Innexons, Membrane Channels for ATP, Control Microglia Migration to Nerve Lesions

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INNEXONS, MEMBRANE CHANNELS FOR ATP, CONTROL MICROGLIA MIGRATION TO NERVE LESIONS

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ATP released upon nerve injury is an important chemotactic activator of microglia; however, the source of the extracellular ATP is uncertain. In large glial cells such as astrocytes, chemical or mechanical stimulation or injury causes an intracellular calcium wave that moves from cell to cell propagated by extracellular ATP through a regenerative ATP-induced ATP-release mechanism. In mammals, a prime candidate for the ATP release channel is the pannexon, a hexamer of the invertebrate gap junction homologue pannexin1, although the pharmacologically similar protein connexin is also a candidate. I hypothesized that upon nerve injury, ATP released through pannexon channels activates microglia and initiates their movement. My experiments were done on leeches (Hirudo sp.) in part because they lack connexins, their microglia are regulated by ATP, and their glia are especially large and produce calcium signals. I found that in the giant glia of leeches the 2 homologues of pannexin1, innexins2 and 3 (HmINX2 and HmINX3), formed innexons with similar properties to pannexons. Innexons opened and released ATP upon depolarization or in elevated extracellular potassium solution, were permeable to dye molecules, and were blocked by carbenoxolone (10 to 30 µM) or saturated CO₂. Pharmacological
experiments showed these channels were essential for the migration of microglia to nerve lesions, but calcium waves in single leech glia did not require innexons or exogenous ATP. In addition, nerve injury typically produces arachidonic acid, which closed the innexons and thereby blocked microglia migration. The results were consistent with those in the leech showing that ATP activates microglia and NO is important for their directed movement. The contribution of these findings to a general understanding of the molecular signals regulating the activation of microglia is discussed.
Dedication

This dissertation is dedicated to my wife, Ilanit, whose never-ending love and encouragement supported me throughout my graduate career and continues to do so.
Acknowledgements

I would like to acknowledge all those individuals that helped me with my research. Jeff Lipitz and Emanuel Ngu, a former graduate student and a post-doc in my laboratory respectively, taught me how to quantify microglia migration and accumulation. Silviu Locovei and Feng Qiu taught me to inject and record from oocytes. Eduardo Macagno from UCSD provided the innexin mRNA constructs. University of Miami undergraduates Maria Rubi, Oscar Cabrerra, and Kavita Patel counted and tracked microglia. Bao Li and Silviu Locovei, two former graduate students in the Dahl laboratory, collaborated on the paper in which my data in Chapter 2 are published. Jeff Lipitz will co-author the paper containing the results of Chapter 3 and 5. Gerhard Dahl, whom I consider a “co-mentor,” was very helpful troubleshooting my experiments and was a constant source of insight at every stage of my research. I would like to thank my committee members Stephen Roper, Michael Norenberg, and Charles Luetje, for their support, challenges, advice, and criticisms; I could not have asked for a better committee. Finally, I would like to thank Ken Muller for mentoring me in science and life. There was no question too big and no task too small that he did not help me with. I am grateful for all the time he put into my training and I hope to emulate his successful career. I would also like to thank Ken for reviewing the manuscript of this dissertation.
# Table of Contents

*Figures list* ................................................................................................................. viii

*List of Abbreviations* ................................................................................................. x

*Definitions* .................................................................................................................. xii

**Chapter 1: General Introduction** ............................................................................. 1

  Gap junction hemichannels ................................................................. 1

  Pannexins/innexins .................................................................................. 2

  Mammalian Microglia .............................................................. 4

  Leech Microglia .............................................................................. 5

  Calcium waves ................................................................................. 6

  Calcium waves in mammalian glia .................................................. 7

  Advantages of the leech nervous system ......................................... 9

  Summary .................................................................................... 12

  Organization of this thesis ........................................................ 12

**Chapter 2: Glial-associated innexin proteins form functional membrane channels in oocytes and in the intact leech nervous system** ............................................ 14

  Summary .................................................................................... 14

  Background .............................................................................. 14

  Methods .................................................................................... 16

    mRNA injected oocyte recordings ....................................................... 16

    Extracellular ATP measurements from oocytes ................................. 18

    In-vivo dye release ........................................................................ 18

  Results ....................................................................................... 20

    Innexins formed ATP permeable innexons when expressed in oocytes 20

    Innexons in vivo ......................................................................... 21

  Discussion .................................................................................. 23

  Figures ...................................................................................... 24
### Chapter 3: Regulated release of ATP after CNS injury in the leech controls

**microglia migration** ................................................................. 29

Summary .................................................................................... 29

Background .............................................................................. 29

Methods ................................................................................... 33

  - Leeches and drugs ................................................................................................................ 33
  - Microglia accumulation studies ............................................................................................. 33
  - Low light video microscopy ................................................................................................... 35
  - Measurement of extracellular ATP ......................................................................................... 35

Results ...................................................................................... 36

  - ATP release after nerve injury ............................................................................................... 36
  - Microglia migration and apyrase ........................................................................................... 38
  - Microglia migration and carbenoxolone ............................................................................... 39

Discussion ............................................................................... 41

Figures ..................................................................................... 46

### Chapter 4: Innexons and ATP are not required for the initiation or propagation of mechanically induced calcium waves in leech glial cells... 50

Summary .................................................................................... 50

Background .............................................................................. 51

Methods ................................................................................... 52

Results ...................................................................................... 54

  - ATP induced calcium waves ................................................................................................. 54
  - Mechanically induced calcium waves .................................................................................... 55
  - Calcium gradient upon injury ................................................................................................. 56

Discussion ............................................................................... 56

Figures ..................................................................................... 59

### Chapter 5: Arachidonic acid inhibits the migration of microglia to lesions in the leech CNS by closing innexon/pannexon channels................. 64

Summary .................................................................................... 64

Background .............................................................................. 64

Methods ................................................................................... 66
Figures list

Figure 1.1: Schematic Diagram of the leech nerve cord............................................... 9
Figure 2.1: Currents recorded from HmlNX2 injected oocytes and the effect of CBX and CO2........................................................................................................... 24
Figure 2.2: Assay with Hm-inx2 injected oocytes ....................................................... 25
Figure 2.3: Morphology of the connective glial cell.................................................... 26
Figure 2.4: Dye loss from the connective glial cell and the effect of CBX................. 27
Figure 2.5: Dye loss from the connective glial cell and the effect of intracellular acidification.................................................................................................................. 28
Figure 3.1: Microglia accumulate at nerve lesions ..................................................... 34
Figure 3.2: Measurements of extracellular ATP after an injury............................... 46
Figure 3.3: The effect of apyrase on microglia migration and accumulation............. 47
Figure 3.4: The effect of carbenoxolone and ATP on the migration and accumulation of microglia ........................................................................................................ 48
Figure 3.5: Microglia in connectives treated with isotonic potassium gluconate (KGlu) and the effect of CBX (10 µM).................................................................................. 49
Figure 4.1: Measurements of calcium waves in connective glia.............................. 54
Figure 4.2: ATP induced calcium waves in the connective glial cell ......... 59
Figure 4.3: Mechanically induced calcium waves and the effect of CBX……60-61
Figure 4.4: The effect of apyrase on mechanically induced calcium waves ....... 62
Figure 4.5: The post-injury calcium response ............................................................ 63
Figure 5.1: The effect of Arachidonic acid and ETYA treatments on the migration and accumulation of microglia ................................................................. 76
Figure 5.2: Dye loss from the connective glial cell and the effect of arachidonic acid and ETYA.................................................................77
Figure 5.3: The effect of ArA and ETYA on the currents of Hm-inx2 and mouse panx1 injected oocytes .................................................................78
Figure 6.1: Proposed signaling pathway for the migration of microglia..........92
Figure 6.2: Schematic representation of the post-injury signals that regulate the microglial response...........................................................................93
List of Abbreviations

2-AG: 2-arachidonylglycerol
ArA: arachidonic acid, eicosatetraenoic acid
ADP: adenosine diphosphate
ATP: adenosine triphosphate
cAMP: 3'-5'-cyclic adenosine monophosphate
COX: cyclooxygenase
CBX: carbenoxolone
CCD: charge coupled device
cGMP: cyclic guanosine monophosphate
CNS: central nervous system
CO₂: carbon dioxide
eNOS: endothelial nitric oxide synthase
ETYA: eicosatetrayenoic acid
Hoechst: Hoechst 33258 dye
Hm-inx2/Hm-inx3: Hirudo medicinalis innexin 2/3 gene
HmINX2/HmINX3: Hirudo medicinalis innexin 2/3 protein
HRP: horseradish peroxidase
ICICR: IP3-dependent calcium-induced calcium release
IL-1: interleukin 1
IL-6: interleukin 6
L-15: Leibowitz-15
L-NAME: Nω-nitro-L-arginine methyl ester
LOX: lipoxygenase

LPS: lipopolysaccharide

NA: numerical aperture

NO: nitric oxide

OR2: oocyte Ringer's solution

P2X receptor, P2XR: purinergic receptor type P2X (a ligand-gated ion channel)

P2Y receptor, P2YR: purinergic receptor type P2Y, (G-protein coupled receptors)

Panx1: pannexin 1 gene

PANX1: pannexin 1 protein

PBS: phosphate buffered saline

RB2: reactive blue 2

TNFα - tumor necrosis factor- alpha
Definitions

Astrocytes: The most abundant glial cell type in the mammalian nervous system that forms the structural architecture of the CNS. These cells release “gliotransmitters” such as ATP and glutamate and have been implicated in essential physiological processes such as the modulation of synaptic transmission and the regulation of cerebral blood flow. Astrocytes are known to have intercellular calcium waves in response to mechanical stimulation and injury.

Calcium waves: The propagation of a calcium signal (i.e. an increase in intracellular calcium) from one cell to another (an intercellular calcium wave) or within a single cell (an intracellular calcium wave). The diffusion of IP3 and the activation of IP3 gated channels on the endoplasmic reticulum propagate intracellular and intercellular calcium waves (in the latter via gap junction channels), although a second mechanism, namely the release of ATP and the activation of P2Y purinergic receptor, is also involved in the propagation of intercellular waves.

Connexins: A family of mammalian proteins that are the molecular substrates of gap junction channels. There are over 20 members of the family in humans and they are named after their molecular weights in kDa (eg. Cx32.1, Cx32.2, Cx36, Cx43, Cx45, etc)

Gap junctions: Morphologically identified plagues consisting of large pore channels that connect the cytoplasm of adjacent cells. Two hemichannels, one
from each cell, dock to from a complete channel. Each hemichannel consists of six connexin or innexin proteins. 

**Glia:** The non-neuronal cells in the central nervous system (CNS). In the mammalian nervous system, the most common of these cells are the astrocytes, oligodendrocytes, and microglia.

**Hemichannels:** Membrane channels formed by half a gap junction channel. These are termed “connexins”, “innexons”, or “pannexons” when the hemichannels is formed by connexins, innexins, or pannexins respectively.

**Innexins:** A family of invertebrate proteins that are molecular substrates of gap junctions and are homologous to the mammalian pannexins. Relevant to this thesis is the fact that the leech has 12 identified innexins (appropriately named HmlNX1, HmlNX2, etc.).

**Microglia:** The resident macrophages of the CNS. They are the first responders to injury in the CNS and have been implicated in a wide variety of neurological and neurodegenerative diseases.

**Pannexins:** A recently discovered family of mammalian gap junction proteins homologous to the invertebrate gap junction family, the innexins.
Chapter 1: General Introduction

One goal of neuroscience is to understand the cellular and molecular signals that mediate the nervous system’s response to injury. Microglia are the first responders to injury in the CNS and understanding the mechanisms that regulate their behavior is crucial. In my research, I have investigated the role of gap junction membrane channels on glial cells as possible regulators of microglia migration through the release of ATP.

Gap junction hemichannels

Gap junctions are composed of large conductance channels that connect the cytoplasms of two neighboring cells thereby allowing for the passage of ionic currents, metabolites, and other small molecules between them (Bennett, 1997). All gap junction proteins are structurally similar, each having four transmembrane spanning regions with their amino and carboxy terminals in the cytoplasm. A hexamer of gap junction proteins pairs with a hexamer in another cell membrane to form the gap junction channel, the pore of which is a conduit that can pass molecules of up to 1 kDa (Sosinsky, 1996). Vertebrates and invertebrates were thought to contain different families of gap junction proteins, known as connexins and innexins respectively (Phelan, 2005). A search of the human genome database uncovered a new group of proteins in vertebrates that were homologous to the innexins and were named pannexins (Panchin et al., 2000; Bruzzone et al., 2003; Baranova et al., 2004).

Besides providing for direct cell to cell communication, unpaired gap junction channels, or “hemicannels”, are now also known to allow for communication
between the cell interior and the extracellular space. Strictly speaking, the “hemichannel” nomenclature is incorrect, since one might infer it is but half a channel when it is in fact a complete channel (Dahl and Locovei, 2006), so I shall refer to hemichannels as connexons, pannexons or innexons. Several types of connexins have been proposed to form functional connexons (Zhao, 2005; Plotkin et al., 2002; De Vuyst et al., 2006). Connexons have been implicated in various systems as the ATP release channel that mediates purinergic calcium waves in non-contiguous cells (Gomes et al., 2005; Leybaert et al., 2003; Stout et al., 2002; Zhao et al., 2005). But some biochemical properties of connexons, such as their voltage sensitivity and their closure in the presence of high intracellular calcium, are the opposite of what would be expected from an ATP release channel (Cotrina et al., 1998a; Hofer and Dermietzel, 1998). Moreover, calcium waves are present throughout phyla that lack connexons (Brehm et al., 1989; Zimmermann and Walz, 1997; Peters et al., 2007).

**Pannexins/innexins**

Pannexins, the recently discovered family of mammalian gap junction proteins that have sequence similarities to the invertebrate innexin family (Baranova et al., 2004; Panchin, 2005), do not appear to form gap junctions but make unpaired pannexons (Dahl and Locovei, 2006; Locovei et al., 2006a). The pannexons have qualities that make them prime candidates for the ATP release channels present in astrocytes and neurons (Dahl and Locovei, 2006). Pannexin1 (*panx1*=gene; PANX1=protein) proteins form functional pannexons in the oocyte expression system, through which molecules smaller than ~1 kDa,
including ATP, cross the membrane (Bruzzone et al., 2005; Bao et al., 2004). Some of the properties of pannexons are similar to connexons, such as their pore size and conductance (~500pS), as well as their closure to cytoplasmic acidification and their sensitivity to gap junction blockers such as carbenoxolone (Bruzzone et al., 2005; Dahl and Locovei, 2006). Unlike connexons made by connexins, however, PANX1 pannexons open in response to increases in intracellular calcium ion. Extracellular ATP opens the pannexons in oocytes co-expressing P2Y purinergic receptors and PANX1 (Locovei et al., 2006b). Moreover, gap junction channel blockers inhibit ATP release from erythrocytes, cells that do not make gap junctions but express PANX1 (Locovei et al., 2006a).

Dykes and colleagues discovered and developmentally mapped the gene expression of twelve leech innexins, designated Hm-inx1 to Hm-inx12 (or HmINX1 to HmINX12 for the proteins) (Dykes et al., 2004; Dykes and Macagno, 2006). A protein Blast search of Homo sapiens PANX1 revealed its closest invertebrate homologs are Hirudo medicinalis innexins HmINX2, HmINX3, and HmINX5, which have 27% identity with 56% similarity, 22% identity with 50% similarity, and 30% identity with 53% similarity respectively at the amino acid level to PANX1 (RID=1159378167-23240-184313320177.BLASTQ2). Hm-inx2 and Hm-inx3 are specifically expressed in glial cells, and HmINX2 was already shown by Dykes et al. 2004 to be responsible for making gap junctions between glial cells in the ganglia. Because of their glial expression and similarity to PANX1, these innexons, therefore, may be the best homologues available to study the properties of the pannexin/innexin family of proteins.
Mammalian Microglia

Microglia are the resident immune cells of the nervous system that, even at rest, actively survey and sample their local area for signals of damage or disease (Gehrmann et al., 1995; Nimmerjahn et al., 2005; Raivich, 2005). They are derived from blood macrophages in the hematopoietic system; they migrate into the nervous system during development and proliferate (Alliot et al., 1999; Perry et al., 1985). In response to a nerve injury the microglia become activated, change morphology (from ramified to ameboid), migrate to the injury site, release cytokines and other molecules that mediate inflammation, and phagocytose debris (Hanisch and Kettenmann, 2007; Prinz et al., 1999; von Zahn et al., 1997). The disregulation of microglia has been implicated in the pathogenesis of stroke, trauma, Alzheimer’s disease, Parkinson’s disease, prion disease, HIV associated dementia, amyotrophic lateral sclerosis (ALS), and multiple sclerosis (Ransohoff and Perry, 2009). Recently, evidence that some activated microglia may support neurogenesis in the adult brain further demonstrates the many and complicated functions of microglia (Ekdahl et al., 2009).

It has recently been recognized that purinergic signaling acts as an important activator and chemotactic regulator of microglia. The two families of purinergic receptors are the ionotropic P2X family (ion channels) and the metabotropic P2Y family (G-protein coupled). In mammals, nucleotides such as adenosine triphosphate (ATP) act via the metabotropic P2Y G-protein coupled receptors on microglia to activate them and cause the release of tumor necrosis factor-alpha (TNFα), Interleukin1 (IL-1), interleukin6 (IL-6) and a variety of other
inflammatory cytokines (Inoue, 2002). The activation of metabotropic P2Y receptors induced outward potassium currents (Wu et al., 2007). The activation of the P2Y_6 receptor has been shown to regulate microglial phagocytosis (Koizumi et al., 2007). ATP released specifically by astrocytes has been shown to activate microglia both in culture and in vivo (Nimmerjahn et al., 2005; Schipke et al., 2002; Verderio and Matteoli, 2001). In vivo studies in mice have shown ATP as the signal responsible for the immediate movement of microglial processes toward a lesion (Davalos et al., 2005). Microglia migrate to damaged neonatal hippocampal neurons in an ATP dependent manner (Kurpius et al., 2007). Using a chemotaxis assay, microglia were shown move in response to ATP through the activation of P2Y Gi/o-coupled receptors (Honda et al., 2001). One specific P2Y receptor, P2Y_{12}, has been identified as the receptor responsible for microglial migration and process extension in response to ATP and injuries in both in vitro and in vivo studies (Haynes et al., 2006).

**Leech Microglia**

Over the past 25 years, several dozen papers have been published on the biology of leech microglia. Microglia are of interest in part because of their involvement in axon sprouting and, possibly, regeneration, which occurs in the leech CNS (Ngu et al., 2007). The ability of microglia to produce laminin after injury may be crucial for axon growth (Duan et al., 2005; von Bernhardi and Muller, 1995). Leech microglia are similar to mammalian microglia in their cytology, physiology, histochemical staining (e.g. with silver carbonate and *Griffonia* lectin), and phagocytic ability (Coggeshall and Fawcett, 1964; Morgese
et al., 1983). Leech microglia migrate rapidly—beginning within seconds to minutes, with a top speed of 7 µm/min—after nerve injury from up to several hundred micrometers away (McGlade-McCulloh et al., 1989; Duan et al., 2005).

Leech microglia respond similarly to their mammalian counterparts to ATP. In a recent publication, Yuanli Duan, a former graduate student in the Muller laboratory, demonstrates that 100 µM ATP, ADP, and UTP, all of which are ligands of P2Y receptors on mammalian microglia, cause leech microglia to move in situ, and RB2 (50µM), a broadly acting P2Y antagonist, inhibits their migration toward a nerve crush and accumulation there (Duan, 2004). The similar activation by purines of both mammalian and leech microglia to initiate movement toward an injury implicates an evolutionarily conserved mechanism for responding to injury and indicates the leech CNS is an appropriate model for investigating the cellular and molecular mechanisms involved in this response.

**Calcium waves**

Calcium is an important intracellular signal implicated in diverse cell functions from embryogenesis to cell death. A calcium wave is a local increase in cytoplasmic calcium that is relayed either to other locations in the same cell or to other cells. Although calcium itself does not diffuse freely within and between cells, there are two mechanisms of intercellular calcium wave propagation described in the literature. These are direct propagation via the diffusion of IP3 and calcium movement through gap junctions (Saez et al., 1989; Boitano et al., 1992), and indirect propagation via the release of ATP, which binds to purinergic receptors on adjacent cells causing an increase in intracellular calcium, leading...
to a further release of ATP—a positive feedback mechanism (Hassinger et al., 1996; Guthrie et al., 1999). This second mode of propagation has sparked a debate in the literature as to the mechanism of ATP release. Although some evidence implicates the vesicular release of ATP as a main mechanism of calcium wave propagation (Bowser and Khakh, 2007), there is much evidence that channel-mediated ATP release, specifically through gap junction hemichannels, can initiate and propagate calcium waves (Bennett et al., 2003; Contreras et al., 2003; Cotrina et al., 1998b; Cotrina et al., 2000). The specific identity of the channel involved in ATP release, whether connexon or pannexon, has not been established. Much of the confusion arises because of their overlapping expression and similar responses to pharmacological agents (Bruzzone and Dermietzel, 2006).

**Calcium waves in mammalian glia**

Calcium waves are known to occur in astrocytes and other mammalian glial cells (Scemes and Giaume, 2006; Newman, 2001; Metea and Newman, 2006a) and perform crucial physiological functions by what were once considered to be only the supportive “glue” of the nervous system. Calcium waves in glial cells can regulate and control synaptic transmission (Perea and Araque, 2005; Fellin and Carmignoto, 2004), neurogenesis and proliferation (Weissman et al., 2004), neuronal differentiation and migration (Komuro and Rakic, 1996; Ciccolini et al., 2003), neuronal metabolism (Bernardinelli et al., 2004), glutamatergic signaling in the cerebellum (Ishiuchi et al., 2001), and blood flow and neuronal activity in the retina (Metea and Newman, 2006b; Newman, 2003). More recently, astrocyte
calcium waves are now recognized to be the mechanism that regulates cerebral blood flow (Koehler et al., 2009; Straub and Nelson, 2007). Given the physiological importance of glial calcium waves, understanding their initiation and propagation mechanisms is absolutely crucial.

The release of ATP from glial cells is now recognized as the primary method by which intercellular calcium waves propagate, although the mechanism of ATP release, either through hemichannels (pannexons or connexons) or through exocytosis, is still controversial (Anderson et al., 2004; Bowser and Khakh, 2007; Cotrina et al., 1998c; Cotrina et al., 1998b; Cotrina et al., 2000; Guthrie et al., 1999; Stout et al., 2002; Suadicani et al., 2004; Suadicani et al., 2006; Dahl and Locovei, 2006; Lohr and Deitmer, 1999). Mechanical stimulation and trauma can induce calcium waves that propagate hundreds of micrometers and are thought to be involved in the generation of glial scars and microglial activation; both are part of the glial response to injury (James and Butt, 2002; Ostrow and Sachs, 2005; Verkhratsky, 2006; Mills et al., 2004).

Much less research has been done on the calcium signals in leech glia; however, calcium signals initiated by glutamate and by ATP have been described (Rose et al., 1995; Muller et al., 2000; Lohr and Deitmer, 1999). This calcium signal comes from intracellular stores, and is likely due to the activation of P2Y G-protein coupled receptors and opening of IP3 gated channels on the endoplasmic reticulum.
**Advantages of the leech nervous system**

The leech (*Hirudo medicinalis*) nerve cord consists of a long chain of ganglia (where the neuronal cell bodies are located) separated by axon tracts called the connectives. Two neuropil and 6 packet glial cells surround the neurons in each ganglion; a separate large glial cell extends the entire length of each of the paired connectives (Figure 1.1). The leech nerve cord is easily removed from the animal and can survive in a petri dish for up to several weeks.

![Figure 1.1: Schematic diagram of the leech nerve cord. Glial cells are outlined (in blue). The ganglia containing the cell bodies are separated by connectives consisting of paired axon bundles, each ensheathed by a single giant glial cell. Each connective glial cell extends the full distance between the ganglia (not drawn to scale). The nuclei are located in the middle of the connectives.](image)

Different animal models and techniques used for studying the migration and behavior of microglia each have their own advantages and disadvantages. In vitro studies (such as chemotaxic assays like the Boyden chamber), while useful for isolating and studying cells in a controlled environment, lack the physiological environment of the nervous system with its interstitial fluid and other cellular contacts. These studies are also fraught with problems of serum activation of the microglia, as well as activation by the plastic or glass of the culture dishes. Consequently, any findings in vitro must be replicated in vivo.
In situ studies, in conjunction with two photon imaging, have provided important insights into the natural behavior of microglia in the intact mammalian nervous system (Davalos et al., 2005; Nimmerjahn et al., 2005). Although these are an attempt to study microglia in their natural environments, they too, have several problems that affect their interpretation. One obvious difficulty with studies of microglia in brain slices is that the cortex has been damaged in order to visualize the cells. The slicing process itself is traumatic and may on its own activate the microglia. Also, there is presently no way to distinguish microglia from infiltrating macrophages, so any conclusions must bear this qualification. Although infiltrating macrophages and microglia are similar in their physiology and behavior, they are not the same, and it would be advantageous to separate the activation of one from the other.

Studying microglia in the leech has the obvious disadvantage that the leech is an invertebrate; to generalize results to mammals, any discoveries must be confirmed in mammals. However, the leech has several experimental advantages. The nerve cord, which lies entirely within a blood vessel, may be removed from the animal intact and free of blood cells. Thus, although the nerve cord is not in the animal, experiments in many respects resemble those in vivo, and even accurate synapse regeneration occurs in the isolated nerve cord. Also, the nerve cord is transparent and easily imaged. Microglia are the most abundant cells in the leech nervous system and their nuclei are the only nuclei found in the long stretches of the connectives (excluding sheath cells, and the two distinctive nuclei of the connective glial cells) that run between ganglia.
Microglial nuclei can be easily observed when stained Hoechst 33258 dye or other fluorescent vital nuclear stains, so their movement can be tracked in living tissue or counted at sites of injury in fixed tissue. Because the migration of microglia to injuries is such an important and evolutionarily conserved behavior, it is likely that basic mechanisms involved in the migration of vertebrate and invertebrate microglia are similar.

Specifically with regard to studying calcium waves, some of the advantages of using the leech nervous system include the clear visibility of the glia in living tissue, the unusually large size of the glia, up to several mm in length, and the suitability of the cells for injection of dyes and other substances for physiological study. By studying an intracellular calcium wave in one large glial cell, it may be possible to attribute any inhibition of innexons that modifies the calcium waves to the function of hemichannels and not gap junctions (or connexons). Because of the great length of the connective glial cell and the quick sequestering of cytoplasmic calcium, one might expect calcium waves in the cell to propagate through a regenerative mechanism, and ATP-induced ATP release would be a candidate.

This dissertation describes my work to uncover mechanisms by which the large glia influence microglia after injury, specifically involving the pannexin/innexin family of proteins. The studies were designed to provide information on interactions of the cells and on cellular responses that may lead to recovery from injury, as occurs successfully in the leech.
Summary

It would be advantageous to isolate an area of CNS that contains only pannexons and not connexons, and to study calcium wave propagation and the response to injury \textit{in vivo} or \textit{in situ}. This task is difficult because of the overlapping expression of pannexins and connexins in mammalian tissue and their similar responses to pharmacological agents. The work described in this dissertation has avoided the pannexin/connexin controversy by focusing on the leech, which has no connexins but in which its innexins are closely related to human PANX1. Added advantages of the leech have been that its glial cells are large - up to 5 mm long - and thus are particularly amenable to experimental manipulation and intracellular injection. Moreover, living microglia can be tracked in vivo and readily distinguished from neurons, large glia and blood macrophages.

Organization of this thesis

This thesis is divided into chapters that are generally organized with the expectation that they will published as independent papers, with the exception of chapter 4. In chapter 2, I present evidence that innexins can form functional innexons (hemichannels) that are permeable to ATP and do in fact form functional channels in the leech glial cell plasmalemma. This research has already been published (Bao et al., 2007). In chapter 3, I present evidence that the innexons in the leech are necessary for the migration of microglia toward a crush lesion in the leech CNS through their ability to release ATP. In chapter 4, I investigate the role of innexons and ATP in calcium waves produced in the large
glial cell of the connectives. As will be discussed, neither innexons nor ATP were required for the initiation or propagation of calcium waves in leech glia and I now hypothesize that these waves are mediated by an IP3 dependent, calcium-induced-calcium-release mechanism. There was, however, a large and sustained calcium gradient observed in the connective glial cell after a crush that may be important in regulating the activity of the innexons. The results of chapter 4 will be included in the publication based on the results of chapter 3. In chapter 5, I show that arachidonic acid is a novel pannexin/innexin channel inhibitor, and may be an endogenous regulator of pannexons/innexons in vivo. The last chapter, chapter 6, is a general discussion of my results and their contribution to the current understanding of the molecular signals involved in the early microglial response to injury. The last chapter also presents models of the signaling pathway and a schematic diagram of the proposed molecular events that occur upon injury.
Chapter 2: Glial-associated innexin proteins form functional membrane channels in oocytes and in the intact leech nervous system

Summary
Pannexins, the mammalian homologues of the invertebrate gap junction proteins innexins, form non-junctioning membrane channels that act as ATP release channels. In this chapter, I show evidence that, in addition to making gap junctions, leech innexins found in glial cells are also able to form ATP permeable membrane channels, or innexons, in the intact leech nervous system and when expressed in oocytes. This is evidence for the evolutionarily conserved function of these proteins.

Background
Gap junction channels are present the electrical synapses that allow for the passage of ionic currents, metabolites, and other small molecules between cells, thereby connecting the cytoplasm of the two cells (Bennett, 1997). All gap junction proteins are structurally similar, each having four transmembrane spanning regions with its amino and carboxy terminals in the cytoplasm. A hexamer of gap junction proteins forms a channel which when paired with a hexamer in another cell membrane forms the gap junction, the pore of which can pass molecules of up to 1 kDa (Sosinsky, 1996).

Besides providing for direct cell to cell communication, gap junction channels when unpaired as “hemichannels” allow communication between the cell interior and the extracellular space. Several types of connexins as hexamers have been proposed to form functional hemichannels, or connexons (Zhao, 2005; Plotkin et

\[1\] This chapter is taken from Bao et al. 2007, but presents only work that I have done.
Connexons have been implicated in various systems as the ATP release channel that mediates purinergic calcium waves in non-continuous cells (Gomes et al., 2005; Leybaert et al., 2003; Stout et al., 2002; Zhao et al., 2005). But some biochemical properties of connexons, such as their voltage sensitivity and their closure in the presence of high intracellular calcium, are the opposite of what would be expected from an ATP release channel (Cotrina et al., 1998a; Hofer and Dermietzel, 1998).

Vertebrates and invertebrates were thought to contain different families of gap junction proteins, known as connexins and innexins respectively, with no primary sequence similarity (Phelan, 2005). A search of the human genome database uncovered three protein members of a new vertebrate gap junction protein family that were homologous to the innexins and were named pannexins (Bruzzone et al., 2003; Baranova et al., 2004).

The pannexin hemichannels, or pannexons, have qualities that make them prime candidates for the ATP release channels responsible for calcium waves (Shestopalov and Panchin, 2007). Pannexin1 (PANX1) proteins form functional pannexons in the oocyte expression system, which allows for the flux of small molecules, including ATP, across the membrane (Bruzzone et al., 2003; Bao et al., 2004). Some of the properties of pannexons are similar to connexons, such as their pore size and conductance (~500pS), as well as their closure to cytoplasmic acidification and sensitivity to gap junction blockers such as carbenoxolone (Bruzzone et al., 2005; Dahl and Locovei, 2006). Although carbenoxolone is known to be an inhibitor of 11-beta hydroxysteroid
dehydrogenase, an enzyme important for the metabolism of cortisol (Duax et al., 2000), it is well established as a gap junction inhibitor and has recently been identified as the most potent inhibitor of pannexons (Ma et al., 2009). Unlike hemichannels made by connexins, however, PANX1 hemichannels open in response to increases in intracellular calcium. Extracellular ATP opens the pannexons in oocytes coexpressing P2Y purinergic receptors and PANX1 (Locovei et al., 2006b). Moreover, ATP release can be inhibited by gap junction blockers in erythrocytes, cells that do not express connexins but express pannexin1 (Locovei et al., 2006a).

In these studies, I investigated whether some invertebrate gap junction proteins, the innexins, can also form membrane channels, or innexons. Glia in mammals express functional pannexons (Huang et al., 2007a; Iglesias et al., 2009), so I investigated whether the glial-associated leech innexins, HmINX2 and HmINX3 (data not shown), can form functional innexons like their mammalian homologues. In this chapter I will provide evidence that leech innexons form functional membrane channels, are permeable to ATP, and present in the intact leech CNS. These innexons have been demonstrated to form gap junctions (Dykes et al., 2004), so this would be an additional and novel function for these proteins.

**Methods**

**mRNA injected oocyte recordings**

*Solutions:* Oocyte Ringer solution (OR2) was composed of 82.5 mM NaCl, 2.5 mM KCl, 1.0 mM MgCl₂, 1.0 mM CaCl₂, 1.0 mM Na₂HPO₄, 5.0 mM HEPES,
and antibiotics (penicillin, 10,000 units/ml; streptomycin, 10 mg/ml), pH7.5). The patch pipette solution (Kglu) was 140 mM potassium gluconate, 10 mM KCl, and 5.0 mM TES, pH 7.5

mRNA injected oocytes: Xenopus laevis oocytes were isolated by incubating small pieces of ovary in 2 mg mL^-1 collagenase in calcium-free OR2 and stirring at 1 turn/second for 3 hours at room temperature. After being thoroughly washed with OR2 (with calcium), oocytes devoid of follicle cells and having uniform pigmentation were selected and stored in OR2 at 18 °C. Hirudo innexins 2 and 3 (Hm-inx2 and Hm-inx3) had been cloned into the expression vector pCR-BluntII-TOPO. mRNA was transcribed by SP6 (Hm-inx2) or T7 (Hm-inx3) RNA polymerase from 10 µg of XbaI- (Hm-inx2) or SpeI- (Hm-inx3) linearized plasmid using the mMessage mMACHINE kit (Ambion). mRNA was quantified by absorbance (260nm), and the proportion of full-length transcripts was checked by agarose gel electrophoresis. 20 nL of mRNA (50 ng/µL) was injected into oocytes. The injected oocytes were then transferred into fresh OR2 medium with elevated Ca^{2+} (5 mM) and incubated at 18 °C for 24-48 hours. For electrophysiological recordings, oocytes were transferred to regular OR2.

Voltage clamp: Voltage-clamp recording was performed with two intracellular microelectrodes, one to pass current and the other to record voltage. The oocytes membrane potential was clamped at a given voltage from which it was depolarized with square 5-100 mV pulses of 5 seconds duration at 0.1 Hz, the amplitude depending on the experiment. Changes in current required for the voltage pulses indicated the conductance. Conductance of the pannexin/innexin
channels was determined by comparison with control oocytes; in practice, most of the conductance was due to pannexons/innexons, as confirmed in later experiments in which CO₂ closed the channels.

**Extracellular ATP measurements from oocytes**

ATP assay solutions (luciferin/luciferase, Sigma, St. Louis) were mixed with supernatants collected from *Hm-inx2* injected and uninjected cells treated with OR2 or OR2 with potassium gluconate in place of sodium chloride (KGlu), in some cases with up to the presence of 100 µM carbenoxolone (CBX). Oocytes were injected 4 days before assay. Innexin expression and cell viability were confirmed electrophysiologically. Cells were pretreated for 10 min in experimental solutions and then isolated for 10 min in 150 µL of the same experimental solutions. A 100 µL sample of supernatant was obtained for each reading. Each condition was measured in quintuplicate. Luminescence readings were obtained with a Victor 1420 multilabel counter (PerkinElmer, Waltham, MA) on a 96 well culture plate. ANOVA with Post-Hoc Fisher test (Statistica, StatSoft Inc., Tulsa, OK) was used for comparison and statistical analysis of luminescence.

**In-vivo dye release**

Nerve cords were dissected in physiological saline and the connectives cut into 1 mm sections, pinned to Sylgard-lined dishes, and kept in L15 culture medium overnight to allow membrane resealing before recording electrically and injecting dye. For the experiments it was considered unimportant whether the piece contained the glial nucleus (Elliott and Muller, 1981). Recording
microelectrodes were pulled from standard omega-dot borosilicate glass and filled with 0.1 M 6-carboxyfluorescein (6-CF, 376 Da), or for the acidification experiments, with 0.2 M Lucifer Yellow CH (a non-pH sensitive dye, 450 Da), via capillary action; the back end was filled with 4 M potassium acetate or 2 M lithium chloride. A voltage shift of -40 to –60 mV was observed when the microelectrode penetrated the connective glial cell, and the resting potential became more negative as the membrane resealed. The dyes were injected into the connective glial cells using a -2 nA current for 3-5 minutes (Figure 2.2b). Photographs of the injected preparations were taken every 5 minutes on a Leica fluorescence microscope with a 10X objective (0.3 NA), mercury arc lamp and FITC and neutral density filters. Exposure time was limited to under a second to minimize bleaching. An aliquot of CBX was added from a 10 mM stock solution directly into the bath away from the tissue for a final concentration of 10 µM. For the wash, the tissue was removed from under the microscope, rinsed 3 times with physiological saline and immediately returned to the microscope. To achieve acidification of the Ringer’s, it was bubbled with CO₂ in the reservoir leading to the Sylgard dish recording chamber; the flow rate was 2 mL/min.

Changes in fluorescence intensity with time were used as a measure of dye loss from the connective glial cell. The fluorescence intensities of entire injected connectives were measured and averaged using Metamorph® (Molecular Devices, Sunnyvale, CA). The adjacent, uninjected connective was also measured, averaged and subtracted from the injected connective measurement to adjust for autofluorescence. The percent dye loss in 5 minutes
was measured every 5 minutes. For each connective, four time points each for the saline (before CBX), CBX treatment, and after 30 minutes of wash were averaged. A repeated-measures ANOVA followed by a post-hoc Fischer test (Statistica, StatSoft Inc., Tulsa, OK) was used for comparison and statistical analysis of the dye loss. Decreases due to bleaching were minimal, as determined by the relatively constant readings in CBX.

**Results**

**Innexins formed ATP permeable innexons when expressed in oocytes**

The glial associated innexins, *HmlNX2* and *HmlNX3* (Bao et al., 2007), both formed functional hemichannels, or innexons, when expressed in oocytes. An example of the channels they both formed is shown in Figure 2.1 for *HmlNX2*. As determined by macroscopic membrane currents, innexons, like pannexons, opened in response to depolarization. The innexons were closed at -30 mV or lower but opened at -20 to -15 mV or higher (Figure 2.1A). The channels were quickly closed by addition of carbenoxone (CBX) or by perfusion with CO$_2$-saturated Ringer’s solutions to cause intracellular acidification (Figure 2.1A-C). The effects of CBX and CO$_2$ were reversible (Figures 2.1B-C).

PANX1 forms an ATP permeable channel in erythrocytes and other cell types (Locovei et al., 2006a; Bao et al., 2004). To test for ATP permeability of innexons, the efflux of ATP from oocytes expressing *HmlNX2* was measured by a luminometric luciferase assay. As reported previously, uninjected control oocytes released some ATP when depolarized by a high potassium solution (KGlu) (Figure 2.2), which probably represented the brefeldin sensitive vesicular
release (Maroto and Hamill, 2001). *Hm*INX2 expressing oocytes had the same basal ATP release as control oocytes. However, when depolarized with high potassium solution a significant increase in ATP release was observed (Figure 2.2). Carbenoxolone inhibited this ATP release in a dose-dependent way.

**Innexons in vivo**

Although measurements using frog oocytes showed that innexins formed innexons, this did not determine whether innexins form innexons in vivo. One measure of the presence of pannexons and other "hemichannels" has been the uptake or loss across the plasmalemma of small molecules of dye that can be specifically blocked by drugs such as CBX (Bruzone et al., 2005), which we showed above blocks *Hm*INX2 innexon activity in frog oocytes. A single ~5 mm-long, glial cell expressing both *Hm*INX2 and *Hm*INX3 ensheathes all the axons of each connective between ganglia along the nerve cord of the leech (Figure 2.3) (Coggeshall and Fawcett, 1964).

To determine whether *Hm*INX2 and *Hm*INX3 form non-junctional innexon channels in the living nervous system as they do in oocytes, dye release studies were done on live connective glial cells. Lucifer Yellow CH (M.W. 457 Da) and 6-carboxyfluorescein (M.W. 376 Da) are both anionic and cross between glial gap junctions in the leech (Elliott and Muller, 1981). The dye 6-carboxyfluorescein (6-CF) was directly injected into the connective glial cell using small negative currents (~2 nA). Injection was restricted to one of the two glial cells of the connective; the second served as a control and the preparation photographed as a through-focus series with a confocal microscope. The series
was reconstructed as a cross-section of the living glial cell injected with 6CF (Figure 2.3C) and compared with a cross-section of glial cell injected with LY and fixed before mounting (Figure 2.3D). The bright cytoplasmic projections of the connective glial cell surrounded the round, dark profiles of axons. The glial cells of the connective were imaged at 5 min intervals to determine the rate of dye loss. In leech saline, approximately 15% of the dye was lost from the connective every 5 min. This dye loss was almost completely blocked with 10 µM CBX in the saline (Figure 2.4A). This effect was reversed by rinsing the CBX from the tissue, as shown for a representative example and the average of 6 experiments in Figure 2.4B. An identical effect was seen with intracellular acidification (by bubbling CO₂), which blocked the loss of Lucifer Yellow, another gap-junction permeable dye, from the connective glial cell (Figure 2.5). Because the fluorescence of carboxyfluorescein is pH-sensitive, Lucifer Yellow was used for this experiment. The tissue s were exposed to the blue excitation light for only a few seconds, and no significant bleaching of the dyes was observed. It is likely that in the resting, uninjured state, the innexons remain closed. Why the dye should leak out so readily from the cells after injection is not known, but it may be due to the injection itself, perhaps from tissue stretching that may occur with the osmotic equilibration of the dye. In any case, the attenuation of dye loss by CBX and CO₂ demonstrated that non-junctional membrane channels were involved in the passage of dye from the cell. These experiments are strong evidence that innexons do indeed form functional channels in the intact leech CNS.
Discussion

The results presented in this chapter have provided evidence that leech innexins can make innexons in oocytes that are ATP permeable. Moreover, a dye loss study showed those channels are present in the intact leech nervous system. Except for the results showing that CO$_2$ blocked dye loss, these data were published in an article in *FEBS Letters* in 2007 which I coauthored (Bao et al., 2007). Additional studies done by my collaborators and published in the article showed that innexons (1) are mechanosensitive, (2) open in response to increases in intracellular calcium, (3) have multiple subconductance states, and (4) have a unitary conductance of ~500 pS, all properties similar to those of the PANX1 channel (Locovei et al., 2006b; Bao et al., 2004).

These results were the first to demonstrate that invertebrate innexins can form functional hemichannels similar to those known to be formed by pannexins. It is unknown whether all innexins have the ability to form innexons, but these data demonstrate that at least the glial-associated innexins in the leech do. These data corroborate the likely role of pannexins as ATP release channels associated with a variety of physiological processes. In deuterostomes, PANX1 does not form gap junctions in vivo, but it has retained the evolutionarily conserved ability of innexons to form membrane channels when the gap junction function was taken over by connexins. The rest of my dissertation will demonstrate the important role the innexons play in the microglial response to injury, via the ability of innexons to release ATP.
Figures

Figure 2.1: Currents recorded from Hm-inx2 injected oocytes and the effect of CBX and CO2.

A

Figure 2.1: A. Current recorded from Hm-inx2 injected oocytes and the effect of CBX and CO2. The membrane potential of an HmINX2 expressing oocyte was held at the various potentials indicated and 15 mV depolarizing voltage pulses were applied (top trace) at a rate of 12/min. The membrane conductance due to the opening of innexon channels, which increased as the cell was held at higher potentials, was eliminated by bath applying carbenoxolone. B. Reversible block by carbenoxolone (CBX) of the macroscopic membrane currents in Hm-inx2 injected oocytes. Oocytes were injected 2 days earlier with mRNA for Hm-inx2. The voltage clamp maintained a positive membrane potential to open the innexons. The membrane potential was shifted from +20 mV to +25 mV for 5 seconds every 10 seconds and the current measured to determine membrane conductance. C. Intracellular acidification by bubbling CO2 reversibly closed the HmINX2 channel expressed in oocytes. The membrane potential was shifted from +10 mV to +20 mV for 5 seconds every 10 seconds to determine membrane conductance. CO2-saturated oocyte Ringer’s solution (OR2) was perfused through the oocyte chamber when indicated. Perfusion with saline saturated with CO2 caused intracellular acidification that reversibly closed innexon channels and reduced the membrane conductance (ΔI/ΔV).
Figure 2.2: ATP Assay with *Hm-inx2* injected oocytes

Release of ATP to the extracellular medium by oocytes was measured by luminometry using a luciferase assay. Treatment of the cells with high potassium using a potassium gluconate saline (KGl, 140 mM) caused little ATP release from uninjected oocytes but significantly larger release from *Hm-inx2* injected oocytes. CBX attenuated this larger release in a dose-dependent fashion. Means ± S.E.M. are given (n = 5). P*** < 0.0001, P** < 0.001, P* < 0.05.
Figure 2.3: Morphology of the connective glial cell

(a) Schematic diagram of the leech nerve cord. The ganglia containing the cell bodies are separated by connective consisting of paired axon bundles, each ensheathed by a single giant glial cell. Each connective glial cell extends the full distance between the ganglia (not drawn to scale). The nuclei of these cells are located in the middle of the connectives. The grey box represents the region used in the present experiments. (b) In 1 mm piece of the connectives, one of the two connective glial cells was iontophoretically filled with 6-carboxyfluorescein (6-CF) (-2 nA for 15 min). Uninjected cell is traced at bottom. Vertical line represents the location of a cross section, such as shown in c. (c) Cross-section of connectives with one glial cell filled with Lucifer Yellow dye through a recording microelectrode, so that it could be fixed, dehydrated, embedded in Epon 812 and sectioned at 2 μm. The glial sheets that wrap axons are visible as highly branched structures. (This image was contributed by Ken Muller) (d) Stacked confocal images transformed to cross sections of connective glial cells injected with 6-CF, top panel, and Lucifer Yellow, bottom panel. In both cases the paired, uninjected connective was present in the field but not visible since it was not fluorescent. Images taken with a 40X water immersion objective (0.75 NA) at 0.4 μm intervals for the living 6-CF injected tissue (top), and with a 60X dry objective (0.95 NA) at 0.2 μm intervals for the fixed (in 4% paraformaldehyde), LY-injected preparation, explaining the greater resolution of the LY tissue.
Figure 2.4: Dye loss from the connective glial cell and the effect of CBX

A. Representative time course of dye loss from a 1 mm piece of connective glial cell injected with 6-CF. Carbenoxolone (CBX, 10 µM) was added and washed as indicated.

B. Quantification of average dye loss before, during and after CBX treatment. (n=6, $P^*<0.01$, $P^{**}< 0.001$)
Figure 2.5: Dye loss from the connective glial cell and the effect of intracellular acidification.

A. Representative time course of dye loss from a 1 mm piece of Lucifer Yellow injected connective glial cell. CO₂ saturated Ringer’s solution was added when indicated.

B. Average dye loss before, during and after CO₂ treatment. Data were analyzed using Metamorph® region analysis. Statistical analysis was performed using Statistica® repeated-measures ANOVA and post-hoc Fischer test (n=5, *P*<0.01, **P**< 0.001).

Figure 2.5: Dye loss from the connective glial cell and the effect of intracellular acidification. A. Representative time course of dye loss from a 1 mm piece of Lucifer Yellow injected connective glial cell. CO₂ saturated Ringer’s solution was added when indicated. B. Average dye loss before, during and after CO₂ treatment. Data were analyzed using Metamorph® region analysis. Statistical analysis was performed using Statistica® repeated-measures ANOVA and post-hoc Fischer test (n=5, *P*<0.01, **P**< 0.001).
Chapter 3: Regulated release of ATP after CNS injury in the leech controls microglia migration

Summary
Injuries to the central nervous system (CNS) attract and activate microglia. ATP release is presumably the signal affecting microglia, and injured glial cells that release ATP through pannexons (unpaired gap junction channels) are considered a source of extracellular ATP. But work on nerve injury in the leech has shown that while ATP activates microglia, a second signal, nitric oxide (NO), directs their movement. Leech innexons, pannexon homologues, were blocked, which decreased injury-induced ATP release and consequent movement of microglia. Directed movement and accumulation were restored by diffuse application of ATP. The results reveal an important role of the pannexin/innexin family of proteins in the innate immune response and support the idea that glial ATP and NO dually regulate the microglial response to injury.

Background
Microglia respond rapidly to CNS injury by extending processes and migrating to the lesion (Gehrmann et al., 1995; Nimmerjahn et al., 2005). Extracellular ATP, a signaling molecule important to glial physiology and pathology (James and Butt, 2002), has been demonstrated to be crucial in the process of microglial activation and migration both in vivo and in vitro (Davalos et al., 2005; Honda et al., 2001; Inoue, 2002). However, migration assays typically do not distinguish between extracellular ATP’s activation of movement and its trophic attraction. Recent work on the leech suggests that activation is separate from the signal that directs migration, which in situ appears to be nitric oxide.
(NO) (Duan et al., 2009) and is produced both directly at the lesion (Kumar et al., 2001) and likely by the glial calcium wave (Li, Sul, & Haydon, 2003).

The sources of ATP that activate microglia remain elusive. While injured neurons and glia undoubtedly release ATP pathologically through torn membranes, there also appears to be a regulated, physiological release of ATP from damaged cells. Wang and colleagues (2004) have shown that ATP is released upon mechanical injury to the spinal cord of an anesthetized rat and persists in the extracellular space for several hours. They also demonstrated that the prolonged release of ATP does not derive from the lesion itself (which actually has reduced ATP release), but from the peri-tramautic region surrounding the lesion from metabolically active, activated cells. Based on what is known about astrocytic ATP release upon mechanical injury and stimulation, the authors hypothesize that astrocytes are responsible for the physiologically regulated release of ATP after injury.

Mammalian astrocytes can activate microglia both in vivo and in co-cultures in vitro (Bianco et al., 2005b; Schipke et al., 2002; Verderio and Matteoli, 2001). However, the exact mechanism of this glia-microglia interaction and how it functions in vivo under physiological conditions is still speculative. Astrocytes release ATP in conjunction with intercellular calcium waves and extracellular ATP is widely accepted as the soluble signal responsible for the propagation of these waves, via its action on P2Y receptors (Guthrie et al., 1999; Salter and Hicks, 1995; Scemes and Giaume, 2006). The mechanism of this release of ATP from the cell is debated, and both vesicular and channel-mediated release have been
proposed (Anderson et al., 2004; Bowser and Khakh, 2007; Cotrina et al., 1998c; Cotrina et al., 1998b; Cotrina et al., 2000; Guthrie et al., 1999; Stout et al., 2002; Suadicani et al., 2004; Suadicani et al., 2006; Dahl and Locovei, 2006; Lohr and Deitmer, 1999).

Pannexin1 (PANX1= protein; panx1= gene), the mammalian homologue of innexins, the invertebrate gap junction proteins, has emerged as a likely candidate to release ATP during the calcium wave (Dahl and Locovei, 2006; Scemes and Giaume, 2006). The large pore PANX1 channel (~500 pS) is calcium sensitive, ATP permeable and has been localized to vertebrate cells that produce calcium waves including astrocytes (Locovei et al., 2006b; Lai et al., 2007; Bao et al., 2004). Furthermore, the immunoreactivity of PANX1 does not co-localize with gap junction plaques in cultured astrocytes, indicating that PANX1 plays a non-junctioning role in these cells (Huang et al., 2007a). In pathological conditions, PANX1 has been implicated in the activation of the inflammasome (Kanneganti et al., 2007; Pelegrin et al., 2008; Silverman et al., 2009) and the P2X7 “death” receptor complex (Pelegrin and Surprenant, 2006; Iglesias et al., 2008; Locovei et al., 2007).

Unfortunately, the similar pharmacological profiles of pannexins and connexins, the mammalian family of gap junction proteins, have made it difficult to distinguish the two. This is not the case in invertebrates, including the leech, which have only pannexin-like proteins, the innexins, and not connexins. We previously found that HmlINX2 present in the leech glia (Dykes and Macagno, 2006) forms ATP permeable membrane channels (innexons) when expressed in
oocytes and is inhibited by carbenoxolone (50% inhibition, ~1 µM) (Bao et al., 2007), which inhibits both connexins and pannexins. Moreover, at similar concentrations carbenoxolone inhibits the leak of 6-carboxyfluorescein from leech glia, evidently through innexons (see chapter 2). One aim of the present study was to determine whether dye is also retained in the glial cell under conditions of cellular acidification, which closes HmINX2 channels in oocytes (Figure S1 of Bao et al., 2007).

The central nervous system of the leech Hirudo, in which microglia were early described and characterized (del Rio Hortega, 1920), has been valuable for understanding basic mechanisms involved in migration of microglia to lesions (McGlade-McCulloh et al., 1989; Duan et al., 2005; Morgese et al., 1983; Ngu et al., 2007; von Bernhardi and Muller, 1995; del Rio Hortega, 1932). Leech microglia are similar to mammalian microglia in their cytology, physiology, histochemical staining (e.g. with silver carbonate and Griffonia lectin), and phagocytic ability (Coggeshall and Fawcett, 1964; Morgese et al., 1983). The leech nerve cord consists of a chain of segmental ganglia containing neuronal cell bodies linked by a pair of axon tracts, each ensheathed by a single large glial cell forming the connectives. The nucleus of each connective glial cell is midway between ganglia. Although the 2 connective glia are each several millimeters long, they are structurally and functionally similar to mammalian astrocytes and oligodendrocytes, but do not form myelin (Deitmer et al., 1999; Coggeshall and Fawcett, 1964; Gray and Guillery, 1963; Kuffler and Nicholls, 1966). The connectives contain thousands of microglia (Kai-Kai and Pentreath, 1981;
Morgese et al., 1983). We hypothesize that the pannexin family of proteins in glial cells is involved both in the release of ATP after an injury and in the subsequent activation of microglia in the medicinal leech. This study was designed to determine whether the pannexin channel inhibitor carbenoxolone (Barbe et al., 2006; Ma et al., 2009) can block microglial migration to a lesion by closing innexon channels of the large glial cells and blocking release of ATP.

Methods

Leeches and drugs

Adult leeches (Hirudo, Leeches USA, Westbury, NY), 3-4g, were maintained in artificial pond water (Forty Fathoms, 0.5 g/L H2O; Marine Enterprises, Towson, MD) at 15ºC. Their nerve cords were dissected and pinned in 35 mm Petri dishes (BD Falcon #351008, Franklin Lakes, NJ) containing 1 mL Sylgard 184 silicone rubber (Dow Corning, Midland, MI). For dissections and experiments, leech nerve cords were kept in physiological saline, or Ringer's (Kuffler and Potter, 1964). Nerve cords can be maintained for several days when incubated in Leibowitz-15 (L-15) culture medium supplemented with 2% fetal bovine serum, 1% glutamine, 0.06% glucose, and 0.0005 % gentamicin. Drugs were administered by bath application at their final concentrations. Carbenoxolone and apyrase were dissolved in water. All drugs were purchased from Sigma-Aldrich (St. Louis, MO).

Microglia accumulation studies

Nerve cords were prepared as described. Connectives were crushed for 8-12 seconds with fine forceps (Dumont #5), and incubated in Ringer's at room
temperature for 4 hours with or without drugs. The tissue was then fixed overnight at 4ºC in PBS with 4% paraformaldehyde (pH 7.2), washed, and mounted on a microscope slide in 80% glycerol with Hoechst 33258 dye (10 µg/mL) to stain nuclei. Microglia nuclei were imaged with a Leica DM RZA2 fluorescence microscope (20X objective, NA 0.7) using an appropriate UV filter set. A series of 10 optical sections was photographed at 2 µm intervals and a combined depth of 18 µm. An image of the connectives was also made with blue illumination and a fluorescein filter set to identify the location of the crush, which had less autofluorescence at fluorescein emission wavelengths (green); this was done to determine where to count microglia without bias, since they were not visible with the fluorescein filter set. Each stack of images showing microglia at the crush was combined and collapsed using the Metamorph program (Molecular Devices, Downingtown, PA); the microglia were counted in a 10,000 µm² region at the edge of each crush (Figure 3.1, below). The counts were statistically analyzed with an ANOVA and post-hoc Fischer Test (Statistica®, StatSoft Inc., Tulsa, OK).

Figure 3.1: Microglia accumulate at nerve lesions. Representative collapsed images (10 in total) of Hoechst stained nuclei at crushes (total depth is 18 µm). The area of the crushes is indicated in orange. Red squares are 100 µm x 100 µm regions of measurement.  “Treatment” tissue was incubated in 10 µM CBX.
Low light video microscopy

Dissected nerve cords were incubated for 30 minutes in Hoechst 33258 dye (0.001%), rinsed 3 times in Ringer’s for 5 minutes and kept overnight in L-15 culture medium with supplements (Ready and Nicholls, 1979) at 15°C to ensure that nuclei were stained and to lower the background. The tissue was then imaged on a Zeiss WL epifluorescence microscope (40X water immersion objective, NA 0.75) using a 12V, 100 W tungsten-halogen lamp operated at 8-10V and UV filter set. For time-lapse studies, Metamorph® imaging software controlled a shutter and acquired images from a CCD camera (XC-77, Hamamatsu City, Japan), averaging 256 frames at video rates every 2.5 minutes for up to 4 hours. Prior to photography, the connectives were crushed and drugs added. Controls were uncrushed connectives and crushed connectives without drug treatment, depending on the specific experimental protocol. Microglia movement during a time-lapse series was measured by double-blind assistants (undergraduate students ignorant of the experimental aims and conditions) who tracked individual microglia nuclei or counted the number of nuclei that moved more than 30 or 50 µm, noting direction of movement. The counts were statistically analyzed by ANOVA and post-hoc Fischer Test using Statistica®.

Measurement of extracellular ATP

Connectives cut from dissected nerve cords were incubated in L-15 overnight. They were then rinsed, crushed, incubated in 150 µL Ringer’s solution for a timed interval ranging from 15 to 120 minutes and 100 µL of the supernatant collected for ATP analysis. A 50 µL sample of the premixed luciferin/luciferase solution (Enlighten® ATP Assay System, Promega, Madison WI) was added to
each 100 µL supernatant. Luminescence measurements were acquired using a Victor 1420 multi-label counter (Perkin Elmer, Waltham, MA). Results were statistically analyzed with ANOVA and post-hoc Fischer test (Statistica®). Standard concentrations of ATP were used to calibrate the assay and determine the concentration of ATP in the tissue. Although the luminescence measurements were consistently of the same order of magnitude, the sensitivity of the assay only allowed the reporting of a range of ATP concentrations. In the 150 µL of supernatant surrounding the crushed tissue, ATP concentrations in crushed tissues were between 1 and 10 nM. Since the tissue volume of ~0.1 µL (100 x 100 x 10,000 µm³) represented a small fraction of 150 µL, and the extracellular space a small fraction of that, ATP concentrations in the extracellular space in tissue were in the upper micromolar to low millimolar range.

Results

ATP release after nerve injury
Leech microglia can be activated by ATP, and P2Y purinergic antagonists block that activation and block their accumulation at lesions (Duan et al., 2009), consistent with injury releasing ATP along the nerve. To confirm the release of ATP after a crush injury, we performed a luciferin/luciferase ATP assay on the leech connectives in both crushed and non-crushed conditions. ATP release after 2 or 3 crushes rose to 10 times that of uncrushed controls, a significant rise (Figure 3.2A). In the 150 µL of supernatant surrounding the tissue, ATP concentrations were between 1 and 10 nM after a crush. Since the tissue volume
was ~0.1 µL and the extracellular space approximately 5% to 10% of that (Nicholls and Wolfe, 1967), the ATP concentrations in the extracellular space were estimated to be in the range of 0.5 to 5 millimolar. The measurements did not distinguish between ATP released directly through damaged membranes and that passing through innexons, nor did it distinguish its originating from glia or from other cells.

Microglia have been reported to continue migrating to crushes many hours after an injury is made (Masuda-Nakagawa et al., 1990; McGlade-McCulloh et al., 1989; Ngu et al., 2007). To determine the time-course of elevated ATP levels following nerve cord damage, ATP was measured at 15, 30 and 120 min from 2 pairs of connectives that were each crushed twice for a total of 4 nerve crushes (Figure 3.2B). Although the extracellular ATP concentration was less at 120 minutes than at 15 or 30 min, it remained significantly higher than uncrushed controls. ATP is broken down by ATPases in the extracellular space, so it is unlikely that such high concentrations would remain in the supernatant without a mechanism for prolonged release of ATP from the injured cells.

The experiments in figures 3.2A and B did not distinguish between ATP released through torn membranes and by physiological processes. Because microglia reportedly migrate to a crush from at least 700 µm away and for several hours (Duan et al, 2005), we hypothesized that an additional mechanism of ATP release was responsible for continued high levels of ATP. To try to distinguish between the ATP released by the damaged tissue and the continued release by physiological processes, the tissue was washed 3 times at 15 minutes after injury.
and 30 minutes later the supernatant removed and ATP measured. Extracellular ATP remained elevated compared to uncrushed controls (Figure 3.2C). This continued release of ATP diminished when the tissue was treated with carbenoxolone (10 µM), implicating innexon channels in the extended release of ATP after an injury (Figure 3.2D).

**Microglia migration and apyrase**

Since activation of purinergic receptors by ATP is evidently required for activation of microglia and their accumulation at lesions, and the data above showed that ATP was released by nerve injury, it was hypothesized that apyrase, an ecto-ATPase that hydrolyzes ATP into ADP and then AMP, would inhibit microglia accumulation at a lesion. Leech connectives were therefore treated with apyrase at 5, 10, and 20 units/mL for 4 hours beginning 10 minutes before the tissue was crushed. Apyrase reduced the number of microglia found at the crushes in a concentration dependent manner (Figure 3.3A). Time lapse studies of crushed connectives confirmed that there were fewer microglia migrating to the injury when the tissue was treated with 20 U/mL of apyrase compared to controls (Figure 3.3B). There was no effect of apyrase on the direction of movement, but apyrase decreased the amount of movement of microglia (Figure 3.3C). This was consistent with earlier work indicating that NO rather than ATP affects the direction of movement of microglia, but that ATP is required for movement itself (Duan et al., 2009).

This experiment showed that extracellular ATP released after nerve crush was necessary for movement of the cells, but it did not rule out the possibility that
an additional signal within the connectives, possibly even a mechanical one, was also required for simple movement. To determine more precisely the involvement of diffusible ATP released from the connective, an unharmed connective was placed in close proximity to an injured connective, the two connectives were pinned next to one another. Time lapse microscopy of one connective was performed before and after its neighbor was crushed (Figure 3.3D). Once it was determined that crushing the neighbor connective increased movement of the microglia on the uncrushed connective, 20 U/mL apyrase was added (Figure 3.3D). Apyrase inhibited the increased movement, supporting the idea that extracellular ATP released by nerve injury activates microglia without mechanical stress directly on the microglia. Although ATP may not be the only signaling molecule released by the connective after an injury, we conclude that its presence is crucial for the microglial response.

**Microglia migration and carbenoxolone**

Because the innexin inhibitor carbenoxolone reduced the continued release of ATP from the connective after a crush injury, it was of interest to determine whether carbenoxolone could also inhibit the accumulation of microglia at the lesion. In the presence of 1, 10, and 100 µM CBX, connectives were crushed and the accumulation of microglia measured (Figure 3.4A). CBX prevented accumulation in a dose dependent manner; with statistical significance at 10 µM. Concentrations of 10 to 30 µM CBX were used in subsequent experiments.
To determine whether CBX might interfere directly with ATP activation of microglia rather than release of ATP, a time lapse study was performed. Separate nerve cords were treated with CBX (30 µM) either before or after addition 100 µM ATP (Figure 3.4B). ATP induced microglial movement was not diminished by carbenoxolone.

We hypothesized that if CBX prevents the release of ATP required for microglial movement, with the direction of movement controlled by NO, then addition of ATP to the bath containing the crushed nerve cord treated with CBX would reverse the effect of CBX, causing microglia to move to the lesion and accumulate there. Figure 3.4C shows the results of an experiment in which 10 µM CBX was added to the Ringer’s bath 15 min before the nerve cord was crushed, which reduced accumulation, and that reduction was reversed by addition of 100 µM ATP to the bath. To determine whether CBX treatment was reversible, the connectives were treated with 10 µM CBX for 4 hours, washed 3X in 15 minutes, and crushed. A time lapse study of microglial migration to a crush demonstrated that in CBX the microglial movement was significantly reduced but recovered in the presence of 100 µM ATP (Figure 3.4D). Thus, CBX inhibited the microglial response to the injury by blocking the release of ATP from the damaged tissue without affecting the ability of microglia to respond to ATP or influencing the signal that directs microglia to the lesion, which is considered to be NO.

Since exogenous ATP in the absence of a lesion causes microglia to move, it might be expected that treatments that open innexons without tissue
damage would release ATP and trigger microglia movement. Experiments with oocytes have shown that an isotonic potassium gluconate saline opens leech HmINX2 channels and releases ATP (Bao et al., 2007). This treatment opens innexon channels even if the cell’s membrane is clamped to negative resting potentials. Therefore, uninjured connectives were bathed in an isotonic potassium gluconate Ringer’s solution (K+glu, 120 mM) and the movement of microglia measured from time lapse photographs (Figure 3.5). The microglia moved more than in control conditions, and the increased microglial movement did not occur in 10 µM CBX. This indicates that opening innexin channels in the leech CNS is sufficient to cause microglial movement.

**Discussion**

In this study, CBX reversibly inhibited the migration and accumulation of microglia to a crush injury. CBX did not directly influence the behavior of the microglia, but rather did so indirectly by decreasing the release of extracellular ATP, required for microglia movement. Furthermore, CBX did not inhibit microglia movement when ATP was present.

There are no connexins in the glial cells of the leech, and a single giant glial cell ensheathes all the axons and surrounds the microglia of the connective extending several millimeters from one ganglion to the next. This arrangement in the leech means that the effect of CBX on the connective glia is targeted to innexons (hemichannels) rather than to block connexons or interrupt a chain of linked glia, as in mammalian CNS. Also, the low concentrations of CBX used in these studies were not sufficient to prevent dye coupling between glial cells (data
not shown) indicating that the gap junction function of the innexins was not blocked.

Microglia can be activated by ATP that is released from neighboring cells such as astrocytes (Verderio and Matteoli, 2001), or perhaps even by their own autocrine release of ATP, as has been described for other immune cells (Yip et al., 2009; Chen et al., 2006). In my studies, although autocrine release of ATP from microglia cannot be dismissed, there is reason to believe that the ATP that activates the microglia comes from neighboring cells. First, it is known that in mammals, ATP signals provided by astrocytes, both in culture and in situ, can activate microglia and cause the release of cytokines, such as interleukin-1-beta (Ferrari et al., 1997; Verderio and Matteoli, 2001; Schipke et al., 2002). Second, the distance that some microglia travel to reach the site of injury (up to several hundred micrometers) is much larger that the size of an individual microglial cell (30-50 µm), indicating that a signal must be carried this distance, otherwise the microglia would not detect the injury. Among the neighboring cells, both neuronal axons and glia are present as possible sources of ATP for the microglia. Both of these cell types have been shown to have innexons that can release ATP and are blocked by CBX (Bao et al., 2007). However, large glial cells are activators of microglia and are known to be involved in the immune response to injury through their release of ATP (Abbracchio and Verderio, 2006; Bianco et al., 2005a; Bianco et al., 2005b). Therefore, the most likely cells responsible for the release of ATP after an injury are the glial cells. This might be confirmed by selectively knocking down the expression of the innexons in the
glial cell using RNA interference technology. However, the possibility remains that once the microglia are activated by external stimuli they then release ATP and further activate themselves.

Inflammasomes are membrane platforms that play a crucial role in detecting foreign pathogens; they initiate and maintain inflammation and the innate immune response by activation of caspase 1 and the release of IL1-beta. (Franchi et al., 2009; Lamkanfi et al., 2007). P2X7 purinergic receptors have been implicated in the activation of the inflammasome as have pannexins (Coutinho-Silva et al., 2009; Kanneganti et al., 2007). Such results are consistent with the idea that pannexin channels themselves are the pore forming component of the P2X7 channels (Locovei et al., 2007). It has recently been shown that pannexin channels on both neurons and glia open in response to injury and PANX1 has recently been implicated in the formation of the inflammasome in a mouse model of spinal cord injury (Silverman et al., 2009). The present work has shown that innexons are necessary for an additional element of the immune response, namely the migration and activation of microglia to the site of injury. Whether there is a relationship between microglia and activation of the inflammasome is not known.

Although pannexon/innexon channels open in response to injury, the stimulus for this opening remains elusive. Both innexons and pannexons open in response to increases in intracellular calcium (Bao et al., 2007; Locovei et al., 2006b). Glial calcium waves that occur upon mechanical stimulation can spread several hundred micrometers. Cocultures of microglia and astrocytes have
demonstrated that microglia respond to astrocytic calcium waves via purinergic signaling (Verderio and Matteoli, 2001). In the next chapter I will show that the glial cells respond to injury with a large and sustained calcium gradient that persists for at least an hour. The trauma-induced increase in cytoplasmic calcium may by itself open the innexon channels, since both pannexon and innexon channels open when intracellular calcium concentrations rise. Alternative mechanisms that could also open the channels upon injury include the increased extracellular potassium and tissue stretching (Bao et al., 2007).

In the leech, nitric oxide (NO) has been identified as a crucial signal for the directed migration of microglia to a lesion (Duan et al., 2003; Chen et al., 2000). NO production increases immediately after injury and has been localized to the lesion (Kumar et al., 2001; Shafer et al., 1998). Other signaling molecules, such as endocannabinoids and opioids, have also been shown to block microglial migration through the regulation of NO (Lipitz, 2008; Yahyavi-Firouz-Abadi et al., 2007). However, despite the strong evidence for the involvement of NO in microglial migration, no evidence has emerged supporting NO itself as an initiating signal of microglial movement. Instead, it appears that NO only provides the directional cue for the microglia (and evidently the stopping cue), while ATP provides the cue for movement (Duan et al., 2009). My data also support this hypothesis because while apyrase and CBX blocked microglial movement, they did not disrupt the direction of movement when ATP was added diffusely. The relationship between the NO signal and the ATP signal, if any, has yet to be discovered. However, it is interesting to note that both eNOS and
innexons are activated by increases in intracellular calcium (Bao et al., 2007; Fleming and Busse, 1999), an event that occurs upon injury.

In conclusion, my results appear to be the first evidence that the inhibition of the pannexon/innexon family of the proteins can influence the cellular response to injury, specifically the migration of microglia activated by ATP. Pannexin/innexin membrane channels are evolutionarily conserved, so their release of ATP in response to cellular stresses and role in activating microglia in the leech supports the idea that they may have a similar function in mammals.
Figures

Figure 3.2: Measurements of extracellular ATP after an injury. A. An ATP luminescence assay (luciferin/luciferase) was used to measure the increase in extracellular ATP after a crush injury. Connectives were dissected the day before the experiment and kept overnight in supplemented L-15. 0, 1, 2 or 3 crushes were made to the connectives that were then incubated at 18°C for 30 minutes in 150 µL Ringer’s solution. 100 µL of the supernatant was analyzed for its ATP content. Because of the variability in the measurements only conditions with 2 and 3 crushes showed statistically significant increases in extracellular ATP, although there appears to be an increase with just one crush. The extracellular ATP is no different between conditions with 2 and 3 crushes, where a ceiling appears to have been reached (n=3, p<0.05). B. Extracellular ATP was elevated for at least 2 hours after injury. 100 µL from supernatant of twice crushed connectives (total volume 150 µL) were collected at various time points after the crush and analyzed using a luciferin/luciferase luminescence ATP assay. Extracellular ATP remained elevated for at least two hours post injury. C. Release of ATP more than 15 minutes after injury and the effect of CBX (10 µM). Connectives were crushed twice, incubated for 15 minutes at room temperature, washed three times with Ringer’s, resubmerged in 150 µL fresh Ringer’s solution, and incubated at room temperature for 30 minutes. 100 µL aliquots of supernatant were analyzed with luciferin/luciferase luminescence assay to measure levels of extracellular ATP. Increased levels of ATP were present even after rinsing the tissue, indicating that there was continued release of ATP even 15 minutes after the injury. D. In another experiment, the continued release of ATP was attenuated by treatment with 10 µM CBX. Control and CBX conditions are displayed as percentages of values for uncrushed tissues. (n=3, p<0.05) (Panels A and B are identical to figures 7 and 8 in Jeff Lipitz’s dissertation, who acknowledged doing the experiments with me.)
Figure 3.3: The effect of apyrase on microglia migration and accumulation. A. Tissues were treated with apyrase at 5, 10, and 20 U/mL at the time of crush and the microglia were given 4 hours to accumulate, after which the tissue was fixed in 4% paraformaldehyde, stained with Hoechst 33258 dye and photographed. “Distal” was a measure of microglia in an uncushed region, approximately 1 mm from any injury, and was used as a baseline for the number of microglia distributed randomly throughout the tissue. The difference between the “control” and the “distal” is the measure of accumulation. Bars represent mean number of microglia at the site of injury in a 100x100x18 µm³ volume. (n=3, p<0.05) B. Hydrolysis of ATP and microglial migration toward a crush. Time lapse images of crushed connectives containing Hoechst stained nuclei were collected. In the approximately 400 µm region adjacent to the crush, the microglia nuclei moving more than 50 µm in two hours were counted. The connectives treated with apyrase, the ATP hydrolyzing enzyme, had approximately 1/4 the number of microglia moving relative to the control; the fraction of those moving that moved toward the crush (i.e. the directional movement) was approximately the same as the control. (n=3, p<0.05) C. Extracellular ATP released after a crush caused microglia (MG) to move in an adjacent tissue. Pairs of connectives were dissected, stained with Hoechst 33258 dye and incubated in L15 culture medium overnight. The next day, one connective was imaged with time lapse microscopy (every 2.5 minutes) while the other connective, placed parallel to it and 40-50 µm away, was crushed. The microglia in the connective that was not touched moved. This movement was blocked by bath applying apyrase, an ATP hydrolyzing enzyme, indicating that extracellular ATP released from the crush was required to cause microglial movement. Movement was measured as translocation of the microglial cell nucleus by more than 30 µm in 1 hour. (n=3, p<0.05) (Panel A is identical to figure 9 in Jeffrey Lipitz’s dissertation, who acknowledged doing the experiment with me.)
Figure 3.4: The effect of carbenoxolone and ATP on the migration and accumulation of microglia.

A. Carbenoxolone (CBX) and microglia accumulation at the site of a crush injury. Tissues were treated with CBX at 1, 10, and 100 µM concentrations at the time of crush and the microglia (MG) allowed to accumulate for 4 hours. Tissues were then fixed (4% paraformaldehyde), and stained (Hoechst 33258 dye). Bars represent mean number of microglia at the site of injury in a 100x100x18 µm³ volume. “Distal” is a measure of the number of microglia in an uncrushed region, approximately 1 mm from any injury, and is used as a baseline for the number of microglia distributed randomly throughout the tissue. The difference between the “control” or other conditions and the “distal” is the measure of accumulation. Mean ± SEM are represented. (n=6, p<0.001)

B. Movement in CBX (30 µM) and 100 µM ATP. Hoechst stained microglia nuclei were tracked during a time lapse recording. All cell nuclei moving more than 30 µm were counted. CBX did not inhibit movement in ATP, although it did inhibit movement induced by crushing the connectives. Bars represent average number of microglia moving in 30 minutes ± SEM. (n=5, p<0.01)

C. Microglia accumulation with CBX and ATP. 10 µM CBX significantly reduced the accumulation of microglia (MG) at the crush. In the CBX washout condition the connectives were incubated in CBX for 4 hours, washed for 1 hour, and then all tissue was crushed at the same time. “DIS” (same as “Distal” above) is a measure of microglia in an uncrushed region. The reduction in CBX was eliminated by washing the CBX from the tissue, showing the effect was reversible. It was also eliminated in the simultaneous presence of 100 µM ATP, consistent with an effect of CBX to block release of ATP but not the direction of movement. (n=3, *p<0.5, **p<0.01)

D. Microglial migration toward a crush and the effects of CBX (10 µM) and ATP (100 µM). Time lapse images of crushed connectives with Hoechst stained nuclei were collected. In the approximately 400 µm long region adjacent to the crush, the microglia nuclei moving more than 50 µm in two hours were counted. Drugs were added 5 minutes before crushing. The CBX with ATP condition was similar to the control condition in both total movement and directional movement, but CBX alone decreased the movement significantly. (n=3, p<0.05)
Figure 3.5: Microglia in connectives treated with isotonic potassium gluconate (KGlub) and the effect of CBX (10 µM)

Hoechst 33258-stained microglia nuclei (MG) were imaged using time lapse microscopy for 1 hour, with the number of nuclei moving 25 µm counted from 30-60 minutes. The Ringer’s solution bathing the tissue was then replaced with a potassium gluconate Ringer’s solution (140 mM) for 30 minutes and the nuclei moving were counted again. Although CBX had no effect on the basal movement of the microglia, it blocked the KGlub-induced movement. (T test, n=3, p<0.05)
Chapter 4: Innexons and ATP are not required for the initiation or propagation of mechanically induced calcium waves in leech glial cells

Summary

ATP is known to act as a paracrine messenger to propagate the calcium waves in mammalian glia. Pannexin, the mammalian homologue of the invertebrate gap junction protein the innexins, is a likely candidate to form the ATP release channel responsible for mediating calcium waves. I demonstrated in chapter 3 that by releasing ATP, innexons are necessary for the migration of microglia to a lesion. In this chapter, I investigated whether innexons are required for mechanically induced calcium waves in glial cells, a microcosm of what occurs during injury. Glial calcium waves were evoked with focally applied ATP or a mechanical stimulus and were seen after intracellular injection of calcium green1 or Oregon green Bapta-2 dye. The calcium waves persisted in the presence of carbenoxolone and apyrase with no significant reduction in amplitude or propagation speed, indicating that neither functional innexons nor ATP was necessary for calcium waves in leech glia. Although I had previously hypothesized that an extracellular ATP release mechanism was involved in the glial calcium waves, the result was consistent with the intracellular nature of calcium waves, since for the large glial cell of the leech the waves were not intercellular. The calcium waves also persisted when the glia were bathed in a Ringer’s solution with no added calcium. These calcium waves may be mediated by an IP3 dependent, calcium-induced-calcium release mechanism, similar to what is known to occur during intracellular calcium waves in astrocytes. The results do not clarify whether intercellular calcium waves in mammalian glia are
ATP independent. But because calcium is greatly elevated after an injury, although innexons do not mediate calcium signals, calcium signals may regulate both innexons and pannexons.

**Background**

Calcium waves occur in astrocytes and other glial cells (Scemes and Giaume, 2006; Newman, 2001; Metea and Newman, 2006a) and perform crucial physiological functions in what were once considered to be only supportive cells. The release of ATP from glia is now recognized as the primary method by which calcium waves propagate between glial cells (Anderson et al., 2004; Guthrie et al., 1999; Hassinger et al., 1996), although the mechanism of ATP release, either through “hemichannels” (pannexons or connexons) or through exocytosis, is still controversial (Bowser and Khakh, 2007; Cotrina et al., 1998b; Stout et al., 2002; Suadicani et al., 2006; Dahl and Locovei, 2006) Among the possibilities, the pannexons have qualities that make them favorable candidates for the ATP release channels responsible for calcium waves (Shestopalov and Panchin, 2007), including their pore size and sensitivity to increases in cytoplasmic calcium.

Mechanically induced calcium waves are a microcosm of what likely occurs during nerve trauma. Mechanical stimulation and trauma can induce calcium waves that propagate many hundreds of micrometers and the waves are thought to be involved in the generation of glial scars and microglial activation (James and Butt, 2002; Ostrow and Sachs, 2005; Verkhratsky, 2006; Mills et al., 2004).
Astrocyte calcium waves trigger responses in microglia (Schipke et al., 2002), most likely through the release of ATP (Verderio and Matteoli, 2001).

In the leech nerve cord, the release of ATP through innexons is necessary for the migration of microglia after an injury (see chapter 3). And, ATP can initiate calcium signals in leech glial cells (Rose et al., 1995; Muller et al., 2000; Lohr and Deitmer, 1999). Because extracellular ATP increases mediate the propagation of calcium waves between separate glial cells in mammalian cells in culture, I hypothesized that ATP release may mediate glial calcium waves in general. That is, that calcium waves in the connective glial cells are propagated by ATP released by innexons, the same ATP that initiates microglial movement to injuries.

**Methods**

Leech nerve cords (with at least 3 mm of connective on each piece) were dissected, pinned in 35 mm petri dishes containing 1 ml silicone rubber (Sylgard 184, Dow-Corning, Midland, MI) and incubated overnight in supplemented L-15 medium (Ready and Nicholls, 1979). Calcium Green 1 or Oregon Green Bapta-2 was then iontophoretically injected (-5 nA for 10 minutes) into cut end of the glial cell. The dye was allowed to diffuse within the glial cell for 1 hour before the experiments began.

The glial cells were imaged on a Zeiss WL epifluorescence microscope (40X water immersion objective, NA 0.75) with a 12V, 100 W tungsten-halogen lamp operated at 8-10V and fluorescein filter set. Metamorph® imaging software controlled a shutter and acquisition of time lapse images from a CCD camera.
(XC-77, Hamamatsu City, Japan), averaging 16 frames stored at 1 second intervals.

To evoke ATP induced calcium waves, an electronic picospritzer (Picospritzer II, General Valve Corp., Fairfield, NJ) was adjusted to deliver puffs of 3mM ATP (pH 7.4) directly to the connective (20 psi for 0.1 seconds). The concentration of ATP used was not damaging, since even nerve cords bathed in 3mM ATP were not measurably harmed, and the bath concentration of ATP was negligible even after several experiments.

A sharp tungsten probe (Hubel, 1957) driven by a piezoelectric device was used to elicit reproducible, mechanically-induced calcium waves in the glial cell. With the probe placed flush against the tissue, a voltage pulse was applied to the piezoelectric device to cause a displacement of the probe and the tissue. The deflection duration was 200 ms and the voltage ranged from 30-100 V, depending on the experiment and the sensitivity of the tissue. The calcium response was seen immediately upon stimulation.

Measurements of the calcium wave intensity and propagation were made using the Metamorph® region analysis tool. Quantification of the calcium change was made by measuring the intensities of 5x5 µm² regions separated by 20 µm along the glial cell, beginning adjacent to the probe and up to 100 µm distant from it (Figure 4.1). Cells were stimulated mechanically at 15 seconds after beginning the time lapse recording. The first 10 images were averaged to provide a resting fluorescence, F, and then the change in fluorescence from that baseline, ∆F, was used to calculate a ratio ∆F/F for every region and time point.
The maximum intensity of the calcium response, or peak value, was determined to be the average of the five consecutive points in any region with the largest average ΔF/F measurement. To measure the velocity of the calcium wave, I calculated the time to half peak, which was the time it took each region being measured to reach half its maximum intensity, and divided that time by the region’s distance to the initiation site. ANOVA (Statistica) was used for statistical analysis of both maximum intensities and times to half peak.

Carbenoxolone and apyrase were purchased from Sigma-Aldrich (St. Louis, MO), dissolved in water as stock solutions, and applied to the bath to reach their final concentrations.

Results

ATP induced calcium waves

By applying small puffs of ATP to the connective using a picospritzer (see methods), I was able to evoke ATP induced calcium waves (Figure 4.2). The propagation speed of the calcium response was calculated as the time to half peak shown in figure 4.2B. Beyond the first 40 µm from the site of initiation,
there appeared to be a delay to the half peak, demonstrating the “wave” nature of the calcium response, although there was no evidence that the propagation was regenerative. I attempted to initiate the calcium wave by ejecting ATP from the pipette at varying distances from the connective (from 50-100 µm away). Only when the pipette was flush against the tissue was I able to produce a calcium wave (data not shown). This indicated that only highly local concentrations of ATP could initiate a calcium wave and that the observed calcium wave was not spread by diffusion of ATP from the pipette outside the cell.

**Mechanically induced calcium waves**

Using a sharp tungsten probe attached to a piezoelectric device, I consistently evoked calcium waves (Figure 4.3A). To my knowledge, traveling waves of calcium had not previously been reported in the leech, but their presence confirmed that their evolutionary conservation. CBX (10 µM), the pannexon/innexon channel inhibitor, did not block or reduce the mechanically induced calcium response (Figure 4.3B,C). Also, the propagation speeds of the calcium waves, determined by the time to half-peak, were not different in CBX from controls (Figure 4.3D). These experiments indicate that innexons are not required for the initiation or propagation of mechanically induced calcium waves.

To determine whether another mechanism of ATP release was involved, I treated the tissue with apyrase (20 U/mL) and attempted to initiate a calcium wave. Apyrase did not influence the amplitude or propagation speed of the calcium waves (Figure 4.4A,B). This same concentration of apyrase is able to blocks microglial migration (see previous chapter) so it is clearly able penetrate
the sheath and hydrolyze the ATP on the glial cell surface where the microglia are laying. These experiments suggest that extracellular ATP is not required for the propagation of the calcium wave.

These results indicate that an extracellular mechanism for propagating the calcium signal is not required for these cells. It is likely that they use the IP3 dependent, calcium-induced calcium release mechanism, known to mediate intracellular calcium waves in astrocytes. This is corroborated by the fact that the calcium waves persist even in calcium-free Ringer’s solution (data not shown).

**Calcium gradient upon injury**

Although functional innexons are not required for the calcium wave, it was important to determine whether calcium increased upon injury and for how long. Figure 4.5 shows the calcium response after an injury at various distances from the site of the crush. The crush caused a substantial increase in intracellular calcium in the area adjacent to it. In the succeeding minutes, the area near the crush continued to increase its calcium response while at greater distances the calcium response began to decrease, creating a spatial gradient. This gradient persisted for more than half an hour and extended more than 400 µm from the crush. This large calcium response and spatial gradient may be necessary for the directed migration of microglia. I attempted to block the post-injury calcium response with BAPTA, but was not successful.

**Discussion**

This chapter has shown that leech glia have calcium waves that resemble those in mammalian glia, in response to either ATP or mechanical stimulation.
These waves persisted in the presence of the pannexon channel blocker carbenoxolone and the ATP hydrolyzing enzyme apyrase, indicating that neither innexons nor ATP were necessary for their initiation or propagation. The waves also persisted in the absence of extracellular calcium, indicating the calcium is entering the cytoplasm from internal stores. Given these findings, it is likely that these calcium waves are mediated by an IP3 dependent, calcium-induced calcium release mechanism.

Intracellular calcium waves, such as those seen in individual astrocytes, are mediated via the IP3 dependent, calcium-induced calcium release (ICICR) mechanism (Fiacco and McCarthy, 2004). In short, any stimulus that causes an increase in calcium, such as an injury, a ligand binding to its receptor or even spontaneous calcium oscillations, causes an increase in calcium to spread across the cell in a process that is dependent on the presence of IP3 (Parri and Crunelli, 2003; Floyd et al., 2001; Salter and Hicks, 1995; Shao and McCarthy, 1995; Kim et al., 1994). Given that the leech glial cell is one large cell, it is reasonable that extracellular signaling is unnecessary for the intracellular calcium wave.

Microglia, which migrate quickly to sites of nerve injury, are known to be regulated by two independent signals in the leech, ATP and nitric oxide (NO) (Duan et al., 2009). While ATP provides the cue to cause microglia to move, NO provides the directional cue. In chapter 3, I demonstrated that the ATP necessary for the proper migration is released by innexon channels, which themselves are sensitive to cytoplasmic calcium. Endothelial nitric oxide
 synthase (eNOS), the enzyme that produces nitric oxide, is also activated by calcium. The finding that the glial cell has a large sustained increase in intracellular calcium after a crush may be the link between these separate mechanisms of regulation. Also, in chapter 5, I will discuss the possible role of arachidonic acid (ArA) as an endogenous regulator of innexons. Phospholipase A2 (PLA2), the enzyme that cleaves ArA from membrane phospholipids, is activated by calcium. High levels of calcium near the crush that may tend to open the innexon channels to release ATP may also cause the synthesis of high levels of ArA that would tend to close the channels preventing abundant movement near the crush, where the microglia accumulate. The calcium response extends several hundred micrometers from the crush, similar to the distance from which microglia travel to the injury. A calcium gradient that activates both innexons, eNOS, and the synthesis of ArA, would be able to coordinate the migration of microglia. Further studies are needed to determine the relationship between the calcium increase upon injury and the migration of the microglia.
Figure 4.2: ATP induced calcium waves in the connective glial cell

A. Glial cells were filled with Oregon Green Bapta-2 and stimulated with a small puff of ATP from a micropipette controlled by a picospritzer (3 mM, 20 psi, see Methods). ∆F/F was determined for six 25 µm² regions located at the distances shown. As the distance from the initiation site increased, the calcium response time increased and the peak amplitude decreased.

B. The calcium signal was delayed as a “wave” beginning 40 µm from the initiation site. The velocity of the calcium wave was determined from the time to reach half the maximum amplitude of the calcium response for any given region. These experiments confirmed that leech glia have ATP induced calcium waves that are similar to those found in mammalian glia.
Figure 4.3 (next page): Mechanically induced calcium waves and the effect of carbenoxolone. **A.** An example of a mechanically induced calcium wave in a glial cell. Glial cells were filled with Calcium Green-1 and stimulated with a piezoelectric controlled tungsten probe (see Methods). $\Delta F/F$ was determined for six $25 \, \mu m^2$ regions located at the distances shown. The wave properties of the calcium response were evaluated by the increased delay and decreased amplitude of the calcium response at distances further from the site of stimulation. **B.** Mechanically induced calcium waves were induced with a sharp tungsten electrode controlled by a piezoelectric device. A 40 V piezoelectric touch was given after 7 seconds to initiate the calcium wave in the Calcium Green-1 filled connective glial cell. Changes in calcium in three $25 \, \mu m^2$ regions located 20 $\mu m$ apart were measured and included in this graph. The calcium response was calculated as the change in fluorescence divided by the resting fluorescence ($\Delta F/F$). The representative time courses of mechanically induced calcium responses in a connective glial cell with and without CBX were not distinguishable. Arrows indicate onset of the stimulus. **C.** Carbenoxolone treatment and the peak calcium responses during mechanically induced calcium waves in connective glial cells. Bars represent the average maximum fluorescent intensities induced by a mechanical stimulation in a $25 \, \mu m^2$ region at the initiation site of the calcium wave. Mechanically induced calcium waves were induced with a sharp tungsten electrode controlled by a piezoelectric device. The stimulus for these experiments was 40 V (~50 $\mu m$ displacement) (n=4). **D.** Propagation speed of the calcium wave did not change with or without carbenoxolone (10 $\mu M$). These measurements were made for the same calcium waves as in panel C. Five $25 \, \mu m^2$ regions located 20 $\mu m$ apart (starting at the site of the poke) were circumscribed and used for this analysis. At each site of measurement, a maximum calcium response (peak average of 5 consecutive seconds) and the time to reach the half-maximum intensity after the stimulus were determined for that region. The times to half peak from several adjacent regions divided into the distance of each region to the site of stimulation were averaged to give a measure of the speed of propagation. There was no significant difference between the control and CBX conditions for the times to half peak or their calculated speeds. (n=4).
Figure 4.3: Mechanically induced calcium waves and the effect of carbenoxolone

A

![Mechanically induced calcium waves and the effect of carbenoxolone](image)

B

![Mechanically induced calcium waves and the effect of carbenoxolone](image)

C

![Mechanically induced calcium waves and the effect of carbenoxolone](image)

D

![Mechanically induced calcium waves and the effect of carbenoxolone](image)
Figure 4.4: The effect of apyrase on mechanically induced calcium waves

A. A representative experiment showing calcium responses for tissue bathed in 20 U/mL apyrase, an ATP hydrolyzing enzyme, and for control tissue without apyrase. A 50 V piezoelectric pulse was used to initiate the calcium wave at time T=0. Intensities at the 25 µm² region located near the initiation site of the calcium waves were used for this graph. The “apyrase 1” and “apyrase 2” experiments were performed after the tissue had incubated in apyrase for 20 minutes and were done 15 minutes apart, enough time for the calcium increase from one pulse to subside.

B. For the calcium waves described in A, the time to half peak was calculated for five 25 µm² regions separated by 20 µm that began at the initiation site of the calcium wave and moved away from it. Apyrase did not alter the propagation speed of the calcium wave.
Figure 4.5: The post-injury calcium response. A Calcium Green-1 injected glial cell was crushed and the fluorescence intensity before and after a crush was measured. The connective was imaged before making the crush, removed from the microscope, crushed, and placed back on the microscope at the time corresponding to the jump in fluorescence. An interval of more than a minute is not indicated but occurred at that time. There was a large increase in the intracellular calcium concentration upon injury, which was maintained more than a half an hour. The calcium response around the area immediately adjacent to the crush and up to several hundred micrometers away developed into a calcium gradient during the first few minutes after the injury and is represented graphically here. Regions 100 µm² and 25 µm apart were measured for their calcium response to injury with the closest region 25 µm from the beginning of the crush. All regions measured had a large increase immediately after the crush but intensity of the regions closest to the crush continued to rise while the regions farther away began to decline, thereby creating a spatial calcium gradient.
Chapter 5: Arachidonic acid inhibits the migration of microglia to lesions in the leech CNS by closing innexon/pannexon channels

Summary
Arachidonic acid is one of the most conserved signaling molecules for all species of animals. However, most of its effects are due to its metabolic byproducts processed by the cyclooxygenase and lipoxygenase pathways, and only a few direct effects of arachidonic acid have been described. Pannexins, homologues of the invertebrate innexin family of gap junction proteins, form non-junctioning membrane channels, or pannexons, that are permeable to ATP and have been implicated in a variety of physiological and pathophysiological processes. The possible role of arachidonic acid rather than its metabolites was investigated as a novel inhibitor of the pannexon/innexon channels. It was found that arachidonic acid inhibited the usual migration of microglia to an injury in the leech CNS by blocking the release of ATP from innexon channels. Furthermore, arachidonic acid blocked dye loss from the connective glial cell. Moreover, arachidonic acid reduced the macroscopic membrane currents of Xenopus oocytes injected with mRNAs encoding for innexin and for pannexin subunits. The possible role of arachidonic acid as an endogenous regulator of pannexons and of innexons is discussed.

Background
Arachidonic acid (ArA) is enzymatically freed from membrane bound phospholipids by phospholipase A2 (PLA2) in response to a variety of physiological and pathophysiological conditions, such as increases in intracellular free calcium (Sun et al., 2005). ArA and its products, via the cyclooxygenase
(COX) and lipoxygenase (LOX) signaling pathways, have a well documented presence at sites of neuronal and brain injury (Gabryel et al., 2007; Sun et al., 2004). They are thought to be involved in such diverse processes as vasodilation, recruitment of immune cells, release of cytokines, and platelet aggregation (Minghetti and Levi, 1998; Minghetti, 2004; Phillis et al., 2006). However, the biological targets of arachidonic acid itself remain largely a mystery, despite the fact that the enzymes needed for animals to produce ArA evolved long before the enzymes to metabolize it.

Worms, unlike such other invertebrates as fruit flies, are able to produce arachidonic acid (Watts and Browse, 2002). However, evidence for the processing of arachidonic acid by the COX and LOX pathways is minimal (Scuri et al., 2005). In this aim, I investigated a novel role for ArA as an inhibitor of pannexon channels.

Arachidonic acid has long been recognized as a potent inhibitor of gap junctions in a variety of tissues (Boger et al., 1999; Fluri et al., 1990; Giaume et al., 1989; Martinez and Saez, 1999; Miyachi et al., 1994). However, its effect on gap junction “hemichannels” has not been assessed. Pannexin 1 (PANX1), a member of a recently discovered mammalian gap junction family homologous to the invertebrate gap junction family the innexins, form non-junctioning membrane channels on the cell surface of neurons and glia. Recently, PANX1 has been implicated in the activation of the inflammasome (Kanneganti et al., 2007; Pelegrin et al., 2008; Silverman et al., 2009). Arachidonic acid was also recently found to inhibit a large conductance channel on astrocytes that is permeable to
ATP in response to oxygen-glucose deprivation, which investigators termed a maxi-ion channel (Liu et al., 2008). While the maxi-anion channel was not inhibited with classic blockers of pannexons such as carbenoxolone, the investigators did not consider whether arachidonic acid might also close pannexon channels.

In chapter 3, it was demonstrated that carbenoxolone, a potent inhibitor of pannexon/innexon channels, reduces the release of ATP and inhibits the migration of microglia after an injury to the leech CNS. Because of reports that gap junction channels might be inhibited by arachidonic acid, experiments in this chapter were designed to determine whether arachidonic acid and its non-metabolizable analog eicosatetraynoic acid (ETYA) reduce the conductance of innexon/pannexon channels and inhibit ATP-dependent migration of the microglia to lesions in the leech CNS.

**Methods**

**Leeches and Drugs:**

Adult leeches (*Hirudo*; Leeches USA, Westbury, NY), 3-4g, were maintained in artificial pond water (Forty Fathoms, 0.5 g/L H₂O; Marine Enterprises, Towson, MD) at 15°C. In preparation for experiments, leech nerve cords were dissected and pinned in 35 mm petri dishes with 1mL silicone rubber (Sylgard 184; Dow Corning, Midland, MI) on the bottom. For dissections and experiments, leeches were kept in leech physiological saline (“leech Ringer’s”) containing contained 115 mM NaCl, 4 mM KCl, 1.8 mM CaCl₂, and 10 mM Tris maleate, pH 7.4 (Kuffler and Potter 1964)(Nicholls and Baylor, 1968). Nerve
cords could be maintained for several days by incubating them in Leibowitz-15 (L-15) culture medium supplemented with 2% FBS, 1% glutamine, 0.06% glucose, and gentamicin (5 µg/mL). Drugs were administered by bath application at their final concentrations. ArA and ETYA were dissolved in ethanol (EtOH 0.1%). All drugs were purchased from Sigma-Aldrich (St. Louis, MO).

Microglia accumulation studies

Nerve cords were prepared as described. Connectives were crushed for 8-12 seconds with fine forceps (Dumont #5) and incubated in leech Ringer’s solution at room temperature for 4 hours with or without drugs. The tissue was then fixed overnight at 4°C in PBS with 4% paraformaldehyde (pH 7.2), washed, and mounted on a microscope slide in glycerol with Hoechst 33258 nuclear stain (10 µg/mL). Microglia nuclei were imaged with a Leica DM RZ2A fluorescence microscope (20X objective, NA 0.7) using a UV filter set. A series of 10 optical sections were photographed at 2 µm intervals and a combined depth of 20 µm. An image of the connective was also taken using the fluorescein filter set, which shows tissue autofluorescence but not the Hoechst 33258 dye, to identify the location of the crush without showing the microglia for selecting the region to count stained nuclei without bias. Each stack of images was combined and collapsed using the Metamorph program (Molecular Devices, Downingtown, PA), and the microglia were counted in a 10,000 µm² region at the edge of each crush. The counts were statistically analyzed with an ANOVA and post-hoc Fischer Test (Statistica®, StatSoft Inc., Tulsa, OK).
**Low light video microscopy**

Nerve cords were dissected as described above and incubated for 30 minutes in Hoechst 33258 nuclear dye (0.001%), rinsed 3 times for 5 minutes and kept overnight in supplemented L-15 medium at 15°C. The following day the tissue was imaged on a Zeiss WL epifluorescence microscope (40X water objective, NA 0.75) with a 12V, 100 W tungsten-halogen lamp operated at 8-10V and a UV filter set. Metamorph® imaging software controlled a shutter and CCD camera (XC-77, Hamamatsu City, Japan) to acquire time lapse images (256 frames averaged at 2.5 minute intervals for up to 4 hours). The imaged connectives were crushed or uncrushed and with or without drug treatment depending on the specific experimental protocol. Microglia movement during the time-lapse was measured by double-blind scorers who tracked individual microglia nuclei and counted the number of nuclei that moved more than 30 µm or 50 µm, depending on the experiment, and their direction of movement, whether toward or away from the lesion. The counts were statistically analyzed with an ANOVA and post-hoc Fischer Test (Statistica®).

**In vivo dye loss**

A 1 mm piece of leech (Hirudo sp.) nerve cord was dissected in physiological saline, pinned to a Sylgard-lined petri dish, and incubated overnight in supplemented L-15 culture medium. The following day the connective glial cell was ionotrophically filled with 6-carboxyfluorescein (MW 376.32, 5 mM, -2 nA for 15 minutes) through a cut end. The dye was allowed to diffuse through the cell for 20 minutes. Time lapse images were collected every five minutes on the Leica fluorescence microscope with a 10X objective (0.3 NA). Drugs were bath
applied at their final concentrations. “Washes” consisted of quickly removing the tissue from the microscope and thoroughly rinsing the tissue three times with leech Ringer’s before placing it back on the microscope in the same location.

Fluorescence intensities of the paired connectives were measured with Metamorph® (Molecular Devices, Sunnyvale, CA) using a region analysis tool. Pixel intensities were averaged to obtain an average intensity. The dye lost during treatment was measured from the intensity of each time lapse image over a linear range of the camera down to background or autofluorescence levels, which were subtracted from the intensity to determine dye fluorescence. The rate of dye loss, as a percentage, was then calculated from values at successive 5 min time points. A repeated measures ANOVA followed by a post-hoc Fischer test (Statistica, StatSoft Inc., Tulsa, OK) was used for comparison and statistical analysis of the dye loss.

**mRNA-injected oocyte recording**

*Solutions:* OR2 solution in mM: 82.5 NaCl, 2.5 KCl, 1.0 MgCl₂, 1.0 CaCl₂, 1.0 Na₂HPO₄, 5.0 HEPES, antibiotics (penicillin, 10,000 units/ml; streptomycin, 10 mg/ml), pH7.5). Patch pipette KGlu solution: 140 mM potassium gluconate, 10 mM KCl, 5.0 mM TES, pH 7.5)

*mRNA injected oocytes:* Xenopus laevis oocytes were isolated by incubating small pieces of ovary in 2 mg/mL collagenase in calcium free oocyte Ringer’s solution (OR2) and stirring at 1 turn/second for 3 hours at room temperature. After being thoroughly washed with regular OR2, oocytes devoid of follicle cells and having uniform pigmentation were selected and stored in OR2 at 18 °C.
Hirudo innexin 2 (Hm-inx2) was cloned into the expression vector pCR-BluntII-TOPO. mRNA was transcribed by SP6 (Hm-inx2) RNA polymerase from 10 µg of XbaI- (Hm-inx2) linearized plasmid using the mMessage mMachne kit (Ambion). mRNA was quantified by absorbance (260nm), and the proportion of full-length transcripts was checked by agarose gel electrophoresis. 20 nl of mRNA (50 ng/µl) was injected into oocytes. The injected oocytes were then transferred into fresh OR2 medium with elevated Ca\(^{2+}\) (5 mM) and incubated at 18 °C for 24-48 hours. For electrophysiological recordings, oocytes were transferred to regular OR2.

**Voltage clamp:** Voltage clamp recording from the oocyte was performed with two intracellular microelectrodes, one that recorded the intracellular voltage and the second that delivered the current. Oocytes were held at constant voltages and given depolarizing oscillations (5 seconds each, 6 times per minutes) between 5-100 mV depending on the experiment. Changes in current required for changes in voltage gave a measure of conductance due to the opening of the pannexin/innexin channels.

**Results**

**Arachidonic acid effect on microglia migration and accumulation**

Very few cellular behaviors have been attributed to the action of ArA itself. Carbenoxolone (CBX), the pannexon channel blocker, inhibits the normal microglial migration to an injury in the leech CNS by blocking the release of ATP from innexon channels. I tested whether ArA (eicosatetranooic acid) and its non-metabolizable analog eicosatetraynoic acid (ETYA) behave like CBX and can
prevent the accumulation of microglia at a nerve crush injury when applied exogenously. Accumulation assays with varying doses of both ArA and ETYA were performed and both compounds significantly reduced accumulation at the 100 µM concentrations (Figure 5.1A,B). Both compounds inhibited the migration of microglia to the site of a crush injury in a dose dependent manner. As I had with CBX, I tested whether ArA’s inhibition of microglia accumulation could be prevented if the tissue was treated simultaneously with 100 µM ATP. Microglia in both the ATP alone and in ATP with ArA accumulated at the lesion similarly to microglia in the control nerve cord that was crushed in Ringer’s solution (Figure 5.1C). In time lapse studies of microglia in crushed connectives in ArA alone the microglia did not move, and in ArA with ATP the microglia moved directly toward the lesion (Figure 5.1D). Thus, ArA inhibition of microglial accumulation occurred because of an overall decrease in microglial movement and not because of abnormal directional movement. The addition of ATP to the ArA treatment restored the overall movement of the microglia, and the movement was directed toward the crush. These studies demonstrated that ArA, and not its metabolites, had a potent effect on the migration of microglia toward an injury.

Effect of ArA on dye loss from the connective glial cell

Since the microglia appeared to respond to ArA and ETYA similarly to their response to CBX, it was important to determine whether ArA and ETYA blocked microglial migration by closing the innexons and preventing the release of ATP, as CBX does. By closing the innexons, both CO₂ and CBX inhibit 6-carboxyfluorescein (6-CF) and Lucifer Yellow dye loss from a dye-injected
connective glial cell. As shown in Figure 5.2A, ArA significantly reduced 6-CF loss compared to the control with vehicle, perhaps with a slight delay. Moreover, the non-metabolizable form of ArA, ETYA, reduced dye loss and the effect persisted for about a half hour after the ETYA was removed, but the effect was reversible (Figure 5.2B). Although the reason for the apparent delay with ArA compared to ETYA is unknown, ArA might be broken down by the membrane of the sheath or glial cells before it reaches its target. Nevertheless, ArA itself blocks dye loss from the connective glial cell in a manner similar to CBX, the pannexon/innexon channel blocker.

**Arachidonic acid effect on the conductance of cells exogenously expressing innexon and pannexon channels**

To determine whether ArA directly closes innexon channels, it was applied to glial-associated innexons (HmINX2) expressed in *Xenopus* oocytes. Two days after injection with *Hm-inx2* mRNA, oocytes were penetrated with two sharp electrodes and the membrane potential clamped at positive voltages to open innexons while the cellular currents were recorded. The conductance resulting from *HmINX2* was quickly and reversibly inhibited by 100 µM ETYA or arachidonic acid (ArA) (Figure 5.3A,D). This inhibition also occurred when the channels were opened by treating the cells with an isotonic potassium gluconate solution (Kglu, 70 mM) (Figure 5.B,C). To determine whether the related mammalian pannexons were inhibited by ArA, both ArA and ETYA were applied to *Xenopus* oocytes expressing mouse PANX1. ArA decreased the pannexon current by approximately 15-20% and ETYA by approximately 45-50%; both effects were significant and reversible (Figure 5.3E). The experiments confirmed
that ArA directly closed innexon and pannexon channels and appeared to do so as effectively as its non-metabolizable analogue, suggesting that its metabolites play little or no role.

**Discussion**

ArA, when applied to a nerve crush, blocked the migration of microglia and the effect was reversible by extracellular ATP. ArA also blocked dye release from the connective glial cell and reduced the conductance of glial-associated innexon channels expressed in oocytes. ArA also reduced the conductance of PANX1-expressing oocytes. Although ArA and ETYA did not reduce the pannexin currents as much as they reduced the innexin currents, the effect may nonetheless important physiologically. Pannexons and innexons have multiple conductance states so even a small reduction in channel conductance may block the ability of dyes and ATP to pass through the channel (Bao et al., 2004). Taken together, these experiments provide evidence for a novel function of ArA as an inhibitor of the the innexon/pannexon family of channels, and as an inhibitor of the microglial response to injury in the leech CNS. The similar effectiveness of ETYA and ArA in all assays was evidence that ArA itself and not necessarily its downstream signaling products, such as through the COX and LOX pathways, were responsible for the actions of ArA. ArA may therefore act as an endogenous regulator of pannexon/innexon channels, preventing their opening.

Arachidonic acid levels measured with MALDI-ToF mass spectroscopy increased in the leech CNS after a crush injury (personal communication from
Karim Arafah in the Salzet laboratory, Lille, France). In the leech CNS, ArA may act as an endogenous inhibitor and regulator of the innexon channels during recovery from injury. High local levels of ArA near the crush may close the innexons opened by elevated Ca$^{2+}$, preventing the release of ATP and slowing the migration of microglia near the injury so they accumulate. The high activity of PLA2 at the site of injury may be due to the Ca$^{2+}$ gradient as well as the high local levels of NO (Duan et al., 2009), which enhances the activity of cytosolic PLA2 (Xu et al., 2008).

In mammals, there are many downstream products of ArA that are involved in inflammation and many of the COX and LOX enzymes are induced by cell injury and activation of PLA2. Pannexon channel activation is part of a cascade of inflammatory signals in neurons and glia (Silverman et al., 2009). The early activation of PLA2 by Ca$^{2+}$ produces high levels of ArA that will be present immediately after cell injury. These high levels of ArA would initially inhibit the activity of the inflammasome during a time that COX2 is induced. If large amounts of COX2 are made as a result of the injury, this could create more ArA metabolites and reduce ArA. This may release the pannexin from inhibition and allow for the simultaneous activity of the prostaglandin and inflammasome activity. The initially high levels of ArA that block the pannexon channels may therefore act as an endogenous checkpoint for determining whether the cells will have a full inflammatory response or not. Overcoming this block by ArA by the inducible enzymes could be the trigger for the cells to have a strong response. However, in milder injuries, ones that would not produce as much COX2, the
pannexon channels would remain blocked because of the ArA and the cells would only have a mild inflammatory response to injury. The ArA effect on pannexons may be a type of molecular switch that determines the quantity and quality of the inflammatory response of damaged cells. More research is needed to determine the role of ArA in regulating the inflammatory and immune response in the mammalian nervous system.
**Figures**

**Figure 5.1:** The effect of arachidonic acid and ETYA treatments on the migration and accumulation of microglia

A. ArA reduced the accumulation of microglia at lesions in a dose dependent manner. (n=3, p<0.01)

B. The ArA non-metabolizable analog eicosatetraynoic acide, ETYA, reduced the accumulation of microglia at a crush in a dose dependent manner (n=3, p<0.01).

C. Microglial accumulations at sites of injury persisted in the combined presence of ArA (100 µM) and ATP (100 µM). (n=3, p<0.01)

D. ATP effect on the arachidonic acid inhibition of microglial movement toward a crush. Time lapse images of crushed connectives with Hoechst stained nuclei were collected and made into a movie. In the region adjacent to the crush (approximately 100-500 µm away), the number of microglia nuclei moving more than 50 µM in two hours were counted. The connectives were treated with either arachidonic acid alone (ArA 100 µM) or arachidonic acid with ATP (ArA+ATP, 100 µM each drug). The connectives treated with arachidonic acid had approximately a third of the number of microglia moving relative to the ArA+ ATP condition. The percentage of directional movement was approximately the same. (n=3, p<0.05)

For microglia accumulations, connectives were treated as indicated at the time of crush and the microglia were given 4 hours to accumulate after which the tissue was fixed (4% paraformaldehyde), stained (Hoechst) and photographed. Bars represent mean number of microglia at the site of injury in a 100x100x18 µm³ volume. “Distal” was a measure of microglia in an uncrushed region, approximately 1 mm from any injury, and is used as a baseline for the number of microglia distributed randomly throughout the tissue. The control accumulation was done in 0.1% EtOH. The difference between the “EtOH” and the “distal” is the measure of accumulation.

- A. ArA reduced the accumulation of microglia at lesions in a dose dependent manner. (n=3, p<0.01)
- B. The ArA non-metabolizable analog eicosatetraynoic acide, ETYA, reduced the accumulation of microglia at a crush in a dose dependent manner (n=3, p<0.01).
- C. Microglial accumulations at sites of injury persisted in the combined presence of ArA (100 µM) and ATP (100 µM). (n=3, p<0.01)
- D. ATP effect on the arachidonic acid inhibition of microglial movement toward a crush. Time lapse images of crushed connectives with Hoechst stained nuclei were collected and made into a movie. In the region adjacent to the crush (approximately 100-500 µm away), the number of microglia nuclei moving more than 50 µM in two hours were counted. The connectives were treated with either arachidonic acid alone (ArA 100 µM) or arachidonic acid with ATP (ArA+ATP, 100 µM each drug). The connectives treated with arachidonic acid had approximately a third of the number of microglia moving relative to the ArA+ ATP condition. The percentage of directional movement was approximately the same. (n=3, p<0.05)
Figure 5.2: Dye loss from the connective glial cell and the effect of arachidonic acid and ETYA.

A. 1 mm lengths of leech connectives were dissected, pinned on Sylgard dishes, and incubated overnight in supplemented L-15. Then they were washed with Ringer’s and iontophoretically injected (-2 nA for 15 minutes) with 6-carboxyfluorescein, a gap junction permeable dye. After approximately 20 minutes, when the dye had equilibrated along the entire tissue, the connective was observed with fluorescence time lapse microscopy for 30 minutes and then for 1 hour in either ethanol (vehicle) or arachidonic acid (ArA, 100 µM). The graph shows dye loss per 5 minutes for the first 30 minutes and the first and second 30 minutes of drug treatment. The arachidonic acid treated tissue was only significantly different from the EtOH treated tissue in the 30-60 min time frame, in which ArA demonstrated a strong inhibition of dye loss (n=4, p<0.05).

B. ETYA, a non-metabolizable analog of arachidonic acid, inhibits 6-CF dye loss from the connective glial cell. Graph represents time course of dye loss from 1 mm piece of connective glial cell given as a measure of percent dye loss in 5 minutes. In Ringer’s and in 0.1% ethanol (ETOH vehicle), there was a substantial loss of dye (~12%), which was significantly reduced when ETYA was added. This effect reached a peaked during the first half hour of “EtOH wash” and then began to recover (n=4, p<0.05).
Figure 5.3: The effect of ArA and ETYA on the currents of Hm-inx2 and panx1 injected oocytes.

A. HmlNX2 channels expressed on oocytes were treated with arachidonic acid (100 µM) and ETYA (100 µM). The membrane potential of an HmlNX2 expressing oocyte was held at the various potentials indicated and 15 mV depolarizing voltage pulses were applied (top trace) at a rate of 12/min. The membrane conductance due to the opening of innexon channels, which increased as the cell was held at higher potentials, was eliminated by bath applying ETYA or ArA. An application 0.1% ethanol (EtOH), the vehicle in which both ArA and ETYA were dissolved, preceded the application of both drugs. B. HmlNX2 channels close in response to arachidonic acid when opened with KGlu. Oocytes expressing HmlNX2 and voltage clamped at low resting membrane potentials were treated with a solution of 50% potassium gluconate oocyte Ringer’s (50% KGlu), a solution known to open the innexon channels. The increased conductance of the oocyte in response to the 50% KGlu was blocked with a treatment of arachidonic acid (50 µM). An application 0.1% ethanol (EtOH), the vehicle in which ArA is dissolved, preceded the application of arachidonic acid. C. HmlNX2 channels close in response to ETYA and CBX when opened with KGlu. Oocytes expressing HmlNX2 and voltage clamped at low resting membrane potentials were treated with a solution of 50% potassium gluconate oocyte Ringer’s (50% KGlu), a solution known to open the innexon channels. The increased conductance of the oocyte in response to the 50% KGlu was blocked with a treatment of arachidonic acid (50 µM). An application 0.1% ethanol (EtOH), the vehicle in which ETYA is dissolved, preceded the application of ETYA. An application 0.1% ethanol (EtOH), the vehicle in which both ArA and ETYA were dissolved, preceded the application of both drugs.

D. Oocytes injected with Hm-inx2 mRNA were voltage clamped and oscillated between -30 and +60 mV, a technique known to stably open pannexon and innexon channels. The large conductance of this oocyte was nearly blocked by arachidonic acid. The current (I) was measured during the intracellular acidification of this cell in panel on the right as a positive control for the closing of the innexon channels. E. Arachidonic acid is an inhibitor of mouse pannexin when expressed in oocytes. Mouse pannexin 1 expressing oocytes were voltage clamped and oscillated between -50 and +50 mV to open the pannexon channels. While arachidonic acid (100 µM) caused a decrease in conductance (relative to carbenoxolone) of approximately 15-20%, its non-metabolizable analog, ETYA, caused a decrease in the cellular conductance of approximately 45-50% (n=4). An application 0.1% ethanol (EtOH), the vehicle in which both ArA and ETYA were dissolved, preceded the application of both drugs.
Chapter 6: General Discussion

*Innexons release ATP and control microglia migration*

Microglia are the nervous system’s highly responsive resident immune cells, which are activated by extracellular ATP, LPS, TNFα, and other activators of inflammation, and are the first responders to injury (Gehrmann et al., 1995; Nimmerjahn et al., 2005; Raivich, 2005). As more is discovered about the signals regulating the behavior of microglia, more is understood about the mechanisms of neuroinflammation and the diseases and conditions associated with it, such as trauma, Parkinson’s disease, Alzheimer’s disease, AIDS and other viral encephalopathies, and multiple sclerosis, to name a few.

The molecular signals that initially activate microglia upon CNS injury have not been completely determined but extracellular ATP is involved in the early migration and activation of microglia (Davalos et al., 2005; Honda et al., 2001; Kurpius et al., 2007). G-protein coupled P2Y receptors on microglia, especially P2Y12, are necessary for their response to ATP (Honda et al., 2001; Haynes et al., 2006). In the leech, extracellular ATP is signal that initiates microglial movement (Duan et al., 2009), a finding that has been confirmed by my own experiments (see chapter 3). Previous studies from our laboratory demonstrated that ATP is necessary for the general movement of microglia, regardless of the direction of movement. The treatment of the uninjured CNS with exogenous ATP (as little as 10 µM) causes non-directional movement of microglia, and the application of the P2Y receptor blocker Reactive Blue-2 inhibits the movement of microglia after a crush injury. My own studies added to these data (in chapter 3)
by confirming the presence of ATP even up to two hours post-injury. I also demonstrated that the ATP-hydrolyzing enzyme apyrase blocks microglia migration and accumulation but does not affect the directional movement, consistent with the previous findings. Although damaged cells can release ATP from injured membranes, the prolonged presence of ATP post-injury implicates another, possibly regulated, mechanism of ATP release, which I hypothesized was mediated by innexon channels. Mine are the first studies to propose that the microglia respond to a regulated source of ATP as opposed to ATP that leaks out of cells through damaged membranes. In this thesis I have provided evidence that, in the leech, the release of ATP through the pannexon/innexon family of channels after injury is crucial for the migration and accumulation of microglia at sites of injury.

Chapter 2 demonstrated that innexins, specifically glial-associated innexins, form membrane channels that are permeable to ATP in a manner similar to their mammalian homologues, the pannexins (Bao et al., 2007). This is significant and is the first report, to my knowledge, of innexons acting other than as gap junctions. The fact that this function is observed in invertebrates is consistent with the hypothesis that pannexins have a similar role in mammals.

Chapter 3 showed that the pannexin channel blocker carbenoxolone inhibited the accumulation and migration of microglia, and that inhibition was reversed by the addition exogenous ATP. As discussed in Chapter 3, the innexons that released ATP were likely located in the connective glial cells that span the entire length of the connective. This idea was consistent with the
findings in chapter 2 that the glial associated innexons formed membrane channels, and with the literature identifying glial cells including astrocytes as a source of ATP for activation of microglia (Bianco et al., 2005b; Schipke et al., 2002; Verderio and Matteoli, 2001).

Chapter 4 showed that functional innexon channels, which release ATP to activate microglia post-injury, are not required to initiate and propagate mechanically induced calcium waves. ATP is the known extracellular signal responsible for mediating intercellular calcium waves in astrocytes, however the mechanism of ATP release is controversial (Anderson et al., 2004; Bowser and Khakh, 2007; Guthrie et al., 1999; Stout et al., 2002; Suadicani et al., 2006). My experiments were aimed at contributing to the discussion by attempting to show that innexon/pannexon channels are involved in glia produced calcium waves in the leech nervous system. Despite demonstrating the presence of glial produced calcium waves initiated by both ATP and mechanical stimulation similar to those found in astrocytes, I was unable to block the waves with CBX or apyrase. The logical interpretation of this result is that neither innexons nor ATP are necessary for the initiation or propagation of these signals. I now hypothesize that these calcium waves are mediated by IP₃ dependent calcium-induced-calcium release, the mechanism known to cause intracellular calcium waves in astrocytes (Fiacco and McCarthy, 2004) (see Discussion in chapter 5). Therefore, no conclusions about the mechanism of intercellular calcium waves, such as those observed in astrocytes, may be made.
I measured the calcium response after an injury and discovered that there was an increase in calcium that stretched several hundred micrometers from the site of the crush, persisted for at least an hour, and created a spatial calcium gradient. The relationship between this spatial gradient and the subsequent migration and accumulation of microglia remains to be determined. It is likely that the calcium gradient opens innexons to release ATP and activates eNOS to synthesize NO, since both processes occur with increases in intracellular calcium.

Chapter 5 showed that arachidonic acid (ArA) and its non-metabolizable analog ETYA inhibit the pannexons/innexons and can block the migration of microglia, acting like CBX. Phospholipase A2 (PLA2), the enzyme that cleaves ArA from membrane phospholipids, is known to become activated upon injury and increases in cytoplasmic calcium (Gabryel et al., 2007; Sun et al., 2005). Although arachidonic acid and its metabolic byproducts have been implicated in a wide variety of pathological conditions (Minghetti, 2004; Phillis et al., 2006), arachidonic acid itself has never been known to be a regulator of immune function. ArA had been known to inhibit gap junctions both directly (Giaume et al., 1989) and through its metabolites (Martinez and Saez, 1999; Miyachi et al., 1994), but its ability to block “hemichannels” had never been studied. ArA and ETYA inhibited the innexon channels expressed in oocytes, prevented dye loss from glial cells, and blocked the migration of microglia. Both arachidonic acid and ETYA mimicked CBX in all these assays. Because the block of migration was reversed by exogenous ATP, the block was evidently a result of closing
innexons. In addition, ETYA and ArA also at least partially closed mouse pannexons expressed in frog oocytes. The results confirmed that ATP release from innexons is required for microglia migration and indicated that the endogenous regulation of the innexons may be physiologically important for this process. ArA is the first identified endogenous regulator of innexon/pannexon channels (besides ATP itself, calcium and voltage) that may play an important role in modulating the microglia response to nerve injury.

**Pannexons/innexons as ATP channels**

Pannexins, which were discovered only a few years ago (Bruzzone et al., 2003), have created a paradigm shift in the field of gap junction protein signaling. Before the discovery of pannexins, it was assumed that any release of ATP that appeared to be from “hemichannels” was assumed to be from connexins. After the discovery of pannexins, the debate surrounding which gap junction proteins are responsible for the “hemichannels” in neurons and glia began. Pannexins seemed to have all the channel properties necessary for being ATP channels, especially those involved in intercellular calcium waves. While strong evidence has emerged from experiments using the oocyte expression system (Bao et al., 2004), erythrocytes (Locovei et al., 2006a), taste buds (Huang et al., 2007b), airway epithelia (Ransford et al., 2009), and retina (Reigada et al., 2008) that pannexins function as ATP channels that release ATP as a signaling molecule, there has been confusion about their role in the brain. This is possibly due to the heterogeneity of cell types as well as the confounding presence of connexins in mammalian tissue. However, recent studies have begun to separate the
functions of connexins and pannexins in nerve tissue, and have discovered that pannexins form functional membrane channels in neurons and glia (Iglesias et al., 2009; Silverman et al., 2009; Thompson et al., 2008) and they may play a role in stroke (Thompson et al., 2006). My results further this research by pointing to the evolutionarily conserved function of this family of proteins in that even the “primitive” innexons function as ATP permeable membrane channels in glia and have an important physiological role in regulating the microglial response to injury. Furthermore, I have demonstrated that arachidonic acid, a known mediator of inflammation, may be an endogenous regulator of innexons/pannexons.

One interesting property of the pannexon channels is that ATP itself closes them (Qiu and Dahl, 2009). This unique ability of a permeant inhibiting its permeation pore creates an inhibitory feedback loop that acts as an endogenous regulator of the pannexin channels. If the pannexons were to remain open and create a high concentration of extracellular ATP, the ATP itself would inhibit the channels. Although this property has not been tested with innexins, it may be hypothesized that a similar regulation occurs. Perhaps this way cells balance the release of ATP so that it maintains a workable extracellular concentration for signaling but avoids desensitization of receptors and does not detrimentally affect the energy metabolism of the cell.

Additional signals involved in microglia migration

Aside from ATP and signals such as ArA controlling ATP release, many other signals controlling migration and activation of microglia have been
discovered, although knowledge of the physiological concentrations, time
courses, and mechanisms of action of these signals and their interactions is
incomplete. These include NO, opioids, and cannabinoids, all of which are
reported to increase after nervous system injury, including in the leech, and are
discussed in turn in the following paragraphs. It should be noted that in
mammals, from mostly in-vitro studies, monocyte chemoattractant protein-1
(MCP-1), chemokine CXCL10, and Epidermal growth factor (EGF) activate
microglia and direct chemotaxis (Hailer, 2008). Also, macrophages, which derive
from a similar lineage as microglia, are activated by a variety of cytokines such
as interleukin 4 (IL-4), interleukin 10 (IL-10), interferon-gamma (IFN-γ) and others
(Ransohoff and Perry, 2009). As of yet, there is no evidence that any of these
molecules play a significant role in regulating leech microglia.

NO synthesized by eNOS in the leech glia slows or stops the microglia at
high concentrations found at the lesion (Chen et al., 2000) and is required for
their directed movement toward the lesion by activating soluble guanylate
cyclase, which is typically regulated by NO and converts GTP to cGMP (Duan et
al., 2009). cGMP generated by soluble guanylate cyclase is known to be
involved in the polarization of monocytes and neutrophils that directs them to
move in a specific direction and has been implicated in the directed movement of
amoeboid of the slime mold *Dictyostelium* (Caterina and Devreotes, 1991). How
cGMP signaling polarizes migrating cells is unknown and beyond the scope of
this dissertation. A full discussion of this topic would include a description of the
many targets of cGMP, including cyclic nucleotide gated ion channels and cGMP dependent kinases (cGKs) (Kleppisch and Feil, 2009).

Two other compounds that modulate the microglial response to injury in the leech, endogenous opioids, cannabinoids, increase the production of nitric oxide (Stefano et al., 1997a). Opioids are known to suppress the activation and chemotaxic behavior of microglia in mammals (Chao et al., 1997; Sheng et al., 1997). Leeches too produce opioids and have opioid receptors, the activation of which modulates the activity of immunocytes (Laurent et al., 2000; Zipser et al., 1988). Recently, activation of kappa opioid receptors in the leech was shown to block the normal migration of microglia in response to a crush injury of the connective (Yahyavi-Firouz-Abadi et al., 2007). This study also demonstrated that the opioids affect the leech microglia by increasing the production of nitric oxide. In practice, therefore, a high concentration of endogenous opioids at a lesion may contribute to microglia accumulation.

Cannabinoids, including 2-arachidonylglycerol (2-AG) produced in the mammalian brain, are thought to regulate neuroinflammation. Both microglia and astrocytes can release cannabinoids (Walter et al., 2002; Witting et al., 2004) and microglia have both types of cannabinoid receptors, CB1 and CB2 (Cabral and Marciano-Cabral, 2005). Cannabinoid receptors are present in the leech, and they are known to be coupled to the production of nitric oxide (Stefano et al., 1997b). Jeffrey Lipitz, a former graduate student in our laboratory, found that the cannabinoid anandamide (AEA) when bath applied to leech tissue inhibited the migration and accumulation of microglia in response to an injury (Lipitz, 2008),
and that its effect were reversed by application of an NO scavenger or NOS inhibitor (L-NAME), indicating that cannabinoid action was mediated by NO.

Another recently discovered compound that also appears to influence microglial chemotaxis in the leech, identified using the leech expressed sequence tag (EST) database, is a homologue of the human complement component C1q, designated \( HmC1q \) (Tahtouh et al., 2009). Human C1q is mostly known as part of the opsonization machinery that prepares apoptotic cells for phagocytosis (Fishelson et al., 2001). However, C1q has been shown to be involved in the chemotactic behavior of dendritic cells (Vegh et al., 2006). Tahtouh and colleagues demonstrate \( HmC1q \) is present in neurons and glia of the adult leech using both in situ hybridization and immunohistochemistry. Furthermore, in vitro chemotaxic assays on leech microglia demonstrate that leech microglia move in response to both human and leech C1q in a manner that is dependent on the production of nitric oxide. While no microglial migration studies in the intact leech nerve cord were performed, this finding strongly suggests the \( HmC1q \), acting through NO, may regulate the microglial response to injury.

Taken together, these studies support the hypothesis that in leeches NO plays an important role in regulating the microglial response to injury and is involved in both the directed movement of microglia and their stopping at the lesion. Although NO is known to be an important molecule for regulating cell motility in invertebrates (Bicker, 2005) and the immune response (Minghetti and Levi, 1998), its role in the migration of microglia has not yet been reported in
mammals. The proposed signaling pathway controlling the migration of microglia upon injury, with both ATP and NO signals, is presented below in figure 6.1.

**Proposed sequence of events upon injury to the leech nerve cord**

A schematic of the proposed sequence of events that occurs upon injury to the leech nerve cord is presented in three panels in figure 6.2. The first panel is the resting condition depicting the normal distribution of microglia and important molecules. Resting microglia in the leech nerve cord have a spindle shape and their cytoplasmic processes extend along axon tracts within the sheaths of connective glial cells. In this state, the innexons are generally thought to be closed, ATP is sequestered in the cytoplasm and calcium is sequestered in the endoplasmic reticulum. Arachidonic acid, cannabinoid, opioid and nitric oxide concentrations are so low they are undetectable.

The second panel shows the response beginning during the first few minutes after injury. Immediately upon injury (on the left of the panel), there is a large and sustained increase in intracellular calcium that forms a spatial gradient near the crush. This calcium gradient may be responsible for opening the calcium sensitive innexon channels, even up to several hundred micrometers away from the crush. Also in this panel, ATP is released from the damaged membrane, and, more important, ATP is released for hundreds of micrometers along the connective through the glia cells’ newly opened innexons. This is the ATP signal that initiates microglial movement at a distance from the lesion. The high speed with which the signal travels from the lesion, is too rapid to be
explained by simple diffusion alone. Activated microglia change morphologies by retracting their cytoplasmic extensions and rounding before moving.

Panel 2 also shows another player controlling microglial migration, NO. NO is generated at the lesion by calcium activation of eNOS in the glial, microglial and muscle cells that are injured at the lesion. But there is also evidence that NO is generated by calcium waves. Because NO itself is rapidly inactivated and is unlikely to diffuse far from the lesion, NO appearing at a distance from the lesion may originate in the glial cell as a result of the calcium wave. The sustained calcium gradient may be expected to contribute to the generation of NO effect, since the endothelial nitric oxide synthase (eNOS) is calcium sensitive. NO provides the directional cue for the microglia, and, at high concentrations at the lesion itself, is a stop cue at the site of injury. It is not known exactly how NO by activating soluble guanylate cyclase influences the directionality, but it is likely due to a spatial gradient of the NO that is higher near the lesion. NO is also produced in response to a variety of molecular signals in the leech, including cannabinoids and opioids (see above).

The third panel shows the microglia that accumulate within hours after the injury and are prevented from leaving by high levels of NO and ArA, which is produced in response to increases in calcium by phospholipase A2. ArA, which I hypothesize would close the innexons and reduce the release of ATP near the crush, may limit the movement signal near the crush thereby preventing microglia that arrive from leaving. As the tissue recovers from the injury, the signals produced by the injury, such as the calcium gradient, NO and extracellular ATP,
likely decrease. The microglia that have migrated are no longer resting and are involved in the phagocytosing cellular debris and producing laminin. These are steps that in the leech precede axon sprouting and regeneration of connections.

**Future Studies**

Although much has been discovered about the initial signals that regulate the microglial response to injury, there are still many problems that remain unsolved. What is the mechanism of the sustained elevation of intracellular calcium in the glial cells after an injury? Is the post-injury calcium increase responsible for both the ATP release and the production of NO? How far do signals travel from the lesion? Do microglia at different distances from the lesion “see” different signals? Are there any other signals that can influence the migration of microglia in the leech that have been found in activate them in mammals, such as leukotrienes, cytokines or chemokines, and, if so, do these other compounds work through the action of ATP or NO?

In addition to these questions regarding leech physiology, a more crucial question is the relevance of these findings to mammalian, and specifically human, physiology. Do pannexons in humans play a similar role in human brains in regulating the release of ATP and controlling the microglial response to injury? A recently published paper has found that pannexons form the ATP permeable hemichannels in mammalian astrocytes (Iglesias et al., 2009). In this study, connexin 43 null mice and panx1 siRNA were used to distinguish the function of connexons and pannexons. Considering the literature discussed in this dissertation indicating that astrocytes activate microglia by releasing ATP and
that astrocytes calcium waves propagate several hundred micrometer after injury and release ATP, astrocytes may be responsible for the activation of microglia at a distance.
Figures

Figure 6.1: Proposed signaling pathway for the migration of microglia

This schematic diagram illustrates two independent signaling pathways, that of ATP and that of NO, necessary for the migration of microglia to lesions. Black lines represent signaling interactions with strong evidence. Light grey lines represent hypothesized interactions that do not yet have strong experimental support in this system. Arrows represent activation and squares represent inhibition. ‘*’ represents the activity of an enzyme.
Figure 6.2: Schematic representation of the post-injury signals that regulate the microglial response

Figure 6.2: Panel 1 illustrates the nerve cord before injury. Panel 2 illustrates the situation upon injury up to several hours after. Panel 3 illustrates the recovery from injury when the microglia have accumulated.

MG = microglia; inx = innexon; P2YR = purinergic receptor
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