

1.5.5.1. Functions of PLC-IP_3

One of the major signalling cascades initiated by Trk activation is the phospholipase C – inositol triphosphate (PLC-IP_3) pathway. PLC, which cleaves phosphatidylinositol (4, 5)-bisphosphate (PIP_2) to form IP3 and diacylglycerol (DAG), is activated by tyrosine phosphorylation of Trk receptors at Y785. This cascade results in liberation of intracellular calcium and can affect cellular processes like transmitter release (He et al., 2000; Kleiman et al., 2000).

In addition to acute effects, long-term changes can be mediated by neurotrophins working through the PLC pathway. In some cases other downstream effector proteins can be activated by PLC activity. These can include kinases like PKCδ, which can increase neurite outgrowth (Corbit et al., 1999). Other long-lasting effects include modulation of transcription. One example of this is PLC-dependent expression of sodium channels following NGF activation of TrkA (Toledo-Aral et al., 1995). Another property of PLC-IP_3 signalling by neurotrophins comes from mutational studies, which looked at the loss of TrkB-mediated PLC activation in vivo. In this study LTP was greatly affected suggesting a strong link between Trk-PLC signalling and learning and memory (Minichiello et al., 2002).

Combined, these studies demonstrate diverse functions for PLC-IP_3 in neurotrophin modulation of cellular processes over both short and long periods of time.

1.5.5.2. PI3-Kinase Signalling and Its Functions

PI3-kinase pathways are one of the most well known cascades employed by neurotrophins since they are associated with cell survival. Activation of PI3-kinase is a multi-step process rather than direct activation as is the case for PLC. Here, activated Trks can lead to Ras activation, which then directly results in PI3-kinase activation (Vaillant et al., 1999). An alternative pathway is through Trk receptor activation of Shc-Grb leading to recruitment of Gab, which once phosphorylated, induce PI3-kinase activity (Holgado-Madruga et al., 1997). Once PI3-kinase is activated, it recruits Akt (PKB), which then leads to phosphorylation of a variety of cell survival and cell death proteins such as NF-kB and Bad respectively. Phosphorylation of Bad results in inhibition of its binding to Bcl-XL, a protein that prevents apoptosis by associating with Bax (Datta et al., 1997). Other proapoptotic pathways inhibited by PI3-kinase are GSK3β (Hetman et al., 2000) and FKHRL1 (Brunet et al., 2002). As well as inhibiting apoptotic pathways, PI3-kinase signalling can also directly result in survival signals through activation of NF-kB. Akt phosphorylates the inhibitory binding protein for NF-kB, IkB, and allows NF-kB to promote gene transcription leading to survival (Foehr et al., 2000; Wooten et al., 2001).

Aside from cell survival PI3-kinase signalling can regulate controlled cell motility (Wang et al., 2002a; Weiner et al., 2002). This may be in part through Akt activation of Cdc-42 and downstream Rac-Rho G-proteins (Yuan et al., 2003). Activation of other pathways such as Ras can also lead to steering of growth cones with neurotrophin gradients (Song et al., 1997; Ming et al., 2002). Finally, Akt may also influence processes involved in

neuronal maturation such as branching and regulation of axon diameter (Markus et al., 2002).

PI3-kinase signalling is a major pathway utilized by both Trk and p75 receptors. These cascades lead to diverse cellular processes and the likely remains many more yet unknown.

1.5.5.3. Results of MAP Kinase Signalling

Mitogen activated protein kinase (MAPK) activation occurs through a series of adapter proteins including Shc, Grb, SOS and finally Ras. Ras goes on to activate MAPK and extracellular signal-related kinase (Erk), a cascade involved in cell differentiation (Xing et al., 1998). Activation of Erk1, Erk2 and Erk5 by neurotrophins can lead to diverse cellular actions. The activation of different Erks seems to be regulated through different requirements for local signalling or transport of the receptors (Watson et al., 2001). The different Erks have different downstream transcription factor targets, which leads to diversity in cellular functions mediated by these proteins (Pearson et al., 2001). Recently, the actions of this pathway on neuron outgrowth and branching have been demonstrated in vivo (Zhong et al., 2007). The MAPK/Erk pathway mediated by neurotrophin signalling is involved largely in shaping developing neurons through modulation of transcription factors.

1.5.6. Neurotrophins at the Neuromuscular Junction

Neurotrophins have long been studied in the function and development of the neuromuscular system. This system has facilitated investigations of gross function and intracellular signalling mechanisms. Culture preparations like the Xenopus nerve-muscle co-culture have acted as an excellent system for investigating the actions of neurotrophins.

1.5.6.1. Neurotrophins in Neuromuscular Development

The family of neurotrophins can have profound effects on development of not only the neuromuscular system, but in many systems. For example, complete knockout of NT-3 results in almost immediate death at birth (Woolley et al., 2005). Those animals that survive for a few days show axon retraction, Schwann cell retraction and eventually complete loss of all NMJs. NT-3 has also been reported to improve morphological maturation in co-cultures (Wang et al., 1995).

In contrast, knockout of NT-4 is not lethal, and the loss of this neurotrophin can be examined on the neuromuscular system (Belluardo et al., 2001). Eventually, these knockout animals display a reduction in AChR clustering, which may be in part due to decreased expression of these receptors. It is likely that the effects of NT-4 loss are mediated by TrkB receptors since Rita Balice-Gordon's group reported similar destabilization of receptor clusters in mice expressing a truncated form of TrkB, that effectively inhibited normal TrkB activation (Gonzalez et al., 1999). Aside from receptor clustering, NT-4 may have roles in presynaptic (Funakoshi et al., 1995) and muscle fibre development (Carrasco and English, 2003). Presynaptically, it is suggested that NT-4 can induce axonal sprouting that may play a role in synapse elaboration. Similarly, BDNF has been reported to increase synaptic morphological maturation (Wang et al., 1995). At the level of the muscle fibre it appears that NT-4 induces maturation of the muscle phenotype. Intramuscular injections of NT-4 were shown to speed the developmental switch from fast to slow twitch fibres in rat soleus muscles (Carrasco and English, 2003).

At the NMJ there is conflicting evidence for NGF and TrkA expression and functions during development and maturation. This neurotrophin-receptor pair has sometimes suggested to not be present or unaltered during different experimental conditions (Wang et al., 1995; Sheard et al., 2002). Others have found evidence for only low levels of NGF

expression in muscle and motoneurons of rat (Heumann et al., 1984). In Schwann cells during early development there seems to be relatively high levels of NGF expression (Bandtlow et al., 1987; Heumann et al., 1987). However, no evidence of TrkA has been reported (Kaplan et al., 1991; Offenhauser et al., 1995). Although there is some limited evidence for NGF expression during development of the neuromuscular system, its importance seems to be narrow.

1.5.6.2. Neurotrophins and Neuromuscular Plasticity

Neurotrophin-induced plasticity was first described at the neuromuscular junction (Lohof et al., 1993). In this study, acute application of NT-3 or BDNF induced potentiation of transmitter release in cocultures. Since this report, other studies have confirmed this action as presynaptic (Liou and Fu, 1997; Liou et al., 1997; Xie et al., 1997) in location and mediated by a combination of depolarization (Boulanger and Poo, 1999) and release of presynaptic calcium from internal stores (He et al., 2000; Kleiman et al., 2000). There is also some evidence that neurotrophins work to increase the influx of extracellular calcium (Li et al., 1999; Kleiman et al., 2000).The neurotrophins seem function in a positive-feedback type of mechanism in which expression in muscle fibres is activity-dependent (Funakoshi et al., 1995; Xie et al., 1997) and this increased expression results in increased presynaptic release. Also, more long-term effects of neurotrophin exposure include increased expression of calcium channels (Baldelli et al., 1999; Nick and Ribera, 2000). This is suggested to result in functional maturation of developing synapses (Wang et al., 1995). The potentiating and maturing influences of NT-4 and BDNF are further supported by results from exogenous application, which resulted in a decrease in failure rate and decreased muscle fatigue (Mantilla et al., 2004).

The effects observed for neurotrophins at the developing NMJ are thought not to continue later in life. To date, no effect of neurotrophins on synaptic plasticity at adult NMJs have been published. This may be due to down-regulation of some of the neurotrophins and their receptors in adults. Certainly the NT-3 receptor TrkC is only observed at very low levels in adult mammals (Hess et al., 2007).

1.5.7. Neurotrophins and Glia

Glial cells of both the CNS and PNS are intimately tied to neurotrophins. They express and release various trophic factors (Bandtlow et al., 1987; Acheson et al., 1991; Cai et al., 1999), and express a variety of receptors (Frisen et al., 1993; Funakoshi et al., 1993; Wang et al., 1998; Climent et al., 2000; Hess et al., 2007) that result in a variety of cellular interactions during development and in mature structures (Roback et al., 1995; Rose et al., 2003; Chan et al., 2004; Yamauchi et al., 2004).

1.5.7.1. Neurotrophin-Mediated Signalling in Glia

As previously discussed, neurotrophins can mediate intracellular signalling through a variety of cascades. Initially, however, glial cells were believed to only passively interact with the neurotrophins through truncated receptors that would provide a mechanism for buffering extracellular levels (Klein et al., 1990; Frisen et al., 1993). Since the introduction of these hypotheses it has been demonstrated that neurotrophin signalling through full-length TrkB receptors can result in MAPK-Erk signalling in primary cultures (Roback et al., 1995). These receptors have also been reported to induce calcium signalling in cultured astrocytes when activated with BDNF (Climent et al., 2000). More recently Arthur Konnerth's group provided convincing evidence that BDNF signalling could induce calcium transients in glia in hippocampal and cerebellar slices (Rose et al., 2003). These results

suggest that neurotrophins could have more active signalling capabilities on glial cells.

1.5.7.2. Neurothophins and Myelination

Myelination is one of the major areas where neurotrophins are acitive in the PNS. In fact during early development, Schwann cell precursors are identified by their expression of p75 neurotrophin receptors (Mirsky et al., 2002) suggesting its involvement in precursor cell differentiation. Later, the neurotrophins regulate Schwann cell migration prior to myelination (Yamauchi et al., 2003, 2004; Yamauchi et al., 2005). This occurs through signalling pathways involving the Rho family of small GTPases. In fact NT-3 postively enhances Schwann cell migration while BDNF inhibits it (Yamauchi et al., 2004). Correspondingly, NT-3 negatively regulates myelination while BDNF promotes it through activation of the TrkC and p75 receptors respectively (Chan et al., 2001; Cosgaya et al., 2002). NGF and TrkA also play a role directly on neurons to regulate axon-Schwann cell signalling that promotes myelination (Chan et al., 2004). These studies indicate diverse roles for neurotrophins in regulating the Schwann cell development and myelination of axons.

1.6. Goals of the Thesis

The dynamic interactions between neurons and glia have received increasing attention since the early 1990's. However, many key points regarding neuron-glial interactions in synaptic plasticity remain unresolved. For instance, although glial cells can positively and negatively modulate synapses it has never been shown that the same glia at the same synapse can do this. In order for glial cells to provide different feedback to the synapse depending on the context of ongoing activity, they themselves must be plastic. I therefore, propose to investigate the following

two processes: 1) the role of glial cells in the generation of different plasticity events at the same synapse, and 2) the plasticity of glial calcium elevations.

These studies will provide insight in to the molecules and mechanisms important for context-dependent glial modulation of synaptic function and some of the processes underlying plasticity of calcium elevations in glial cells themselves.

2. CHAPTER 2 (RESEARCH ARTICLE 1)

2.1. INTRODUCTION TO CHAPTER 2

Glial involvement in the modulation of synaptic efficacy has been demonstrated in a number of different preparations starting about 10 years ago. Although it is known that glial cell can up, and down regulated synaptic efficacy, the mechanisms for how this occurs, in an acute time frame, remain elusive. I, therefore, will investigate the bidirectional modulation of synaptic efficacy by glia.

It is known that different patterns of synaptic activity can influence plasticity. Also, we know that glial cells can respond differently to different frequencies of neuronal input (Pasti et al., 1997), as well as to physically different terminals (Perea and Araque, 2005). However, it is unknown whether they will detect differences in synaptic pattern. I, therefore, propose to investigate the impact of different patterns of stimulation at the same frequency on glial activity and neuron-glial communication. Furthermore, I will investigate the resulting modulation of synaptic function that arises following glial activation.

The results that I present here demonstrate that glial cells can decode the pattern of synaptic activity and reflect that with differences in calcium elevations. Furthermore, these differences in calcium elevation are important for the direction (potentiating or depressing) of the modulatory feedback provided to the synapse. These results provide evidence that glial cells can acutely modify synaptic function dependent on the prior pattern of synaptic activity and subsequent glial activity.

In the following pages readers will find the article:

Todd, KJ and Robitaille, R (2008) Glial Cells Decode Patterns of Neuronal Activity to Govern Synaptic Plasticity. Submitted to Science April 1, 2008 #1158522

2.2. ARTICLE: GLIAL CELLS DECODE PATTERNS OF NEURONAL ACTIVITY TO GOVERN SYNAPTIC PLASTICITY

Keith J. Todd and Richard Robitaille

Département de physiologie and Groupe de recherche sur le système nerveux central, Faculté de médecine,
Université de Montréal, C.P. 6128 Succ. Centre-ville,
Montréal, Québec, Canada, H3C 3J7

Corresponding Author: Dr. Richard Robitaille
Département de physiologie, Faculté de médecine,
Université de Montréal, C.P. 6128 Succ. Centre-ville,
Montréal, Québec, Canada H3C 3J7
Phone : (+1) 514-343-6111 ext. 1964
Fax : (+1) 514-343-2111
E-mail: [redacted]

Acknowledgments:

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2.2.1. Abstract

In the nervous system, the induction of plasticity is coded by the frequency and the pattern of synaptic activity. Whereas it is known that glial cells can detect differences in the frequency of the synaptic signal, nothing is known concerning the pattern. Here we provide direct evidence that glia decode the pattern of synaptic activity, and provide context-dependent feedback to determine the direction of synaptic plasticity. This effect was reproduced by selective and direct activation of glia and completely abolished by chelation of glial calcium increases. These results demonstrate for the first time that glia decode the pattern of synaptic activity and provide bidirectional feedback to synapses based on the initial pattern of synaptic activity.

2.2.2. Body

Neuronal information processing in the brain is not only coded by the frequency of neuronal activity but also by the pattern of action potential firing in neurons. This frequency and pattern coding greatly influences synaptic plasticity of CNS synapses as well as the neuromuscular junction (NMJ) (Magleby and Zengel, 1976; Guyonneau et al., 2004; Harris, 2005; Nicoll and Schmitz, 2005). It is now increasingly recognized that glial cells are dynamic partners in a wide variety of brain functions including the induction and modulation of various forms of synaptic plasticity (Haydon and Carmignoto, 2006). Although glial cell activation (indicated by calcium elevation) varies according to different frequency stimuli (Pasti et al., 1997), it is still unknown whether glial cells can decode different patterns of neuronal activity (e.g. differences in burst duration), a critical property that would allow them to fully integrate the neuronal information and participate appropriately in the different forms of neuronal coding. Therefore, we set out to investigate the impact of different patterns of neuronal activity on glial cells and their subsequent modulation of synapses.

To this end, we took advantage of the NMJ, a relatively simple synapse with the closely associated perisynaptic Schwann cells (PSCs), which are non-myelinating glial cells that surround the synapse and have similar roles to astrocytes in the CNS (Auld and Robitaille, 2003b). We chose two physiological patterns of stimulation, for the *soleus* muscle, similar to *in vivo* motoneuronal activity, which produced two forms of post-tetanic of plasticity. The first stimulation paradigm consisted of bursts of activity at 20 Hz with a total of 1800 pulses that induced three periods synaptic depression separated by brief periods of recovery (Supp. Fig. 1A), and closely replicates a form of endogenous activity 20 Hz bursts of 20 stimuli were delivered 30 times with 1 second of rest in between. 3 trains were given with this bursting pattern interspersed by 20 s of rest.(Hennig and Lomo, 1985). The second

pattern used continuous delivery of 1800 pulses at 20 Hz, causing a sustained depression during stimulation (Supp. Fig. 1B). This is the frequency normally seen at the *soleus* muscle (Hennig and Lomo, 1985), and a pattern typical for studies of synapse-glia interactions at the NMJ (Rochon et al., 2001). This provides us with two different patterns of stimulation (bursting and continuous) at the same frequency (20 Hz) using the same number of pulses. We simultaneously recorded post-synaptic potentials (PSPs), and monitored glial calcium responses using Fluo-4 AM, while applying the different stimulation paradigms to the innervating *tibial* nerve. Interestingly, during burst stimulation oscillatory calcium activity was observed in PSCs where 4-10 calcium transients with average duration of 11.2 ± 1.6 s, and amplitude of 47.2 ± 6.3 % $\Delta F/F_0$ occurred in 27 of 29 cells from 10 of 11 synapses. In addition this stimulation induced an underlying increase in basal calcium that lasted on average 94.5 ± 6.6 s (Fig. 1A). As shown in Figure 1A, this pattern of stimulation induced post-tetanic depression of PSP amplitude that develops over a period of 10 minutes. Post-tetanic depression was 83.5 ± 5.1% at 15 min in comparison to control PSPs evoked using test-pulses delivered at 0.2 Hz (n=5; P=0.001; two-tailed t-test).

Unlike bursting, continuous stimulation always resulted 1-2 calcium peaks, that lasted on average 27.3 ± 4.8 s and had an average amplitude of 115.4 ± 28.7 % $\Delta F/F_0$ (Fig. 1A). Also, rather than a post-tetanic depression, we found that continuous stimulation induced a post-tetanic potentiation of 111.8 ± 1.8% at 15 min in comparison to control stimulation (Fig. 1A; n=8; P<0.0001; two-tailed t-test). Hence, the two stimulation patterns induced different PSC activation and post-tetanic plasticity. More importantly, the difference in PSC calcium elevations induced by the two protocols of stimulation indicates that they are differentially activated by these patterns and, thus, that glial cells decode the pattern of neuronal activity.

We next turned our attention to testing whether the pattern of the glial activity caused the selective expression of short-term post-tetanic plasticity.

We developed an approach using photo-labile substances to directly inhibit or activate glial cells. The UV photolysis in the absence of any caged compound had no effect on the glial cells or synaptic transmission (Supp. Fig. 2). This technique allowed us to specifically target PSCs overlying the presynaptic nerve terminal (Supp Fig. 3) by using precise and specific placement of the fiber-optic probe, and by selecting an appropriately oriented NMJ. Indeed, we were able to selectively and precisely target the PSCs or the presynaptic nerve terminal based on the positioning the optic fiber and the orientation of the NMJ (Supp. Fig. 3). Furthermore, we ensured that no contribution to the synaptic modulation was derived from the postsynaptic muscle fiber through specific loading with caged compounds (Supp. Fig. 4).

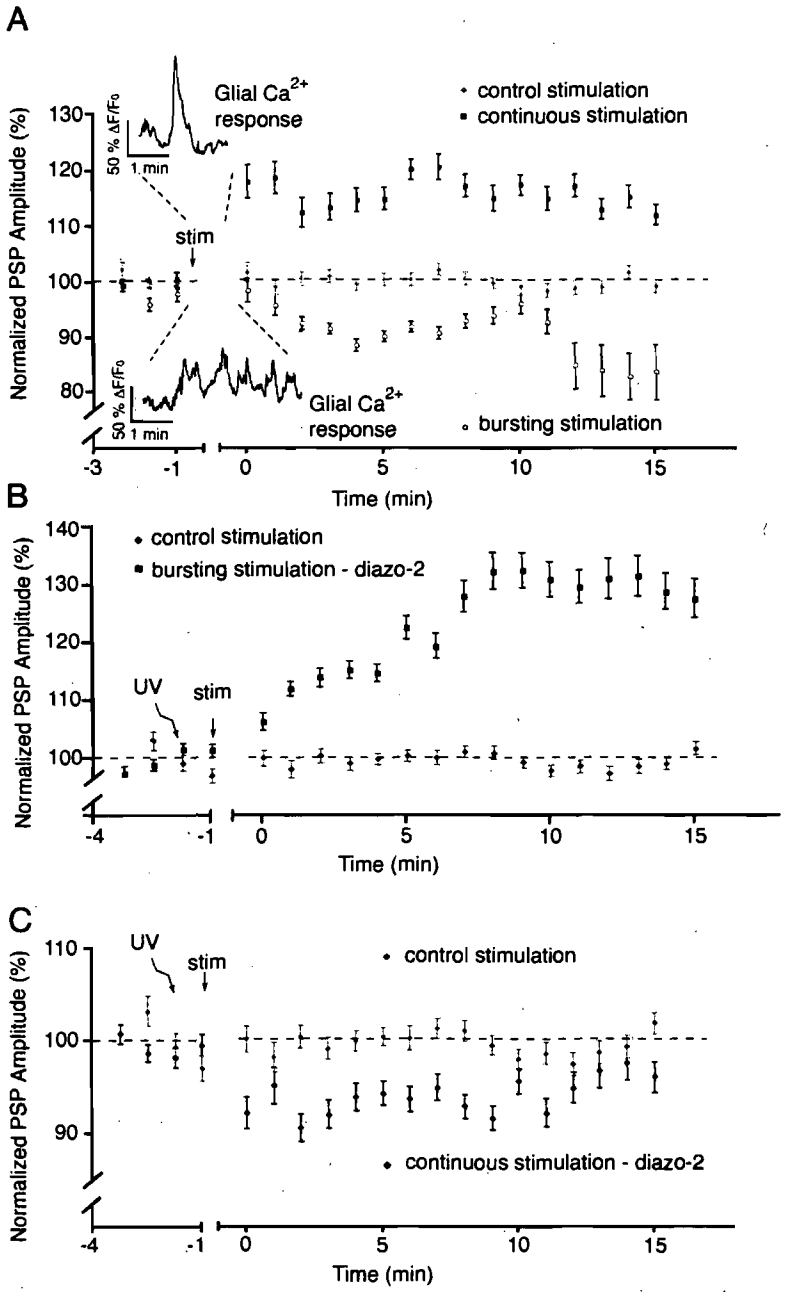

Figure 1. Different stimulation paradigms induce different glial calcium signals that determine post-tetanic plasticity.

(A) Stimulation using bursts of activity induced oscillatory calcium signals recorded from a glial cell and resulted in post-tetanic depression. Conversely, continuous stimulation induced single calcium responses in a glial cell and post-tetanic potentiation. (B) Photolysis of diazo-2 (caged BAPTA) in glial cells during burst stimulation prevented post-tetanic depression, revealing a potentiation. (C) Photolysis of diazo-2 in glial cells during continuous stimulation blocked all post-tetanic potentiation.

We first employed the caged calcium chelator diazo-2 AM (Molecular Probes), a BAPTA derivative that rapidly buffers calcium upon photoactivation with UV light (Kamiya and Zucker, 1994). We placed the UV fiber-optic probe to specifically target glial cells without having any direct effect on the presynaptic terminal, and effectively blocked PSC activity through inhibition of calcium elevations (Supp. Fig. 5). As shown in Fig 1B, chelation of glial cell calcium during bursting stimulation resulted in a potentiation in PSP amplitude, rather than a depression in 12 of 14 preparations: PSP amplitude was 129.1 ± 3.1% at 15 min in comparison to control stimulation ($n=8$; $P<0.0001$; two-tailed t-test). Conversely, photolysis of diazo-2 prior to continuous stimulation prevented post-tetanic potentiation (Fig. 1C; 97.6 ± 1.8%; $n=7$; $P=0.045$; two-tailed t-test). These results clearly indicate the importance of glial activation (via calcium) in the expression of synaptic plasticity. Notably, these data also suggest that glial calcium elevations are not all-or-none events, but contain specific information concerning the ongoing activity of the synapse. This allows glial cells to govern the outcome of synaptic plasticity in a context-dependent manner.

We next turned to understanding the mechanisms underlying glial control of synaptic strength. In order to do this, we needed a method of directly activating glial cells since neurotransmitters released during synaptic activity, such as ATP, acetylcholine and adenosine, all act both on the presynaptic terminal and PSCs (Rochon et al., 2001; Todd et al., 2007). This means that any pharmacological manipulation of the presynaptic terminal during evoked activity would also affect glial activation, thus making interpretation of any changes in synaptic plasticity impossible. Therefore, we designed protocols using photolysis of caged calcium o–nitrophenyl-EGTA (NP-EGTA AM) to mimic endogenously evoked calcium responses, and the underlying synaptic changes. These two different photolysis protocols (Fig. 2A) elicited either multiple calcium responses to mimic oscillatory responses

seen with bursting stimulation or a single calcium response similar to that seen with continuous stimulation. Multiple calcium transients (4-10) were elicited with an average amplitude of 38.5 ± 5.1 % $\Delta F/F_0$ and average duration of 8.2 ± 1.0 s. Single calcium elevations were larger and longer with average amplitudes of 122.9 ± 14.2 % $\Delta F/F_0$ and average duration of 51.9 ± 9.7s. PSP amplitudes were depressed (85.2 ± 2.7%, $P<0.0003$, two-tailed t-test) in comparison to controls following multiple photo-activations (Fig. 2A), and were not statistically different from those evoked by endogenous 20 Hz burst stimulation ($P>0.05$; Student's t-test). In contrast, evoking a single calcium response induced a potentiation in comparison to controls (106.6 ± 2.0%, $P=0.05$; two-tailed t-test) of PSP amplitude similar to that seen following continuous stimulation ($P>0.05$; Student's t-test). Thus, this activation mimics endogenous activation of glial cells and the resulting changes in synaptic efficacy. More importantly, these results demonstrate that direct, differential activation of glial cells is sufficient to selectively induce post-tetanic potentiation and depression.

Knowing that glial cells were responsible for controlling bidirectional plasticity we investigated the mechanisms involved. A number of receptor systems offered the possibility of an opposing regulation of synaptic efficacy such as muscarinic and purine receptor systems (Correia-de-Sa et al., 1996; Dudel, 2007). Since ATP is a prominent gliotransmitter involved in a number of neuronal regulations by glial cells (Fields and Burnstock, 2006), we tested the involvement of purines in our model of glial regulation of synaptic activity. We began by using the 5'-ectonucleotidase inhibitor ARL 67156 (50 μM) that prevents the degradation of ATP into its metabolites. As shown in Fig 2B, we observed a potentiation of 110.1 ± 2.1% in comparison to controls ($n=4$; $P<0.0001$; two-tailed t-test) in the presence of ARL 67156 following the multiple photolysis protocol, rather than a depression of PSP amplitude. Conversely, when we performed a single photolysis of caged calcium in the presence of ARL (Fig. 2C), which normally produced potentiation of PSP

amplitude, we observed no post-tetanic plasticity (101.3 ± 4.0%; n=4; P>0.05). It is unlikely that these results can be explained by the accumulation of ATP since our experiments with calcium buffering in PSCs that should prevent the release of glial ATP, had similar effects on synaptic plasticity (see Fig 1). This suggests that the endogenous modulation of synaptic plasticity is due to the degradation of astrocytic ATP into adenosine.

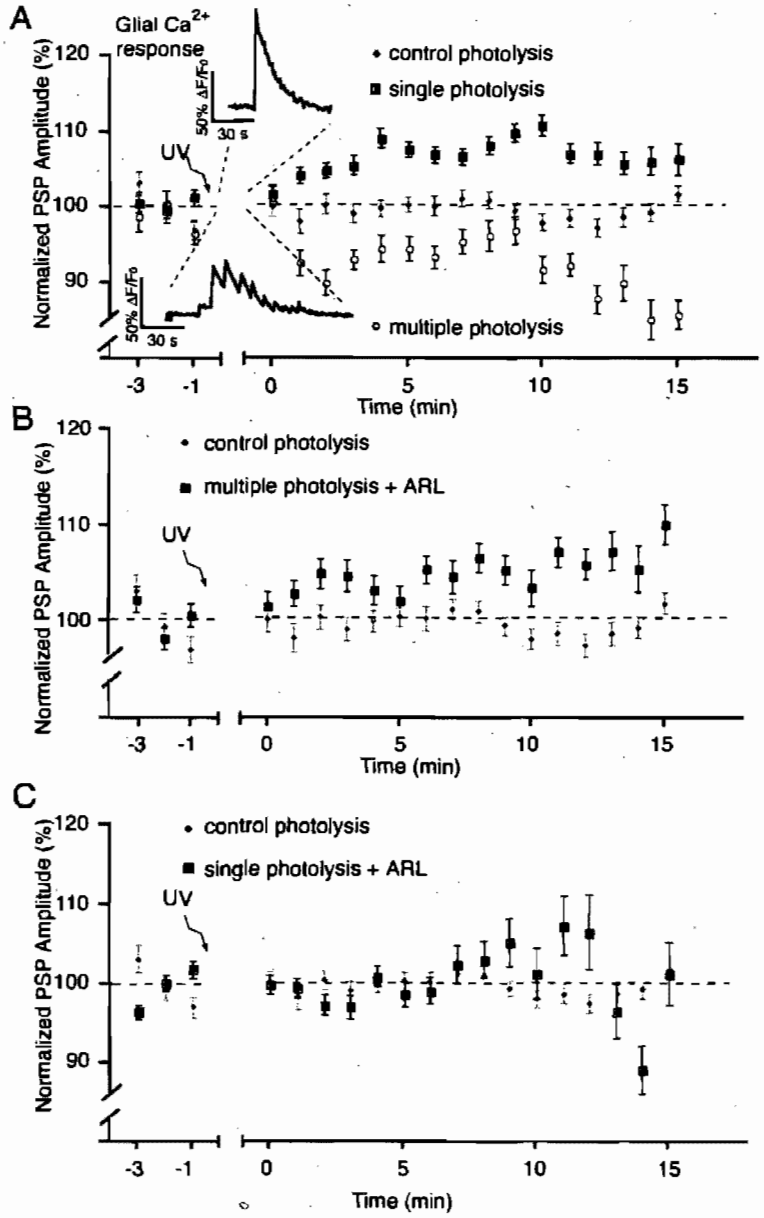

Figure 2. Glial calcium and purine regulation determines the outcome of synaptic plasticity.

(A) Multiple photolysis of NP-EGTA (caged calcium) in glial cells induced a prolonged elevation of glial calcium and post-tetanic depression similar to burst stimulation while a single calcium elevation induced with photolysis of NP-EGTA in glia resulted in a post-tetanic potentiation similar to continuous stimulation. (B) Blockade of ATP hydrolysis with ARL-67156 while applying the multiple photolysis protocol prevented post-tetanic depression. (C) Single photolysis of NP-EGTA in the presence of ARL resulted in an occlusion of post-tetanic potentiation.

Hence, we tested the possibility that the blockade of ATP hydrolysis resulted in the absence of adenosine, and an imbalance in A_1 and A_{2A} receptor regulation. Interestingly, the A_1 and A_{2A} adenosine receptors mediate depression and potentiation, respectively, throughout the nervous system (Correia-de-Sa et al., 1996; Lopes et al., 2002). Addition of the A_1 adenosine receptor antagonist PSB-36 (5 nM) prior to photolysis resulted in a post-photolysis potentiation of PSP amplitude rather than a post-tetanic depression (107.0 ± 1.9%, n=8; P=0.02, two-tailed t test). Furthermore, the PSC-induced depression was occluded by prior activation of A_1 receptors with the selective A_1 agonist CCPA (0.1 μM; 103.5 ± 2.1%; P>0.05; two-tailed t-test Supp. Fig 6). The involvement of A_1 receptors in post-tetanic depression was further confirmed through the use of A_1 receptor knockout mice. We observed no depression in A_1 -/- (100.4 ± 2.5 %, n=8) NMJ preparations (Fig. 3B), whereas it was normal in +/+ controls (89.1 ± 2.9 %) following the multiple photolysis protocol (P=0.004). Finally, performing the multiple photolysis protocol in the presence of SCH-58621 (50 nM), an A_{2A} receptor antagonist, resulted in depression (92.6 ± 1.7%) from control (3C, P<0.0001). Depression was also significant at NMJs from A_{2A} -/- mice in comparison to +/+ controls (Fig. 3D, 91.3 ± 2.0%; P=0.0001) did not affect the PSC-induced depression. As a whole, these experiments indicate that the endogenous pattern of activity

leading to glial-mediated post-tetanic depression involves A_1 receptor activation following ATP degradation.

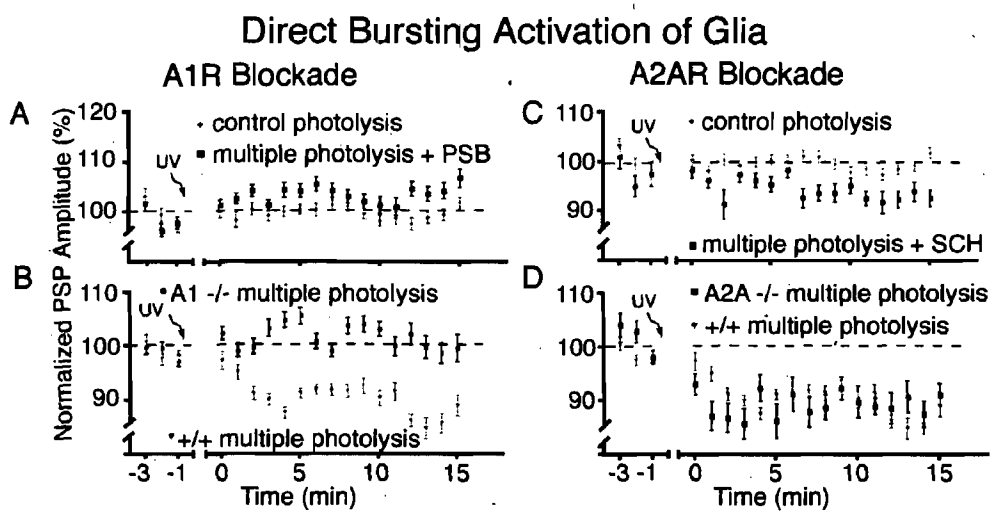

Figure 3. Post-tetanic depression is regulated by A1 adenosine receptors.

(A) Multiple photolysis of NP-EGTA in glial cells in the presence of the A1 receptor antagonist PSB-36 occluded post-tetanic depression (B) No post-tetanic depression was elicited by multiple photolysis at NMJs of A1 -/- animals. (C) Presence of the A2A receptor antagonist SCH has no effect on post-tetanic depression. (D) Post-tetanic depression was evoked by the multiple photolysis protocol at NMJs from A2A -/- mice.

Because our hypothesis was based on the possibility that the different forms of short-term plasticity were due to a balance between A_1 and A_{2A} we next tested the involvement of A_{2A} receptor activation in the modulation of glial-mediated potentiation. Thus, we used the single photolysis protocol to test for the involvement of A_{2A} receptors in the glial-mediated post-tetanic potentiation. The presence of an A_{2A} antagonist SCH-56821 (50 nM) blocked the expression of post-tetanic potentiation and was not significantly different from controls (Fig. 4A; 96.6 ± 2.4%; n=5; $P>0.05$, two-tailed t-test). Prior application of CGS-21680 (30 nM), an A_{2A} agonist, occluded the PSC-induced potentiation and resulted in a depression (90.6 ± 1.6%) that was significantly reduced from controls ($P<0.0001$), further confirming the involvement of A_{2A}

receptors in potentiation (Supp. Fig. 6). Furthermore, post-tetanic potentiation was occluded at NMJs of A_{2A} -/- mice (98.5 ± 2.1%, P>0.05; Fig. 4B). Finally, use of the single photolysis protocol in the presence of the A_1 receptor antagonist PSB-36 (5 nM) resulted in potentiation (108.6 ± 1.6%; P<0.0001; n=6) of PSP amplitude (Fig. 4C), as did the use NMJs from A1 -/- mice (Fig. 4D; 110.4 ± 3.0%; P=0.001). Hence, it appears that A_{2A} receptor activation mediated the glial-dependent potentiation. Taken together, the results strongly implicate a glial-regulated activation of A_1 and A_{2A} receptors in the generation of pattern-dependent induction of depression and potentiation at the NMJ.

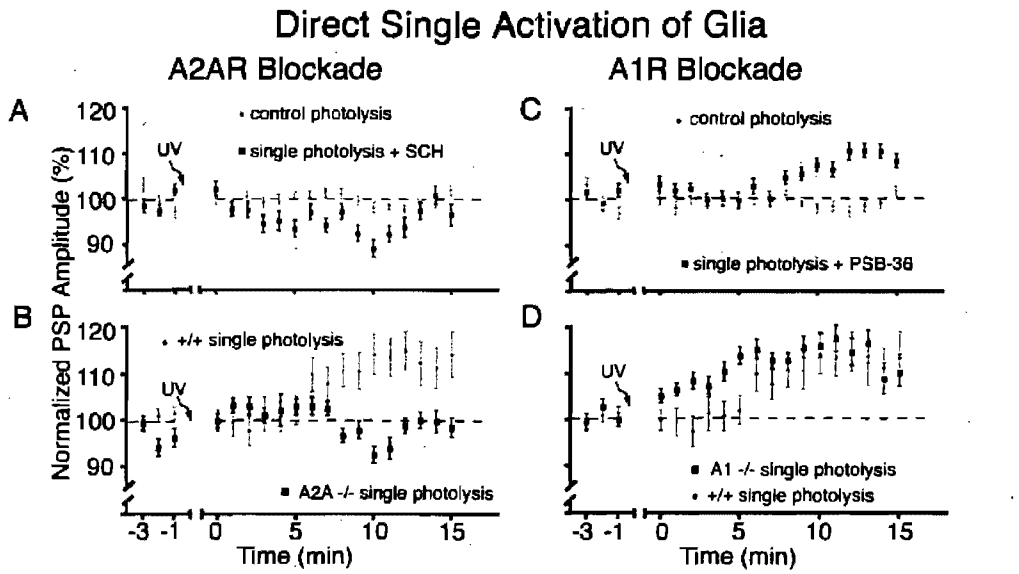

Figure 4. A2A receptors regulate post-tetanic potentiation.
(A) Single photolysis of NP-EGTA in glial cells in the presence of the A2A receptor antagonist, SCH, prevented post-tetanic potentiation. (B) No post-tetanic potentiation was elicited with the single photolysis protocol at NMJs of A2A -/- animals. (C) Addition of the A1 receptor antagonist PSB-36 had no effect on post-tetanic potentiation. (D) Post-tetanic potentiation was induced by the single photolysis protocol at NMJs from A1 -/- mice.

Glial cells display finely tuned responsiveness to neuronal activity. They can detect subtle changes in the frequency of activity (Pasti et al., 1997) and even discriminate between different synaptic inputs (Perea and Araque,

2005). Now, our evidence indicates that glia differentiate the pattern of synaptic activity which is an important element regulating synaptic plasticity. That is, they integrate and decode the pattern of neuronal and synaptic activity to, in turn, influence synaptic transmission. Our data indicate that this regulation is likely to occur through a processing of incoming information and subsequent feedback to provide altered and tuned modulation to a given synapse. Not only are glial cells necessary for this modulation, but also their direct activation with specific patterns is sufficient to regulate changes in synaptic efficacy. Our data have two immediate impacts on the understanding of neuronal communication in the nervous system. First, neurons are no longer the only cells that can decode information from the pattern of neuronal activity, and process it in a way that allows them to influence the outcome of subsequent synaptic communication. Second, owing to their decoding capability, glial cell regulation of short and long-term synaptic plasticity should no longer be seen as an all or none event, but rather as an adaptable regulation dependent on the context of previous neuronal activity. Therefore, this implies that glial cells have the capacity to mediate and regulate a large array of synaptic events.

In this study we have used a PNS synapse as model to reveal cellular interactions that are likely applicable to synapses throughout the nervous system. Indeed, it has previously been demonstrated that direct activation of retinal glial cells can induce both positive and negative changes in neuronal activity (Newman and Zahs, 1998), suggesting similar roles to those described here. Furthermore, Panatier and colleagues recently described a phenomenon similar to ours, occurring in the hypothalamus (Panatier et al., 2006). They showed that glial cells determined the outcome of synaptic plasticity (potentiation or depression) as a result of changes in the glial synaptic coverage and release of D-serine. Here we have built on the current knowledge of glial involvement in synaptic function by providing evidence that glial cells, acutely responding to neuronal activity, can supply context-

dependent feedback to synapses by decoding the pattern of neuronal activity. Hence, our data indicate that glial regulation of neuronal plasticity is not only a matter of slow long-term modification to the glial environment, but that it occurs over a matter of minutes. Interestingly, morphological changes at synapses can occur within a time window of a few minutes (Matsuzaki et al., 2004) which is an appealing possibility to explain the long-term changes induced by the brief glial activation.

Studies have found that activation of A_1 and A_{2A} receptors results in inhibition and activation of different types of presynaptic calcium channels to regulate transmitter release at synapses in general and at the mammalian NMJ in particular (Correia-de-Sa et al., 1996; De Lorenzo et al., 2004; Oliveira et al., 2004; Silinsky, 2005). Moreover, an adenosine-dependent long-term depression has been reported (Redman and Silinsky, 1994) and involves a down-regulation of presynaptic calcium channels (Silinsky, 2004, 2005). For the first time, our data indicate that the balanced A_1-A_{2A} regulation can be controlled, not by the presynaptic pool itself in an auto-regulation manner, but rather by the perisynaptic glial cells. We propose a model (Supp. Fig. 7) that takes into consideration the properties of A_1-A_{2A} receptor interactions (Johansson et al., 2001; Cunha, 2008) and the ability of glial cells to release ATP (Fields and Burnstock, 2006). We propose that small and sustained accumulation of glial calcium elicited by bursting synaptic activity would induce the release of a smaller amount of ATP, leading to lower concentrations of adenosine in the synaptic cleft, the activation of A_1 receptors and post-tetanic depression. Conversely, post-tetanic potentiation would be induced by large glial calcium responses elicited by sustained synaptic activity causing the release of a larger quantity of ATP leading to more adenosine and the activation of A_{2A} receptors.

Our data show that the PSC regulation leads to sustained changes in synaptic potency that would facilitate or reduce motoneuronal control of the NMJ, based on the pattern of neuronal activity itself. Interestingly, the PSC-

mediated adenosine regulation could be a target for possible treatments of muscular diseases (e.g. myasthenia gravis) or conditions that lead to weakened synapses (e.g. aging) by regulating the pattern of activity to favor the PSC-dependent potentiating pathway to strengthen the efficacy of the neuromuscular synapse.

We demonstrate for the first time that glial cells are both necessary and sufficient to govern the outcome of synaptic plasticity based on their ability to decode the patterns of neuronal communication. These results indicate that glial cells are pattern detectors in the nervous system, a role that could influence many CNS functions.

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2.2.4. Supporting Online Material

Materials and Methods

Animals and Preparation

All experiments were performed in accordance with the guidelines of the Canadian Council of Animal Care and the Animal Care Committee at the Université de Montréal. Juvenile (P21-28) male CD-1 mice (Charles River) were killed by decapitation. Soleus muscles, with the tibial nerve intact, were removed and pinned in Rees saline (D. Rees, J Physiol 278, 8P, 1978) (in mM): NaCl, 110; KCl, 5; MgCl2, 1; NaHCO3, 25; CaCl2, 2; glucose, 11; glutamate, 0.3; glutamine, 0.4; BES buffer, 5; cocarboxylase, 0.4 μM; choline chloride, 36 μM and bubbled with 95% O2/ 5% CO2.

A1 and A2A KO Mice

The A1 -/- mice were a kind gift from Bertil Fredholm (Karolinska Institute, Stockholm, Sweden) and Stephen Tilley (University of North Carolina, Chapel Hill, USA). These mice have previously been described (Gimenez-Llort et al., Eur J Neurosci 16, 547, 2002). A2A -/- mice were as previously characterized (Chen et al., J Neurosci 19, 9192, 1999) and kindly provided by Jiang-Fan Chen (Boston University) and Michael Schwarzschild (Massachusetts General Hospital).

Electrophysiological recordings

The tibial nerve was stimulated through a suction electrode filled with extracellular saline. Muscle contractions were prevented with partial blockade of the postsynaptic ACh receptors using d-tubocurarine chloride (2.9-4.4 μM, Sigma). Intracellular recordings of post-synaptic potentials (PSPs) were performed using glass microelectrodes (1.0mm OD; WPI) pulled to 50-70 MΩ (filled with 3M KCl) with a Brown-Flaming micropipette puller (Sutter

Instruments). Recordings were amplified (200x) using an AM Systems 1200 amplifier connected to a WPI external amplifier, digitized using a National Instruments BNC 2110 board and acquired using WinWCP software (John Dempster, Strathclyde University). The continuous stimulation paradigm consisted of 1800 pulses delivered at 20 Hz. The bursting stimulation paradigm (Supp. Fig. 1A) consisted of 30 repetitions of 20 pulses at 20 Hz repeated every 2 s. This was repeated 3 times with 20 s of rest between repetitions. Synaptic efficacy was monitored with test pulses delivered at a frequency of 0.2 Hz, a frequency known to have no effect on synaptic efficacy. PSP amplitude before, during high-frequency stimulation, and following were normalized to the PSP amplitude of test pulses obtained during the baseline period, prior to 20 Hz stimulation. Recordings were discarded when the holding potential changed by more than 5 mV.

Calcium Imaging and Analysis in Glia

Dissected soleus muscles were incubated in 10 μM Fluo-4AM (Molecular Probes) containing 0.02% pluronic acid (Molecular Probes) for 1.5 hr at room temperature. Neuromuscular junctions were located under bright-field optics and were located on the surface of muscle fibers. Evoked calcium responses were obtained by stimulating the tibial nerve with one of the stimulation paradigms described above. Epi-fluorescent images were acquired on a Nikon E600N upright microscope fitted with a Princeton Instruments CCD-1300 camera. Digital acquisition was performed using MetaFluor software (Perkin Elmer) driving a Lamda 10-2 shutter wheel (Sutter Instruments). Images were acquired at a rate of 1 image per second. Fluorescence was quantified by subtracting the background fluorescence from the neighboring muscle fiber and then performing the calculation $(F-F_0/F_0)*100$ to give $\Delta F/F_0$ %.

UV Photolysis

Caged compounds (diazo-2-AM, NP-EGTA-AM; Molecular Probes) were loaded with the following protocol. Muscles were incubated first with Fluo-4-AM (10 μM) alone for 30 min and then with both Fluo-4-AM and caged compound (20 μM) for 45 min followed by another period of 45 min with fresh solution. Photolysis was performed using a Laser Science Nitrogen Pulsed UV laser (337 nM) operating at 50-60 Hz, 10-30 pulses with pulse durations of 2-10 ms. The UV pulses were aimed at the preparation using a fiber-optic probe with a diameter of about 30 μm inserted in a pipette for guidance. For multiple uncaging events 60 Hz, 10 pulses for 2 ms were used repeatedly. For a single large event 15 pulses of 10 ms at 50 Hz were given. Two or more PSCs were targeted for photolysis during all experiments.

A protocol was designed to specifically target PSCs based on a number of criteria. First, we chose NMJs that were on the surface of the muscle fiber. Second, NMJs where PSC somata were clearly overlaying the nerve terminal were selected. Third, alignment of the fiber-optic was performed using visible light passed through the fiber-optic such that the cone of light covered at least two PSCs and not the presynaptic terminal. Possible direct presynaptic effects were monitored in each experiment by monitoring rapid changes (within seconds; Kamiya, Zucker, Nature 371, 603, 1994) in transmitter release and PPR (see Supp Fig 3). Experiments were discarded when direct presynaptic effects were observed.

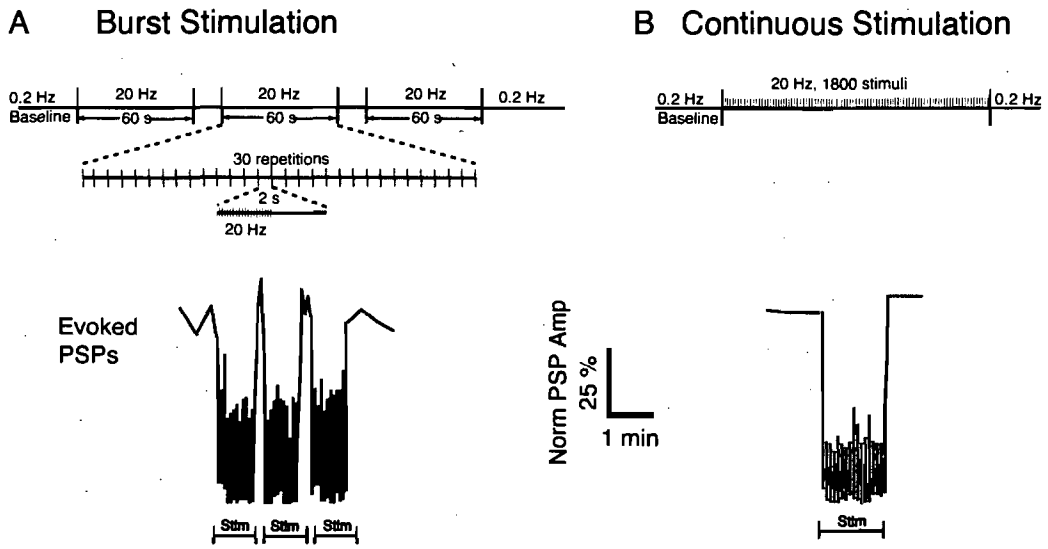
Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) except for ARL 67156, CGS 21680, Dipyridamole, and PSB-36 (Tocris, Ellisville, MO, USA).

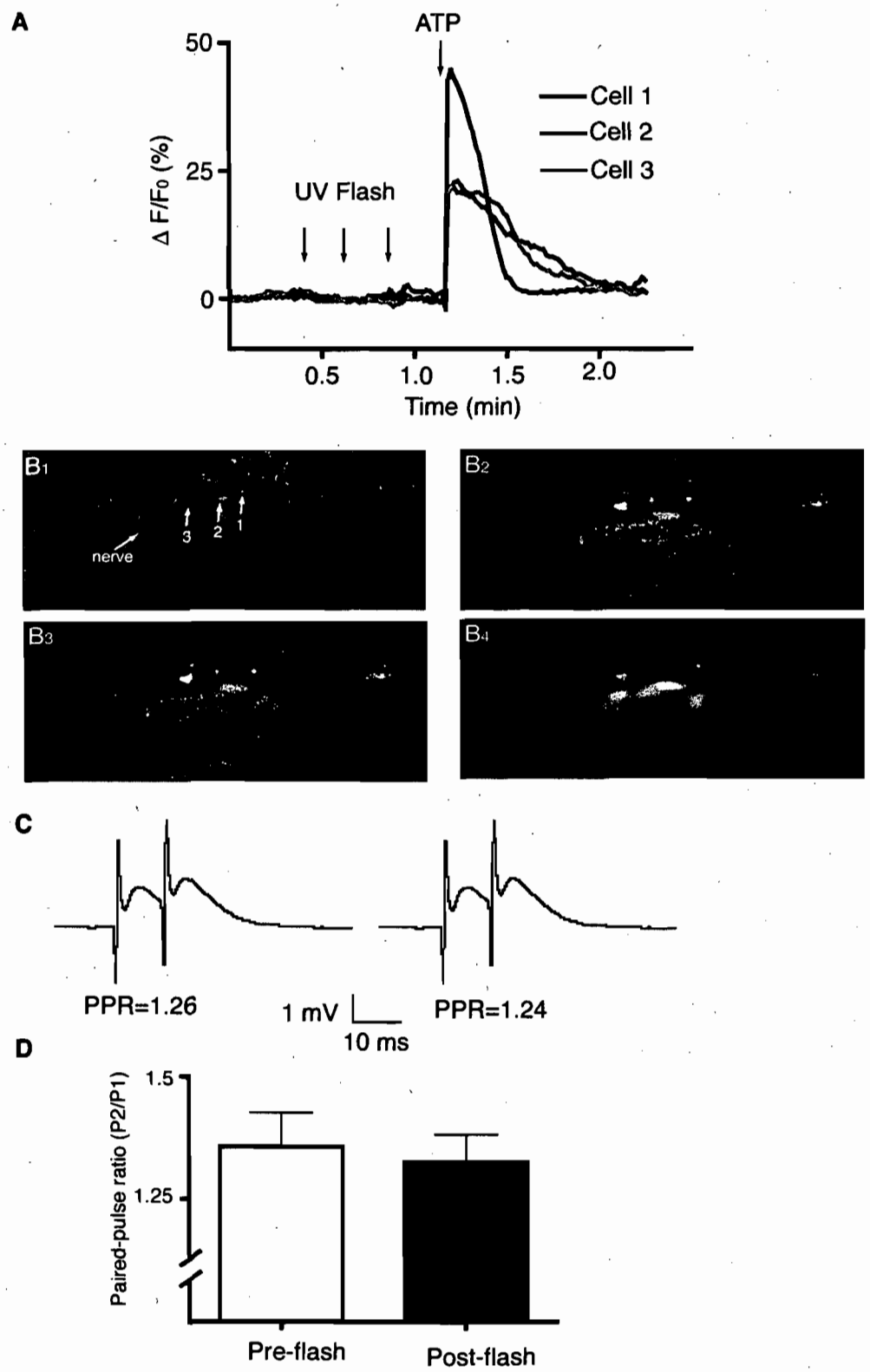
Statistical Analysis

PSP values were compared to the hypothetical mean of 100% using one-sample t-tests. Two groups were compared using Students' T-tests.

When data were found not to conform to normality, Mann-Whitney U tests were used. Analyses were deemed significant at $P \leq 0.05$.

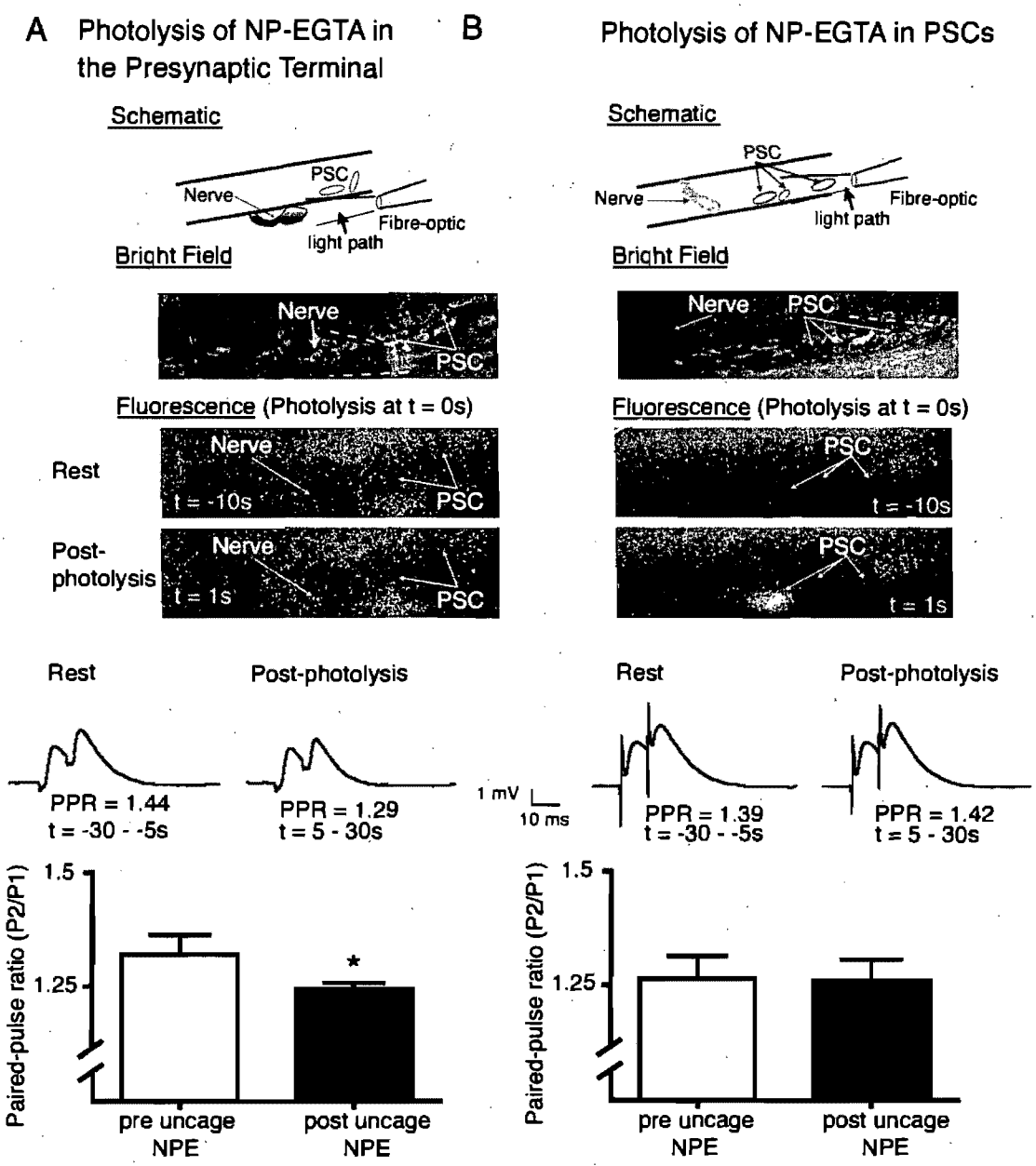


Supplemental Figure 1. Two patterns of stimulation used in this study. (A) The top panel illustrates the burst stimulation, which involves thirty (30) 1 s bursts at 20 Hz with a 1 s pause in between, repeated 3 times. The resulting depression that occurred is shown in the bottom panel. (B) The top panel illustrates the continuous stimulation paradigm that consisted of 1800 pulses at 20 Hz. The depression induced by the stimulation is shown in the bottom panel.


A
ATP
50
25
0
ΔF/F0 (%)
UV Flash
Cell 1
Cell 2
Cell 3
0.5
1.0
1.5
2.0
Time (min)
B1
3
2
1
nerve
B2
B3
B4
C
PPR=1.26
1 mV
10 ms
PPR=1.24
D
Paired-pulse ratio (P2/P1)
1.5
1.25
Pre-flash
Post-flash

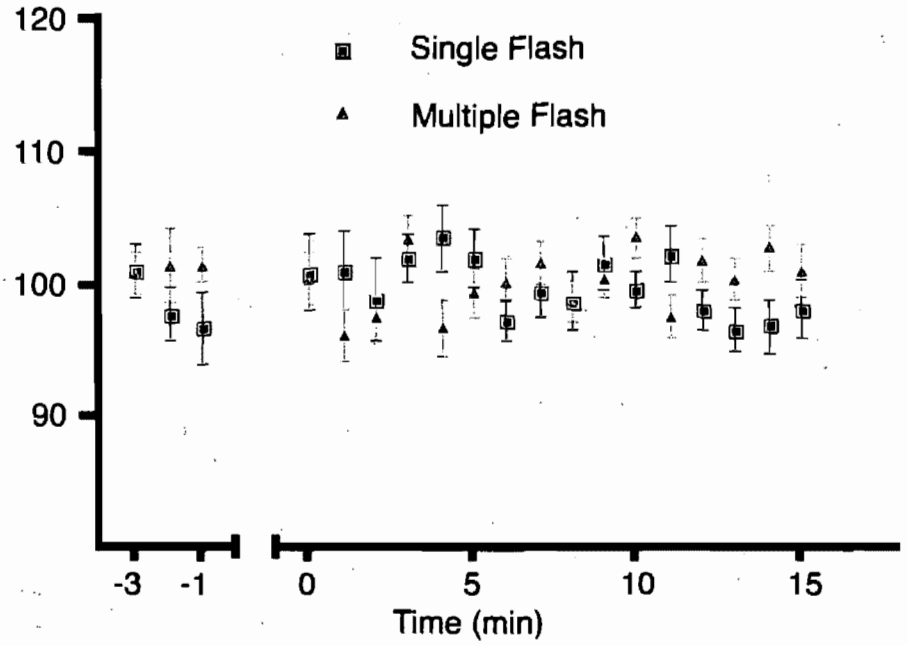
Supplemental Figure 2. UV photolysis alone does not affect glial activation or synaptic transmission.

(A) Large UV photolysis had no direct effect on the resting fluorescence of PSCs in the absence of any caged compound. Cell viability was confirmed by the ability of ATP to induce calcium responses that were similar to the ones elicited without prior exposure to UV flashes. (B1) Bright field image of same neuromuscular junction as in A) with cells 1, 2, and 3 indicated. (B2) Fluorescent image taken during the baseline period prior to the UV flashes. (B3) Fluorescent image taken immediately following the second UV flash. (B4) Fluorescent image taken immediately following the local application of ATP. (C), PSPs evoked by paired-pulse stimulation in control (left) and following UV photolysis in the absence of any caged compound. (D) Histogram showing that paired-pulse ratio was not affected by UV photolysis in absence of any caged compound (n=4).



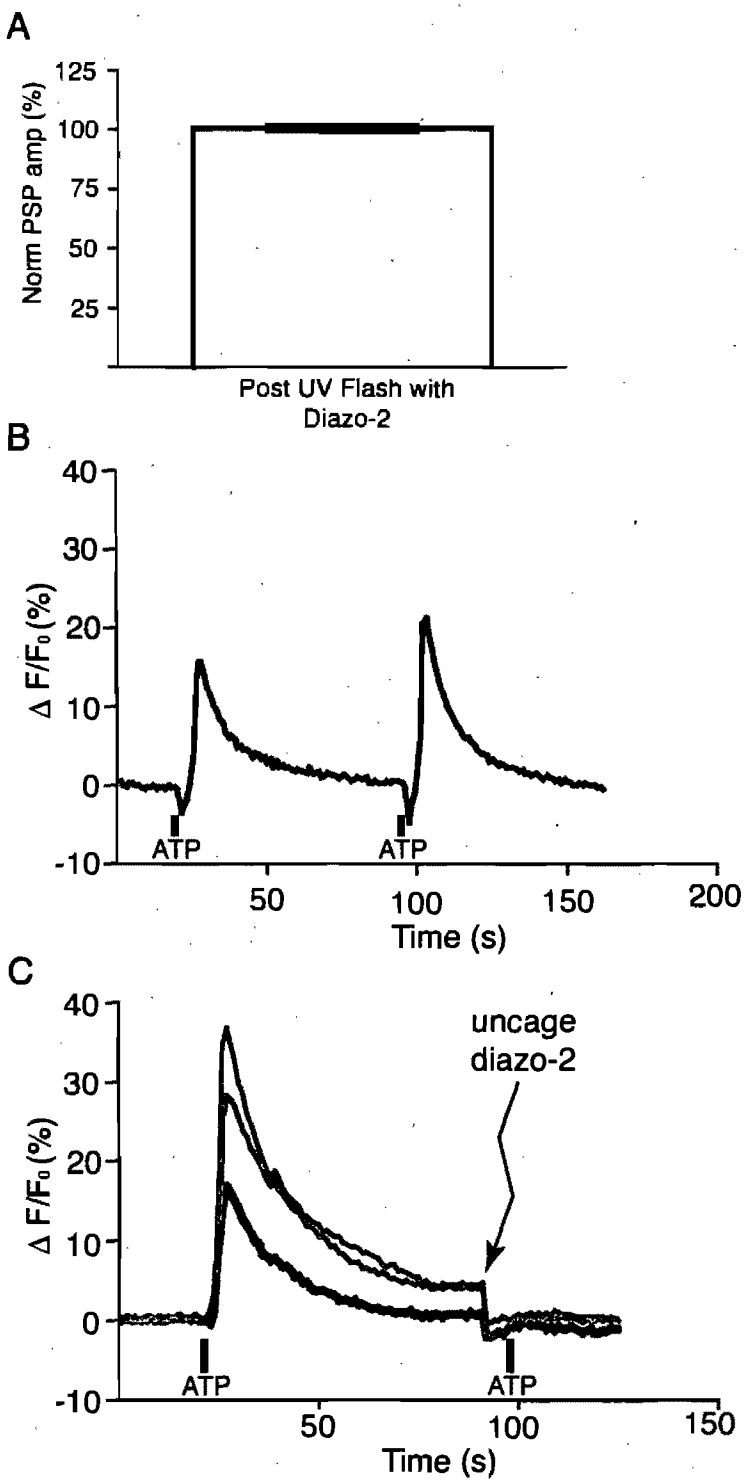
Supplemental Figure 3. Selective and precise activation of PSCs. NMJs were chosen according to the criteria described in the methods to favor either a selective presynaptic effect or a selective glial effect. Owing to the tight relationship between calcium and the release of neurotransmitter we reasoned that any effects that take place presynaptically would occur rapidly (within a few tens of milliseconds) following the photolysis of calcium-related molecules (Bollmann, Sakmann, Nat Neurosci 8, 426, 2005; Kamiya, Zucker, Nature 371, 603, 1994; Millar et al. J Neurosci 25, 3113, 2005). This rate is several orders of magnitude faster than the glial modulation tested here that occurs in the time frame of several minutes. (A) Schematic, bright field

and fluorescence images of an NMJ selected so that the presynaptic terminal could be selectively activated (top panel). Recording PSPs evoked with a paired-pulse protocol during the 30 seconds following the photolysis of NP-EGTA revealed the rapid effect on presynaptic transmission. Uncaging calcium in the presynaptic nerve terminal would result in an immediate increase in release probability (Bollmann, Sakmann, Nat Neurosci 8, 426, 2005), which is inversely related to paired-pulse ratio (PPR). As shown in the bottom panel, UV photolysis on the nerve terminal induced a reduction in PPR indicating a presynaptic effect without the activation of glia (fluorescent images, no calcium response in PSCs). (B) Schematic, bright field and fluorescence images of an NMJ selected so that the PSCs could be selectively activated (top panel). In these conditions we were able to elicit a calcium elevation in PSCs without directly affecting the presynaptic terminal as measured by paired-pulse ratio measured shortly after photolysis (0 – 30 sec; bottom panel). This is consistent with the latency of the slower time course of the glial modulation in comparison to a direct presynaptic effect. Significance measured at $P<0.05$, $n=6$.



Supplemental Figure 4. Postsynaptic muscle fibers have no effect on synaptic plasticity following photolysis on glial cells.

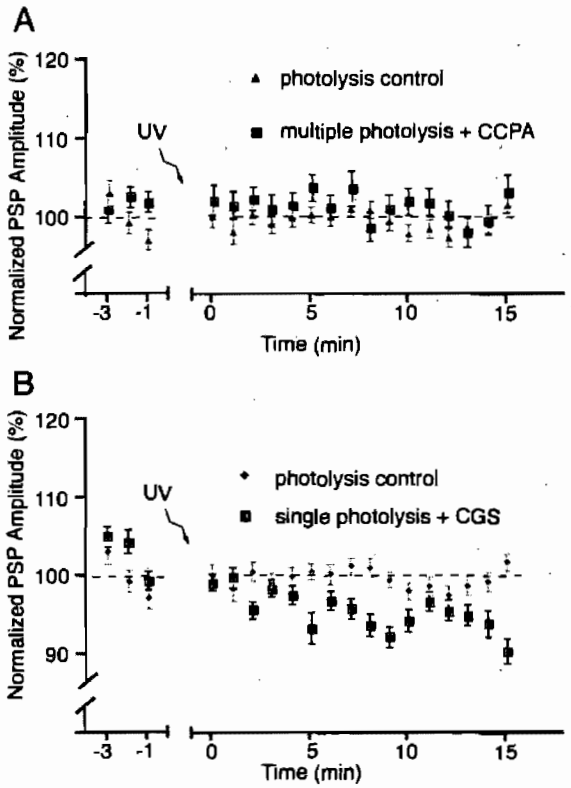
Bulk loading with Fluo-4 AM was performed then postsynaptic muscle fibers were loaded with cell impermeable NP-EGTA salt through iontophoresis (2nA, 200ms, 0.2 Hz for 10min) and photolysis was performed on glial cells as described in Supp. Fig. 3. We observed no calcium elevation in glial cells and saw no change in PTP following photolysis.



Supplemental Figure 5. Specific glial photolysis of diazo-2 has no direct presynaptic effects and selectively blocks glial calcium elevations.

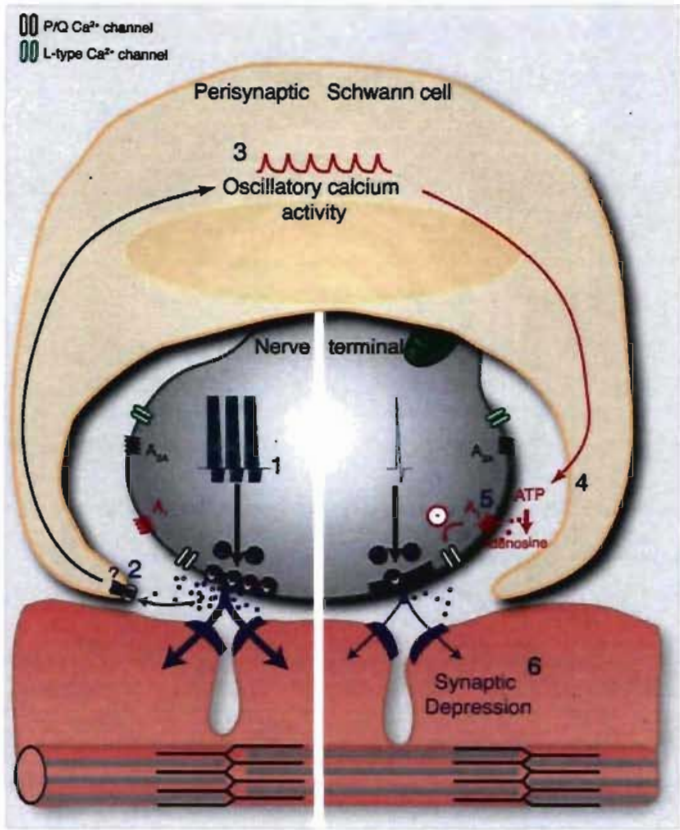
A) Using the same criteria as described in Supp Fig 2, we carefully chose surface NMJs, where overlying PSCs covered the innervating nerve. We were able to selectively activate PSCs with photolysis without any direct effect on presynaptic nerve activity as revealed by

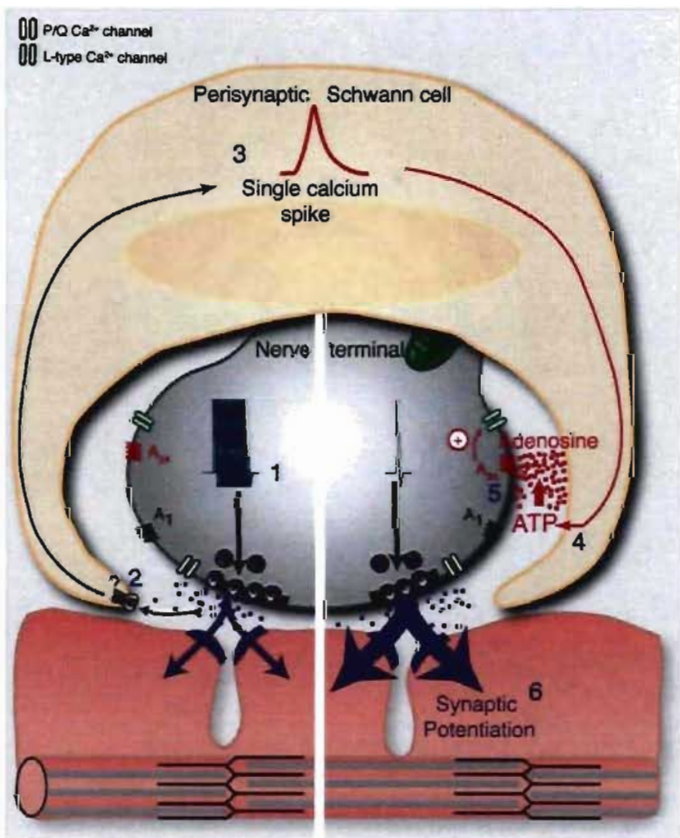
the lack of effect on PSP amplitude and paired-pulse facilitation for up to 60 seconds immediately following photolysis. B) ATP-induced calcium responses in PSCs show little desensitization and are reproducible. C) After a first ATP-induced calcium response, diazo-2 was photo-activated and ATP locally applied again on the same cells. The second response was completely prevented when diazo-2 was uncaged prior to local application.



Supplemental Figure 6. Agonist application occludes synaptic plasticity.

A) application of the A1 receptor agonist (CCPA) occludes depression.
B) application of the A2A agonist (CGS) occludes potentiation.


P/Q Ca²⁺ channel
L-type Ca²⁺ channel
Perisynaptic Schwann cell
3
Oscillatory calcium activity
Nerve terminal
1
2
4
ATP
5
Synaptic Depression
6


P/Q Ca²⁺ channel
L-type Ca²⁺ channel
Perisynaptic Schwann cell
3
Single calcium spike
Nerve terminal
1
2
Adenosine
5
ATP
4
Synaptic Potentiation
6

Supplemental Figure 7. Model of glial-mediated bidirectional modulation of synaptic plasticity.

(A) Bursts of activity (1) induce the release of neurotransmitter to activate perisynaptic Schwann cells (2) and the postsynaptic terminal. Receptor activation on PSCs leads to oscillatory calcium elevations (3) and the release of a lesser amount of glial-derived ATP (4) that is degraded to adenosine. Relatively low levels of synaptic adenosine leads to synaptic depression through activation of A1 receptors and a decrease in presynaptic calcium entry through P/Q-type calcium channels (5) and (6). (B), continuous presynaptic activity (1) induces the release of neurotransmitter to activate perisynaptic Schwann cells (2) and the postsynaptic terminal. Receptor activation on PSCs leads to a single calcium elevation (3) and the release of a larger amount of glial-derived ATP (4) that is degraded to adenosine. Relatively high levels of synaptic adenosine lead to activation of A2A receptors, activation of L-type calcium channels (5) and synaptic potentiation (6).

2.3. Discussion to Chapter 2

Here we have shown that glial cells have the capacity to detect differences in neuronal activity, as reflected in their altered calcium responses. Importantly, these differences in calcium response are responsible for the expression of synaptic plasticity through balanced regulation of A1 and A2A receptor activation.

One important unresolved question from this study is what leads to the differences in glial activation. The calcium responses are clearly different, however, what leads to the initiation of these differences? It could be due to release of different neurotransmitters or perhaps different contributions from internal and external calcium.

There are a number of different possibilities that could result in differential activation of these two receptors. Since they are both considered to be high affinity receptors, this is thought not to be a major factor. Rather, the receptor density, synaptic location and receptor crosstalk are believed to be influential in regulating the activation of one instead of the other (Sebastiao and Ribeiro, 2000). Also, it is possible that there is altered regulation of purines in the synapse through differences in release, uptake, or degradation. Indeed it is known that the pattern of neuronal activity changes the release and production of adenine nucleotides and subsequently receptor activation (Sebastiao and Ribeiro, 2000). Considering this it is possible that the differences in glial activation (i.e. calcium responses) could also influence release and or production of adenosine.

These results greatly advance our view of glial-synapse interactions since they demonstrate for the first time the ability of glial cells to distinguish differences in the pattern of neuronal activity. This discrimination results in a change in their modulation of the synapse. It is thought-provoking to hypothesize that glial cells throughout the nervous

system may be information processing cells that actively aid in the regulation and synthesis of information.

Although the details of the synaptic modulation uncovered here are likely specific for this synapse, the role that glial cells play is hypothesized to be similar throughout the nervous system. Further investigations into the ability of glial cells to distinguish differences in synaptic activity could provide new and exciting perspectives on the influence of glial cells in nervous system function.

3. CHAPTER 3 (RESEARCH ARTICLE 2)

3.1. INTRODUCTION TO CHAPTER 3

As demonstrated in Chapter 2, the type of glial calcium elevation is important for the outcome of changes in synaptic strength. It is therefore, critical to understand the mechanisms involved in generating differences in glial activation, and calcium elevations. At the NMJ key transmitters involved in glial activation are the purines, and ACh.

Interestingly, in the CNS it has recently been shown that neurotrophins can induce calcium elevations in glial cells (Rose et al., 2003). Furthermore, the neurotrophins are known for the roles in modulating short-, and long-term events in neurons and different types of glia. I therefore, wondered if the neurotrophins could be implicated in the induction or modulation of glial calcium signalling at the NMJ, since they are well known for their involvement in the development and plasticity of this synapse.

In this article I investigate the ability of neurotrophins to activate glial calcium signalling. Furthermore, I study the involvement of neurotrophins in modulating glial calcium signalling, a process that could profoundly influence neuron-glial interactions and synaptic function.

In the following pages readers will find the article:

Todd, KJ, Auld, DS and Robitaille, R (2007) Neurotrophins modulate neuron-glia interactions at a vertebrate synapse. European Journal of Neuroscience 25: 1287-96.

3.2. ARTICLE: NEUROTROPHINS MODULATE NEURON-GLIA INTERACTIONS AT A VERTEBRATE SYNAPSE

Keith J. Todd, Daniel S. Auld, and Richard Robitaille

Département de physiologie and Centre de Recherche en Sciences Neurologiques, Faculté de médecine,
Université de Montréal, C.P. 6128 Succ. Centre-ville,
Montréal, Québec, Canada, H3C 3J7

Corresponding Author: Dr. Richard Robitaille
Département de physiologie, Faculté de médecine, Université de Montréal, C.P. 6128 Succ. Centre-ville,
Montréal, Québec, Canada H3C 3J7
Phone : (+1) 514-343-6111 ext. 1964
Fax : (+1) 514-343-2111
E-mail: [redacted]

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3.2.1. Abstract

Neurotrophins are important modulators of synaptic function at both developing and mature synapses in the CNS and PNS. At the neuromuscular junction (NMJ), neurotrophins, as well as perisynaptic Schwann cells (PSCs) are critical for the long-term maintenance and stability of the synapse. Considering this correlation and the acute interactions that occur at the synapse between PSCs and the nerve terminal, we wondered if neurotrophins could also be involved in neuron-glia signalling. To test if neurotrophins were able to signal to PSCs we used brief applications of NT-3, BDNF or NGF (100ng/ml). *Soleus* muscles of mice were incubated with the Ca^{2+} indicator Fluo-4AM and Ca^{2+} responses in PSCs were elicited through nerve stimulation (50 Hz, 30s). Our results indicate that acute application of both NT-3 and BDNF, but not NGF, increased PSC Ca^{2+} responses. Investigation of the mechanisms involved in these increases revealed distinct pathways for BDNF and NT-3. BDNF increased PSC responsiveness through potentiation of ATP responses while NT-3 modulated muscarinic acetylcholine receptor signalling. Using local applications of the neurotrophins, we found that both neurotrophins were able to elicit Ca^{2+} responses in PSCs where BDNF used a PLC-IP_3 mechanism, while NT-3 required extracellular Ca^{2+}. Our results demonstrate a neurotrophin-dependent modulation of neuron-glia signalling through differential mechanisms employed by NT-3 and BDNF. Hence, neurotrophins precisely and differentially regulate PSC functions through modulation of either purinergic or cholinergic signalling pathways.

3.2.2. Introduction

Neurotrophins are important modulators of synaptic function at both developing and mature synapses in the CNS and PNS. They regulate

transmitter release and modulate short and long-term synaptic plasticity events. In addition, they regulate synaptic morphlogical plasticity and stability. Glial cells are an important source of neurotrophins in the CNS and PNS (Alderson et al., 2000; Lessmann et al., 2003), suggesting that they might be in part responsible for neurotrophin-mediated regulation of neuronal function. This is in line with recent evidence demonstrating an active role for glial cells in the regulation of neuronal activity and synaptic transmission (Auld and Robitaille, 2003b; Haydon and Carmignoto, 2006). Hence, these observations point to the possibility that the interactions between glial cells and synapses may be modulated or governed by neurotrophins.

The neuromuscular junction (NMJ) is an excellent model for investigating this question. Indeed, neurotrophins are expressed and released in an activity-dependent manner in immature and culture preparations (Liou and Fu, 1997; Xie et al., 1997; Loeb et al., 2002). Neurotrophin receptors are important for acetylcholine receptor clustering and maintenance of the NMJ structure (Gonzalez et al., 1999) and interference with neurotrophin expression can lead to severe pathology (Belluardo et al., 2001). Among the receptors expressed by PSCs are the pan-neurotrophin receptor $p75^{NTR}$, TrkC and truncated TrkB (Frisen et al., 1993; Funakoshi et al., 1993; Gonzalez et al., 1999; Alderson et al., 2000).

In addition, perisynaptic Schwann cells (PSCs), glial cells at the NMJ, participate in short- and long-term functions of the NMJ (Auld et al., 2003; Feng et al., 2005). They respond to nerve activity (Jahromi et al., 1992; Reist and Smith, 1992; Rochon et al., 2001) and in turn, rapidly modulate NMJ function (Robitaille, 1998; Castonguay and Robitaille, 2001). Also, PSCs are essential for long-term support and maintenance of the NMJ (Reddy et al., 2003). Furthermore, PSCs are sensitive to long-term loss of synaptic activity and respond through sprouting of processes and altered gene expression (Reynolds and Woolf, 1992; Georgiou et al., 1994; Son and Thompson, 1995b). Interestingly, support for the involvement of glial cells in neurotrophin-

mediated synaptic function and maintenance has emerged (Elmariah et al., 2005a; Pitts et al., 2006). Thus, based on these observations, we hypothesized that neurotrophins modulate neuron-glia interactions and PSC signalling knowing that activity-dependent release of neurotrophins is important for the stability of pre-, post- and perisynaptic elements of the NMJ.

Using the mouse *soleus* muscle preparation, we found that short (30 min) applications of BDNF and NT-3, but not NGF, increased amplitude and duration of evoked PSC Ca^{2+} responses. Investigation of the pathways involved revealed that ATP signalling was increased in response to BDNF, whereas NT-3 increased mAChR responses. Furthermore, BDNF and NT-3 signalled through different Ca^{2+}-mediated pathways. These results demonstrate differential neurotrophin modulation of glial responses to neurotransmission and provide evidence for acute neurotrophin modulation of the tripartite synapse. Some of these results have been previously published in abstract form (Todd and Robitaille, 2005).

3.2.3. Materials and Methods

Animals and Dissection

All experiments were performed in accordance with the guidelines of the Canadian Council of Animal Care and the Université de Montréal. Adult (P40-60) male CD-1 mice (Charles River) were injected with a lethal dose of Ketamine/Xylazine cocktail. Following loss of pain reflexes, *soleus* muscles were removed and pinned in oxygenated saline (in mM): NaCl 124, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2, $NaHCO_3$ 1, $NaPO_4$ 1.25, glucose 10 and HEPES 25.

Ca^{2+} Imaging

Dissected *soleus* muscles were incubated in 10 μM Fluo-4AM (Molecular Probes) containing 0.02% pluronic acid (Molecular Probes) for 1.5 hr at room temperature. For 30 min applications of neurotrophins, 100 ng/ml of BDNF, NT-3 or NGF was added to the loading solution for the final half-hour. Muscles were then washed with saline containing 20 μM TPEN (Molecular Probes) for 10-20 min. Perfusion saline was then heated to 32-34 °C for the duration of experiments. NMJs and PSCs were located under bright-field optics for positioning of pipettes used for local drug applications. All junctions used were on the surface of muscle fibers. Local applications of agonists (200 msec, 10-20 PSI) were performed using a Picospritzer II (Parker Instruments) and glass micropipettes with tip diameter of 3-4 μm. Evoked Ca^{2+} responses were obtained by stimulating the tibial nerve at 50 Hz, 30 s with glass suction electrodes at 2X threshold for contraction. Epi-fluorescent images were acquired on a Nikon E600N upright microscope fitted with a Princeton Instruments CCD-1300 camera. Digital acquisition was performed using MetaFluor software (Perkin Elmer) driving a Lamda 10-2 shutter wheel (Sutter Instruments). Images were acquired at a rate of 1-2 images per second. Peak fluorescence change was calculated as relative

change from baseline using the formula: $\Delta F/F\% = (F-F_0)/F_0 * 100$. For all experiments 4-8 muscles were used with a minimum of 10 NMJs.

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except atropine (RBI Chemicals, MA), adenosine, CPA, ω-conotoxins and K252a (Calbiochem, La Jolla, CA). NT-3 and BDNF were kindly provided by Regeneron Pharmaceuticals, Inc. (Tarrytown, NY).

Statistical Analysis

Data conforming to tests of normality were treated with Student's t-test for comparison of two values or ANOVAs for multiple treatments with post hoc Tukey test to compare pairs. Significance was assessed at P=0.05.

3.2.4. Results

BDNF and NT-3 modulate PSC responses

To investigate the capacity of neurotrophins to modulate neuron-glia interactions we used Ca^{2+} imaging to monitor glial responsiveness. The neurotrophins BDNF, NT-3 and NGF were applied individually to *soleus* muscle preparations for 30 minutes at a concentration of 100 ng/ml. Following this application, Fluo-4AM-loaded PSCs were imaged during stimulation of the tibial nerve at 50 Hz for 30 seconds. Ca^{2+} responses were recorded and analyzed for amplitude, duration, time to peak, latency of response onset and area under the curve. Figure 1A depicts examples of evoked Ca^{2+} responses. We found that the $\Delta F/F_0$ amplitude of PSC Ca^{2+} responses (6.8 ± 1.2, n=9) was increased significantly (ANOVA, $p \leq 0.05$) by NT-3 application (12.8 ± 2.0, n=14) but not by BDNF (9.6 ± 1.1, n=12) (Fig. 1B). Furthermore, the duration of these responses (14.2 ± 2.8s) were increased significantly (ANOVA, $p \leq 0.05$) by BDNF but not NT-3 (BDNF, 26.7 ± 3.8s; NT-3, 21.6 ± 4.0s; Fig. 1C). However, when the overall size of the Ca^{2+} responses was investigated using analysis of the area under the curve, both BDNF (117.7 ± 25.8 U^2) and NT-3 (133.9 ± 37.9 U^2) significantly increase responses over control (42.1 ± 3.6 U^2; Fig. 1D, ANOVA $p<0.05$). Time to peak was increased slightly by both neurotrophin applications but did not reach significance (ANOVA, $p>0.05$) (control, 3.4 ± 1.0s; BDNF, 8.9± 1.8s; NT-3, 7.6 ± 1.8s). Similarly, the latency for response onset was decreased by both neurotrophins but not significantly (control 9.8 ± 3.0s; BDNF, 3.2 ± 1.0s; NT-3, 7.2 ± 2.3s) (Fig. 1).

To investigate the involvement of the Trk and $p75^{NTR}$ in the modulation of glial Ca^{2+} signals we bath applied NGF (100 ng/mL, 30 min). Since TrkA receptors have never been reported at the NMJ, this neurotrophin should only activate $p75^{NTR}$. Therefore, if $p75^{NTR}$ were involved in modulating glial Ca^{2+} signals we would observe a similar effect with NGF as seen with BDNF or NT-3. However, NGF had no effect on glial Ca^{2+} responses as they had

similar kinetics to those in control conditions (Fig. 1A). The $\Delta F/F_0$ amplitude of PSC Ca^{2+} responses (8.6 ± 1.5, n=8) was not different from control nor was the duration (13.6 ± 3.0s) or area under the curve (55.6 ± 14.8 U^2). These results indicate that the $p75^{NTR}$ is likely not solely involved in modulating glial Ca^{2+} signals at the NMJ.

Hence, these results demonstrate that neurotrophins potentiate the responsiveness of PSCs and alter neuron-glia signalling through increasing PSC Ca^{2+} responses elicited by synaptic activity and acute synapse-glia interactions.

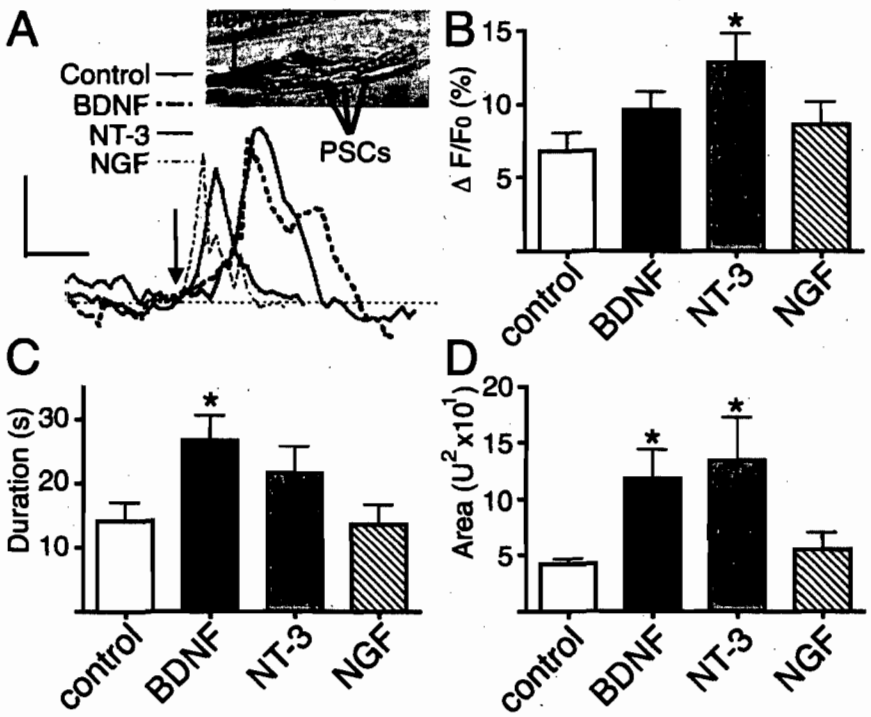


FIGURE 1. EVOKE CA^{2+} RESPONSES IN PSC ARE MODULATED BY NEUROTROPHINS. (A) Representative examples of evoked Ca^{2+} changes recorded in PSCs in control (black line), following BDNF (dashed black line) and NT-3 (grey line) and NGF (grey dashed line) application. Responses were aligned by their onset (arrow). Scale bars are 5% $\Delta F/F_0$ and 10 s duration. The horizontal dashed grey line indicates the origin. Inset, representative *soleus* NMJ with nerve and PSCs indicated. (B) Histogram of the $\Delta F/F_0$ amplitude of evoked Ca^{2+} responses in PSCs.

Responses are increased following 30 minute applications of NT-3 and to a lesser degree of BDNF with no effect of NGF. (C) Histogram of the duration of evoked Ca^{2+} responses in PSCs. Responses are increased by BDNF application for 30 minutes and to a lesser degree by NT-3 with no effect of NGF. (D) Histogram of the area under the curve (arbitrary units), a measure of overall Ca^{2+} response. Both BDNF and NT-3 significantly increased overall Ca^{2+} responses in PSCs, however, NGF had no effect. * indicates significance at $p<0.05$.

BDNF and NT-3 modulation is target specific

To further investigate what mechanisms were responsible for the changes in PSC responses, we tested whether neurotrophin treatment modulated responses to the major receptors involved in eliciting Ca^{2+} responses. These include ATP (P2) and adenosine (P1) receptors, as well as muscarinic acetylcholine receptors (mAChRs). To determine if neurotrophins might alter the function of these receptors we bath applied either vehicle, BDNF or NT-3 (100 ng/ml) for 30 minutes to Fluo-4-loaded muscles. We then locally applied agonists for the different receptors.

When ATP (20 μM) was locally applied following incubation with BDNF, responses were significantly increased in amplitude ($\Delta F/F_0$; control, 33.6 ± 2.0 %, n=44; BDNF, 45.3 ± 2.4 %, n=58; ANOVA, $p<0.001$) and duration (control, 41.1 ± 3.3s; BDNF, 55.0 ± 3.8s; ANOVA, $p<0.001$) (Fig. 2A). Furthermore, BDNF increased the overall Ca^{2+} response as revealed by analysis of the area under the curve (control, 746.4 ± 107.6 U^2; BDNF, 1381.0 ± 130.6 U^2; ANOVA, $p<0.001$). However, NT-3 application had no effect (post hoc Tukey $p>0.05$) on response amplitude (29.9 ± 1.8 % $\Delta F/F_0$, n=34), duration (37.1 ± 2.4s) or area under the curve (507.4 ± 40.7 U^2). Finally, neither neurotrophin caused changes in response rise time or latency (data no shown).

PSC Ca^{2+} responses evoked by local application of adenosine (20 μM), were unaltered by BDNF in terms of amplitude, rise time or latency. However, response duration was decreased (control, 31.9 ± 3.0s, n=31; BDNF, 17.0 ± 1.6s, n=27; ANOVA, $p<0.001$) (Fig. 2B). This reduction in response duration

translated into an overall decrease in Ca^{2+} response as indicated by the reduction of the area under the curve (control, 375.0 ± 56.4 U^2; BDNF, 214.8 ± 26.1 U^2; ANOVA, $p<0.05$). NT-3, however, had no effect (post hoc Tukey, $p>0.05$) on adenosine-induced Ca^{2+} signals in PSCs (duration = 24.9 ± 2.5s, n=28, area under the curve = 271.4 ± 32.5 U^2).

Although NT-3 had no effect on purinergic Ca^{2+} signalling, it did significantly alter mAChR-mediated Ca^{2+} responses. Application of muscarine (20 μM) following incubation with NT-3 resulted in increased duration (ANOVA, $p=0.002$) of PSC responses (control, 14.6 ± 1.3s, n=28; NT-3, 26.8 ± 2.6s, n=35) (Fig. 2C). Figure 2C also illustrates that the increase in duration was mirrored in the overall size of Ca^{2+} responses (control, 164.4 ± 22.0 U^2; NT-3, 242.8 ± 34.5 U^2; ANOVA, $p\leq0.05$). The time to peak and latency of muscarine-induced PSC responses were unchanged by either neurotrophin (data not shown). Furthermore, BDNF had no effect on response duration (22.2 ± 2.9s, n=25) or the area under the curve (193.0 ± 36.8 U^2, Fig. 2C). Hence, these results demonstrate that NT-3 and BDNF differentially modulate glial responsiveness.

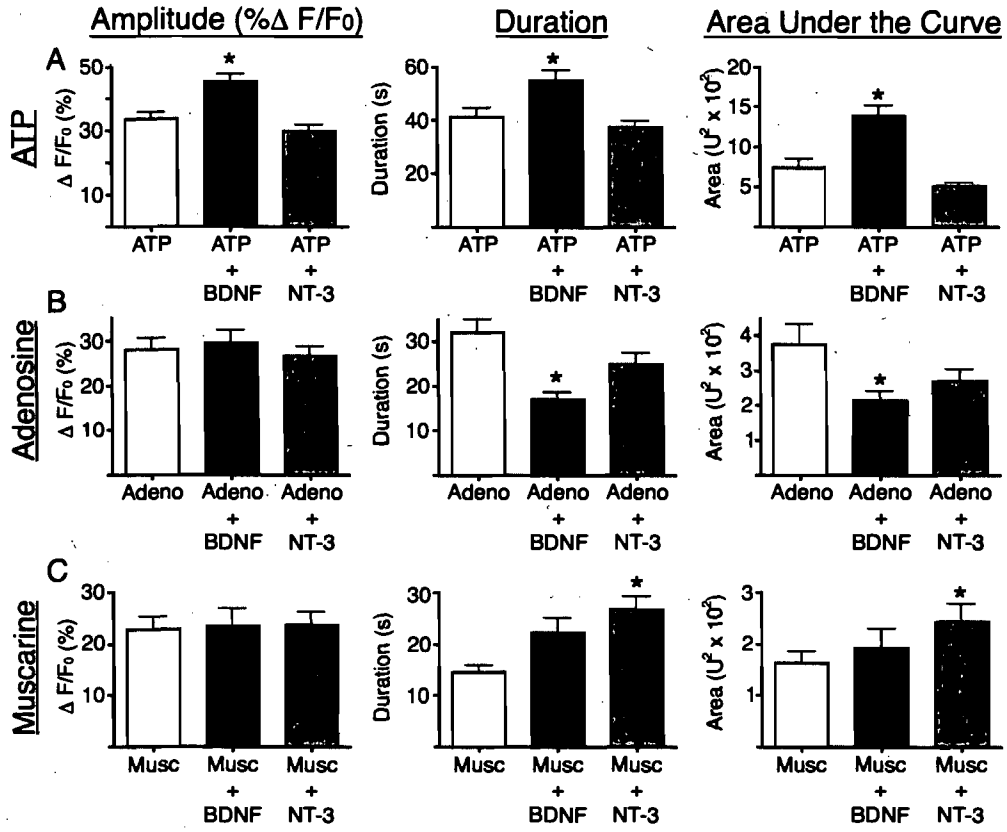


FIGURE 2. BDNF AND NT-3 DIFFERENTIALLY ALTER PSC RECEPTOR SIGNALLING. (A) Histograms of Ca^{2+} responses elicited by local application of ATP (20 μM) were increased in $\Delta F/F_0$ amplitude and duration by BDNF but not NT-3. This resulted in an overall increase of Ca^{2+} responses (area under the curve) by BDNF. (B) Histograms of adenosine-evoked responses show a decrease in the duration and overall Ca^{2+} response (area under the curve) with BDNF but not NT-3 application. (C) Histograms of Ca^{2+} responses evoked by application of muscarine (20 μM) show increased duration and overall response (area under the curve) with NT-3 but not BDNF application. Significance measured as $p<0.05$.

Neurotrophins activate Ca^{2+} signalling pathways

We next wanted to determine the signalling mechanisms used by BDNF and NT-3 to modulate PSC Ca^{2+} responses, as they are important for many glial functions. A potential signalling pathway is through Ca^{2+} itself as

Trk receptors contain a phospholipase C activation domain. Such a pathway can initiate Ca^{2+} signalling in cerebellar glia (Rose et al., 2003). To investigate neurotrophin-induced Ca^{2+} signalling in PSCs, we used local applications of neurotrophins, which enabled us to control the onset of responses.

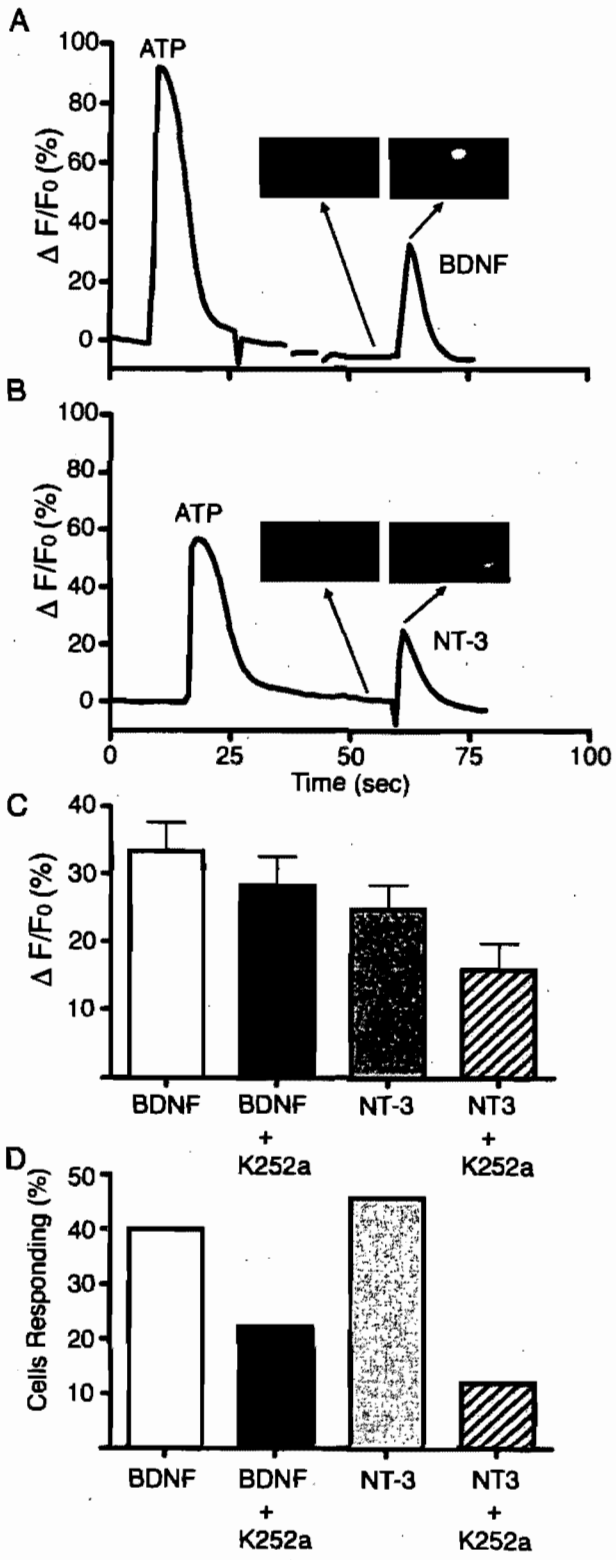
First, we monitored PSC Ca^{2+} elevations in response to brief (200ms) local applications of NT-3 and BDNF (100ng/ml in pipette). PSC Ca^{2+} responses induced by local application of ATP (20μM in the pipette) were used as positive controls since 100% of PSCs responded robustly and repetitively to ATP application (Rochon et al., 2001). To ensure that ATP was not interacting with neurotrophin signalling, which has been described in other preparations (Arthur et al., 2005), we measured Ca^{2+} responses with and without the initial ATP application. Prior application of ATP did not affect PSC responses to NT-3 or BDNF (data not shown).

As shown in Figure 3(A, B), acute, local applications of BDNF or NT-3 both elicited Ca^{2+} responses in PSCs. These responses had average amplitudes (Fig. 3C) of 33.4 ± 4.3% for BDNF (n=24) and 24.9 ± 3.6% for NT-3 (n=27) with average durations of 18.6 ± 2.6 s (BDNF) and 17.1 ± 2.3 s (NT-3), respectively. Neurotrophin-evoked responses were not significantly different from one another but were significantly smaller (ANOVA, $p<0.05$) than ATP responses, both in amplitude (70.2 ± 3.7 %ΔF/F) and duration (34.4 ± 2.2 s). Also, as shown in Figure 3D, 24 of 60 cells responded to BDNF application while 27 of 59 responded to NT-3 representing 40 and 46 % respectively, under control conditions. These results suggest that not all PSCs responded to our local neurotrophin applications in an identical fashion. For example, at a given NMJ not all PSCs responded to the neurotrophins with Ca^{2+} increases, whereas at others all would respond, and at others still there would be no responders.

Neurotrophin receptors have been shown to occur in full-length and truncated forms (Klein et al., 1990; Tsoulfas et al., 1993; Valenzuela et al., 1993). Truncated receptors lack intracellular kinase domains, but retain

certain signalling functions (Baxter et al., 1997; Yacoubian and Lo, 2000; Rose et al., 2003). To inhibit full-length Trk receptor tyrosine kinase signalling we investigated whether PSC responses to BDNF or NT-3 were altered in the presence of the tyrosine kinase inhibitor K252a (200nM). As shown in Figure 3C, we found that the amplitude of Ca^{2+} responses was not significantly altered by K252a (BDNF+K252a 28.3 ± 4.3%, n=8; NT-3+K252a 15.8 ± 3.9%, n=6) (T-test, $p>0.05$) in cells that displayed a Ca^{2+} increase in response to neurotrophins. Intriguingly, however, the percentage of cells responding was reduced. In the presence of K252a, BDNF induced responses in 8 of 36 cells in comparison to 24 of 60 cells. This represents a reduction of 44.5% from control. Also, NT-3 induced responses in only 5 of 42 cells, compared to 27 of 59 cells in control, representing a reduction of 73.8% in responding cells (Fig. 3D).

Together, these results demonstrate that PSCs rapidly respond to NT-3 and BDNF with increases in intracellular Ca^{2+}. Moreover, our results suggest that TrkB and TrkC associated tyrosine kinase activity is critical for generating a Ca^{2+} response to BDNF or NT-3, respectively, in many PSCs. Interestingly, those PSCs that do respond to BDNF or NT-3 in the presence of the Trk-selective tyrosine kinase inhibitor K252a do so with a normal amplitude, suggesting that in these cells Trk tyrosine kinase activity does not contribute, even in part, to generating the Ca^{2+} response. The PSCs, whose neurotrophin-induced Ca^{2+} responses are not sensitive to K252a likely respond to BDNF through truncated TrkB receptors, which are not sensitive to inhibition by K252a.


A
ATP
BDNF
Δ F/F0 (%)
100
80
60
40
20
0
B
ATP
NT-3
Δ F/F0 (%)
0
25
50
75
100
Time (sec)
C
Δ F/F0 (%)
40
30
20
10
BDNF
BDNF + K252a
NT-3
NT3 + K252a
D
Cells Responding (%)
50
40
30
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BDNF
BDNF + K252a
NT-3
NT3 + K252a

FIGURE 3. NT-3 AND BDNF INDUCE Ca^{2+} RESPONSES IN PSCs.
Representative Ca^{2+} responses induced by a focal application of (A) BDNF (100ng/ml) or (B) NT-3 (100ng/ml). Neurotrophins were pressure ejected from glass micropipettes (200msec, 10-20 PSI). The responses to neurotrophins were preceded by a local application of ATP. The neurotrophin responses were compared to ATP responses evoked on the PSCs. (C) Histogram representing the amplitude of Ca^{2+} responses (%ΔF/F) of cells responding to neurotrophin application before and after bath application of the Trk receptor antagonist K252a (200nM). There was no significant difference in the average response amplitude observed in the presence of K252a for either BDNF or NT-3 (T-test, $p>0.05$). (D) Histogram representing the number of cells responding to neurotrophin application before and after bath application of the Trk receptor antagonist K252a (200nM). Note that the number of responsive cells was reduced by K252a.

BDNF signals through the PLC-IP_3 cascade

We next investigated the signalling pathway associated with BDNF responses. To test if BDNF utilizes an intracellular source of Ca^{2+}, we removed extracellular Ca^{2+}, which should have no effect on signalling if intracellular stores are employed. In the presence of this solution the amplitude of BDNF-induced responses was similar to that induced in normal Ca^{2+} solution (38.0 ± 4.3 %, T-test $p>0.05$) whereas 28 of 64 cells responded, a rate similar to control (Fig 4). This result suggests that BDNF does not require extracellular Ca^{2+} and is operating through internal stores. To further investigate this possibility, we applied cyclopiazonic acid (10 μM), an inhibitor of Ca^{2+}-ATPase pumps that empties internal Ca^{2+} stores. Under these conditions, no cells responded to BDNF application (0 of 29 cells, Fig. 4). These results confirm that BDNF utilizes internal stores to induce Ca^{2+} elevations.

Of the potential intracellular sources of Ca^{2+}, it has previously been shown that Trk receptors can activate PLC resulting in the production of IP_3 and DAG (Huang and Reichardt, 2003). Furthermore, this pathway was shown to be recruited in response to BDNF in other glia (Rose et al., 2003). For these reasons it is likely that the PLC-IP_3 cascade is employed in BDNF

signalling, downstream of TrkB. To test this possibility, the PLC inhibitor U73122 (5 μM) was bath applied. In these conditions, only 1 of 43 cells responded (Fig. 4A), representing a reduction of 94.3% from control. The inactive form of the PLC inhibitor (U73343) had no effect with 22 of 52 cells responding (Fig. 4A) and showed no significant difference in the amplitude of BDNF responses (43.4 ± 4.8%; $p>0.05$; Fig. 4B). Finally, we bath applied the IP_3 receptor inhibitor 2-APB (50 μM) prior to applying BDNF. In the presence of 2-APB, 10 of 45 cells responded, representing a reduction of 44.5% from control. The amplitude of Ca^{2+} responses was unchanged in the presence of 2-APB (35.6 ± 8.5%; $p>0.05$; Fig. 4B). These data indicate that the PLC-IP_3 pathway mediates BDNF-induced Ca^{2+} responses in PSCs.

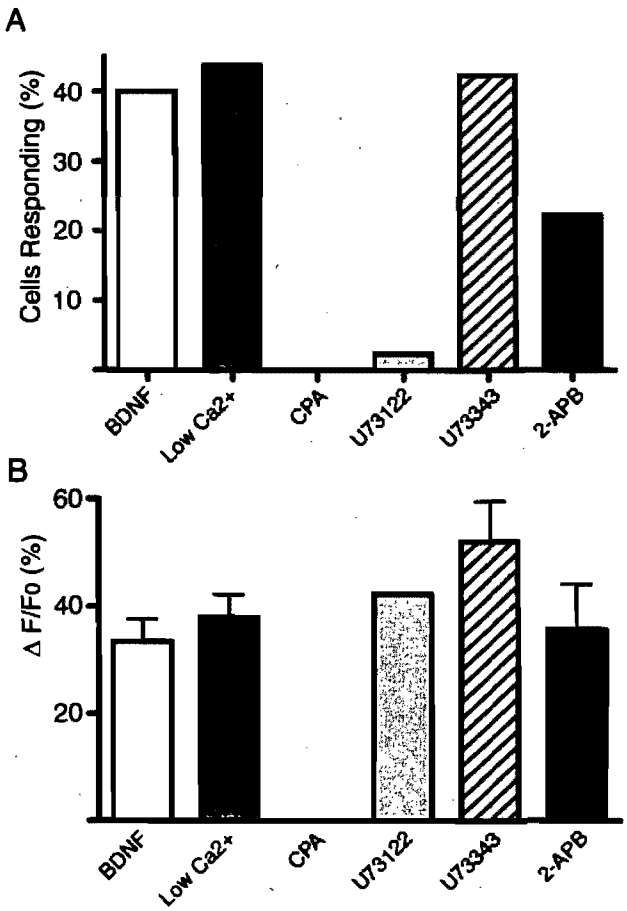

FIGURE 4. BDNF SIGNALS THROUGH ACTIVATION OF PLC-IP_3 CASCADE.
(A) Histogram representing the number of cells responding to BDNF application before and after various treatments. The application of BDNF in low Ca^{2+} extracellular solution had no effect on the percentage of cells responding, suggesting an intracellular source of Ca^{2+}. Bath application of CPA (10 μM) emptied internal stores and prevented BDNF-induced responses. Percentage of cells responding to BDNF-induced responses was reduced in the presence of the PLC inhibitor U73122 (5 μM), but not by the inactive form U73433 (5 μM). Fewer cells also responded to BDNF in the presence of 2-APB (50 μM). (B) Histogram representing the amplitude of Ca^{2+} responses (%ΔF/F) of cells responding to BDNF application before and after bath application of the same treatments as in A. No significant effect on average response amplitude was seen in the presence of any of the inhibitors, although no responses were seen in the presence of CPA and only one response was recorded in the presence of U73122 (ANOVA, $p>0.05$).

NT-3 signals indirectly through extracellular Ca^{2+}

Next, we investigated the NT-3-mediated signalling pathways involved in PSC activation. We first removed Ca^{2+} from the bath solution to investigate the role of intracellular stores in NT-3 signalling. Unlike BDNF, application of low Ca^{2+} extracellular solution had a pronounced effect on the ability of NT-3 to induce Ca^{2+} responses in PSCs (Fig 5A). Only 6 of the 39 cells tested responded to NT-3, (a 66% reduction from control), indicating that NT-3 signalling requires influx of Ca^{2+} from the extracellular space. However, the amplitude of Ca^{2+} responses among cells that did respond to NT-3 in the presence of low extracellular Ca^{2+} (29.1 $\pm$ 3.8%) was not significantly different from control (Fig. 5B, $p>0.05$).

We investigated the involvement of voltage-gated Ca^{2+} channels in NT-3 signalling since, it has been shown that neurotrophins can activate them (Jimenez et al., 1997; Kovalchuk et al., 2002). We therefore, investigated their involvement in NT-3 signalling. Since only L-type Ca^{2+} channels have been described on frog PSCs (Robitaille et al., 1996), we thought it likely that they would be involved in this process. Indeed, only 7 of 34 cells responded to

local application of NT-3 in the presence of 10 μM nifedipine, an L-type Ca^{2+} channel blocker, representing a reduction of 56% (Fig. 5A). In addition to the L-type Ca^{2+} channels, the contribution of N and P/Q types was investigated as well since these channels are important for controlling the release of neurotransmitters at the mouse NMJ (Uchitel et al., 1992). Interestingly, the N-type Ca^{2+} channel blocker ω-conotoxin GVIA (1 μM) was also effective, and in its presence only 15 of 56 cells responded to NT-3, a reduction of 41% from control with NT-3 only (Fig. 5A). However, the most pronounced effect was observed with the P/Q-type channel blocker ω-conotoxin MVIIC (0.5 μM), where only 6 of 52 cells responded to NT-3, a reduction of 75% from control (Fig. 5A). There was no significant difference in the amplitude of NT-3-evoked Ca^{2+} responses recorded in the presence of the different channel blockers (Fig. 5B; ANOVA, $p>0.05$). These results suggest that NT-3 is acting through a variety of Ca^{2+} channels to activate PSCs.

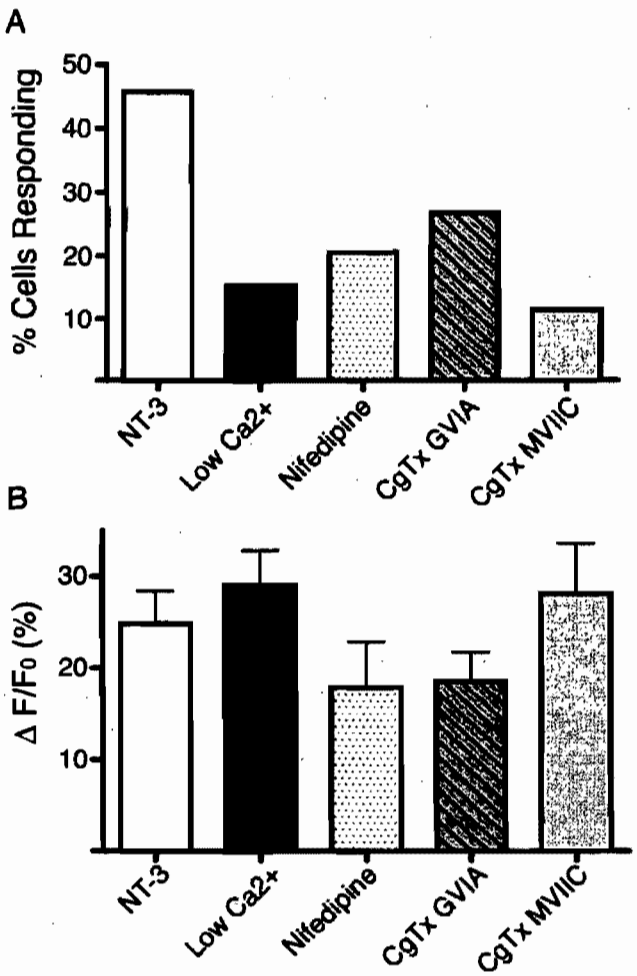

FIGURE 5. NT-3 REQUIRES EXTRACELLULAR Ca^{2+}.

(A) Histogram representing the number of cells responding to NT-3 application in control, in low Ca^{2+} solution and in the presence of various Ca^{2+} channel blockers. The percentage of cells responding to NT-3 was reduced in Ringer with no-Ca^{2+} added. Application of the L-type Ca^{2+} channel blocker nifedipine (10 μM) reduced responding cells as well as in the presence of the N-type Ca^{2+} channel blocker ω–conotoxin GVIA (1 μM). The greatest reduction was observed in the presence of the P/Q-type Ca^{2+} channel blocker ω–conotoxin MVIIC (0.5 μM. (B) Histogram representing the amplitude of Ca^{2+} responses (%ΔF/F) of cells responding to NT-3 application before and in the same conditions as in A. No significant change in amplitude was observed in the presence of any of the channel blockers (ANOVA, $p>0.05$).

Indirect effect of NT-3

Since our results suggest that NT-3 acts through modulating Ca^{2+} channel activity we thought that this might be an indirect effect involving different cell types since only L-type channels were found on amphibian PSCs (Robitaille et al., 1996). Hence, we tested whether NT-3-induced Ca^{2+} responses in PSCs were elicited by a neurotransmitter substance released from either the pre- or postsynaptic compartments. We hypothesized that acetylcholine or ATP may be the transmitter mobilized by NT-3 as these are the two most efficacious activators of PSCs (Rochon et al., 2001) and NT-3 has been shown to induce acetylcholine release at immature nerve muscle-synapses in culture (Lohof et al., 1993; Wang et al., 1995). Acetylcholine would act through mAChRs whereas ATP could activate purinergic P1 and P2 receptors.

To test if the NT-3-induced Ca^{2+} responses involved mAChRs we bath applied the general antagonist atropine (20 μM), known to block muscarinic receptors of mouse PSCs (Rochon et al., 2001). This antagonist had no effect on the number of cell responding (23 of 49) in comparison to control. Furthermore, the amplitude of responses was also unchanged (36.9 ± 4.7%; Fig. 6). To investigate if ATP was the intermediary transmitter, we perfused the general P2 receptor antagonist suramin (200 μM) for 15 minutes before and during the NT-3 application. In the presence of suramin, only 5 of 44 cells responded, representing a reduction of 75.1% from control (n=44; Fig. 6A). The five cells that did respond in the presence of the antagonists showed no significant difference in amplitude (16.0 ± 1.9; Fig 6B; ANOVA $p>0.05$). These results suggest that NT-3 acts indirectly through mobilization of ATP to activate PSC purinergic receptors. ATP mobilization could occur from a number of sources including the presynaptic nerve terminal, the postsynaptic muscle fiber or from PSCs themselves.

In order to test the involvement of the presynaptic terminal in this phenomenon we performed electrophysiological recordings from muscle fibers while applying NT-3. However, consistent with previous work by

Ribchester and colleagues (1998) both spontaneous activity and evoked responses were unaltered by NT-3 (data not shown).

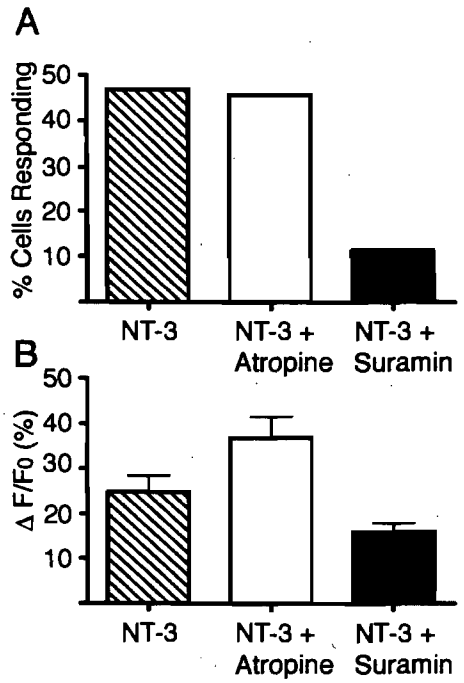

FIGURE 6. NT-3 ACTIVATES PSCS INDIRECTLY THROUGH PURINERGIC RECEPTORS. (A) Histogram of the percentage of cells responding to focal application of NT-3 in control and in the presence of the muscarinic receptor blocker atropine or the purinergic receptor antagonist suramin. 42 % of cells responded in the presence of atropine (20 μM; n=49), while suramin (200 μM) reduced responses to 11.4% (n=44). (B) Histogram of the amplitude of Ca^{2+} responses (%ΔF/F) of responding cells elicited by NT-3 application in control and in the same conditions as in A. No significant difference in the amplitude was observed in the presence of either antagonist (ANOVA, $p>0.05$).

3.2.5. DISCUSSION

We have demonstrated for the first time the ability of neurotrophins to modulate neuron-glia interactions in an acute manner. Additionally, we have found that in the case of PSCs at the NMJ, BDNF and NT-3 seem to act through different mechanisms and have different down-stream targets (Fig.

7A). We have also shown that different neurotrophins use distinct Ca^{2+} signalling pathways (Fig. 7B).

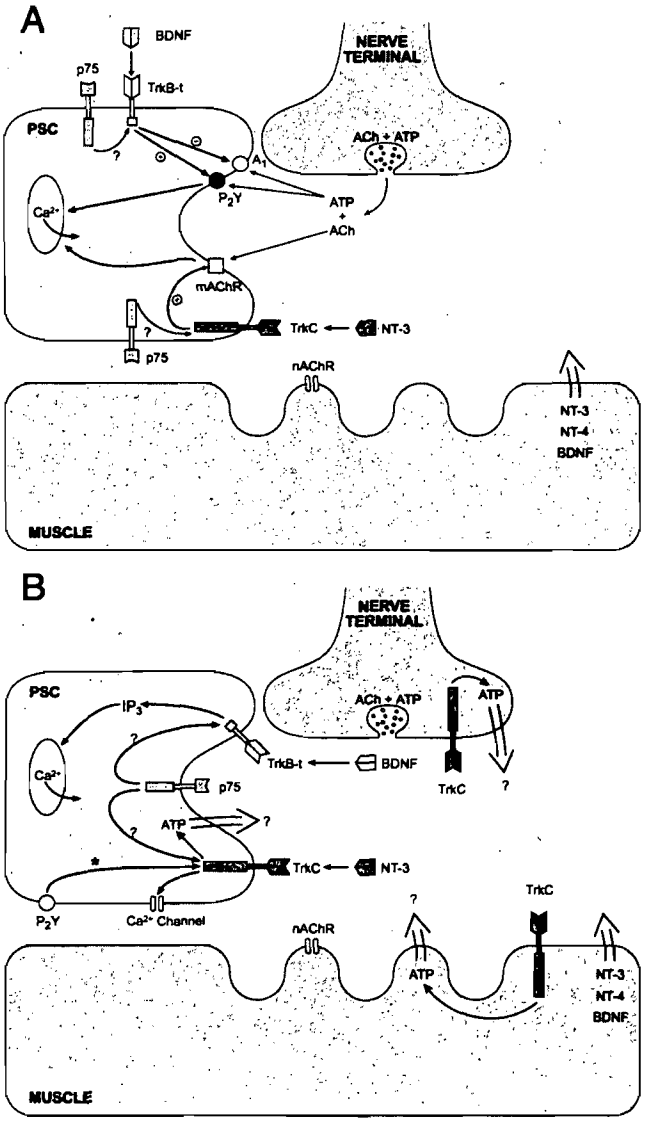

FIGURE 7. NEUROTROPHINS MODULATE PSC Ca^{2+} SIGNALLING AND INDUCE PSC Ca^{2+} RESPONSES.

(A) application of BDNF induces a reduction in adenosine-mediated Ca^{2+} signals in PSCs. In addition BDNF potentiates P2 receptor-mediated Ca^{2+} signals, resulting in an overall increase in PSC response to nerve activity. Application of NT-3 differentially modulates PSC responsiveness to nerve activity through potentiation of mAChR responses. It cannot be ruled out that p75NTRs are co-activated with

either Trk to mediate the signalling observed. (B) BDNF and NT-3 differentially induce PSC Ca^{2+} responses through IP_3 and external Ca^{2+} respectively. BDNF seems to directly induce Ca^{2+} responses through production of IP_3. Ca^{2+} responses induced by NT-3 are indirect requiring ATP. One possibility is that NT-3 induces the release of ATP from one of the 3 cellular compartments that then induces a Ca^{2+} response in PSCs. Another possibility is that NT-3 signalling is potentiated through trans-activation mediated by P2Y receptors (*).

Modification of PSC signalling

Here we report that not only do neurotrophins modulate PSC Ca^{2+} signalling during evoked activity, but also directly induce Ca^{2+} signalling. Interestingly, NT-3 and BDNF seem to have different modulatory actions on PSCs. Although each neurotrophin seems to change different properties of the Ca^{2+} responses (amplitude and duration by BDNF and only duration by NT-3), both result in increased Ca^{2+} signals as revealed by analysis of area under the curve. This indicates that neurotrophins increase PSC responsiveness to synaptic transmission at the mouse NMJ.

Importantly, each neurotrophin targets different mechanisms in PSCs: while NT-3 modulates mAChRs, BDNF interacts more effectively with the purinergic receptors (Fig. 7A). Not only does BDNF increase ATP-mediated signalling, it also decreases adenosine signalling. This further illustrates the fine level of regulation mediated by neurotrophins. The BDNF effect on modulating purinergic receptors is not too surprising since reciprocal neurotrophin-purinergic interactions has previously been described in multiple systems (Lee and Chao, 2001; Arthur et al., 2005). However, to our knowledge this is the first description of a modulation of mAChR function by neurotrophins. Although the interaction between these specific receptors is novel, many others have reported interactions between receptor tyrosine kinases (RTKs) and G-protein coupled receptors (Kotak et al., 2001; McLaughlin and Chavkin, 2001; Dalle et al., 2002; Nielsen et al., 2004).

Since NT-3 and BDNF clearly function by modulating different receptors, it stands to reason that these activations have different down-

stream effects on PSCs. Modulation of one cascade could occur independently from the other, in part through differential release of the neurotrophins (Brigadski et al., 2005). We hypothesize that the release of different neurotrophins is likely to be associated with changes in neuron-glia communication. These changes in neuron-glia communication may subsequently influence the long-term condition of the NMJ. In keeping with this, it has been shown that PSCs are critical for NMJ maintenance (Reddy et al., 2003) and PSCs themselves are dynamically regulated in reponse to synaptic activity (Georgiou et al., 1994; Georgiou et al., 1999; Love et al., 2003). Our data indicate that the presence of NT-3 or BDNF changes neurotransmission-associated signal transduction in PSCs, and would thus be likely to modulate responses dependent on synaptic activity that influence the stability and condition of the NMJ.

Do neurotrophins signal through Trk or p75NTR?

Knowledge of the receptors involved in neurotrophin modulation of PSC function would aid in further elucidating these processes. Our data showing that the selective Trk-kinase inhibitor K252a inhibits the Ca^{2+} response in the majority of PSCs in response to both NT-3 and BDNF suggests that full-length, kinase competent TrkC and TrkB receptors, respectively, are involved. This is consistent with the evidence that full-length TrkC has previously been reported in Schwann cells (Funakoshi et al., 1993; Offenhauser et al., 1995). However, there is no evidence for full-length TrkB receptor expression in Schwann cells (Gonzalez et al., 1999). The somewhat smaller effect of K252a on BDNF signalling may reflect a greater involvement of truncated TrkB or non-Trk receptor signalling in response to BDNF application. It is possible that full-length TrkB receptors are expressed by PSCs, but at a low level. Alternatively, it is possible that TrkB-mediated Ca^{2+} signalling is occurring through a similar mechanism to that reported by Rose and colleagues in Bergmann glia (Rose et al., 2003). These authors also

propose that $p75^{NTR}$ or some other signalling protein (Kryl and Barker, 2000) may be associated with truncated TrkB receptors.

However, the lack of effect of NGF suggests that sole activation of $p75^{NTR}$ is not sufficient to modulate glial Ca^{2+} signals. This would also suggest that pro-neurotrophins do not mediate the effects we observe. Owing to the presence of $p75^{NTR}$ in PSCs (Bandtlow et al., 1987; Heumann et al., 1987), a possibility might be that these receptors were jointly activated with Trk receptors. However, considering that NT-3 and BDNF mobilize extracellular and intracellular Ca^{2+} pools, respectively, it seems unlikely that activation of the same $p75^{NTR}$ receptor would be associated with such a radical difference in signalling. Accordingly, our data do not support the involvement of $p75^{NTR}$ in both modulations observed.

Distinct Ca^{2+} signals for NT-3 and BDNF

The mechanisms used by BDNF and NT-3 to induce Ca^{2+} signalling in PSCs are different, and provide a way for specific alteration of purinergic and muscarinic receptor pathways respectively (Fig. 7B).

The BDNF-dependent Ca^{2+} signals were somewhat expected considering that Trk receptors contain conserved PLC activation domains (Huang and Reichardt, 2003) and that a similar pathway was reported in cerebellar glia (Rose et al., 2003). However, the NT-3 mechanism suggested by our results is more novel.

Indeed, our results indicate that NT-3 signalling on PSCs is indirect, requiring the activation of purinergic receptors likely following the release of ATP (Fig. 7B). Furthermore, the Ca^{2+} elevation in PSCs requires the contribution of diverse Ca^{2+} channels. The presynaptic terminal is a possible site for NT-3 action since ATP is co-released with ACh at NMJs (Redman and Silinsky, 1994). However, in the case of our manipulations and those of others (Ribchester et al., 1998) no effect on vesicular release was found for either NT-3 or BDNF. One alternative might be that ATP is released by non-

vesicular mechanisms such as anion transporters (Liu and Bennett, 2003) or other non-selective ion channels such as certain types of purinergic receptors (Reisin et al., 1994). Another possibility is that it could involve the release of ATP from PSCs themselves (Fields and Burnstock, 2006), which would then act in an autocrine fashion. A third possibility is that ATP is released from postsynaptic muscle fibers acting as a retrograde signal to PSCs (Smith, 1991; Cunha and Sebastiao, 1993; Santos et al., 2003). Another consideration is that release of ATP and P2Y receptor activation during NT-3 signalling may results in transactivation of TrkC receptors (Fig. 7B). A similar process has been shown for TrkA receptors (Arthur et al., 2005) and this could lead to a potentiation of NT-3 signalling. This would explain the reduction in the presence of a purine receptor inhibitor.

It would have been interesting to test whether the Ca^{2+}-dependent mechanisms acutely activated by the neurotrophins are at play in the modulation observed on the synaptic-mediated interactions between the synapse and PSCs. However, this proved to be a difficult task owing to the fact that the same cellular Ca^{2+} mechanisms are involved in the genesis of the Ca^{2+} responses induced by the neurotrophins and by ATP and muscarine in PSCs.

The finding that ATP is involved in the NT-3 mediated Ca^{2+} signals would suggest that this pathway may be involved in the modulation of muscarine-induced Ca^{2+} responses. However, this is quite unlikely since ATP has never been found to modulate mAChR signalling in PSCs. At this point it is unclear what signalling cascade is responsible for the observed modulation of mAChRs, however, it is most likely distinct from the Ca^{2+}-dependent pathway we described here.

Significance of acute neurotrophin signalling

The observed potentiation of PSC responsiveness to synaptic activity strongly suggests that neurotrophins regulate the modulation that PSCs exert

at the NMJ. A first possibility might be that neurotrophins alter glia-to-neuron signalling as a consequence of the modulation of neuron-to-glia communication. Our laboratory has previously demonstrated that PSCs can alter synaptic function at the amphibian NMJ (Robitaille, 1998; Castonguay and Robitaille, 2001). It would be of great interest in future works to investigate the involvement of neurotrophins in similar PSC modulation of synaptic function. Although our results and those of others found no clear effect of neurotrophins on synaptic function at mature NMJs, modulation may occur at different developmental stages. For instance, neurotrophins can potentiate synaptic transmission at immature NMJs (Lohof et al., 1993; Wang et al., 1995; Liou and Fu, 1997). In such a situation perhaps neurotrophins are involved in altering PSC-mediated modulation of synaptic function to favor establishment and consolidation of the synapse.

A second possibility might be that the modulation of PSCs could alter the regulation of transcription or phosphorylation processes. For instance it is known that mAChRs can regulate the expression of glial fibrillary acidic protein (GFAP) in PSCs (Georgiou et al., 1994; Georgiou et al., 1999) whereas ATP receptors have no effect on this process, high-lighting the discrete actions of these two receptors. Also, although GFAP expression was not altered by purinergic receptor activation, it would be of interest to determine whether other genes may be regulated by purinergic receptor signalling as is the case for myelinating Schwann cells (Stevens and Fields, 2000). The end result of these modulations may be related to the long-term maintenance of the NMJ and may relay information concerning the functional state of the synapse.

Concluding Remarks

As a whole, the results presented here strongly argue for a fine and complex neurotrophin-mediated regulation of synapse-glia interactions by modulating the responsiveness of PSCs at the NMJ. Hence, it will be

particularly interesting to determine what impacts this neurotrophin-mediated modulation have on the functions associated with PSCs and their involvement in short-term and long-term regulation of the NMJ.

3.2.6. References

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3.3. Discussion of Chapter 3

These results demonstrate for the first time that neurotrophins are involved in acute synapse-glial interactions. One troublesome point that arose through the course of this study was the lack of a high percentage of junctions and PSCs that responded to the local neurotrophin application. Interestingly, at about the same time that my study was published, a report came out indicating that very little TrkC was expressed in adult PSCs (Hess et al., 2007). These results could explain the difficulty I had getting cells to respond to local application of the neurotrophins. It would also suggest that, when considering the results I did obtain, further investigation of neurotrophin signalling to glial cells during NMJ development could uncover even more in-depth interactions. A similar problem could also be associated with BDNF-TrkB signalling as it has been suggested that little of this full-length receptor is expressed in adult PSCs (Gonzalez et al., 1999). Considering this information it is likely that further investigation of neurotrophin involvement in neuron-glial interactions would be best completed in the context of development or perhaps injury.

An interesting aspect on neurotrophin signalling to PSCs comes from work done on myelinating Schwann cells. There are diverse functions for the neurotrophins in Schwann cell maturation, migration, and myelination. This would suggest that PSC development could also be regulated by neurotrophin signals especially considering that PSCs and myelinating Schwann cells are developmentally quite similar (Mirsky et al., 1996).

Avenues of study regarding neurotrophin signalling could include investigating their involvement in PSC migration or proliferation during early postnatal life as there is evidence for the involvement of NT-3 in this (Hess et al., 2007). Other potential roles could include regulating process elongation and elaboration. One problem with performing these studies is that it is next to impossible to isolate only PSCs and any sort of dissociation and sorting procedure would result in inclusion of myelinating Schwann cells as well as

PSCs. This limits these investigations to observations of what is occurring in intact systems and makes it difficult to perform experiments looking at the molecular pathways involved in the processes. However, efforts should be made to find ways of furthering our understanding of how neurotrophins are involved in neuron-glial interactions at the NMJ.

These results are most directly applicable to the role of neurotrophin signalling in modulation of glial cells throughout the nervous system. Although the mechanisms and cellular interactions may differ, the fact remains that neurotrophins are capable of acutely altering glial responsiveness and signalling. As shown in Chapter 2, subtle changes in glial calcium elevations can result in quite pronounced differences in synaptic function. Continued study in this area will likely reveal more diverse roles for the neurotrophins in glial cell function and neuron-glial interactions.

4. General Discussion and Conclusion

In this thesis I have addressed two main questions pertaining to neuron-glial interactions. The first, a more global question regards the sensitivity of glial cells to differences in neuronal activity. This project asked whether glial cells could: 1) distinguish between patterns of neuronal activity, which I showed that they can with different calcium elevations, and 2) what impact differences in glial activation has on synaptic function. These results impact the fields of synaptic plasticity and neuron-glial communication by indicating that glial responses are not simply all or none, but that these responses contain precise information that affects the expression of synaptic plasticity.

The second problem investigated in this thesis involved the plasticity of glial cell calcium elevations. I investigated this in the context of neurotrophin signalling to PSCs. I found that not only do glial cells of the NMJ respond acutely to neurotrophins, but also, longer exposure to them can alter glial signalling through other receptor systems. These interactions involving the neurotrophins could significantly influence neuron-glial communication and glial modification of synaptic function.

4.1. Neuron-Glial Interactions in Synaptic Plasticity

Expression of synaptic plasticity is dependent on a number complex processes. For instance, we now have a plethora of information indicating the importance of glial cells in these processes (Allen and Barres, 2005; Todd et al., 2006). The roles that glial cells have in plasticity include both long- and short-term events and occur in every region of the nervous system investigated thus far.

4.1.1. Glial Cells in Information Processing and Plasticity

In this section I will discuss the importance of information processing at the cellular level for synaptic function. A number of different events can be involved in processing synaptic information including rate and temporal coding, spike timing-dependent plasticity, changes in signal to noise ratio and synchronous activity. These phenomena and the potential roles of glial cells will be addressed in the following section.

4.1.1.1. Detection of Patterns

Processing of information by the brain is a complex task. Current thinking appears to be moving towards an inclusive view of coding being rate and temporally dependent (Magee, 2003; Harris, 2005). Aside from information coding, rate and temporal synaptic information has long been known to induce plasticity in neurons. The frequency of stimulation used can influence the outcome of plasticity (potentiation or depression), as can the pattern of neuronal activity in both CNS and PNS synapses (Magleby and Zengel, 1976; Nicoll and Schmitz, 2005). This control over plasticity is employed widely throughout studies of the nervous system.

The expression of plasticity, i.e. changes in synaptic efficacy, is normally thought to be a neuronal process as is information coding. However, we know that glial cells can be involved in plasticity events like synaptic scaling (Stellwagen and Malenka, 2006). Another mechanism of plasticity is spike timing-dependent plasticity, where the timing of incoming activity onto a postsynaptic neuron influences (i.e. increases or decreases) the strength of subsequent activity between those neurons (Sjostrom and Nelson, 2002). A recent modelling study illustrated that an individual neuron, integrating many inputs can "learn" a pattern by decreasing response delay to that repeated pattern (Masquelier et al., 2008). This shows that neurons can be sensitive to particular patterns of activity.

However, in the intact nervous system there are more cells involved in synaptic function than just the postsynaptic cell, and my work suggests that glia are also able to detect patterns of activity.

One interesting idea in neuronal coding is that since there is always some basal, background activity, as with the modelling study mentioned about these coding processes therefore must occur through detection of a particular signal occurring in the context of the underlying activity. This means that the signal must be sorted out of the "noise". With this in mind, it would be of great interest to know how glial cells would respond to a complex signal containing underlying oscillatory activity with a particular pattern imbedded in it. Would glial cells respond rhythmically, asynchronously or not at all to a background oscillatory pattern of activity? Additionally, would glial cells, like neurons in the modelling study mentioned above, then "tune" their responsiveness to a particular pattern of activity? One new piece of evidence that might be important for these questions comes from work on the somatosensory cortex. Carola Schipke showed that astrocytes of the barrel cortex respond to specific neuronal activity in their own barrel if it occurred in layer IV (Schipke et al., 2008). However, the same cells did not respond to spontaneous activity occurring in layers II/III. This suggests that they can distinguish "important" from "unimportant" information and are "tuned" to particular activity as they respond to the normal input pathway but not the background oscillations occurring in this region.

4.1.1.2. Synchrony

Synchronous activity is something that is thought to be important for information transfer. This is seen in neuronal oscillations and more recently, also as glial oscillations.

In the thalamus it has been shown that astrocytes have an intrinsic pacemaker rhythm of calcium elevations (Parri and Crunelli, 2001). Interestingly, the astrocyte oscillations appear to be coordinated in a localized region and include about 5 astrocytes that display coincidental activation (Crunelli et al., 2002). This stimulates the question of whether oscillations in astrocytes are at all coordinated with oscillations occurring in the neuronal network? From *in vivo* work we do know that glial cells of the cortex are intimately linked to ongoing neuronal oscillations (Seigneur et al., 2006) suggesting that they may indeed display coordinated activity with neurons. Either way it would be interesting since independent glial and neuronal networks, when aligned, could be a source of resolution in a noisy system. However, it seems more likely that oscillatory patterns in glial cell and neuronal networks would be linked in some way and a particular pattern occurring in the context of oscillations could be decoded by glia, postsynaptic neurons and perhaps by presynaptic neurons as well. All cellular components, perhaps, provide different bits of information to a complex puzzle. Importantly, however, what are the signals that lead to gliotransmission in a situation where glial oscillations are occurring or are they simply liberating gliotransmitter at each calcium elevation? Probably they are, since only about 4% of the thalamocortical astrocytes display this pacemaker-like activity it seem likely that they are providing drive for other astrocytes and/or neurons. Would these downstream glial cells then be synthesizing incoming information from a number of different cells, glial and neuronal alike? It seems probable that they could integrate signals from a number of different cells, since my data indicates that they can decode differences in neuronal activity, and that their signalling is modulated by different transmitters. Furthermore, Perea and Araque (2005) showed that glial cells respond differently to different inputs, therefore, suggesting that they are constantly integrating information to respond appropriately to a given context.

At the level of neurons, the glial calcium oscillations are reported to induce large, slow depolarizations of neighbouring neurons through activation of NMDA receptors (Parri et al., 2001). Recent reports from other laboratories have demonstrated that these large, slow NMDA receptor currents, mediated by astrocytes have the capacity to synchronize neurons (Angulo et al., 2004; Fellin et al., 2004). Although the relevance of this *in situ* has recently been called into question (Fiacco et al., 2007), it remains a tantalizing mechanism of regulating network function.

Oscillations of glial calcium oscillations may provide drive for neighbouring glia and neurons leading to synchronous activation. This synchrony may provide valuable information to ongoing processes.

Glial cells are capable of processing incoming information from neurons in a pattern-dependent manner. Studies from other labs further suggest glial involvement in the decoding of information from underlying noise and in the generation of synchronous oscillatory activity, which could be critical for information transfer.

4.1.2. From Detection to Perception: Glia Solve for Differences in Neuronal Activity

It has been over 15 years since the first reports of glial cells responding to nerve activity (Jahromi et al., 1992; Reist and Smith, 1992). These glial responses were later shown to be responsible for modifying synaptic function in a number of preparations. Additionally, one of these demonstrated that glial cells could bidirectionally modulate neuronal activity, however, the mechanisms were not determined (Newman and Zahs, 1998). Similarly, separate studies on the NMJ have shown that neuronal function can be both positively (Castonguay and Robitaille, 2001) and negatively (Robitaille, 1998) regulated by PSCs depending on the manipulation. These kinds of results lead to a working hypothesis that glial

calcium responses were all or none events that, when present, lead to a particular change in synaptic function. This would suggest that a calcium response in a particular glial cell could only be involved in one form of plasticity. This is somewhat contradictory to glial cells being active partners in synaptic function since it is clear that synapses must both up-, and down-regulate under different conditions. Therefore, other possible methods of neuron-glial communication have been suggested.

One hypothesis regarding the underlying control of positive versus negative modulation was derived from the two studies from Richard Robitaille's laboratory. It was suggested that glial calcium elevations were responsible for potentiation, while activation of other G-protein coupled receptors, for example Gi coupled receptors that are linked to adenylate cyclase activity, could be responsible for depression. This would certainly be a feasible mechanism for the separation of positive or negative signals at the receptor level. Certainly these mechanisms could be active and would be difficult to detect since our primary method of studying glial-synapse interactions involves calcium imaging, which may not be occurring under all conditions. However, my results suggest that glial cells can separate neuronal signals and provide positive and negative feedback to the synapse based solely on differences in the glial calcium signal. This arrives through the generation of single spike and oscillatory calcium elevations in response to different presynaptic activities. The surprising and most interesting discovery was that these differences in calcium elevation apparently translate the neuronal signal into tangible differences in how the glial cells respond. This type of idea is quite different from all-or-none responses where glial activation "state" might provide a more accurate indication of how synaptic function will be modified.

The idea that glial cells could be involved in coding and information processing in the brain is an intriguing possibility. It would revolutionize our thinking about many processes such as sensory perception and neuronal

network function. It seems that retinal Muller glial cells might be performing these functions (Newman and Zahs, 1998), although it has not yet been defined in this way. This would be an interesting system to investigate the ability of glial cells to perform discrimination tasks on different stimuli since the application of varieties of stimuli is relatively uncomplicated.

These results shift how we look at glial cell responses from being all-or-none to being varied with substantial differences in the resulting synaptic modulation. This provides a mechanism for glial cells to process incoming information and alter their functioning accordingly. Similar methods are probably employed by astrocytes in the brain, although the specifics are likely different. This evolution in our understanding of glial function moves these cells from bits of "glue" to intimately involved information processors.

4.1.3. Glial Cells Bidirectionally Modulate Synaptic Function

Over the past decade a number of studies have demonstrated the involvement of glial cells in synaptic function (Volterra and Meldolesi, 2005). As mentioned above, they can both positively and negatively modulate synaptic function, something that is dependent on glial decoding of synaptic information. Following this decoding glial cells provide feedback to synapses in a variety of ways.

4.1.3.1. Acute Feedback

Acute signalling by glial cells is known to modulated synaptic function. As mentioned earlier this occurs through the release of gliotransmitters. Most recently researchers have begun to investigate glial-to-neuron signalling at the single synapse level using direct activation of glial cells (Perea and Araque, 2007). This study revealed the ability of glial calcium elevations to potentiate an individual synapse in the hippocampus. Interestingly, similar to spike timing-dependent plasticity discussed in

neurons, coincidence of the glial signal and postsynaptic activation lead to prolonged potentiation. This suggests the involvement of glial cells in a process, previously thought to only to involve neurons.

Prior to this study, a number of different studies have shown the ability of glial cells to increase and decrease synaptic function (Auld and Robitaille, 2003b). Most of the information gained to date, however, provides evidence of how glial cells at a given synapse modulate synaptic efficacy in one direction or the other, but not both. However, for synapses to function properly, they must be able to rapidly modulate up and down, not just continually in one direction. The results that I present in Chapter 2 reveal this ability in glial cells. In the case of my study we suggest that glial cells are releasing different amounts of ATP to cause the differences in plasticity. However, it is also possible that glial cells could release different transmitters under different conditions, depending on how they were activated. This acute feedback to synapses has the possibility to rapidly modulate synaptic function.

4.1.3.2. Long-term Modulation

Interestingly, plasticity can be regulated over longer periods of time through changes in expression or through structural changes. It is now apparent that glial cells can influence these longer changes as well as the acute ones mentioned above. A good example of this was shown to occur in the hypothalamus where synaptic coverage by astrocytes is changed during lactation. These changes can have potent effects on how neurons communicate and can dramatically alter their activity (Piet et al., 2004; Panatier et al., 2006).

There is a certain amount of dendritic morphological plasticity in the normal brain (Grutzendler et al., 2002; Zuo et al., 2005; Xu et al., 2007) one could hypothesize that there is also a certain level of accompanying

glial plasticity as suggested in work on hippocampal slice culture (Haber et al., 2006). However, knowing that the choice of preparation greatly affects dendritic morphological plasticity (Xu et al., 2007), it is reasonable to suspect similar effects on glia. It would, therefore, be important to investigate the role of anatomical rearrangement between neurons and glia *in vivo*, an approach that would more strongly implicate glial synaptic associations in directly shaping synaptic plasticity. These adjustments could in fact alter the direction or extent of plasticity seen at synapses as well as the plasticity of neighbouring synapses.

Distinct calcium signals in glial cells could have a long-term influence on glial and synaptic morphological plasticity. As will be discussed below, calcium signalling could be important for regulating the actin cytoskeleton and the interaction of a number of effectors with it. This is one area where the neurotrophin modulation of glial cell calcium signals could have an undiscovered effect. These factors are known for their involvement in outgrowth and long-term changes, they therefore, could be involved in morphological plasticity of glia and synapses.

One surprising piece of information, however, regarding glial cells and synaptic function comes from anatomical studies that investigate the interaction between astrocytes and neurons of the hippocampus (Ventura and Harris, 1999). This study suggests that glial cells surround only a portion of all synapses in the hippocampus. This would, therefore, suggest that glia would have a limited impact on synaptic function since they may only influence up to half of them. Interestingly, current thought is that glial cell association with synapses is occurring primarily at developed, functional synapses and it is possible that glial cells do not as commonly associate with developing synapses, but arrive at a time when they can provide stability or perhaps in some cases, instability (Murai et al., 2003).

Although these interactions are not entirely resolved it is clear that glial cells can modify synaptic function in both an acute and chronic manner.

This can occur through the release of different gliotransmitters and through reorganization of synaptic compartments.

4.1.4. Glial Cells Modulate Plasticity Through Purines

There is more and more evidence for the involvement of glial cells in synaptic plasticity. As previously mentioned, glial cells can influence synapses through direct cell-cell contact as well as through release of a number of different factors. One group of gliotransmitters that continues to surface as an influential molecule in neuron-glial interactions are the purines. ATP and adenosine are known to influence axon-glial communication and myelination (Fields and Stevens-Graham, 2002). Furthermore, for a long time purines have been known to influence neuronal function throughout the nervous system. It is relatively recently that research has demonstrated the involvement of glial cells in many of these processes (Cunha, 2008).

Some of the best understood purine-mediated mechanisms involve adenosine receptor actions on neurons. The mechanisms of A_1 receptor-mediated inhibition of synaptic transmission are well understood. At the presynaptic terminal these receptors can interact with K^+ channels (Trussell and Jackson, 1985) and Ca^{2+} channels (MacDonald et al., 1986; Silinsky, 2004) to inhibit release. Also, at the NMJ A_{2A} receptors seem to be linked to L-ype Ca^{2+} channels to enhance release (Oliveira et al., 2004). Interestingly, the activation of one receptor type, versus the other, is not the only way in which these receptors regulate transmission. In fact, there is significant interaction between adenosine receptors where activation of one can influence the activation of the other. For instance, in the hippocampus PKC activation by A_{2A} receptors can lead to a functional down-regulation of A_1 receptors (Lopes et al., 1999). Additionally, it has recently been reported that A_1 and A_{2A} receptors can form functional

heteromers that crosstalk. This was shown to result in A_{2A} mediated inhibition of A_1 receptors that occurred as synaptic adenosine levels rose (Ciruela et al., 2006). This degree of crosstalk may, in fact, play a role in the phenomena I investigated, where activation of one receptor is dependent on the other. Another scenario is that activation of the A_{2A} leads to inhibition of the A_1 receptors and ultimately synaptic potentiation. Aside from purinoceptor crosstalk, these receptors also interact with glutamate receptors to regulate glutamate receptor-dependent plasticity (Rebola et al., 2008). Taken together, these reports suggest complex and important roles for purinoceptors in synaptic function throughout the nervous system. Interestingly, more and more evidence is pointing to roles for glial cells in the regulation of purine signalling.

Through all of these complex interactions one theme is beginning to take precedence, which is the involvement of glial cells in the generation of these adenoceptor functions. It is well known that glial cells throughout the nervous system can release ATP (Fields and Burnstock, 2006). For instance, in the mammalian retina, Muller glia release ATP, an activity that subsequently decreases neuronal activity (Newman, 2003). In hippocampal culture, astrocytic ATP can also depress neuronal function (Koizumi et al., 2003). These effects were further shown to occur in slices and are important for rapid (Zhang et al., 2003) and more long lasting (Pascual et al., 2005; Serrano et al., 2006) modulation of synaptic function. My work presented here suggests that glial cells at the NMJ perform similar roles, and release ATP to modify synaptic function. The major advancement, however, that we propose is that glial cells decode neuronal activity and modify their activity to differentially affect synapses. It is likely that glial cells in other parts of the nervous system are similarly capable of detecting differences in neuronal activity and modifying their function accordingly.

4.2. Neurotrophins and Plasticity

Synaptic plasticity is dependent on a number of different factors depending on the context and region of the nervous system involved. As discussed above, glial cells are involved in many of these processes. One concept that surfaced during my work was the importance of the type of calcium elevations in the glial cells and the impact those differences can have on synaptic function. The generation of different calcium responses appears to be a critical element of neuron-glial interactions, however, we do not know what leads to the differences in glial activation. What is known is that anything modulating glial calcium signalling could have profound effects on neuron-glial communication and synaptic function.

One group of molecules that appeared to hold interesting modulatory capabilities was the neurotrophins. These molecules can have short-term functions in cell signalling and induce calcium elevations through IP3, but also have long-lasting effects such as cell morphology and gene regulation. Furthermore, recently BDNF was shown to induce calcium elevations in CNS glia (Rose et al., 2003). This suggested that neurotrophins could potentially do the same in PSCs at the NMJ, something that could lead to altered neuron-glial communication.

I investigated the involvement of neurotrophins in the function of PSCs at the NMJ, and found similar calcium signalling, induced by neurotrophins as to that reported in the CNS (Rose et al., 2003). I also revealed potential functional implications for the neurotrophin signalling on PSC, and NMJ function through their modulation of other signalling pathways. When put in the context of my first project, it is possible that the neurotrophin-mediated modulation of glial calcium signalling could have profound effects on synaptic function as well as neuron-glial communication.

4.2.1. Modulation of Glial Calcium Responses

As mentioned, differences in calcium regulation and signalling in glial cells appears to be a major route for regulating different cellular processes. At the NMJ, neurotrophins are able to modify the PSC calcium elevations. In addition, many other mechanisms exist for the modulation of glial calcium signals.

Glial cells are heterogeneous, similar to neurons. There are many different types of glial cells and with each glial cell type are correspondingly different properties. One area of particular relevance is the heterogeneity in calcium handling. It is well known that different glial cells express different receptors and this is partially responsible for differences in calcium signalling. For example, calcium responses can be simple single spikes, biphasic or complex depending on the receptors activated and the source of calcium (Verkhratsky and Kettenmann, 1996).

Although it is apparent that activation of different receptors could lead to different calcium signals, signalling cascades can also be regulated. Here I have shown that the neurotrophins do just that, by increasing and decreasing various properties of purine and acetylcholine-dependent signals. This modulation could be achieved through a number of different mechanisms. Considering the time frame (30 min) it is possible that the number of receptors on the surface were altered. Also, since the responses investigated were coupled to metabotropic receptors it is possible that a step in the signalling cascade was modified. For example, perhaps the availability of PIP_2, the precursor of IP_3 was changed. This could occur through altered sequestering to membrane-associated proteins, which can be regulated by PKC (Larsson, 2006). These processes tie together the acute calcium signalling with longer-term events such as receptor expression.

In fact, all of these molecular cascades could be implicated in more long-term aspects of glial cell regulation. For example, glial stability could

also be affect by changes in calcium signalling. This could occur since PIP_2 and a number of related effectors are linked to actin dynamics (Logan and Mandato, 2006). Therefore, modulation of PLC activity and the amount of PIP_2 available could have important consequences. Another example of proteins linked to this pathway is GAP-43, a protein linked to the actin cytoskeleton and known to be upregulated in PSCs following denervation (Woolf et al., 1992). This upregulation may be involved in PSC sprouting and is likely involved, in part, in sequestering PIP_2 to the membrane (Tong et al., 2008). One possibility, therefore, is that neurotrophin signalling to PSCs is involved in the regulation of proteins associated with the cytoskeleton. The observed change in calcium signalling by purinoceptors and mAChRs could be a side effect related to changes in functions associated with more long-term stability or simply due to short-term regulation of the receptor signalling itself. Perhaps, as with the postsynaptic density of the NMJ (Gonzalez et al., 1999), neurotrophins are important for the stability of PSCs and their processes. Therefore, investigating the long-term effect of specific perturbations to neurotrophin signalling in PSCs could reveal new roles for these molecules.

Despite all these possible roles for neurotrophin signalling in PSCs, I was unable to observe any modulation to synaptic function. This could be due to a number of reasons. One primary aspect that should be addressed in subsequent work is the age of animals used. Since I used adult animals it is quite possible that decreased expression of receptors complicated my results. Furthermore, it is likely that changes to synaptic structure and function could be observed over longer periods of time. For example Hess et al. (2007) reported changes in PSC number when NT3 was overexpressed. Considering this, it seems likely that neurotrophins are have significant impact on neuron-glial communication and the long-term structure of the NMJ even though I observed no short-term modification to synaptic function.

4.2.2. Crosstalk Between Signalling Cascades

Neurotrophin receptors interact with a number of different signalling systems. My work suggests that not only do the neurotrophin receptors interact with purinoceptors, but that they also interact with the cholinergic system operating in PSCs. This leads to changes in the calcium signalling in PSCs, which I propose, may play a role in PSC stability.

Neurotrophin receptors are known to interact with purinoceptors (Lee and Chao, 2001; Arthur et al., 2005) and other G-protein coupled receptor systems (Lee et al., 2002; Rajagopal et al., 2004). These previous studies have demonstrated that activation of purinoceptors can lead to activation of Trk receptors in the absence of neurotrophins. This seems to occur through signalling by P2Y2 and A_{2A} receptors. These data lead to the interesting question of whether activation of presynaptic A_{2A} receptors at the NMJ, what I showed to induce a potentiation, affects neurotrophin signalling in any way. Also, a recent report suggests that activation of the A_{2A} receptors is important for the facilitatory effects of BDNF on neuromuscular transmission (Pousinha et al., 2006). This indicates even further interaction between these two signalling systems in a functional context.

Interaction between different signalling cascades can occur at many different levels. For instance with the neurotrophins, interactions can be observed between the receptors themselves. Also, downstream from the receptors there are any number of possible interactions. For instance it could be that Trk activation leads to a phosphorylation event that influences other receptor signalling. Alternatively, it could be more modulation of some target or substrate in the cascade of the other receptor that is altered. As mentioned in the last section, one potential target could be PIP_2. Crosstalk between receptor systems could occur at many places in the cascades to cause the observed modulation of calcium signalling.

Perhaps at the developing NMJ or even in the adult, A_{2A} receptors and neurotrophins interact to modulate plasticity and stability of PSCs and the NMJ as a whole.

4.2.3. Implications of Neurotrophins in the Tripartite Synapse

The involvement of neurotrophins in PNS development, stability, and myelination are well established (Pitts et al., 2006). As more information surfaces regarding this group of molecules we better understand their importance, not only for neurons, but also for glial cells. Furthermore, their impact on glial cell function becomes increasingly complex.

The neurotrophins have prominent functions during development. For example it is now known that glial-derived BDNF can influence inhibitory neuron synaptic development in the hippocampus (Elmariah et al., 2005b). At the NMJ the evidence for neurotrophins in development is limited to the neuronal elements and myelinating Schwann cells except for some recent evidence that NT-3 could influence PSC proliferation (Hess et al., 2007). However, considering the importance of neurotrophins for other aspects of myelinating Schwann cell development and synaptic development it seem likely that they could have other influences on PSCs as well. For instance in myelinating Schwann cells neurotorphins influence cellular elongation (Chan et al., 2001), something similar occurs in PSCs and could also be influenced by neurotrophins.

In terms of synaptic development, neurtrophins seem to have important roles in strengthening immature synapses (Lohof et al., 1993). To date no information exists on the influence neurotrophins have on the development of perisynaptic glia other than that mentioned above. However, it is likely that they induce some changes in glial cells during development, whether directly or indirectly. Since the synapse develops as a whole, the absence

of neurotrophins during these early stages would probably affect more than just neurons.

The demonstration that neurotrophins could induce rapid calcium signalling in glial cells provides an attractive link between the neurotrophins and perisynaptic glia (Rose et al., 2003). It also raises the question of whether neurotrophin signalling to glial cells during development plays a role in regulating synaptic maturation. Another intriguing question regards the involvement of neurotrophins in PSC stability. Although both TrkB and TrkC seem to be expressed at only low levels in adult PSCs (Gonzalez et al., 1999; Hess et al., 2007) it is possible that p75 still has functions. Alternatively, rather than providing signals to maintain stability, perhaps receptor expression increases following denervation, and is involved in PSC instability and outgrowth. It does, however, seem unlikely that the neurotrophins play any role in the plasticity of mature NMJs since we found no evidence of this, in agreement with others (Ribchester et al., 1998). Considering this, I suggest that neurotrophin signalling in PSCs at mature synapses, if they have a role, is likely in a more long-term phenomenon other than modulation of transmitter release.

4.3. Conclusion

In this thesis I have addressed basic aspects of cellular communication and synaptic function and have also investigated the influence of specific molecules on glial cell function. To this end, I have provided, what I feel, is novel insight into neuron-glial communication and glial cell function in the context of synaptic transmission. The idea that glial cells could be able to process information and distinguish differences in the pattern of neuronal activity is an intriguing possibility when viewed in the context of whole brain functioning. It is most certainly a topic that deserves further investigation, since the implications are extensive.

The major advancement that I have provided through my work on neuron-glial interactions and the involvement of neurotrophins in those interactions is the idea that glial calcium signals are varied, and modifiable. More importantly, these properties are critical for the outcome of neuron-glial interactions and synaptic function. It is clear that different patterns of nerve activity can lead to different glial activation. How exactly this arrives is still unknown, however, my work on the neurotrophins points to one group of molecules that can influence glial calcium signalling in this way.

Taken together the results that I have presented here advance our understanding of how synaptic communication occurs in the nervous system. It is my hope that these studies will influence future work on neuron-glial communication and synaptic transmission in general.

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In the following pages readers will find the article:

Todd, KJ, Serrano, Alexandre, Lacaille, Jean-Claude, and Robitaille, R (2006) Glial Cells in Synaptic Plasticity. Journal of Physiology (Paris) 99: 75-83

6. Appendix I Review : Glial Cells in Synaptic Plasticity

Keith J. Todd*, Alexandre Serrano*, Jean-Claude Lacaille and Richard Robitaille

Centre de Recherche en Sciences Neurologiques
and
Département de physiologie
Université de Montréal
C.P. 6128 Succursale Centre-Ville, Montréal, Québec
CANADA, H3C 3J7

*These authors contributed equally to this work.

Corresponding author:
Dr. Richard Robitaille
Département de physiologie, Faculté de médecine,
Université de Montréal, C.P. 6128 Succ. Centre-ville,
Montréal, Québec, Canada H3C 3J7
Phone : (+1) 514-343-6111 ext. 1964
Fax : (+1) 514-343-2111
E-mail: [redacted]

6.1. ABSTRACT

Plasticity of synaptic transmission is believed to be the cellular basis for learning and memory, and depends upon different pre- and postsynaptic neuronal mechanisms. Recently, however, an increasing number of studies have implicated a third element in plasticity; the perisynaptic glial cell. Originally glial cells were thought to be important for metabolic maintenance and support of the nervous system. However, work in the past decade has clearly demonstrated active involvement of glia in stability and overall nervous system function as well as synaptic plasticity. Through specific modulation of glial cell function, a wide variety of roles for glia in synaptic plasticity have been uncovered. Furthermore, interesting circumstantial evidence suggests a glial involvement in multiple other types of plasticity. We will discuss recent advances in neuron-glial interactions that take place during synaptic plasticity and explore different plasticity phenomena in which glial cells may be involved.

Key words: synaptic plasticity; neuron-glia interactions; glial cells; synapse; transmitter release

6.2. INTRODUCTION

Although glial cells are important for structural and metabolic maintenance of the nervous system, there are now numerous reports demonstrating the ability of glia to respond to, and send signals to neurons and synapses in the central and peripheral nervous systems (CNS and PNS). Evidence further suggests that glial contributions to synaptic communication and plasticity are complex and heterogeneous. However, glial cells remain relatively understudied in comparison to their 'spiking' counterparts. Through the use of calcium imaging in glia, it has become easier to investigate interactions between glial cells and neurons. Here, we will discuss plasticity events from the perspective of the tripartite synapse (glia plus neuronal elements) and suggest roles for glia in synaptic plasticity.

Synaptic plasticity is defined as a change in efficacy of synapses that is mediated by various pre- and postsynaptic mechanisms. However, we will present evidence that glia modulate or even mediate synaptic plasticity and propose areas of possible glial involvement in a wider range of neuronal phenomena. It is well established that glia can interact with neurons in a variety of ways with a variety of outcomes. However, is there one or several reoccurring theme(s) that can be obtained from what we know about specific neuron-glial interactions?

6.3. SYNAPTIC PLASTICITY

Efficacy of synaptic transmission is variable and changes with experience. This ability of synapses to modulate their efficacy, termed synaptic plasticity, encompasses a large number of cellular phenomena that modify synaptic function. A wide variety of changes can occur presynaptically to alter transmitter release properties and/or postsynaptically to modify the responsiveness to transmitter release such as receptor trafficking. These

changes occur in a frequency-dependent manner and can result in increased efficacy, termed potentiation, or in a reduction of synaptic efficacy, termed depression. Additionally, these changes in synaptic efficacy can be accompanied and supported by morphological changes such as extrasynaptic density and synaptic coverage.

Different types of synaptic plasticity can be distinguished based on their temporal properties. The former lasts for seconds to minutes and transmitter release returns to control levels with no apparent long-term modification in synaptic efficacy. For the latter, changes in synaptic transmission last several minutes to hours, days and weeks. Synaptic plasticity phenomena can also be characterized according to the complexity of the synaptic pathways involved, with homosynaptic plasticity representing modifications confined to a single afferent pathway and heterosynaptic plasticity involving interactions between two or more distinct afferents. The properties of plasticity have traditionally been attributed solely to neurons. However, recent evidence has come to the fore-front suggesting that not all plasticity events can be explained by neuronal elements and that glial cells provided an essential contribution.

6.4. GLIA AND SYNAPTIC PLASTICITY

6.4.1. GLIA, SYNAPTOGENESIS AND SYNAPTIC EFFICACY

Glial cells are known to be plastic structures, able to change their phenotypes under various conditions such as following injury or during development [46]. Because of this plasticity, and their ability to release trophic factors and glio-transmitters, it has been proposed that glial cells may be important for synapse formation during development. Experimental data have been obtained in the past decade in support of such a role [5, 26, 36, 51]. Pfrieger and Barres [36] demonstrated the importance of glial cells on synaptic development in retinal ganglion cell (RGC) cultures. An increase in

frequency of excitatory postsynaptic currents (EPSCs) and a decrease in failure rate were observed when RGCs were cocultured with glial cells. In the same culture system, Ullian et al. [51] demonstrated that an increased number of functional synapses are formed in the presence of glia. These effects were thought to be due to diffusible factors released by glial cells. The same group recently demonstrated similar results in motoneuron cultures, suggesting that this may be a conserved mechanism in CNS synaptogenesis [50]. Further experiments with RGC cultures demonstrated that cholesterol by itself was sufficient to induce the same changes as glial-conditioned media [26]. This supports the hypothesis that glia can be directly involved in synaptic efficacy through release of diffusible factors that alter the molecular and functional phenotype of neurons.

In a separate study, Beattie and colleagues [5] demonstrated an important role for cytokines in synaptic strength. Initial experiments were performed on hippocampal cultures where they found that tumour necrosis factor-α (TNFα) increased AMPA receptor trafficking and surface expression. Interestingly, it was shown that TNFα was a factor released by glial cells. In culture, a decrease in EPSC frequency and amplitude was observed following the addition of a functional antagonist (soluble TNF receptor 1). These authors found similar results when using hippocampal slices, where EPSC frequency was also reduced.

Recently, exciting results from the work of Barres and colleagues demonstrated a role for glia and glial-released factors in synaptic function in vivo [11]. A family of extracellular matrix proteins called thrombospondins were shown to be released from astrocytes in culture and to increase expression of pre- and postsynaptic proteins in RGC cultures. These authors went on to show that not only were thrombospondins widely expressed in astrocytes in developing brain, but that knockout mice had fewer synapses than controls. The temporally restricted expression of thrombospondins around the time of synaptogenesis is thought to be important for synapse

formation in many brain regions. This may be a general mechanism by which glia can influence synaptogenesis in the CNS.

Similar results to those found in RGC cultures [26] were found in Xenopus nerve-muscle coculture by Ko and colleagues [34]. This group demonstrated that addition of Schwann-cell conditioned media (SCCM) to cultures could increase the number of nerve-muscle contact points that were apposed to nicotinic cholinergic receptor (AChR) clusters. It appears that SCCM is able to change neurites from an outgrowth mode to a more stable mode capable of undergoing synaptogenesis. The contents of the SCCM were not determined in these experiments. However, it would be interesting to see if the factors are similar to those found in other culture systems, such as cholesterols or cytokines. Although many of these results were obtained in culture, some were tested and reproduced in more intact preparations [5, 11]. This would suggest the existence of a common role for glial cells in synapse formation and stabilisation.

Increasingly, results are coming to light that demonstrate a high level of morphological plasticity in CNS neurons [15, 17]. Morphological changes can occur in a matter of seconds [15, 17] and are dependent on neuron-neuron contact and synaptic activity [14, 40]. However, other results have demonstrated a role for glia in balancing dendritic plasticity and stability. The membrane bound ephrin ligands bind to tyrosine kinase Eph receptors and regulate various cell-cell interactions. Murai and colleagues [28] found that ephrin-A3 was expressed on astrocytic processes in the hippocampus. Expression of ephrin-A3 by glia serves to negatively regulate dendritic spine expansion. Therefore, when astrocytes do not surround a dendritic spine, they do not make the necessary contact to initiate Eph signaling, thereby allowing further elaboration of the spine. This work suggests an ability of glia to directly regulate morphological plasticity in the intact brain.

The above-mentioned examples are indicative of the importance of glial cells in synaptic function, plasticity and development. Furthermore, not

only are glia involved in the molecular modification of synaptic function, they can also induce morphological changes at the cellular level. This is a fascinating avenue of research since glial cells are known to have stabilizing roles at synapses [3]. This potentially indicates the capacity for glial cells to stabilize synapses under a certain set of conditions and to induce modification under another. However, the question remains; do such glial-mediated molecular and morphological synaptic modifications occur in vivo outside the context of synapse formation?

6.4.2. Glia, Architectural Plasticity and Neurotransmitter Clearance

The examples discussed above demonstrate the ability of glia to modify synapses. However, neuronal changes may not be the only aspect of glial-mediated synaptic plasticity. Recently it has been found that glial cells not only modify neuronal plasticity, but they are themselves also plastic and can change in response to neuronal activity [31, 37]. Furthermore, these studies demonstrate that glial plasticity can occur in adult systems where changes in glial structure alter both the immediate synaptic environment as well as the surrounding extracellular space. These changes could have profound effects on synaptic function and plasticity.

Clear evidence was obtained in the supraoptic nucleus (SON). Indeed, this region of the brain undergoes dramatic architectural changes due to hormonal regulation during lactation in adult females [48]. Increased levels of neuronal activity, observed during lactation, result in decreased glial coverage of these glutamatergic synapses. The possibility that these changes could alter glutamate clearance and thus modulate synaptic efficacy was investigated [31]. At this synapse, presynaptic neurons release glutamate, activating postsynaptic AMPA receptors. However, the presynaptic neuron also expresses metabotropic glutamate receptors (mGluRs), which decrease transmitter release when activated. To investigate the influence of glial

coverage on synaptic function, Oliet and colleagues [31] tested synaptic function during different degrees of synaptic-coverage. In virgin rats, where synapses are tightly ensheathed by glia, blockade of glial glutamate transporters decreased the amplitude of evoked currents and increased paired-pulse facilitation (PPF) ratio. This is due to presynaptic mGluR activation by high levels of synaptic glutamate. Interestingly, blocking glial transporters had a reduced effect in lactating rats where glial coverage is reduced. These results suggest that glutamate handling in the SON is markedly changed during lactation. In virgin animals, glial cells tightly regulate the concentration of glutamate in the synaptic cleft, keeping it relatively low. However, during lactation, withdrawal of glial cell processes from the synapse results in decreased glutamate clearance and higher levels glutamate in the synapse.

The decrease in glial coverage and higher levels of glutamate in the synaptic cleft could affect the spillover of transmitter from the synaptic cleft. Not only does glial morphology affects this phenomenon, but their transporters are also important for removal of glutamate from synapses [6, 44, 47]. Synaptic spillover may have implications for synaptic crosstalk, heterosynaptic plasticity and overall functioning of the brain. Synaptic crosstalk was investigated in the SON in the context of heterosynaptic depression [37]. This was an extension of the above model, where synaptic crosstalk between SON glutamatergic synapses causes heterosynaptic depression in neighbouring GABAergic neurons through presynaptic mGluRs. Piet and others (2004) recorded the amount of GABAergic depression due to glutamate spillover and activation of presynaptic mGluRs. They observed that extracellular diffusion was facilitated and that depression was twice as pronounced in lactating animals in comparison to virgin ones. This suggests reduced glial coverage, which agrees with the electrophysiological data that indicate a greater level of glutamate diffusion, and increased heterosynaptic depression in lactating rats.

These experiments indicate a clear role for glial cells in the regulation of synaptic function and reveal the complexity of such plasticity events involving non-neuronal elements. In this system, glial-dependent homosynaptic plasticity occurs in a glutamatergic pathway while more complex heterosynaptic depression can also occur in a different GABAergic pathway. This is an interesting example of how glial cells can directly impact not only a single synapse, but also, in more complex fashion on multiple synapses. The question that now remains is; what are the functional implications of a neuro-glial environment and neuro-glial plasticity in a given brain region?

6.5. GLIA AND SHORT-TERM SYNAPTIC PLASTICITY

Short-term plasticity of synaptic efficacy is widely expressed in the nervous system and is a form of plasticity for which a glial contribution has been found and well characterized. In the following section we will discuss evidence for glia-mediated short-term plasticity in simple and complex synaptic environments.

6.5.1. SHORT-TERM PLASTICITY IN THE PNS

The neuromuscular junction (NMJ) is a useful model of a simple synapse that demonstrates purely homosynaptic plasticity in an intact preparation. Importantly, it allows specific manipulation of all three of the synaptic compartments: the presynaptic terminal, the postsynaptic muscle fibre, and the perisynaptic Schwann cell (PSC), the glial cell at this synapse. The NMJ has been used as a reliable model for studying the tripartite synaptic function (pre, post and glia). The first studies of neuron-glia interactions at the NMJ demonstrated that PSCs could respond to nerve activity with Ca^{2+} elevations [19, 42] and these elevations were frequency-

dependent [19]. Is PSC activation at the NMJ a simple response to high activity or could they reciprocally modulate nerve activity? This was investigated by Robitaille [43] by monitoring NMJ plasticity while specifically activating or inactivating glial cells. He showed that interfering with the G-protein-coupled receptors on PSCs disrupted the NMJ high frequency-induced depression (Fig 1A). Also, G-protein activation of PSCs caused a decrease in nerve activity during 0.2 Hz stimulation. However, the injection did not have any effect on spontaneous release or PPF. These results suggest that PSC activation can modulate homosynaptic plasticity during high frequency depression at the NMJ.

A later study, performed by the same group, demonstrated a further role for PSCs in the modulation of NMJ function. This was done by direct and specific Ca^{2+}-mediated activation or inactivation of glial cells. Direct activation of the IP_3 cascade caused a potentiation of neurotransmission at 0.2 Hz [10]. Furthermore, during 10 Hz stimulation, injection of PSCs with the Ca^{2+} chelator BAPTA induced greater depression (Fig. 1B). This suggests that the release of Ca^{2+} from internal stores results in a potentiation of transmitter release. These results suggest that not all PSC G-protein signaling operates through the same mechanisms, and that the capacity exists for the PSCs to mediate bi-directional modulation of homosynaptic plasticity in neuromuscular function.

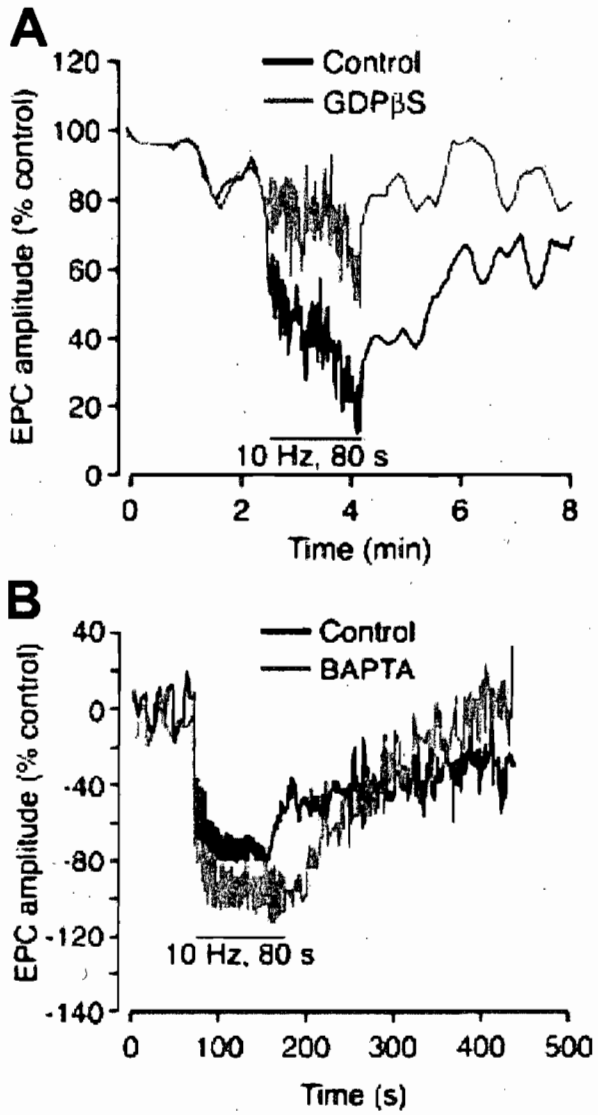

Figure 1. Bidirectional modulation of synaptic transmission by perisynaptic Schwann cells at NMJs.

A) Injection of GDPbS to inactivate PSC G-protein signaling results in a reduction in synaptic depression. Modified from Robitaille 1998. B) Injection of the calcium chelator BAPTA into PSCs results in greater depression at the NMJ, revealing a reduction in the potentiation. Modified from Castonguay and Robitaille 2001.

Recent work by this group proposes that glutamate may be the glio-transmitter involved in the PSC-mediated depression. Pinard et al. [38] demonstrated the presence of mGluR mediated depression of neuromuscular

transmission and investigated evoked (EPPs) and spontaneous end-plate potentials (MEPPs) at the frog NMJ. They found a decrease in evoked EPP amplitude and MEPP frequency but not in MEPP amplitude in the presence of glutamate or a glutamate agonist. This suggests that glutamate reduced transmitter release. However, immunohistochemical staining for mGluR subunits suggested that these receptors were located on the muscle fibre. How then is glutamate having a presynaptic effect on neurotransmitter release?

An earlier study from the same laboratory demonstrated that nitric oxide (NO) scavengers could decrease the level of high-frequency depression at the NMJ [49]. Furthermore, evoked EPP amplitude and spontaneous EPP frequency were both decreased in the presence of NO donors. These effects were similar to what was observed in the presence of glutamate [38]. Since NO is thought to be produced in the muscle and PSCs [13], it is possible that the glutamate and NO signaling pathways are coupled. A potential model for this system is one where presynaptic release of acetylcholine activates PSCs, inducing release of glutamate from the PSCs, which activates mGluRs on the muscle fibre. Activated mGluRs then stimulate the production of NO, modulating high-frequency depression. It is thought that activation of NO synthase is most likely calcium-dependent since it appears that neuronal NO synthase is present in muscle fibres, but also in PSCs [13].

Recently, Reddy and colleagues [41] developed a technique for specifically eliminating PSCs at frog NMJs using an antibody-compliment ablation technique. They observed that the absence of PSCs lead to a reduction in synaptic efficacy after five to seven days. However, they reported no effect of PSC ablation on synaptic transmission and plasticity minutes after the ablation [41]. At first, these results may appear in contradiction with those obtained by Robitaille and colleagues. However, such results were somewhat expected. Indeed, data obtained from neuron-myoblast co-cultures in the absence of Schwann cells, revealed that synapses displayed

all forms of plasticity, in particular depression, observed at mature NMJs when PSCs are present. Hence, what the culture and ablation experiments indicate is that the mechanisms of short-term plasticity are presynaptic in origin and are modulated by PSCs when these cells are present. This is also consistent with the observation by Robitaille [43] that blockade of G-protein-coupled receptors in PSCs did not totally prevent synaptic depression. These results indicated that there was an important, fully active presynaptic component. Furthermore, since PSCs can potentiate and depress transmitter release it is possible that the effect of PSC ablation may result in a null effect where the lack of potentiation and depression would balance out.

6.5.2. SHORT-TERM PLASTICITY IN THE CNS

The NMJ has proven to be a useful model for investigations of simple synaptic interactions. However, is there evidence for glial involvement in synaptic plasticity in the CNS where synaptic organization and plasticity can involve multiple pathways and complex network interactions? The hippocampus is a laminar structure with well-defined function, characterized cellular interactions and synaptic plasticity. The main cellular elements involved in synaptic plasticity are the pyramidal cells and the inhibitory interneurons [35]. However, the functional and morphological characteristics of glial cells are consistent with a possible role for these cells in the modulation of certain forms of synaptic plasticity. For instance, it is known that single hippocampal astrocytes contact thousands of synapses [9] and that they are connected together to form large, interconnected networks (Fig 2.) [12]. Furthermore, these cells express receptor types that are similar to the ones present on neurons, are activated by hippocampal activity, and can release glio-transmitters and neuroactive substances known to be involved in various forms of plasticity.

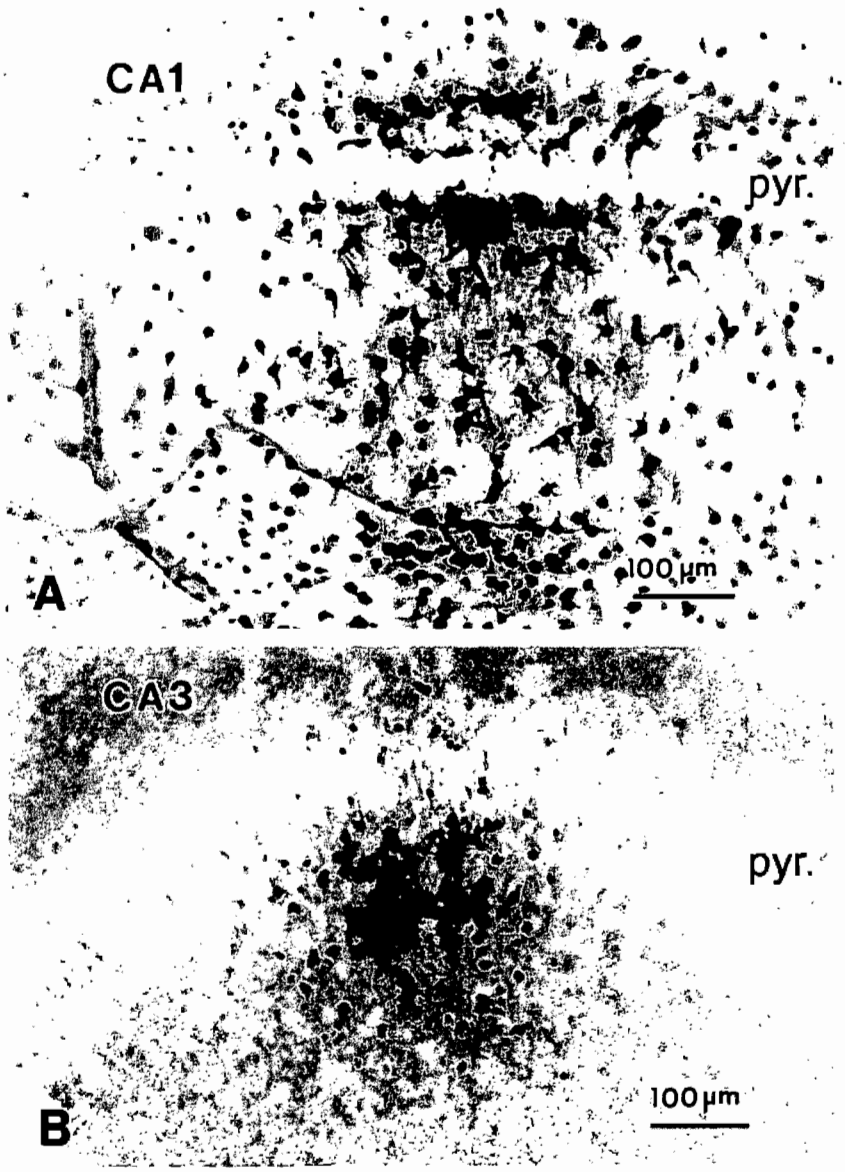

Figure 2. Heterogeneity of hippocampal glial networks.

A) Electrically coupled astrocytes in area CA1 form larger networks than those in area CA3 (B). Pyr indicates the pyramidal cell layer. Adapted from D'Ambrosio et al. 1998 with permission from the Society for Neuroscience. Copyright 1998 Society for Neuroscience.

6.5.2.1. Hippocampal Plasticity

In the hippocampus, activation of pyramidal neurons has been shown to induce elevations in glial Ca^{2+} levels [39]. In addition, elevations in glial Ca^{2+} levels have been shown to be involved in signaling to neurons through glutamate release [33]. The impact of glial-to-neuron signaling was investigated in hippocampal culture where the effect of glial glutamate release was investigated during neuronal activity [2]. These experiments showed that action potential-evoked synaptic transmission was depressed following astrocyte stimulation. This depression involved an increase in glial intracellular Ca^{2+} since it was blocked by BAPTA injection in astrocytes [2]. Interestingly, impeding the Ca^{2+} activation of glial cells also perturbed the presynaptic mGluR-induced depression. Thus, these results suggest that astrocytic glutamate release following Ca^{2+} elevations diffuses presynaptically to induce homosynaptic depression. This is a good example of glial modulatory capabilities in the CNS. However, glial cells were stimulated exogenously in these experiments and not by neuronal activity. This leaves the problem of whether glia can respond to neuronal activity and signal back in an intact preparation.

The involvement of glial cells in relation to inhibitory interneurons was investigated by studying the effect of astrocyte signaling on inhibitory currents in hippocampal slices [20]. In this system, interneurons synapse onto principal neurons of the hippocampus and provide GABA-mediated inhibitory input. The authors demonstrated that stimulation of astrocytes located in close proximity to interneurons could, in turn, stimulate these interneurons through glutamate release. This stimulation resulted in increased frequency of miniature inhibitory postsynaptic currents recorded in pyramidal neurons. They also showed that astrocytes could be activated directly by GABA release from interneurons acting on glial $GABA_B$ receptors. Furthermore, application of $GABA_B$ antagonists disrupted potentiation of inhibitory synaptic

transmission [20]. These data suggest that GABAergic activation of astrocytes potentiates inhibitory synaptic activity through a glia-interneuron to pyramidal neuron loop. It was later shown that this regulatory loop was mediated by astrocytic glutamate signaling to kainate interneuronal receptors [24]. These results indicate that astrocytes can modify interneuron activity that subsequently impacts on CA1 pyramidal neuron excitability. In addition, these data indicate that astrocytes can functionally interact with not only excitatory, but also inhibitory synaptic circuits in the hippocampus.
Further support for an involvement of glia in complex synaptic interactions was provided by Poo and colleagues who investigated the role of glial cells in purinergic-mediated depression [53]. At CA3-CA1 hippocampal synapses, high frequency activity results in synaptic suppression, a rapid and short-lasting form of presynaptic depression of transmitter release. This group showed that this depression was indeed a glial-mediated mechanism since it was abolished in the presence of glial inhibitors (fluoroacetate and octanol) or in the absence of glial cells in culture preparations [53]. Additionally, they showed that during high levels of activity synaptically released glutamate induced intracellular calcium rises in glial cells, triggering ATP release. This extracellular ATP acted homo- and heterosynaptically to induce presynaptic suppression. Hence, glial cells seem to be a necessary element of ATP-mediated synaptic suppression in hippocampus.

Recent results from our laboratory uncovered a glial involvement in adenosine-mediated heterosynaptic depression at Schaffer collateral synapses [45]. We reported that in rat hippocampi, Schaffer collateral tetanization recruits interneurons through NMDA receptors and induces GABA release. Surrounding glial cells show a calcium-dependent activation via $GABA_B$ receptors. It is thought that the Ca^{2+} increase induces ATP release from glial cells, which degrades into adenosine. We demonstrated that adenosine, acting through A1 receptors, heterosynaptically induces depression on Schaffer collaterals. Interestingly, we observed that blocking

Ca^{2+}-mediated activation of glial cells disrupted the heterosynaptic depression of Schaffer collateral synapses. This suggests that glial cells not only act as complementary elements in neuronal modulation, but are an integrated and necessary components in network plasticity. Based on our results and those of Zhang et al. [53] we propose that homosynaptic modulation and plasticity that occur locally probably involve a small number of astrocytes and do not require communication within a glial network, whereas, heterosynaptic plasticity phenomena are based upon glial communication that occurs throughout a glial syncitium. Although the mechanisms involved in these two studies are different, they suggest that glial cells are essential elements of hippocampal plasticities.

6.5.2.2. Retinal Modulation by Glial Calcium Waves

The data presented above indicate that astrocytes can modulate synaptic plasticity over a large area owing to a large degree of glial interconnectivity. This suggests that glial cell networks could be important for signaling over long distances. Glial morphology and interconnectivity vary in different brain regions [12] suggesting a differentiall influence on local brain function. Evidence for neuronal modulation through glial networks has also been obtained by Newman and colleagues in the retina [29]. In the isolated retina, astrocytes and radial Muller glia form networks that can propagate Ca^{2+} waves over long distances (400 μm) [29]. This in situ preparation responds to light stimuli with changes in neuronal firing rate. Newman and Zahs (1998) investigated whether glial calcium waves could alter neuronal activity, therefore, implicating glial cells in regional modulation. Initial experiments demonstrated that neuronal firing rates were either increased, or decreased at the time a calcium wave reached a glial cell neighbouring the recorded neuron [29]. Increases in firing rate were only observed in ON cells, whereas decreases were observed in all cell types, suggesting that glial cells

could differentially modify neuronal function based on the neuronal phenotype. Furthermore, changes in neuronal activity were not observed when glial calcium waves did not reach the recorded neuron or in the presence of glycine, GABA or glutamate antagonists. Modulation of firing rate was observed at an average distance of 60μm indicating that glia could affect neuronal activity over moderate distances.

6.5.2.3. Neuronal Synchrony

Other results have recently come to light that suggest that glia could be involved in synchronization of neural activity. Two studies were published showing that in the hippocampal CA1 region, astrocytic release of glutamate synchronously activated neighbouring neurons through extrasynaptic NMDA receptors [1, 16]. In one of the studies, Carmignoto, Haydon and colleagues [16] showed that inward currents could occur synchronously in two pyramidal neurons when astrocytes are stimulated by an mGluR agonist. Interestingly, this effect was observed in the presence of TTX, suggesting that glial cells mediated the effect. These results were also reproduced by specifically elevating Ca^{2+} in astrocytes. These authors showed that the synchronization by glial cells was activated by neuronal activity itself, indicating the involvement of a reciprocal neuron-glia regulatory feedback loop. Additionally, both of these studies indicated that synchronous activation was spatially restricted to neurons with less than 100 μM separation [1, 16]. This suggests some intrinsic property associated with the regional glial network that imparts a spatially limited degree of synchronization.

Overall, work with CNS synapses in cultures and slices reveal that glial cells can receive, send and dynamically modulate neuronal function. This is occurring in direct homosynaptic neuron-glial interactions, and in more complex synaptic loops. These results challenge classical ideas as they strongly implicate glial cells in many forms of synaptic plasticity. It is also

interesting to note the similarities that exist in the studies that have used semi-intact models. For instance, glial cells seem able to modulate homosynaptic events as seen in the SON, NMJ and during high levels of activity in the hippocampus through release of ATP. These events can occur through glial plasticity, through sensing neuronal activity and modulating it in return. Furthermore, glia can be important for interactions between different groups of neurons and in heterosynaptic plasticity as reported by Kang et al. [45] and Serrano et al. [45]. Based on the evidence for frequency-dependent neuron-glial interactions in various forms of plasticity, it appears that ideas of neuronal networks should consider glial cells as interconnected partners. The extent of the glial interconnectivity of a given region is likely to be important in determining the degree of the glial modulation. For example, it is known that glial cells in the hippocampal CA1 region form larger networks than those in the CA3 region (Fig. 2) [12]. Therefore, could glial modulation be more spatially restricted in the CA3 region and what could be the functional consequences of such a difference?

The impact and the function of glia on local and regional environments are diverse and appear to adjust to the complexity of the environment. Hence, it is likely that perisynaptic glial cells play many more roles than have been investigated yet. This leaves many unaddressed questions surrounding the field of synaptic plasticity and neuron-glial interactions. Below we will briefly discuss some areas of study that may be promising for elucidating the role of glia in synaptic plasticity.

6.6. FURTHER GLIAL INVOLVEMENT

There are many other instances of plasticity where glial cells are well positioned anatomically and functionally to respond and signal to neurons. However, addressing the question of glial involvement is not always the easiest task. Thus we will next discuss some plasticity events where glial

cells have not been directly implicated, but in which their involvement is possible considering available information and general glial cell properties.

6.6.1. Cerebellar Plasticity and Glia

The cerebellum is a well conserved, highly ordered structure. Long-term depression (LTD) is a well-characterized form of cerebellar plasticity that is proposed to be important for fine motor control [8]. Cerebellar LTD is observed at glutamatergic synapses between granule cell parallel fibres (PFs) and Purkinje cells (PCs). The induction of LTD in the cerebellum has been shown to be at least partially dependent on NO [22, 23] that acts postsynaptically on PCs. The source of NO remains to be determined although it appears not to originate from the PCs themselves [23]. Obviously, there are important pre- and postsynaptic mechanisms involved in the induction of LTD, but could there be a role for Bergmann glia? For instance, Bergmann glia show the highest levels of NO synthase activity [21], they are closely associated with PF-PC synapses and can respond to PF activity [18]. Glial modulation of long-term plasticity would not mean that neuronal processes are not important. Simply that both glial and neuronal influences likely have different roles in the full expression of cerebellar LTD. In this system glial cells may modify or support the neuronal processes.

6.6.2. Long Term Changes in the Hippocampus

It is a daunting task to address the question of glial involvement in long-term potentiation (LTP) or LTD in the hippocampus. Moreover, it is not until recently that evidence was obtained to support such a role for glia. Two signaling pathways have been found by which glial cells can modulate NMDA-dependent glutamatergic transmission. The first series of evidence involves D-serine, a co-agonist acting on the glycine site at NMDA-type glutamate receptors, known to be necessary for the induction of hippocampal

LTP [4, 32]. NMDA receptor activation can be a necessary step in activity-dependent LTP induction [7]. Additionally, both glutamate and glycine sites on NMDA receptors must be filled for activation. Using mixed cultures and hippocampal slices, it was reported that glial release of D-serine was necessary to induce NMDA-type LTP [52]. In the presence of glycine site blocker, or in the absence of glial cells, LTP could not be induced. Thus, these results suggest that astrocytes also play a key role in long-term synaptic plasticity. The second series of experiments is related to TNFα. As discussed previously, TNFα is a protein that increases surface expression of neuronal AMPA receptors. It has been shown that TNFα, released by glial cells, increases synaptic efficacy. Glial release of TNFα is another key mechanism of glial control of glutamatergic plasticity since continuous presence of this molecule is necessary to maintain synaptic strength. Given the importance of AMPA and NMDA receptors in LTP and LTD [25], these results suggest that glial cells can release at least three glio-transmitters that can be involved in these types of long-term plasticity: glutamate, D-serine and TNFα. The above results are consistent with observations from glial specific knockout mice where LTP was modified, providing further support for a glial involvement in LTP [27, 30]. It will be of interest to investigate in more detail the putative involvement of glial cells in NMDA and AMPA receptor-mediated types of plasticity.

6.7. Conclusion

In this review, evidence has been presented demonstrating that glial cells not only play a supportive role for neuronal activity, but that they are also involved in the regulation of neuronal network plasticity. From simple homosynaptic modulation to complex hippocampal plasticity, the tripartite synapse is the basis of these interactions and modulations. Owing to the plasticity of glial cells, their heterogeneity and their exquisite sensitivity to

neuronal activity and synaptic transmission, it is fascinating to hypothesize that glial cells could have central roles in many different forms of plasticity. Evidence suggests that glia and neurons should no longer be considered independent cellular elements in the nervous system, but rather two interrelated, interconnected pathways involved in information processing and plasticity.

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