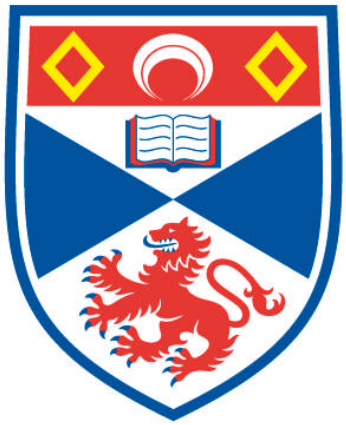
PURINERGIC AND CHOLINERGIC MODULATION OF SPINAL MOTOR NETWORKS IN MICE

Emily Witts

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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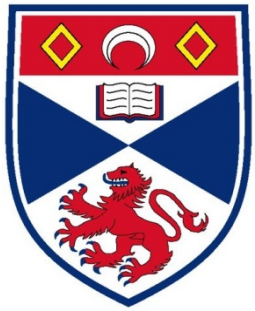

This thesis is submitted in partial fulfilment for the degree of Doctor of Philosophy at the University of St Andrews

September 2015

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I, Emily Witts, hereby certify that this thesis, which is approximately 40,000 words in length, has been written by me, and that it is the record of work carried out by me, or principally by myself in collaboration with others as acknowledged, and that it has not been submitted in any previous application for a higher degree.

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ABSTRACT

Neuromodulation allows flexibility within networks of neurons controlling rhythmic motor behaviours. This thesis explores how purinergic and cholinergic neuromodulation contributes to the functioning of spinal motor networks in mice.

Purinergic modulation in the spinal cord was investigated using neonatal mouse *in vitro* spinal cord preparations in which locomotor-related bursts of activity were pharmacologically induced. Ventral root recordings from these preparations showed that glia release ATP, which is broken down to adenosine and binds to A_1 adenosine receptors to reduce the frequency of locomotor-related bursts. Whole-cell patch-clamp recordings showed that adenosine opens leak potassium channels in ventral horn interneurons, leading to general network inhibition. Interestingly, although adenosine reduces synaptic inputs in interneurons and motoneurons, interneurons show a reversible hyperpolarisation and reduction of miniature postsynaptic currents (mPSCs) in response to adenosine, while motoneurons show a reversible depolarisation and no change in mPSCs. It was therefore concluded that adenosine opens leak potassium channels in ventral horn interneurons which reduces the speed of locomotor-related output from the whole network. However, motoneuron activity is prevented from falling so far as to cause muscle contraction to cease.

Cholinergic modulation of mouse spinal motor networks was also investigated. Pitx2+ cells, known to be the source of cholinergic C-bouton inputs to motoneurons, were selectively transfected with channelrhodopsin and light was used to activate the Pitx2+ cell population. Pitx2+ cell activation was found to reduce rheobase and range of firing in small numbers of spinal motoneurons. The role of Pitx2+ cells in behaviour was also investigated by testing adult mice before and after Pitx2+ cell ablation. No difference in performance was observed in reaching or ladder walking tasks. It therefore seems likely that activation of Pitx2+ cells modulates motoneurons via C bouton synapses, but this modulation is task-dependent and is not critical for goal-directed movements requiring fine motor control.

ACKNOWLEDGEMENTS

There are many people who have made this experience possible and/or enjoyable. First and foremost, I would like to thank my supervisor, Dr Gareth Miles, for his guidance and encouragement, and for always finding time for me somehow! This would not have been possible without his input. Secondly, thanks to Professor Keith Sillar, for showing equal enthusiasm for science and silliness (sometimes both at the same time) and to members of his lab for tadpolian inspiration. I would also like to thank members of the Miles lab, past and present, for the technical support, advice and encouragement. Similarly, thanks to the SCaM group and many support staff for helpful encouragement and warm community. Thanks also to many friends and family - all have supported me in different ways, none more than my parents. And finally, thanks to my wonderful husband Peter, who makes life immeasurably more enjoyable and who makes me a better person.

COLLABORATION STATEMENT

The third chapter of this thesis was performed in collaboration with colleagues at Columbia University and the Academy of Athens. I am extremely thankful to colleagues at Columbia for a warm welcome, patient teaching, and for being incredibly generous with their time and resources. I would like to particularly thank Eiman Azim, Andy Murray, Andrew Fink and Tom Jessell. Similarly, thanks must go to Laskaro Zagoraiou and Ismini Rosani for their help with anatomy and continuously collaborative attitude. Parts of this work were also performed in collaboration with colleagues in the Miles lab, and some experiments presented here were performed by Filipe Nascimento and Kara Panetta. Some sections are adapted from previously published work (Witts et al., 2012, Witts et al., 2014, Witts et al., 2015). All collaborations and publication adaptations are detailed at the beginning of each chapter.

CONTENTS

INTRODUCTION

Parts of this section are adapted from previously published work (Witts et al., 2014).

MOTOR CONTROL

One of the greatest remaining goals of science is a complete understanding of the functioning of the nervous system, as this underlies how we move, think and feel, and holds the key to what makes us who we are. Groups of neurons in the nervous system work together to produce a huge range of different outputs that are adaptable and dynamic. Movement is a particularly interesting aspect of neural control as although it is relatively simple in comparison to higher processes, the neural control of movement remains poorly understood. As movement is essential to many aspects of everyday life, and many of the most debilitating diseases involve some loss of movement, there is great value in understanding how movement is controlled and executed. As well as being an important area of study in and of itself, the motor output of the nervous system involves the same basic structures and interactions as the most complex processes of thinking. In many cases, research into higher processes comes with difficulty in directly measuring the output or consequences of whatever is being manipulated. Movement, on the other hand, can be easily observed and objectively measured, and is controlled by neurons that are similar to those involved in higher processes, but spatially distinct. The study of motor control, therefore, provides an objective, easily measurable model in which to study the basic building blocks and processes which also underpin all reasoning, emotions and development.

The networks of neurons which underlie rhythmic movements such as walking, swimming, flying, feeding and breathing are known as central pattern generators (CPGs). They produce a basic rhythm which can then be adapted to suit many different environmental situations and external stimuli. Rhythmic movements were once thought to be created by chains of reflexes (Sherrington, 1906) and the first suggestion that central circuits underlie rhythmic activity is attributed to T.G. Brown (Brown and Sherrington, 1911). He described antagonistic neural activity in the spinal cord, in the absence of descending or sensory input, as the basis for rhythmic movements. His ideas still form the foundations of current circuit models. Traditionally, CPGs are divided into pacemaker-driven CPGs, where intrinsically oscillating neurons generate rhythmic activity, and half-centre CPGs, where inhibitory neurons on each

side connect to contralateral excitatory neurons leading to oscillatory firing. In many CPGs oscillatory activity may depend on a combination of both pacemaker activity and half centre-like connectivity, and therefore CPGs may not fit neatly into one category or the other (Selverston, 2010).

CPGs are normally defined in terms of pre-motor interneurons, although motoneurons are sometimes included. CPGs can be extremely simple, consisting of few neurons, or very complex, consisting of many neurons and separate rhythm and pattern generating layers (Marder and Bucher, 2001, Guertin, 2009). The specific neurons within complex CPGs are sometimes hard to define because individual neurons can be part of more than one CPG and can switch between CPGs, and there can also be interaction between CPGs (Selverston, 2010). CPGs controlling locomotion are one example of those that can range from very simple to incredibly complex, often depending on the complexity and variety of movements needed. The focus here will be the neural control of vertebrate locomotion.

Vertebrate locomotor CPG

The neurons which make up the locomotor CPG are well characterised in some systems but largely unknown in others. The neurons of the vertebrate locomotor CPG are known to be located primarily in the spinal cord in most vertebrates. Even when separated from the brain and sensory input, it is possible for neurons in the spinal cord of many organisms to produce rhythmic activity, known to underlie motor output. This therefore provides an excellent model to study neural processes as the basic properties of neuronal networks can be investigated without complications from sensory inputs and higher functions.

During development, neurons in the embryonic vertebrate spinal cord begin to form distinct groups. Commissural and association neurons differentiate dorsally in the neural tube while motoneurons and ventral interneurons differentiate ventrally (Jessell, 2000). Genetic marking of cell types has been particularly useful in defining the neurons of the locomotor CPG,

and progress has begun to be made towards understanding some of the pieces which make up these networks.

Although CPGs controlling swimming differ between species, there are distinct types of neurons which are present in lamprey and *Xenopus* tadpole swim networks, which are among the most well understood vertebrate swim systems (Dale and Kuenzi, 1997, Grillner, 2003, Roberts et al., 2010). These networks, as well as zebrafish swim networks, all receive descending input from the brain and execute movements via motoneurons which innervate adjacent muscle blocks. In addition, there are three main types of interneurons that are conserved between the swim CPGs of these organisms: excitatory interneurons which provide rhythmic drive to motoneurons and other CPG neurons during swimming; inhibitory commissural interneurons which ensure muscles on opposite sides of the body contract out of phase with one another; and ipsilaterally-projecting inhibitory interneurons which provide inhibitory signals to motoneurons and commissural interneurons to relay sensory feedback (Grillner, 2003, Goulding, 2009, Li et al., 2009).

Mammalian locomotion shows some conservation of organisation and neuronal properties from swim networks, for example commissural interneurons secure coordination between the left and right sides of the spinal cord. However, there appears to be a greater level of complexity within CPGs controlling limbed locomotion. Knowledge of the developmental genetics underlying the formation of neuronal subtypes within the spinal cord has led to significant advances in our understanding of the neural components of the locomotor CPG in mice. Gradients of sonic hedgehog and bone morphogenetic proteins result in the ventricular zone of the mouse spinal cord being subdivided into discrete dorsoventral progenitor domains (Goulding, 2009). Five early classes of neurons, V0, V1, V2, V3 interneurons and motoneurons, then develop from progenitor domains established within the ventral neural tube (Figure 0.1).

V0 interneurons express the transcription factor Dbx1 (Pierani et al., 2001) and are mostly commissural. They can be subdivided into the more ventrally derived $V0_V$ interneurons which make up one third of the population and the more dorsally derived $V0_D$ interneurons

which make up two thirds of the population. $V0_V$ interneurons are mostly glutamatergic and express Evx1, while $V0_D$ interneurons are inhibitory, using GABA and/or glycine as transmitters, and do not express Evx1 (Moran-Rivard et al., 2001). The $V0_V$ interneuron population also contains glutamatergic $V0_G$ interneurons and cholinergic $V0_C$ interneurons which both express Pitx2. While the other neurons in the V0 population are commissural, Pitx2+ V0 interneurons can project both ipsilaterally and contralaterally (Zagoraiou et al., 2009). V0 interneurons are thought to play a role in the control of left-right alternation as loss of V0 interneurons results in a hopping-like gait in rodents (Lanuza et al., 2004, Talpalar et al., 2013).

V1 interneurons express the transcription factor En1, and are largely inhibitory. V1 axons project locally within the ipsilateral spinal cord and some synapse directly onto motoneurons. The V1 population includes Renshaw cells and Ia inhibitory interneurons, both of which form well-defined circuits with motoneurons (Sapir et al., 2004, Alvarez et al., 2005, Benito-Gonzalez and Alvarez, 2012, Stam et al., 2012). V1 interneurons are thought to be involved in controlling speed of locomotion as mice lacking V1 interneurons show a slowing of stepping movements and appear unable to produce fast locomotor activity (Gosgnach et al., 2006). They also play a role in flexor-extensor coordination (Zhang et al., 2014).

V2 interneurons express the transcription factor Lhx3 and their axons project intersegmentally in the ipsilateral spinal cord. They can be subdivided into V2a interneurons which express Chx10 and are excitatory and V2b interneurons which express Gata2/3 and are inhibitory (Al-Mosawie et al., 2007, Lundfald et al., 2007, Dougherty and Kiehn, 2010). V2a interneurons are known to be involved in the control of left-right alternation, as loss of V2a interneurons leads to loss of left-right alternation, particularly at higher locomotor frequencies (Crone et al., 2008, Crone et al., 2009, Zhong et al., 2010). Along with V1 interneurons, V2b interneurons are known to control flexor-extensor alternation as ablation of V1 and V2b interneurons leads to a synchronous pattern of flexor and extensor activity and a deficit in limb joint control and limb-driven reflex movements (Zhang et al., 2014).

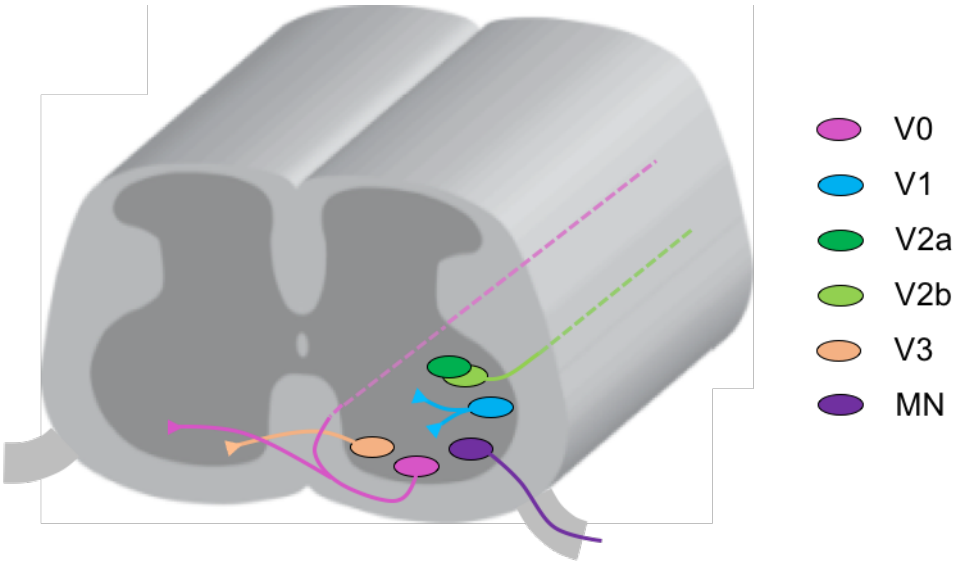

Figure 0.1: Projection patterns of ventral horn neurons in the spinal cord. V0 interneurons project contralaterally, V1 interneurons project locally within the ipsilateral ventral horn, V2 interneurons project intersegmentally, most V3 interneurons project contralaterally and motoneurons have axons that project out of the spinal cord via a ventral root.

V3 interneurons express the transcription factor Sim1 and are excitatory. Most project contralaterally but around 15% have an ipsilaterally-projecting axon (Zhang et al., 2008). They can be divided into two subpopulations; ventral and dorsal. Ventral V3 interneurons fire rapidly, display few processes and are thought to be premotor interneurons. Dorsal V3 interneurons have a slower average spike frequency, more complex morphology and are thought to be relay neurons which mediate sensory inputs (Borowska et al., 2013). Suppression of V3 synaptic transmission disrupts both the regularity and left-right coordination of locomotor activity. V3 interneurons are therefore thought to be responsible for ensuring a robust and organised locomotor rhythm as well as being involved in the control of left-right alternation (Zhang et al., 2008).

It is highly likely that these basic interneuron groups can be subdivided into many more functionally discrete populations. Understanding more about the complexities of these classifications may help to address some of the many unanswered questions in the functioning of mammalian locomotor CPGs. For example, research has provided evidence regarding the control of left-right alternation (Lanuza et al., 2004), but flexor-extensor co-ordination remains very poorly understood. In addition, the development of CPG neurons is not set in stone and, in some cases, activity can alter the normal development pattern of neurons. For example, enhancing or supressing activity in tadpoles changes transmitter phenotype in a homeostatic fashion; increased activity causes more neurons to express inhibitory transmitters while reduced activity increases the number of neurons expressing excitatory transmitters (Borodinsky et al., 2004). The complexity and intricacy of these systems means much more research will be needed in order to fully understand the neural control of basic locomotion, particularly in mammals.

Neuromodulation of the locomotor CPG

A further level of complication is added when one takes into account the wide range of different environments and circumstances that vertebrates face. CPGs need to be adaptable in

order to respond to the biomechanical demands of varying motor tasks. For example, a change in speed may be required to escape a predator or increased strength of muscle contraction may be needed when travelling uphill. One important process in this adaptability is modulation, which involves changing intrinsic properties of individual neurons, modifying the synaptic connections between them, or a combination of the two. Modulation of these elements can come from a range of sources both within the spinal cord, known as intrinsic modulation, and from out with the spinal cord, known as extrinsic modulation (Miles and Sillar, 2011). When considering neuromodulators, a number of factors are important in determining their action on network output. The same modulator can have a variety of effects depending on the location of its source neurons, the cells that it acts upon, the receptor subtype activated and the downstream pathway initiated. These different factors can change the way a neuromodulator acts, for example whether it has an excitatory or inhibitory effect and whether ion channels or synaptic release machinery are targeted.

Extrinsic Neuromodulators

Descending inputs from the brainstem are known to have profound modulatory effects on vertebrate locomotion. They serve as examples of the key features and actions of modulatory systems and can aid in understanding basic principles of modulation. The most widely studied and well understood extrinsic modulators in vertebrate locomotion are the biogenic amines noradrenaline, serotonin and dopamine.

1. Noradrenaline

The main source of neuromodulatory noradrenergic input to the mammalian spinal cord is from neurons in the locus ceruleus of the brain stem (Nygren and Olson, 1977). Noradrenaline influences both motoneurons and interneurons, and adrenergic receptors are widespread throughout the mammalian spinal cord (Roudet et al., 1994, Noga et al., 2011). Noradrenaline has a role in activating motor circuits, and it is able to induce locomotor activity

in some, but not all, mammals (Barbeau and Rossignol, 1991, Gabbay and Lev-Tov, 2004). Noradrenaline is also able to modulate the activity of ongoing motor output, and can change both the frequency and amplitude of locomotor activity (Sqalli-Houssaini and Cazalets, 2000). The dominant effect of noradrenaline appears to be excitatory but it can have varying effects depending on which receptor subtypes are activated (Chau et al., 1998, Kiehn et al., 1999). In the rat, activation of β-adrenoreceptors increases the amplitude of neural activity underlying muscle contraction, while α1-adrenoreceptor agonists increase, and α2-adrenoreceptor agonists decrease, the frequency of pharmacologically induced, locomotor-related ventral root output (Sqalli-Houssaini and Cazalets, 2000). In the neonatal mouse, α2-adrenoreceptor activation completely blocks electrically-induced, locomotor-related rhythmic ventral root activity, while α1-adrenoreceptor activation increases the amplitude and cycle period of this activity (Gordon and Whelan, 2006). This variation in effects may be due to the variety of downstream pathways that noradrenaline can influence, which may alter both cellular properties and synaptic activity. Noradrenaline is known to affect a variety of currents including persistent inward currents (Conway et al., 1988, Lee and Heckman, 1999) and inwardly rectifying potassium currents (Tartas et al., 2010), and can also affect action potential thresholds (Fedirchuk and Dai, 2004, Theiss and Heckman, 2005). In addition, noradrenaline is able to influence sensory (Jankowska et al., 2000) and descending (Hammar et al., 2007) inputs to spinal interneurons. The scope and variety of the effects and targets of noradrenaline show that it is capable of powerful and complex neuromodulation of locomotor networks in the mammalian spinal cord.

2. Serotonin

The vast majority of serotonin in the mammalian spinal cord originates externally; it is transported via descending pathways from the raphe nuclei in the brainstem (Lakke, 1997). Serotonin is capable of both initiating locomotor output and modulating locomotor activity in mammals (Cazalets et al., 1992, Nishimaru et al., 2000, Madriaga et al., 2004). There are a large

number of serotonin receptors in the ventral horn of the mammalian spinal cord, and serotonin is known to affect both interneuron and motoneuron activity. Serotonin generally has inhibitory effects when acting via 5-HT_1 receptors (Beato and Nistri, 1998) and excitatory effects when acting via 5-HT_2 or 5-HT_7 receptors (Liu and Jordan, 2005). In many interneuron subtypes, serotonin depolarizes resting membrane potential and reduces action potential threshold. (Fedirchuk and Dai, 2004, Carlin et al., 2006, Zhong et al., 2006, Dai et al., 2009). In motoneurons, serotonin can change the amplitude of locomotor output by the modulation of persistent inward currents (PICs) which often underlie periods of sustained action potential firing observed during rhythmic locomotion (Schmidt and Jordan, 2000).

3. Dopamine

Dopaminergic inputs in the spinal cord descend predominantly from a group of dopaminergic neurons in the hypothalamic A11 region (Bjorklund and Skagerberg, 1979, Hokfelt et al., 1979). All five subtypes of dopamine receptors are found within the ventral horn of the mammalian spinal cord, and dopaminergic inputs are widely distributed in this area (Holstege et al., 1996, Zhu et al., 2007). Both motoneurons and some subpopulations of interneurons are known to receive dopaminergic input (Weil-Fugazza and Godefroy, 1993). In comparison to noradrenaline and serotonin, dopamine is less important in the initiation of locomotion (Barbeau and Rossignol, 1991, Kiehn and Kjaerulff, 1996, Lapointe et al., 2009). However, it appears to play a role in shaping locomotor output, as dopamine levels increase during locomotor activity (Gerin et al., 1995). Dopamine is thought to be involved in sculpting the rhythmic activity produced by mammalian locomotor CPGs, as it can increase the amplitude of locomotor-related activity as well as slow and smooth the rhythmic output (Barbeau and Rossignol, 1991, Whelan et al., 2000, Barriere et al., 2004). Dopamine has been shown to increase excitability of motoneurons by modulating potassium currents. Application of dopamine can reduce afterhyperpolarisation (AHP) via blockade of calcium-dependent

potassium currents as well as reduce latency to first spike via inhibition of I_A channels (Han et al., 2007).

Intrinsic Neuromodulators

Intrinsic modulators are those which arise from within the spinal cord. They are less well understood than the classic descending extrinsic modulatory systems but there are several intrinsic classes of neuromodulators known to influence vertebrate locomotion. Amino acids such as glutamate and GABA act as neuromodulators as well as classic neurotransmitters in many locomotor systems (Bertrand and Cazalets, 1999, Iwagaki and Miles, 2011), and acetylcholine receptor activation has also been shown to alter locomotor-related motoneuron output (Miles et al., 2007). Nitric oxide is known to act as both a neuromodulator (McLean and Sillar, 2000, Kyriakatos and El Manira, 2007), and a metamodulator, modulating the activity of other neuromodulators (McLean and Sillar, 2004a). There is also a large number of peptides which modulate mammalian locomotor activity, the most researched being substance P (Barthe and Clarac, 1997, Parker et al., 1998) although many others have been shown to have the capacity to alter locomotor output (Pearson et al., 2003, Barriere et al., 2005). Finally, the purines ATP and adenosine have been widely researched in a large number of systems over many years, and are now known to modulate the activity produced by locomotor CPGs (Dale and Gilday, 1996, Brown and Dale, 2000).

Here, the focus will be on purinergic and cholinergic modulation. Firstly, purines are known to occur extremely widely and have been shown to have many roles in homeostasis and modulation in a large number of systems. However, their role in motor control is less well understood. Purinergic modulation of tadpole swimming (Dale and Gilday, 1996) and rodent respiration (Miles et al., 2002, Lorier et al., 2007) has been investigated, but the role, if any, of purines in mammalian locomotion is currently unknown. This work will therefore attempt to address this gap in our knowledge.

Secondly, acetylcholine is known to be the neurotransmitter used by motoneurons, but beyond this there are relatively few cholinergic neurons in the spinal cord. However, the C bouton is a surprisingly abundant cholinergic synapse found on motoneurons which appears to originate from within the spinal cord, as C boutons function remains intact following complete spinal transection (McLaughlin, 1972). C bouton synapses have been known to exist since 1969 (Conradi and Skoglund, 1969), but there still remain many unknowns surrounding the mechanism and circuitry of this intriguing system. Individual elements of synaptic transmission at the C bouton synapse have been investigated (Miles et al., 2007) and recent work has defined the source cells of C boutons (Zagoraiou et al., 2009). However, the C bouton synapse and its circuitry have never been studied directly using electrophysiological recordings. This work will therefore attempt to develop a method to directly study the mechanism and circuitry of the C bouton synapse, and then use this method to explore the mechanisms of synaptic transmission at the C bouton synapse. In addition, behavioural experiments will be performed to investigate the role of the C bouton synapse in fine motor behaviours.

PURINERGIC MODULATION

Purines are a family of organic compounds consisting of carbon, hydrogen, nitrogen, and sometimes oxygen atoms. Each purine is a heterocyclic compound, consisting of a pyrimidine ring fused to an imidazole ring. Key purines include adenine and guanine, which, along with the pyridamines cytosine and thymine, form the basis of DNA. Purines have roles in many biological processes, including energy transduction, cellular signalling, nitrogen disposal and antioxidation (Burnstock et al., 2011).

One group of purines particularly important in the nervous system is adenosine and its derivatives (Figure 0.2). Adenosine consists of the purine adenine linked to a ribose sugar via a glycosidic bond, and can be phosphorylated up to three times to produce adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP). The

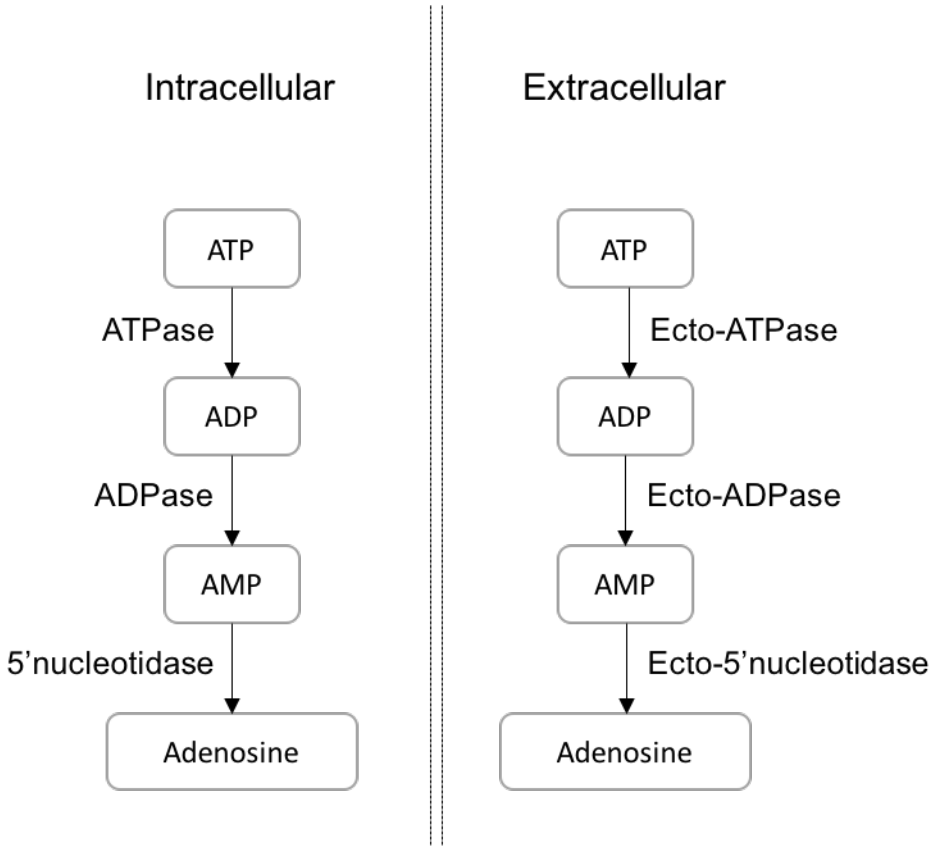

Figure 0.2: Biochemical pathway of adenosine production. The ectonucleotidase inhibitor used in chapter 1, ARL67156, is a selective ecto-ATPase inhibitor, preventing the breakdown of ATP to ADP.

production and metabolism of this family of purines occurs in many neurons and glial cells throughout the nervous system (Fields and Burnstock, 2006).

Adenosine exerts its effects via a family of receptors known as P1 or A1, of which there are 4 subtypes (A_1, A_{2A}, A_{2B}, and A_3). A_1 and A_{2A} receptors occur most frequently in the spinal cord and are distributed throughout the ventral horn (Geiger et al., 1984, Choca et al., 1987, Burnstock et al., 2011). Activation of A_1 receptors generally has inhibitory effects as these receptors are often linked to the Gi/Go protein pathway. Activation of A_{2A} receptors generally has an excitatory effect as they are linked to Gs protein pathways. Adenosine normally reduces neuronal activity predominantly by inhibition of neurotransmitter release, but also by hyperpolarisation through activation of potassium conductance (Li and Henry, 1992, Thompson et al., 1992). Neurotransmitter release is blocked by A_1 receptor-mediated inhibition of N-type calcium channels independently of cAMP (Scanziani et al., 1992) and/or by A_{2A} receptor-mediated inhibition of P-type calcium channels via cAMP and PKC (Mogul et al., 1993, Gubitz et al., 1996). A_{2A} receptors have varying roles in different regions of the CNS; for example they facilitate the release of GABA in the hippocampus (Cunha and Ribeiro, 1999) but inhibit GABA release in the striatum (Kirk and Richardson, 1995).

ATP acts via P2 receptors which are divided into two major families: ionotrophic P2X receptors of which there are seven subtypes ($P2X_{1\text{-}7}$) and multiple splice variants (Burnstock, 2007), and G-protein-coupled P2Y receptors of which there are at least 8 subtypes. The P2Y receptor subtypes can be divided into two groups based on the G-protein they couple to; $P2Y_{1,2,4,6,11}$ couple to $G_{q/11}$ while $P2Y_{12,13,14}$ couple to $G_{i/}G_o$. Due to the large number of receptor subtypes, ATP can have a wide range of effects, most often excitatory but also inhibitory. The most well-characterised P2X receptors are mixed cation channels, often with high calcium permeability. Most P2Y receptors either inhibit cAMP production or activate phospholipase C. As with adenosine receptors, the same receptor can have different effects in different regions and organisms (Abbracchio et al., 2006).

Adenosine and ATP have many roles in the body. In stressful situations, intracellular adenosine levels rise, resulting in adenosine being released to the extracellular environment (Meghji et al., 1989, Dulla et al., 2009). This reduces metabolic rate via A_1 receptors and signals the metabolic imbalance to neighbouring cells. In this way, homeostasis can be maintained. Adenosine is also known to be involved in learning and memory (Cunha et al., 1996, Wieraszko, 1996), sleep and arousal (Huston et al., 1996), feeding (Capogrossi et al., 1979) and mood and motivation (Nehlig, 2010, Burnstock et al., 2011).

ATP is best known for being the main energy storage molecule in the body, but it also frequently acts as a signalling molecule, especially in nocioception (Sawynok, 1998, Cook and McCleskey, 2002) and stimulus detection (Spyer and Thomas, 2000, Gourine et al., 2005). Purines were first discovered to be involved in signalling in the nervous system in 1972, when it was shown that ATP acted as a neurotransmitter in non-adrenergic, non-cholinergic inhibitory signalling (Burnstock et al., 1972). ATP is now known to act widely as a fast excitatory neurotransmitter and both ATP and adenosine have been shown to act as neuromodulators in a wide range of systems.

In many cases, ATP and adenosine have complex effects on complicated systems, where output is often not simple to record. In order to try and uncover the role of this family of purines in a more straightforward system, motor control provides an objective and easily measurable behaviour in which to look at cause and effect relationships.

One motor system where purinergic signalling is known to be important for mammals is respiration. The effects of ATP on the inspiratory rhythm-generating pre-Bötzinger complex (preBötC) have been investigated using rhythmically active slices from the medulla of the neonatal rat (Lorier et al., 2007). ATP was applied to the slices, and was found to cause a rapid increase in inspiratory frequency, with the largest and most rapid increase occurring when ATP was applied at the preBötC site. The effect was sensitive to the $P2Y_1$ receptor antagonist 2'-deoxy-N^6-methyladenosine-3',5'-bisphosphate (MRS2179) suggesting that the excitation is mediated by $P2Y_1$ receptors.

ATP was also found to cause a rapid increase in C4 ventral root tonic discharge and inspiratory burst amplitude in neonatal rats. This was followed by a reduction in burst amplitude, thought to be caused by adenosine as a non-hydrolysable analogue of ATP, ATP-γ-S, evoked only the excitatory effect. The excitatory effect is thought to be mediated by P2X receptors due to the extensive distribution of these receptors in phrenic motoneurons (Miles et al., 2002).

A similar biphasic effect has been observed in hypoglossal motoneuron inspiratory activity (Funk et al., 1997). ATP was applied to mouse medullary slices containing the hypoglossal nucleus and to anesthetized adult rats. ATP caused a rapid excitation of hypoglossal nerve activity followed by a slower reduction of inspiratory output. The initial excitation after ATP application is blocked by the P2 receptor antagonist suramin while the later inhibition is sensitive to the A1 antagonist theophylline, suggesting the effects are caused by ATP and adenosine respectively.

This work led to the proposal of a three-part purinergic signalling system in the control of respiration, consisting of adenosine, ATP and ectonucleotidases (Funk et al., 2008). In this model, ATP initially has an excitatory effect which increases inspiratory burst amplitude and is then broken down to adenosine by ectonucleotidases which causes a reduction in burst amplitude. More recent work has supported ATP as an excitatory molecule and adenosine as inhibitory in respiration, and extended previous work to include the ATP metabolite ADP, which also acts as an excitatory modulator of inspiratory rhythm (Huxtable et al., 2009).

It is unknown whether purinergic signalling is involved in mammalian locomotion, but the roles of purines in tadpole locomotor activity has been studied. It has been demonstrated that ATP increases, and adenosine decreases, the excitability of motor circuits in *Xenopus* tadpoles (Dale and Gilday, 1996). High levels of ATP at the onset of swimming block potassium channels to maintain high excitability. Over the course of swimming, ATP is broken down to adenosine, which activates A1 receptors, blocks calcium channels and slowly reduces excitability, eventually resulting in the cessation of swimming (Brown and Dale, 2000). This

mechanism was further investigated by measuring purine levels during activity. Swimming was briefly induced by sensory excitation and an adenosine sensor on the outside of the spinal cord was used to detect changes in adenosine levels during swimming. Changes in adenosine levels ranging from 10 to 650nM were detected, and adenosine rose during swimming and fell after the termination of swimming, returning to baseline once run-down was complete. This suggests that adenosine is involved in the run-down and termination of swimming, and the time course is consistent with ATP conversion to adenosine. Application of an inhibitor of adenosine uptake, nitrobenzylthioguanosine (NBTG), increased adenosine levels during activity and slowed recovery after termination of activity, suggesting that uptake is an important mechanism in the control of the effects of this neuromodulator. The study also highlighted the importance of ectonucleotidase levels, and how feed forward inhibition of 5'-ectonucleotidase is responsible for the delayed production, and therefore effects, of adenosine (Dale, 1998).

Taken together these results suggest that ATP binds to P2 receptors, which leads to potassium channels being blocked, causing an increase in excitation by inhibiting the delayed rectifier. ATP is then broken down by ectonucleotidases to adenosine which reduces excitation and slows the CPG rhythm by reducing calcium currents. This process is evident in respiration and tadpole swimming, but has not as yet been investigated in mammalian locomotion.

Work described in this thesis will therefore investigate the potential role of ATP and adenosine in the neural control of walking in mice, using electrophysiological recordings taken from *in vitro* spinal cord preparations. Ventral root recordings will be used to obtain the neural output which would underlie muscle contraction in the intact animal, and therefore obtain information about purinergic modulation of the whole CPG network. Whole cell patch clamp recordings will help to elucidate the effects of adenosine in individual neurons, and provide information about the mechanism of action of any purinergic modulation.

CHOLINERGIC MODULATION

Acetylcholine is a widespread excitatory neurotransmitter in the CNS. Cholinergic neurons in the cortex are known to be involved with sensory input, attention and memory (Giocomo and Hasselmo, 2007, Lawrence, 2008, Pauli and O'Reilly, 2008). Acetylcholine is also known to modulate dopaminergic signalling to control both sensory and motor pathways (Wang et al., 2006, Mena-Segovia et al., 2008). The spinal cord contains several discrete groups of cholinergic neurons. Firstly, motoneurons have long been known to use acetylcholine as their primary transmitter at the neuromuscular junction and they also form cholinergic synapses by contacting one another via recurrent motor axon collaterals (Lagerbäck et al., 1981). In addition, there are several discrete interneuron groups which provide sources of acetylcholine in the spinal cord: partition cells located between the border of the dorsal and ventral horns; central canal cluster cells closely associated with the central canal; and neurons scattered in the dorsal horn (Barber et al., 1984, Phelps et al., 1984, Arvidsson et al., 1997, Huang et al., 2000, VanderHorst and Ulfhake, 2006). There are also large cholinergic inputs found on the soma and proximal dendrites of motoneurons, known as C boutons, and these will be the focus of the second section of the current work.

C bouton inputs to motoneurons were first reported in electron microscopy studies over 40 years ago (Conradi, 1969, Conradi and Skoglund, 1969). This work described large (2-6μm in diameter) synaptic inputs on the soma and proximal dendrites of motoneurons which were associated with highly specialised postsynaptic structures. Perhaps the most distinctive of these postsynaptic structures were the sub-surface cisternae which are flattened membrane disks positioned just below the narrow synaptic cleft, typically extending the entire length of the synapse. These large, distinctive synapses were given the label C-type to distinguish them from synapses with spherical vesicles (S-type), flattened vesicles (F-type), dense postsynaptic "Taxi" bodies (T-type) and synapses contacted by smaller presynaptic boutons (M-type). Following the first description of C boutons in cats, these specialised synapses have been reported in a wide

range of mammals (Conradi and Skoglund, 1969, Bodian, 1975, Bernstein and Bernstein, 1976, Hamos and King, 1980, Pullen et al., 1992, Wilson et al., 2004).

Although motoneurons were already known to receive cholinergic inputs (Lewis and Shute, 1966), C boutons were not definitively shown to be cholinergic until many decades after their first description. The cholinergic identity of C boutons was eventually confirmed using antibodies against choline acetyl transferase, acetylcholinesterase and vesicular acetylcholine transporter (Arvidsson et al., 1997). It is now known that choline acetyltransferase catalyses the production of acetylcholine from choline and acetyl-CoA within the synaptic bouton (Connaughton et al., 1986, Nagy et al., 1993, Li et al., 1995, Arvidsson et al., 1997).

Once released into the synaptic cleft, ACh binds to receptors on the soma and proximal dendrites of motoneurons. There are two types of ACh receptors: ionotropic receptors which act as sodium and potassium ion channels, and G-protein-coupled metabotropic receptors. Ionotropic receptors are fast acting, and are known as nicotinic receptors due to their high affinity for nicotine. Nicotinic acetylcholine receptors have only been found presynaptically at the C bouton synapse (Khan et al., 2003), suggesting an absence of ionotropic transmission at C boutons. Metabotropic receptors are slower acting and are called muscarinic receptors as they preferentially bind muscarine. Metabotropic m2-type muscarinic receptors are known to cluster postsynaptically at C bouton synapses (Hellstrom et al., 2003), suggesting that these receptors are the target of ACh released from C boutons. ACh binding to m2 receptors is known to activate a G_i protein pathway, leading to a reduction in cAMP, which can have a number of downstream effects. Metabotropic muscarinic receptors at the C bouton synapse could act via several potential downstream targets; $K_v2.1$ potassium channels, SK potassium channels, P/Q-type calcium channels (Deardorff et al., 2014) and N-type calcium channels (Wilson et al., 2004) which are all found postsynaptically at the C bouton synapse.

$K_v2.1$ channels are delayed rectifier voltage gated potassium channels. They are thought to play an especially prominent role in regulating the intrinsic excitability of vertebrate neurons (Misonou et al., 2005). At the C bouton synapse in mice, they are clustered with m2 receptors.

The clustering behaviour and kinetics of $K_v2.1$ channels change in response to a variety of stimuli, and phosphorylation can also affect their functioning (Romer et al., 2014). This means that $K_v2.1$ channels are dynamic and flexible, and so are good candidates for mediating a range of modulatory effects on motoneurons.

SK channels are small conductance calcium-activated potassium channels. They underlie AHP in neurons by slow leakage of potassium ions into the extracellular space, causing hyperpolarisation of the cell after action potential firing. They are known to be involved in modulating the frequency of firing of motoneurons. Interestingly, in mice, the SK2 subtype of Ca^{2+}-dependent K^+ channels is found on all alpha motoneurons while the SK3 subtype is only found on a small subpopulation of alpha motoneurons. In comparison, SK3 channels are found on all cat motoneurons, while the exact distribution of SK2 channels is not clear (Deardorff et al., 2012). These data support, at least in rodents, some degree of motoneuron-subtype specificity in C bouton signalling.

N-type calcium channels are high voltage activated calcium channels. They are most commonly found presynaptically in the nervous system, where they are opened by depolarization, causing an influx of calcium which initiates vesicle fusion and therefore transmitter release. P/Q-type calcium channels are also high voltage activated and were first found in Purkinje neurons. They play a similar role to N-type calcium channels, but have a different pore-forming subunit. At the C bouton synapse, both N-type and P/Q-type calcium channels are found postsynaptically. This may be because SK channels require high voltage activated calcium currents in order to generate the AHP (Deardorff et al., 2014).

Sigma 1 receptors have also been observed postsynaptically at C bouton synapses, in the membrane of the subsurface cisternae (Mavlyutov et al., 2012). In contrast to other proteins at the synapse, sigma-1 receptors appear to be located beneath the postsynaptic plasma membrane, on subsurface cisternae. In support of a functional role for these receptors, the enzyme which converts tryptamine to the sigma-1 receptor agonist dimethyltryptamine is also localized in close proximity to C bouton synapses. Sigma 1 receptors are known to act as

chaperone proteins, so may be involved in the assembly or rearrangement of some of the other components of the C botuon synapse. They are also known to modulate calcium signalling so may interact with the calcium channels known to be present postsynaptically at C botuon synapses (Mavlyutov et al., 2012).

There is also some evidence to support the presence of presynaptic P2X7 receptors at the C bouton (Deng and Fyffe, 2004). P2X7 receptor expression occurs throughout the central nervous system, and these receptors are known to have a wide variety of roles, although it is likely that they play some sort of feedback role, due to their presynaptic location. It is important to note that there has been some debate regarding the specificity of P2X7 antibodies (Kaczmarek-Hajek et al., 2012) and so the presence of P2X7 receptors on the C bouton may need further confirmation.

Given the clustering of postsynaptic m2 receptors at C bouton synapses, pharmacological activation of muscarinic receptors on motoneurons was used to indirectly investigate the effects of C bouton activation. Data obtained from whole-cell patch-clamp recordings of motoneurons in neonatal mouse spinal cord slice preparations revealed that activation of m2 receptors reduces the action potential AHP which increases motoneuron excitability (Miles et al., 2007). Analysis of the mechanisms underlying reductions in AHP amplitude revealed a direct blockade of Ca^{2+}-dependent K^+ (SK) channels, with no effect on voltage-activated Ca^+ channels (sources of Ca^{2+} for SK channel activation). Physiological experiments demonstrating the effects of m2 receptor activation on motoneurons thus provide an explanation for postsynaptic clustering of m2 receptors, SK channels and N-type Ca^{2+} channels at C bouton synapses. However, the roles of other protein clusters remain less clear.

Perhaps most strikingly, despite their specific localisation to C bouton synapses, the roles of Kv2.1 channels remain unknown. Evidence of K^+ channel modulation by C boutons was also provided by experiments activating m2 receptors on motoneurons; a small hyperpolarising current likely reflecting facilitation of a resting K^+ current was observed in some, but not all, motoneurons (Miles et al., 2007). However, given that Kv2.1 channels mediate delayed rectifier

currents which are not normally active at rest, it is unlikely that their facilitation could underlie any m2-induced hyperpolarisation. Kv2.1 channels are nonetheless known to control neuronal excitability, perhaps fulfilling a homeostatic role to limit hyperexcitability (Misonou et al., 2005). Interestingly, the voltage-dependent activation properties and clustering of Kv2.1 channels depend on their phosphorylation state. Dephosphorylation of Kv2.1 channels, which can be induced by glutamatergic simulation, leads to the dispersion of Kv2.1 channel clusters and a large (~30mV) leftward shift in their activation curve (Misonou et al., 2005). Conversely, phosphorylation of Kv2.1 channels, which might be induced by m2 receptor activation (Zhou et al., 2003), leads to a rightward shift in their activation curve (Murakoshi et al., 1997). It is thus plausible that following activation of C bouton synapses, greater depolarisation is required to open Kv2.1 channels providing another mechanism by which C boutons synapses might increase motoneuron excitability besides blocking the AHP. In addition to mediating delayed rectifier currents, Kv2.1 channels have the potential to fulfil non-conducting roles at C bouton synapses. Kv2.1 channels are predominantly non-conducting when clustered on the plasma membrane (Fox et al., 2013). In this clustered, non-conducting state they may instead act as cell surface insertion platforms for ion channel trafficking (Deutsch et al., 2012).

Another protein found at C bouton synapses for which a function remains to be ascribed is the sigma-1 receptor (Mavlyutov et al., 2012). One potential role for these receptors is the regulation of Ca^{2+} availability at C bouton synapses. This possibility is supported by their localisation to subsurface cisternae, which form intracellular Ca^{2+} stores (Berridge, 1998), and their known association with IP3 receptors (Kourrich et al., 2012). Sigma-1 receptors might therefore contribute to the control of Ca^{2+} release from subsurface cisternae which could in turn contribute to the activation of SK channels and AHP generation (Berridge, 1998). Alternatively, sigma-1 receptors could modulate the function of ion channels at C bouton synapses to regulate motoneuron excitability (Kourrich et al., 2012). For example, given their direct interactions with Kv channels, sigma-1 receptors could be involved in the redistribution of Kv channels from intracellular compartments to the plasma membrane (Kourrich et al., 2013).

In addition to modulating the intrinsic properties of motoneurons, C boutons might also modulate synaptic input to motoneurons. Two recent studies in rodents have provided indirect evidence of this. The first showed that the activation of projections from cholinergic neurons located near the central canal potentiates commissural glutamatergic inputs to motoneurons via muscarinic receptor-dependent mechanisms (Bertrand and Cazalets, 2011). Conversely, the second study showed that muscarinic receptor activation can inhibit synaptic currents mediated by postsynaptic AMPA receptors on motoneurons (Mejia-Gervacio, 2012). Further work will be required to determine whether either of these cholinergic modulatory effects on synaptic transmission involves the C bouton system.

In addition to research focussed on the structure and function of C boutons, some studies have investigated their neuronal source. Initial work found that C boutons remained following complete spinal transections in cats supporting a intraspinal rather than supraspinal origin for C boutons (McLaughlin, 1972). This was further reinforced by a comprehensive description of bulbospinal connections in mice, showing that cholinergic neurons of the brainstem do not project to the spinal cord (VanderHorst and Ulfhake, 2006).

Research next focussed on which class of cholinergic neurons within the spinal cord give rise to C boutons. Motoneuron axon collaterals were considered as a potential source. However, given differences in synaptic proteins at C boutons versus motoneuron terminals on Renshaw cells (Hellstrom et al., 1999), a motoneuronal source for C boutons appeared unlikely. The definitive exclusion of motoneurons as a C bouton source was later demonstrated using a Chat-GFP mouse line in which GFP was expressed by all motoneurons, but not all cholinergic interneurons. In this mouse line C boutons did not express GFP (Miles et al., 2007), demonstrating that they must arise from cholinergic interneurons and not motoneurons.

With motoneurons excluded as the source of C boutons, attention shifted to cholinergic interneurons of the spinal cord. Several classes of cholinergic spinal interneurons have been described including: partition cells located between the border of the dorsal and ventral horns; central canal cluster cells closely associated with the central canal; and neurons scattered in the

dorsal horn (Barber et al., 1984, Phelps et al., 1984, Arvidsson et al., 1997, Huang et al., 2000, VanderHorst and Ulfhake, 2006). Research utilising knowledge of the molecular identity of C boutons revealed that the most likely source of C boutons is a medially positioned population of partition cells (Miles et al., 2007). Both C boutons and these interneurons are fluorescently labelled in mice in which YFP expression is dependent on the transcription factor Dbx-1 (a marker of the developmentally defined V0 interneuron cohort (Jessell, 2000). In addition, both Dbx-1 expressing partition cells and C boutons lack expression of neuronal nitric oxide synthase (nNOS). Several years later, the paired-like homeodomain transcription factor Pitx2 was also found to label medially positioned partition cells (Zagoraiou et al., 2009). Pitx2 expression was found to define a small cluster of V0 ($Dbx1^{+}$) interneurons which form a longitudinal column near the central canal of the spinal cord and can be further subdivided into cholinergic ($V0_C$) and glutamatergic ($V0_G$) subtypes. Remarkably, molecular genetic techniques suggest that this small cluster of $V0_C$ interneurons, which is outnumbered by motoneurons by a factor of ~10:1, represent the sole source of C bouton inputs to all spinal motoneurons.

It is clear that there is considerable divergence in the output of $V0_C$ interneurons, with individual cells likely to form ~1000 synaptic contacts with motoneurons (Zagoraiou et al., 2009). Transynaptic viral tracing experiments have shown that the majority of $V0_C$ interneurons project to ipsilateral motoneurons, although up to a third of cells have contralateral projections (Zagoraiou et al., 2009). Other research has suggested that a proportion of $V0_C$ interneurons may project bilaterally to functionally equivalent motoneurons on each side of the spinal cord. However, it should be noted that this work concentrated on premotor, cholinergic interneurons near the central canal, but did not define these cells as $Pitx2^{+}$, $V0_c$ interneurons (Stepien et al., 2010). As well as suggesting bilateral output from $V0_C$ interneurons, this work also raised the possibility that motoneurons receive C bouton inputs from a number of $V0_C$ interneurons distributed throughout the rostro-caudal axis of the spinal cord. In addition to innervating motoneurons, the axons of $V0_C$ interneurons also appear to synapse onto other interneurons within the intermediate zone of the spinal cord (Zagoraiou et al., 2009). Although the full

complement of interneurons contacted remains to be determined, there is evidence that $V0_C$ interneurons project to Ia inhibitory interneurons (Siembab et al., 2010).

To determine the circuitry of the C bouton system it is of course also important to consider the inputs that $V0_C$ interneurons receive. Although data are limited, there is anatomical evidence of descending serotonergic inputs and a lack of primary afferent input to $V0_C$ interneurons. Physiological experiments demonstrating oligosynaptic, but not monosynaptic, dorsal root-evoked input to $V0_C$ interneurons confirm a lack of primary afferent input (Zagoraiou et al., 2009). In addition, this work also demonstrated that $V0_C$ interneurons receive input from the spinal locomotor central pattern generator (CPG) but are themselves unlikely to be involved in generating the locomotor rhythm (Zagoraiou et al., 2009). These CPG inputs appear to be very closely related to those driving the locomotor activity of motoneurons. Taken together, data regarding inputs to $V0_C$ interneurons suggest that they may receive copies of the descending, sensory and local synaptic drive received by motoneurons, perhaps enabling the C bouton system to control motoneuron activity in a manner appropriate for on-going motor activity (Zagoraiou et al., 2009).

Advances in our understanding of the circuitry of the C bouton synapse have been paralleled by recent insight into their function in spinal motor networks and motor behaviour. Research regarding the function of the C bouton system has primarily focused on their contribution to the control of locomotion. Early evidence that the C bouton system contributes to locomotor control came from work in cats which demonstrated that fictive locomotor activity induces c-*fos* expression in cholinergic interneurons near the central canal (Huang et al., 2000). It was later demonstrated that the activity of $V0_C$ interneurons is indeed tightly phase locked to motoneuron activity during fictive locomotion in isolated neonatal mouse spinal cord preparations (Zagoraiou et al., 2009). Initial work investigating the role of C boutons in the control of locomotor-related motoneuron output was directed by knowledge of postsynaptic m2 receptor clustering at C bouton synapses. This work utilised an m2 receptor antagonist to block C bouton signalling and a cholinesterase inhibitor to turn up C bouton activation during on-

going, fictive locomotor activity recorded from isolated neonatal mouse spinal cord preparations (Miles et al., 2007). Blockade of m2 receptors reduced the amplitude of locomotor-related bursts of motoneuron output. Conversely, cholinesterase inhibition increased tonic and phasic locomotor-related motoneuron output. Neither treatment affected the frequency or pattern of locomotor-related output, indicating a specific role for C boutons in the modulation of motoneuron activity, but no effect on spinal interneurons within locomotor CPG circuitry.

In addition, recent research has demonstrated a functional role for the C bouton system in whole animal behaviour (Zagoraiou et al., 2009). This work utilised mice in which the cholinergic output of V0c interneurons is inactivated due to the conditional knockout of the enzyme responsible for the biosynthesis of acetylcholine (choline acetyltransferase). The motor performance of mutant and control animals was assessed during locomotor behavioural assays designed to uncover task-dependent modulation in the activation of hind limb muscles. The degree of muscle activation was monitored via recordings of electromyographic (EMG) activity while animals were subjected to sequential walking and swimming tasks. In rodents, swimming elicits greater activation of some hind limb muscles compared to walking. However, in mutant animals in which C boutons were inactivated the enhancement of muscle activation during swimming was significantly diminished compared to controls (Zagoraiou et al., 2009). This suggests that C boutons modulate motoneuron activity during locomotion to match the intensity of muscle activation to the biomechanical demands of different motor tasks. It remains unclear how V0c interneuron activity is adjusted to suit different locomotor tasks; possible mechanisms include feedback from sensory systems, or feed-forward control originating from higher motor control centres.

Many of the questions that remain regarding the function of the C bouton system relate to our incomplete knowledge of the inputs received by $V0_C$ interneurons and the pattern by which they innervate motoneurons. As discussed above, there are varying levels of specificity with respect to C bouton connectivity to motoneurons, including preferences based on the muscle types that motoneurons innervate (Hellstrom et al., 2003). In addition, it has recently

become apparent that although ubiquitous, C bouton synapses are not completely uniform. Some degree of specialisation in C bouton signalling is, for example, indicated by variability in the complement of SK channel subtypes clustered at C bouton synapses on different motoneurons (Deardorff et al., 2012). Although the functional significance of such motoneuron subtype-specific features remain to be elucidated, it seems likely that C bouton-mediated modulation does more than simply set a global tone of motoneuron excitability, but rather fine tunes the excitability of functionally distinct groups of motoneurons to help orchestrate complex motor behaviours.

RESEARCH FRAMEWORK

Although considerable progress has been made in the study of purinergic and cholinergic modulation in mammalian motor control, we do not know how purinergic signalling affects mouse locomotion, nor do we understand the mechanism and circuitry of the C bouton system. This work will therefore attempt to address these gaps in current knowledge.

Firstly, *in vitro* spinal cord preparations will be used to study the role of adenosine and ATP in mouse locomotion. Locomotor-related bursting activity will be chemically induced in the isolated spinal cord using NMDA, serotonin and dopamine. Suction electrodes will be used to record the activity of ventral roots in order to study the neural output that would control muscle activity in the intact animal. Application of P1 and P2 receptor agonists and antagonists will provide information about the role of ATP and adenosine in mouse walking. Single cell recordings will also be performed using slices of *in vitro* spinal cord preparations, in order to investigate the effects of ATP and adenosine in individual cells.

Secondly, the mechanism and circuitry of the C bouton synapse in the mouse spinal cord will be studied. The relationship between Pitx2+ cell activation and motoneuron activity will be directly investigated by stimulating the entire Pitx2+ cell population while recording the activity of individual motoneurons. The behavioural implications of Pitx2+ cell activity will also be

investigated by studying the behaviour of mice in a range of motor tasks before and after Pitx2+ cell ablation.

Learning more about the modulation of motor patterns and individual neurons is important for both understanding the healthy nervous system and for interventions when things go wrong. The ability to externally control locomotor networks could aid recovery from spinal cord injury and understanding the control of motoneuron excitability may provide avenues to explore treatments for motor diseases. Understanding the basic output of locomotion could also provide a platform for research into higher brain functions, and neurodegenerative diseases affecting more complicated neural systems.

CHAPTER 1

PURINERGIC MODULATION OF SPINAL MOTOR NETWORKS

This chapter is adapted from previously published work (Witts et al., 2012).

Six of the 75uM adenosine experiments were performed by Kara Panetta.

INTRODUCTION

Although purines have been widely studied, relatively little is known about purinergic modulation of mammalian locomotion. The focus of this chapter will therefore be to investigate the effects of adenosine and ATP on locomotor output recorded from ventral roots of isolated mouse spinal cord preparations. Although primarily an energy source, ATP can be released by neurons and glia to act as a signalling molecule via binding to P2 receptors, which exist as two main subtypes - ionotropic P2X receptors and G-protein coupled metabotropic P2Y receptors (Burnstock, 2007). Adenosine, which is mainly derived from the ectonucleotidase-mediated hydrolysis of extracellular ATP, also acts as a signalling molecule via its binding to P1 receptors, of which there are four subtypes – A_1, A_{2A}, A_{2B} and A_3 (Cunha, 2001, Burnstock, 2007). The downstream effects of P2 and P1 receptor activation are diverse and typically oppose each other. P1 receptor activation most often leads to neuronal excitation while P2 receptor activation normally inhibits synaptic transmission and neuronal activity (Burnstock, 2007).

Data concerning a role for purines in the control of locomotion are limited to studies of *Xenopus* tadpoles where ATP facilitates swimming by increasing the excitability of neurons within the locomotor CPG, most likely via the activation of P2Y receptors. (Dale and Gilday, 1996, Brown and Dale, 2002). In contrast, adenosine, most likely derived from the ectonucleotidase-mediated breakdown of ATP, reduces CPG excitability via the activation of A_1 receptors (Dale and Gilday, 1996, Brown, 2000). Extracellular ATP levels, which are linked to activity, are highest at the beginning of locomotor episodes, while adenosine production is delayed by feed-forward inhibition of ectonucletidases. Because of their opposing actions, a gradually changing balance between ATP and adenosine, is thought to contribute to the run-down and eventual cessation of swimming in *Xenopus* tadpoles (Dale and Gilday, 1996, Dale, 1998).

Although the role of purines in the control of mammalian locomotion remains to be addressed, purines have been shown to modulate mammalian respiration, another CPG-driven rhythmic motor behaviour. In medullary slice preparations obtained from neonatal mice, ATP

acts via P2Y receptors to increase the frequency of inspiratory-related activity recorded from motor nerve roots (Lorier et al., 2007). As in the tadpole locomotor system, adenosine-mediated inhibition appears to follow ATP-mediated excitation of respiratory activity (Lorier et al., 2007). In addition, adenosine is reported to have a tonic depressive effect on the frequency of mammalian respiration which appears strongest at foetal stages (Kawai et al., 1995, Schmidt et al., 1995, Herlenius and Lagercrantz, 1999, Mironov et al., 1999, Huxtable et al., 2009). Adding to growing evidence of an important role for purinergic gliotransmission in the modulation of neuronal networks (Fellin et al., 2009), glial cells appear to contribute to the ATP-sensitivity of the respiratory CPG (Huxtable et al., 2009) as well as central respiratory chemosensitivity (Gourine et al., 2005). In addition to modulating the frequency of respiratory activity, purines also modulate the intensity of respiratory-related motor output. ATP excites both phrenic and hypoglossal motoneurons (Funk et al., 1997, Miles et al., 2002), most likely via activation of P2X receptors, to increase their inspiratory-related output. This effect is again followed by inhibition thought to reflect the actions of adenosine derived from the breakdown of ATP.

Given the importance of purines in the control of tadpole locomotion and the demonstration that purines can modulate mammalian CPG networks, an attempt was made to determine whether purines also contribute to the control of mammalian locomotion via modulation of spinal locomotor networks. Using rhythmically active isolated mouse spinal cord preparations, the results presented in this chapter demonstrate that endogenous purines modulate the locomotor CPG. Furthermore, these data provide evidence that this modulation involves gliotransmission.

METHODS

In vitro whole spinal cord preparation

All methods required to obtain tissue for *in vitro* experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 under personal licence 60/12934. Spinal cord preparations were obtained from postnatal day (P)0-5 C57BL/6 mice

using techniques similar to those described previously (Jiang et al., 1999). Animals were killed via cervical dislocation, decapitated and the front paws were used to pin the preparation ventral side up in a petri dish containing Sylgard resin. Ventral skin was removed and mice were eviscerated, taking care not to damage the spinal column. The preparation was then transferred to a dissecting chamber containing artificial cerebrospinal fluid (aCSF; equilibriated with 95% oxygen, 5% carbon dioxide; ~4^{o}C). The mouse was pinned into Sylgard resin at the bottom of this chamber, ventral side up and using all four paws. Vertebrae were removed and then the spinal cord was isolated by cutting through both dorsal and ventral roots. Once isolated, the spinal cord was pinned at both ends into the Sylgard resin. Dura mater and dorsal roots were removed. Ventral roots were trimmed, leaving enough to allow suction electrodes to be attached and the cord was trimmed at both ends to leave just mid-cervical to upper sacral segments. This preparation was pinned ventral side up to Slygard resin in a recording chamber superfused with aCSF (equilibrated with 95% O_2, 5% CO_2; room temperature), with a flow rate of 8-10 ml/min.

Ventral Root Recordings

Glass suction electrodes were attached to the 1st or 2nd lumbar (L1/L2) ventral roots on both the left and right sides of isolated spinal cord preparations. N-methyl-D-aspartic acid (NMDA; 5μM), 5-hydroxytryptamine (5HT; 10μM) and dopamine (DA; 50μM) were added to the aCSF to induce rhythmic, left-right alternating bursts of locomotor-related ventral root activity (Jiang et al., 1999, Miles et al., 2007). Locomotor-related activity was allowed to stabilise (~1 hour) before subsequent drug applications. All signals were amplified and filtered (30 - 3000Hz), (Qjin Design, ON, Canada) before being acquired at 1 kHz using a Digidata 1440A A/D board and AxoScope software (version 7.0, Molecular Devices, Sunnyvale, CA). A subset of signals was also rectified and integrated (Qjin Design) prior to acquisition such that both raw and rectified/integrated traces were recorded for each ventral root.

Data analysis

Data were analysed offline using DataView software (courtesy of Dr W.J. Heitler, University of St Andrews). Integrated traces were used to detect locomotor bursts. The frequency and peak-to-peak amplitude of these bursts were then measured from raw traces. Unless otherwise stated in the results section, all sample sizes (n) refer to the number of preparations in which drugs were applied. For time course plots, mean values are presented normalised to control levels ± SE to facilitate comparison between preparations. Time course plots were constructed from data averaged in 1 minute time bins. Statistical comparisons between different experimental conditions were performed on raw data averaged over 10 minute periods and these data presented in bar graph form. Differences in means were assessed from raw data using paired, two tailed Student's t-tests (Microsoft Excel) or analysis of variance with Tukey post hoc tests (SPSS). Values of $p < 0.05$ were considered significant. The phase relationship between activity recorded from the left and right sides of the spinal cord was assessed using circular plots and Rayleigh's test for uniformity (Kjaerulff and Kiehn, 1996, Zagoraiou et al., 2009); statistiXL software, Nedlands, WA, Australia). In circular plots the point labelled 0 represents the beginning of the locomotor cycle (defined as the onset of left ventral root activity). If left and right ventral root activity is out of phase and therefore appropriate alternation is present, left ventral root activity should terminate and right ventral root activity should begin near point 0.5 on the circle. The individual data points plotted around the circle represent the onset of individual locomotor bursts recorded from right ventral roots. Arrows point to the mean burst onset time and their length represents the concentration of the data points around the mean.

Solution and drugs

The aCSF used for dissecting and recording contained (in mM): 127 NaCl, 3 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, 26 $NaHCO_3$, 10 glucose. The adenosine A_{2A} receptor antagonist SCH58261 and the ectonucleotidase inhibitor ARL67156 were obtained from Tocris, all other

drugs were obtained from Sigma-Aldrich, UK: N-methyl-D-aspartic acid (NMDA), serotonin hydrochloride (5-HT), dopamine hydrochloride (DA), adenosine, adenosine triphosphate disodium salt (ATP), theophylline (non-selective adenosine receptor antagonist), bicuculline methiodide ($GABA_A$ receptor antagonist), strychnine (glycine receptor antagonist), glutamine, cyclopentyldipropylxanthine (DPCPX; adenosine A_1 receptor antagonist), fluoroacetate (FA; glial aconitase inhibitor) and methionine sulfoximine (MSO; glial-specific glutamine synthetase inhibitor). All drugs were made up fresh using aCSF, apart from bicuculline methiodide and strychnine which were stored as frozen aliquots prior to their use.

RESULTS

Modulation of spinal locomotor networks by exogenously applied purines

To determine whether activation of P2 (ATP) receptors modulates the activity of spinal motor networks controlling mammalian locomotion, ATP was bath applied at a variety of doses (10μM, n=3 preparations; 50μM, n=8; 75μM, n=5; 100μM, n=6; 150μM, n=4; duration 30 mins) to isolated mouse spinal cord preparations while recording pharmacologically-induced (5μM NMDA, 10μM 5-HT, 50 μM DA) rhythmic, left-right alternating locomotor-related activity from lumbar ventral roots. At doses of 75 - 150μM, application of ATP caused a significant decrease in the frequency of bursts of locomotor-related ventral root activity, while lower doses had no significant effect (Figure 1.1A, C & E). Burst frequency was maximally reduced by ATP at a concentration of 100μM (24 ± 4.9% reduction, n = 6; Figure 1.1C & E). Locomotor burst frequency decreased gradually throughout the application of 75 - 150 μM ATP, reaching a minimum toward the end of the drug application, before returning to control levels after drug washout (Figure 1.1C). At all doses utilised, ATP application had no significant effect on the amplitude of locomotor bursts (Figure 1.1A & D). Circular plots were used to assess the degree of alternation between activity recorded from segmentally aligned left and right ventral roots in control conditions and in the presence of ATP. Rayleigh's test for uniformity (Kjaerulff and Kiehn, 1996, Zagoraiou et al., 2009) was used to statistically analyse the phase relationships of

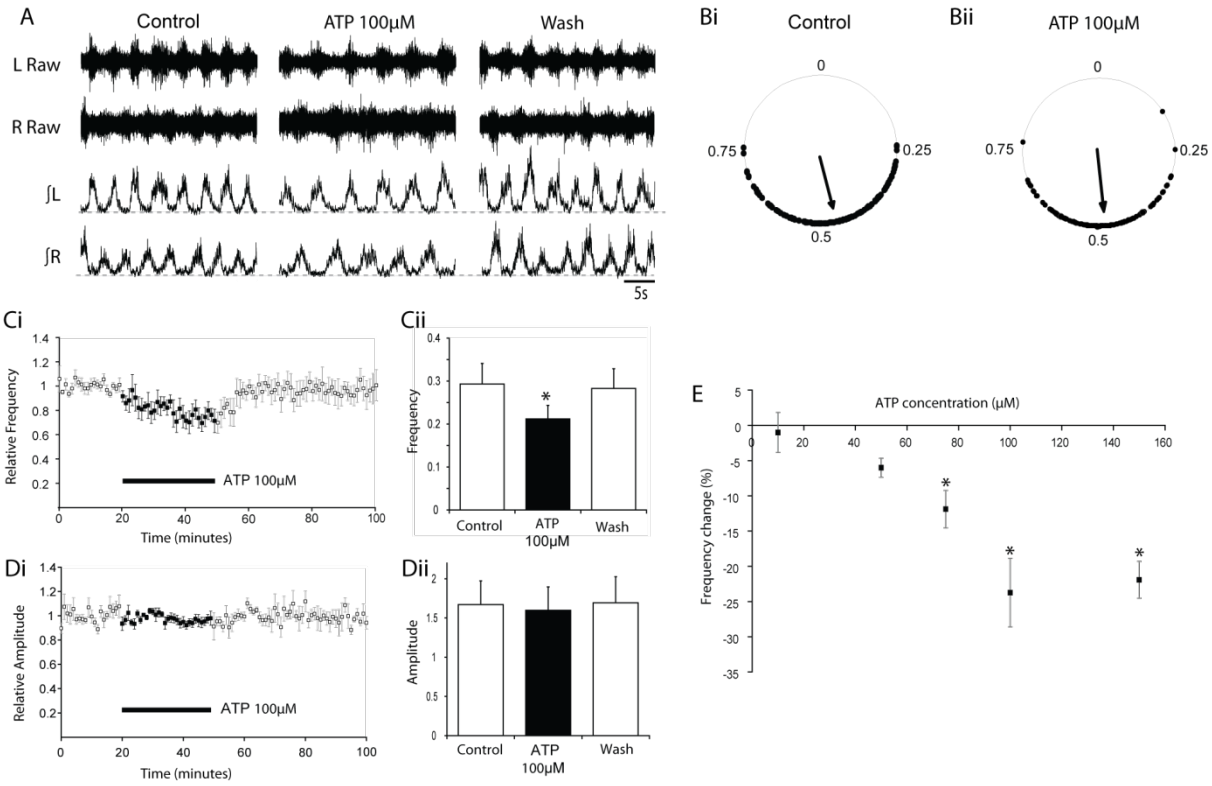

Figure 1.1: ATP reduces locomotor burst frequency but does not affect burst amplitude or left-right alternation. (A) Sample raw (top panel) and rectified/integrated (bottom panel) L2 ventral root recordings during control, 100µM ATP application and washout. Traces represent 30 seconds of each condition. **(B)** Circular plots depicting the phasing of the onset of locomotor bursts recorded from the right L2 root in relation to the onset of activity recorded from the left L2 root in control conditions **(Bi)** and during 100µM ATP application **(Bii)**. In both cases the points are clustered around 0.5 suggesting that bursting alternates between the left and right sides with little change upon the application of ATP. **(Ci)** Pooled time course plot showing changes in the normalised frequency of locomotor bursts due to ATP application (n=8). **(Cii)** Graph showing average burst frequency during the final 10 minute period of each condition (control, 100µM ATP application and washout; n=8). A significant decrease in frequency ($p<0.01$) was observed in the presence of ATP. **(D)** Time course plot **(Di)** and bar chart **(Dii)** showing no change in the amplitude of locomotor bursts following the application of ATP (n=8). **(E)** Dose-response relationship for ATP showing the dose-dependency of the reduction in locomotor-related burst frequency. * = significantly different to control.

at least 100 bursts during both control and drug conditions within individual spinal cord preparations. These analyses revealed that left-right coordination of locomotor activity remained in the presence of ATP (n=3 preparations; Figure 1.1B). Together these results indicate that exogenously applied ATP affects components of the CPG for locomotion to alter locomotor frequency, but not the intensity of locomotor-related output or the left-right coordination of motor activity.

Interestingly, a reduction in locomotor burst frequency contrasts the excitatory effects of ATP on the respiratory rhythm in mice (Lorier et al., 2007) and locomotor rhythm in *Xenopus* tadpoles (Dale and Gilday, 1996). However, in both of these systems adenosine derived from the breakdown of ATP has inhibitory effects. It was therefore hypothesised that the reduction in locomotor frequency, and lack of any transient excitatory effects, observed following the application of ATP might involve breakdown to adenosine and subsequent activation of adenosine receptors. To investigate this hypothesis, adenosine was exogenously applied at a range of doses (10μM, n=3; 25μM, n=4 50μM, n=5; 75μM, n=12; 100μM, n=3; 150μM, n=5; duration 30 mins), to assess its effects on locomotor activity recorded from isolated spinal cord preparations. Similar to ATP, application of adenosine (25-100μM) caused a significant reduction in locomotor burst frequency, while lower doses had no significant effect (Figure 1.2A, C & E). The maximum effect of adenosine on burst frequency was reached at a concentration of 75μM (32 ± 3.7%; n=12; Figure 1.2C & E). At all doses utilised, adenosine had no significant effect on burst amplitude (Figure 1.2A & D). Left-right alternation, assessed via the analysis of at least 100 locomotor bursts under each experimental condition, within 3 different preparations, also remained intact in the presence of adenosine (75μM; Figure 1.2B). The effects of adenosine on locomotor bursts followed a faster time course than those mediated by ATP. The effect of adenosine (75μM) plateaued approximately 7 minutes after drug application, while the maximum effect of ATP (100μM) occurred approximately 18 minutes after the drug was applied (Figure 1.2C versus Figure 1.1C).

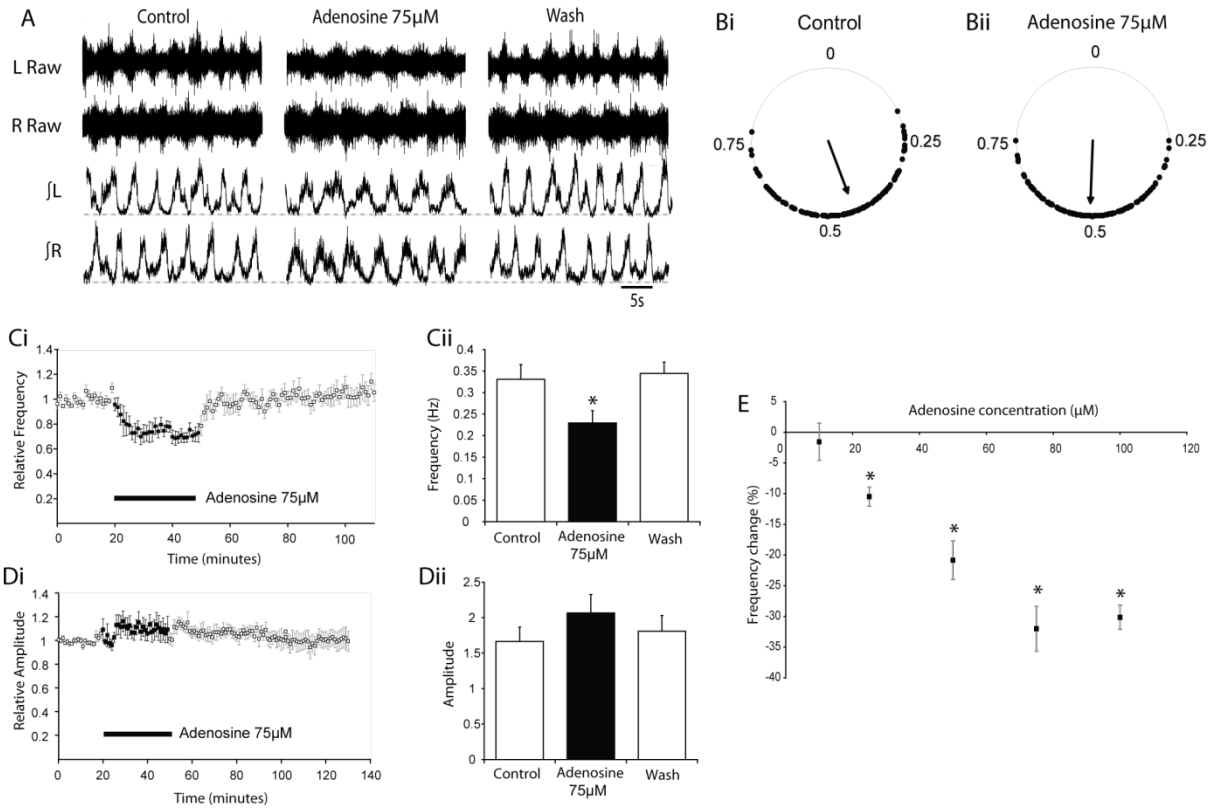

Figure 1.2: Adenosine reduces locomotor burst frequency but does not affect burst amplitude or left-right alternation. (A) Sample raw (top panel) and rectified/integrated (bottom panel) L2 ventral root recordings during control, 75µM adenosine application and washout. Traces represent 30 seconds of each condition. **(B)** Circular plots demonstrating that left-right alternation is unaffected by adenosine application. **(Ci)** Pooled time course plot showing changes in the normalised frequency of locomotor bursts following the application of adenosine (n=12). **(Cii)** Graph of the average burst frequency during 10 minute periods of control, 75µM adenosine application and washout (n=12). Adenosine caused a significant reduction in frequency ($p<0.01$). **(D)** Time course plot **(Di)** and bar chart **(Dii)** showing no significant change in burst amplitude following the application of adenosine. **(E)** Dose-response relationship for adenosine showing the dose-dependency of the reduction in locomotor-related burst frequency. * = significantly different to control.

These data demonstrate that activation of adenosine receptors modulates components of the spinal locomotor CPG to regulate locomotor frequency. Modulation of frequency appears to be the dominant effect of adenosine and although amplitude and rhythmicity were measured in all experiments in this chapter, no changes in either of these parameters were observed. For this reason, only frequency data is presented from here onwards. Given that applications of adenosine and ATP both led to a decrease in locomotor frequency and that the effects of ATP had a slower time course than those of adenosine, it seemed likely that ATP-mediated effects reflect the breakdown of ATP to adenosine and subsequent activation of adenosine receptors. To test this further, ATP (100μM) was applied in the presence of the general adenosine receptor antagonist theophylline (20μM). When adenosine receptors were blocked by theophylline, ATP had no significant effect on locomotor burst frequency (n=6; Figure 1.3A & B). In addition, when the ectonucleotidase inhibitor ARL67156 was applied to reduce ATP breakdown, ATP still had no effect on locomotor activity (n=6; Figure 1.3C & D). Thus, adenosine, rather than ATP, seems to be the primary purinergic modulator of the mammalian locomotor CPG.

To begin to elucidate the mechanisms by which adenosine receptor activation modulates the locomotor CPG, an investigation was made into whether adenosine affects excitatory or inhibitory components of spinal motor circuitry. Given that isolated spinal cord preparations can generate a motor rhythm in the absence of inhibition (Bracci et al., 1996, Taccola et al., 2004), an attempt was made to assess whether the modulatory actions of adenosine remain when inhibitory transmission is blocked. When NMDA (5μM), 5-HT (10μM) and dopamine (50μM) were applied in conjunction with blockers of inhibitory transmission (strychnine, 1μM and bicuculline, 10μM), we recorded slow, rhythmic bursts of synchronous activity from left and right lumbar ventral roots (Figure 1.4A). Application of adenosine (75μM; duration 30 mins; n=8) had no significant effect on the rhythmic activity recorded under these conditions (Figure 1.4A, B & C). In addition to blocking GABAergic transmission, bicucullline has also been shown to affect the AHP in spinal neurons (Khawaled et al., 1999). Thus, the lack of an adenosine effect in the presence of bicuculline may suggest that adenosine modulates the

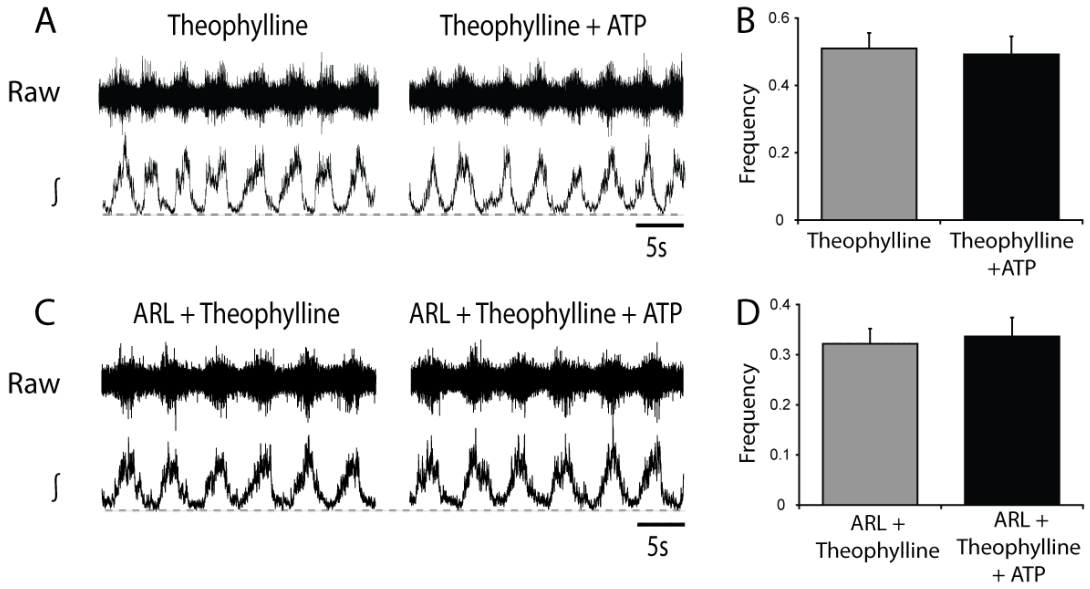

Figure 1.3: The effects of exogenously applied ATP reflect breakdown to adenosine and activation of P1 receptors. (A) Raw (top) and rectified/integrated (bottom) L2 ventral root recordings in the presence of the adenosine receptor antagonist theophylline (20µM) and during the subsequent application of ATP (100µM). **(B)** Averaged frequency values from the final 10 minutes in theophylline alone and ATP with theophylline showing no significant change in burst frequency (n=6). **(C)** Raw (top) and rectified/integrated (bottom) L2 ventral root recordings in the presence of theophylline and the ectonuclotidase inhibitor ARL67156 (50µM) and then upon addition of ATP. **(D)** Averaged data showing no change in frequency with ATP applications in the presence of both theophylline and ARL67156 (n=8). * = significantly different to control.

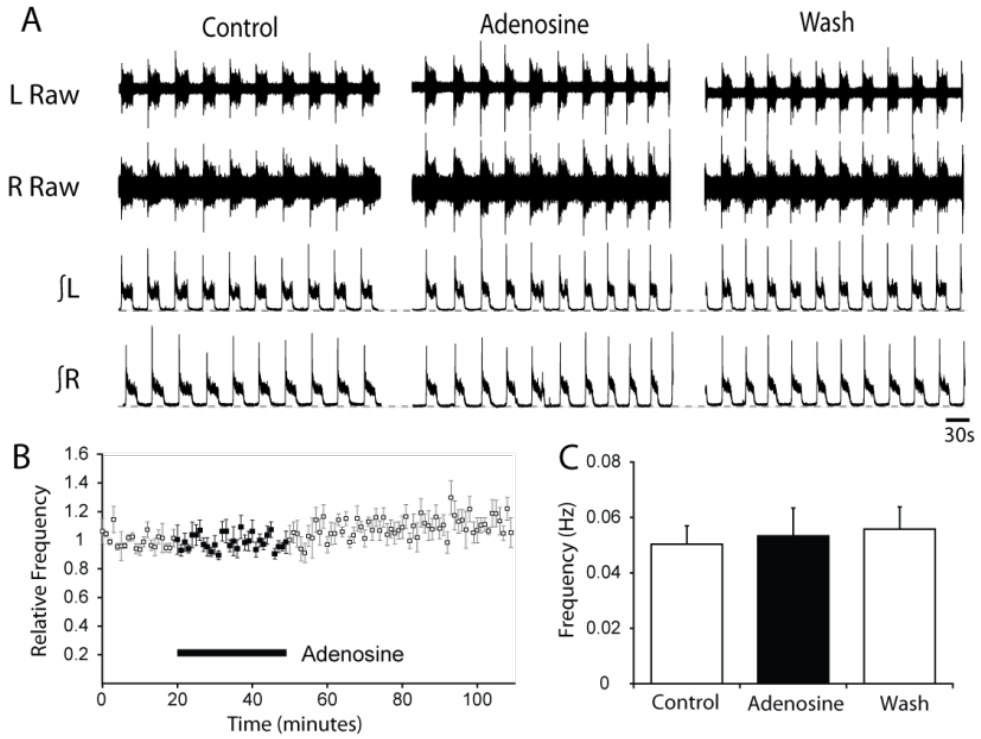

Figure 1.4: Adenosine modulates mammalian locomotion via effects on inhibitory transmission. **(A)** Sample raw (top) and rectified/integrated (bottom) traces showing the lack of effect of adenosine (75µM) on rhythmic activity recorded from ventral roots when the locomotion inducing drugs (NMDA, 5-HT and Dopamine) were applied in the presence of the $GABA_A$ receptor antagonist bicuculline (10µM) and the glycine receptor antagonist strychnine (1µM). **(B)** Pooled time course plot of burst frequency over the course of the adenosine application (n=8). **(C)** Graphical representation of average frequency, during the final 10 minutes from control, 75µM adenosine application and washout, again showing no change in the frequency of activity.

AHP rather than inhibitory transmission. To control for this possibility we substituted bicuculline with the $GABA_A$ receptor antagonist picrotoxin, which does not affect the AHP in spinal neurons (Pflieger et al., 2002). Slow, rhythmic bursts of synchronous activity from left and right lumbar ventral roots were again observed in the presence of strychnine (1μM), and picrotoxin (60μM). In these preparations adenosine still had no effect on locomotor frequency (n=4; data not shown). Together these data suggest that adenosine receptor activation affects the locomotor CPG by modulating inhibitory transmission or the activity of inhibitory interneurons.

Modulation of spinal locomotor networks by endogenously-derived adenosine

Having demonstrated that the activation of adenosine receptors by exogenous adenosine can modulate the mammalian locomotor CPG, it was next assessed whether endogenously released adenosine contributes to the control of the locomotor CPG. This was investigated by observing the effects of adenosine receptor antagonists on locomotor activity recorded from isolated spinal cord preparations. Firstly, the effects of the general adenosine receptor antagonist theophylline were examined. Application of theophylline (20μM; duration 30 mins) caused a significant increase in locomotor burst frequency (21 ± 4.5%; n=6) which persisted throughout the drug application and reversed following drug washout (Figure 1.5A & B). Theophylline had no significant effect on burst amplitude or left-right coordination (data not shown; n=6).

Next, the actions of antagonists specific for the two adenosine receptor subtypes most prevalent in the CNS, A_1 and A_{2A}, were investigated (Cunha, 2001). Bath application of the selective A_1 receptor antagonist DPCPX (50μM; duration 30 mins) caused a significant increase in locomotor burst frequency (22 ± 1.7%; n=6) which persisted throughout the drug application and reversed following drug washout (Figure 1.5C & D). The magnitude of the effects of DPCPX and theophylline were very similar, suggesting that adenosine-mediated modulation of locomotor activity primarily involves A_1 receptors. To test this further, theophylline (20μM) was applied in the presence of DPCPX (50μM). When A_1 receptors were blocked by DPCPX,

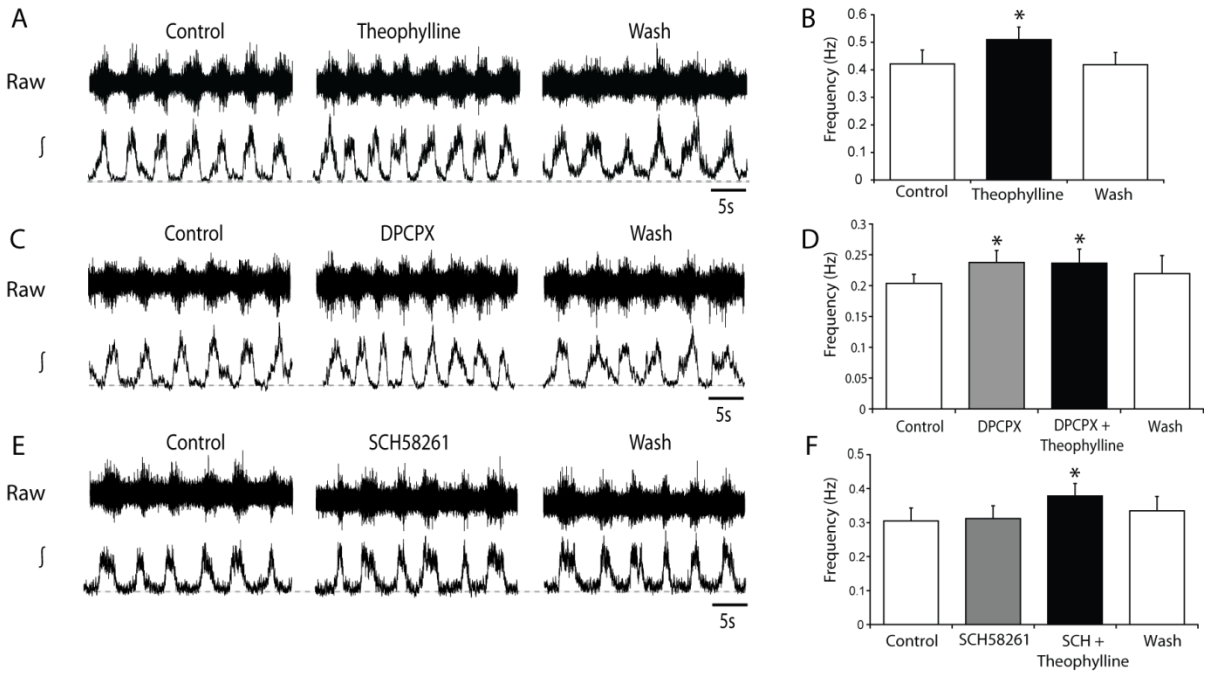

Figure 1.5: Endogenous adenosine modulates the locomotor CPG via activation of A_1 receptors. **(A)** Sample raw (top) and rectified/integrated (bottom) ventral root recordings in control, theophylline (20µM) application and wash out. **(B)** Averaged data of the final 10 minute periods of control, theophylline application and wash out, showing a significant increase in locomotor frequency in the presence of theophylline (n=6). **(C)** Traces showing the effects of the A_1 receptor antagonist DPCPX (50µM) on locomotor activity. **(D)** Average frequency data showing an increase in locomotor frequency in the presence of DPCPX and no further change with the subsequent addition of theophylline (20µM; n=6). **(E)** Traces showing that the A_{2A} receptor antagonists SCH58261 (25µM) has no effect on locomotor activity. **(F)** Average frequency data also shows no significant effect of SCH58261 on locomotor frequency, while the subsequent addition of theophylline (20µM) led to a significant increase in frequency (n=6). * = significantly different to control.

theophylline had no significant effect (n=6; Figure 1.5D), confirming that exogenous adenosine primarily acts via A_1 receptors. Furthermore, although theophylline has non-specific effects [for example on phosphodiesterases (Banner and Page, 1995) and internal calcium stores (Munakata and Akaike, 1994)], these data demonstrate that the actions of theophylline on locomotor frequency are specific to the blockade of adenosine receptors. Involvement of A_{2A} receptors was also investigated using the A_{2A} specific antagonist SCH58261. Application of SCH58261 (25μM, duration 30 mins) had no significant effect on locomotor burst frequency (n=6; Figure 1.5E & F). The subsequent addition of theophylline (20μM), in the presence of SCH58261, led to a significant increase in burst frequency (24 ± 3.7%; n=6; Figure 1.5F). Together these data reveal that release of adenosine from endogenous sources within the spinal cord regulates the frequency of on-going locomotor activity. Furthermore, the effects of endogenous adenosine appear to be primarily mediated by A_1 receptor activation.

Glia as a source of neuromodulatory adenosine

Next, the source of endogenous adenosine which modulates the spinal locomotor network was investigated. Extracellular adenosine is thought to be primarily derived from the enzymatic breakdown of extracellular ATP (Cunha, 2001) which can be released from either neurons or glia via exocytosis (Burnstock, 2007). Given the established link between purinergic signalling and glia within mammalian respiratory control networks (Gourine et al., 2010, Huxtable et al., 2010) it was hypothesised that ATP is released from glia and then converted to adenosine which in turn modulates components of the spinal locomotor network. To examine whether inhibition of ATP breakdown removes the endogenous modulatory actions of adenosine, the ectonucleotidase inhibitor ARL67156 (50μM) was bath applied. This caused a significant increase in locomotor frequency (20 ± 6.5%; n=9; Figure 1.6A). When theophylline (20μM) was subsequently applied in the presence of ARL67156, no further change in frequency was observed (n=9; Figure 1.6A). These data support that neuromodulatory adenosine is primarily derived from the breakdown of extracellular ATP.

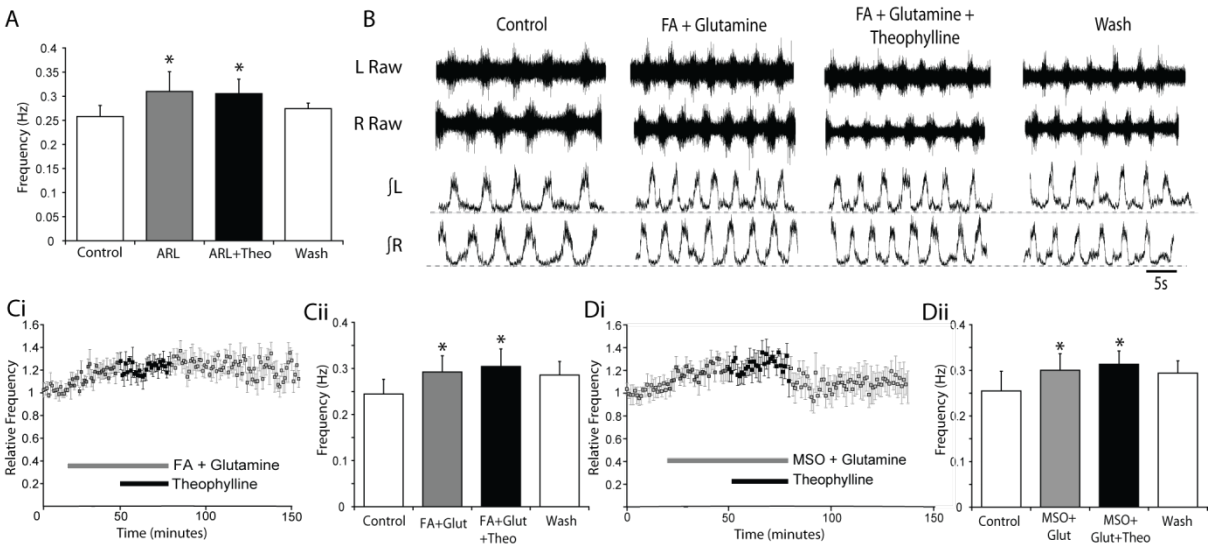

Figure 1.6: Glia-derived ATP is the source of endogenous modulatory adenosine. (A) Bar chart showing averaged frequency data from the final 10 minutes of control, ARL67156 (50µM) application, addition of theophylline (20µM) and washout. ARL67156 increases burst frequency while theophylline has no effect in the presence of ARL67156 indicating that breakdown of ATP is the primary source of adenosine. **(B)** Sample raw (top) and rectified/integrated (bottom) ventral root recordings in control, during co-application of the glial toxin FA (5mM) and glutamine (1.5mM), the addition of theophylline (20µM) and then wash out. **(Ci)** Pooled time course data showing the frequency of locomotor activity during control, the application of FA and glutamine, addition of theophylline and wash out (n=6). **(Cii)** Averaged data of the final 10 minute time periods from each condition (control; FA and glutamine; FA, glutamine and theophylline, washout). **(Di)** Pooled time course plot showing the frequency of locomotor activity during control, the application of MSO (100µM) and glutamine, addition of theophylline and wash out (n=6). **(Dii)** Averaged data of the final 10 minute time periods from each condition (control; MSO and glutamine; MSO, glutamine and theophylline, washout). Theophylline has no effect on the frequency of locomotor activity when applied in the presence of the glial toxins FA and MSO suggesting that ATP is released from glia then converted to adenosine to provide tonic P1-receptor mediated modulation of the locomotor CPG.

Next, the idea that glial cells represent the primary source of extracellular ATP which is converted to adenosine was investigated. Two compounds which disturb glial cell function were utilised; fluoroacetate (FA), a glial aconitase inhibitor (Fonnum et al., 1997), and methionine sulfoximine (MSO), a glial-specific glutamine synthetase inhibitor (Ronzio et al., 1969). Bath application of either FA (5mM; n=6) or MSO (100μM; n=6) caused a gradual decline in both the amplitude and frequency of drug induced locomotor activity (data not shown). Activity was disrupted after 30 minutes and eventually ceased after 120 minutes of FA or MSO application. Given that loss of glial function will perturb the glutamate-glutamine cycle, an attempt was made to recover locomotor activity using glutamine (1.5mM). When FA or MSO were applied together with glutamine, stable locomotor activity, indistinguishable in pattern from the activity recorded without the toxins, could be maintained for the duration of recordings (Figure 1.6B). Co-applications of FA or MSO with glutamine were associated with a significant increase in locomotor frequency (FA, 19 ± 3.5%, n=6; MSO, 18 ± 8.8%; n=6; Figure 1.6B, C & D) which is likely to reflect increases in extracellular glutamate but may also reflect the loss of gliotransmission. Once stable locomotor activity was obtained in the presence of FA or MSO and glutamine, theophylline (20μM) was bath applied to assess whether the effects of endogenous adenosine were blocked. Theophylline had no significant effect on locomotor activity in the presence of FA (Figure 1.6B & C; n=6) or MSO (Figure 1.6D; n=6), suggesting a lack of endogenous adenosine when glial function is perturbed. In a subset of experiments, it was verified that theophylline increased locomotor activity in control conditions prior to the application of glial toxins (n=2, data not shown). It was also assessed whether exogenously applied adenosine could modulate locomotor-related activity in the presence of glial toxins. When adenosine (75μM) was applied in the presence of MSO and glutamine, a rapid and significant reduction in locomotor frequency was observed (9 ± 1.2%; n=6; data not shown). Although this reduction was smaller than that observed in the absence of glial toxins, it may represent an underestimation due to the gradual increase in locomotor frequency observed in the presence of MSO and glutamine (as seen in Figure 1.6Di), which continued during the

application of adenosine. Together these data demonstrate that, most likely via the release of ATP, glia are involved in the production of endogenous adenosine which modulates spinal locomotor networks.

DISCUSSION

Purines are known to modulate CPGs controlling *Xenopus* tadpole swimming (Dale and Gilday, 1996) and mammalian respiration (Lorier et al., 2007). The data in this chapter provides the first report of purinergic modulation of the mammalian locomotor CPG. These data demonstrate that endogenous adenosine modulates the frequency of locomotor-related activity generated by spinal motor networks in mice. This neuromodulatory adenosine appears to derive from the breakdown of extracellular ATP which is itself released by glial cells.

Previous work in *Xenopus* tadpoles and rodents suggest that the purines ATP and adenosine work in concert, with opposing effects, to modulate rhythmic motor patterns (Dale and Gilday, 1996, Lorier et al., 2007). In tadpole swimming ATP excites CPG neurons, leading to an increase in the frequency of rhythmic motor activity while adenosine, derived from ATP-breakdown, then inhibits CPG neurons causing a secondary slowing in motor rhythm (Dale and Gilday, 1996). Initial studies of the respiratory control network in rats indicated that purines have similar, biphasic effects on respiratory frequency, with ATP-mediated excitation preceding adenosine-mediated inhibition (Lorier et al., 2007). However, more recent studies suggest that purinergic modulation of the respiratory rhythm is complicated by species-specific ectonucleotidase expression such that ATP-mediated excitation dominates in rats while adenosine-mediated inhibition dominates in mice (Huxtable et al., 2009, Zwicker et al., 2011). In the present study, no evidence was found of ATP-mediated modulation of spinal locomotor circuitry in mice, even when ATP breakdown was reduced using an ectonucleotidase inhibitor and potentially opposing effects of adenosine receptor activation were blocked using a general adenosine receptor antagonist. Adenosine therefore appears to be the primary purinergic modulator of the mouse locomotor CPG. Although these findings contrast those of tadpole

locomotion and rat respiration, adenosine-dominated modulation is consistent with studies of respiratory rhythm generation in mice and purinergic modulation in other regions of the CNS including the hippocampus (Zhang et al., 2003, Pascual et al., 2005), retina (Newman, 2003), and cortex (Fellin et al., 2009).

The application of adenosine to isolated mouse spinal cord preparations demonstrated significant modulatory effects of this purine on rhythmic, locomotor-related output. Activation of adenosine receptors was associated with a rapid and persistent reduction in the frequency of locomotor activity. These data indicate that adenosine acts on neurons within the locomotor CPG which are involved in controlling the frequency of rhythmic output from the network. In contrast, adenosine receptor activation had no effect on the amplitude of bursts of locomotor-related motoneuron output recorded from ventral roots. Thus, adenosine does not appear to directly modulate the activity of motoneurons which determine the intensity of motor output from the CNS. Data demonstrating modulatory effects of adenosine on interneurons involved in locomotor rhythm generation parallel findings in the mammalian respiratory system where activation of adenosine receptors within the rhythm generating Pre-Bötzinger complex of the medulla also leads to a slowing in the frequency of respiratory activity (Lorier et al., 2007). However, in contrast to the locomotor system, adenosine also modulates the output of respiratory motoneurons to affect the intensity of respiratory related-motor output and the activation of respiratory muscles (Funk et al., 1997, Miles et al., 2002).

It was also found that applications of the general adenosine receptor antagonist theophylline led to an increase in the frequency of locomotor activity. These data demonstrate that spinally-derived, endogenous adenosine modulates the locomotor CPG and highlight adenosine as an important intrinsic modulator of locomotor circuitry. By using adenosine receptor subtype-specific antagonists (DPCPX, A_1; SCH58261, A_{2A}), it was then established that the modulatory actions of endogenous adenosine predominantly result from activation of A_1-type adenosine receptors. Consistent with a primary role for A_1 receptors in adenosine-

mediated modulation, significant expression of A_1 receptors has been reported throughout the rodent spinal cord, including in the ventral horn (Deuchars et al., 2001).

In the present study, exogenously applied adenosine had no effect on the frequency of rhythmic activity recorded from isolated spinal cord preparations in which inhibitory transmission was blocked. Despite indirect evidence supporting the involvement of common motor circuitry in the generation of disinhibited bursting and fictive locomotor activity (Bracci et al., 1996, Kiehn, 2006), it remains unknown whether the same populations of neurons underlie these two rhythmic outputs. Nevertheless, data presented here suggest a link between the activity of inhibitory interneurons and/or the strength of inhibitory transmission within spinal motor circuitry and the modulatory effects of adenosine on locomotor output. Given that endogenous adenosine reduces locomotor frequency without affecting left-right coordination (Figure 1.2B), adenosine may alter locomotor frequency by modulating ipsilaterally projecting inhibitory interneurons. Interestingly, the ablation or inactivation of the V1 class of ipsilaterally projecting inhibitory interneurons (Alvarez et al., 2005) leads to a decrease in the frequency of locomotor activity (Gosgnach et al., 2006). Adenosine could therefore modulate locomotor activity by reducing the excitability of V1 interneurons or inhibiting their synaptic transmission. An alternative mechanism by which inhibition might be involved in adenosine-mediated modulation has been described in the hippocampus where GABA, released by inhibitory neurons, activates $GABA_B$ receptors on glial cells to simulate the release of ATP. Glial-derived ATP is then broken down to extracellular adenosine which inhibits synaptic transmission (Serrano et al., 2006). Similar mechanisms could contribute to adenosine-mediated modulation of spinal locomotor circuitry since glycine- and GABA-activated currents have been observed in spinal glial cells (Pastor et al., 1995). In order to provide more definitive information regarding the role of inhibitory transmission in adenosine-mediated modulation of spinal locomotor circuitry, and to identify the exact cellular mechanisms involved, future studies will need to focus on individual classes of inhibitory interneurons within the locomotor CPG. Such experiments will become more feasible with continued advances in the definition of distinct

subclasses of ventral horn interneurons using developmental genetics (Goulding, 2009, Kiehn et al., 2010).

The primary source of extracellular adenosine is thought to be the breakdown of ATP by ectonucleotidases. However, adenosine may also be released directly from neurons and glial cells via equilibrative nucleoside transporters or possibly by exocytosis (Wall and Dale, 2008, Hamilton and Attwell, 2010). The present study sought to determine the source of the endogenous adenosine which modulates spinal locomotor circuitry. In the presence of the ectonucleotidase inhibitor ARL67156, the general adenosine receptor antagonist theophylline had no effect on the frequency of locomotor activity. Thus, when ATP breakdown is reduced, endogenous adenosine-mediated modulation is lost, indicating that spinal neuromodulatory adenosine is derived from the breakdown of ATP.

ATP can be released by neurons, often as a co-transmitter (Burnstock, 1995) and from glial cells, specifically astrocytes (Hamilton and Attwell, 2010). Compounds which selectively interfere with glial function, commonly referred to as glial toxins, were used to probe involvement of glial cells in the purinergic modulation of spinal motor circuitry. The first of these was fluoroacetate (FA) which, via its toxic metabolite fluorocitrate, acts as a glial aconitase inhibitor leading to accumulation of citrate and a reduction in the formation of glutamine and, of particular relevance to the present study, ATP (Fonnum et al., 1997). The second was the glial toxin methionine sulfoximine (MSO), a glial glutamine synthetase enzyme inhibitor (Ronzio et al., 1969). Both of these glial toxins have been widely used to demonstrate the glial origin of purines and other signalling molecules and the role of glial cells in neuronal function (e.g. (Clarke, 1991, Fonnum et al., 1997, Zhang et al., 2003, Huxtable et al., 2010).

In keeping with studies of respiratory rhythm generating networks (Hülsmann et al., 2000, Huxtable et al., 2010) FA and MSO inhibited locomotor circuitry such that rhythmic activity eventually ceased in the presence of glial toxins. Locomotor activity could, however, be rescued by the co-application of glutamine, most likely because exogenous glutamine replaces the glial-derived glutamine that is normally taken up by neurons and converted to glutamate for

subsequent release (Hülsmann et al., 2000). In the presence of glial toxins (supplemented with glutamine) the adenosine receptor antagonist theophylline no longer affected locomotor activity. Thus, glial toxins prevent the endogenous modulatory influence of adenosine, supporting that neuromodulatory adenosine is derived from ATP which is released from glial cells.

It should be noted that FA and MSO have additional effects within the central nervous system which may not relate to their actions on glia. Such effects include increasing extracellular potassium levels (Largo et al., 1997), affecting calcium chelation (Clarke, 1991) and causing glycogen deposition in cranial motoneurons (Young et al., 2005). In addition, although FA and MSO are primarily taken up by glial cells, they can also cross neuronal membranes (Hassel et al., 1992). Studies in the rodent respiratory system have shown that FA depolarizes glia but not hypoglossal motoneurons (Hülsmann et al., 2000), and that modulation of the respiratory rhythm by substance P, which is thought to act neuronally, remains intact in the presence of FA and MSO (Huxtable et al., 2010). In the experiments presented here, it was found that adenosine could still modulate the locomotor-network in the presence of glial toxins. Together these data support the glial specificity of FA and MSO and suggest that the function of respiratory and locomotor networks is not substantially perturbed by the potential non-glial actions of these toxins.

The data presented in this chapter highlight purinergic gliotransmission as an important intrinsic modulatory system affecting spinal motor circuitry and add another level of complexity to the regulation of the mammalian locomotor CPG by supporting a role for non-neuronal cells. Purinergic gliotransmission, particularly that culminating in adenosine receptor activation, is emerging as an important mechanism for the control of neuronal networks throughout the brain, including those of the retina (Newman, 2003), hippocampus (Zhang et al., 2003, Pascual et al., 2005) and cortex (Fellin et al., 2009). At the behavioural level, purinergic gliotransmission has recently been shown to be important in regulating sleep homeostasis (Fellin et al., 2009, Halassa et al., 2009). However, the exact stimuli that induce the release of purines from glia, the

mechanisms of purine release, and the temporal relationship between purine release and neuronal activity, remain unclear. In the case of the spinal locomotor CPG, tonic neuromodulation by adenosine may act as a negative feedback mechanism, limiting and stabilising the frequency of locomotor-related output. Alternatively, given the demonstration that glial-derived adenosine contributes to activity-dependent heterosynaptic depression in hippocampal circuits (Pascual et al., 2005, Serrano et al., 2006), temporally dynamic control of adenosine levels within the spinal cord could help to reduce synaptic noise and ensure the synchronicity of neuronal components of the locomotor CPG. In order to fully understand the role of glial-derived adenosine in the control of spinal locomotor circuitry it will be important to determine the exact stimuli which lead to purine release from glia and to study the activity patterns of glia during locomotion. Given the neuroprotective potential of adenosine (Cunha, 2001) and the involvement of glial cells in neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (Di Giorgio et al., 2007, Nagai et al., 2007), a greater understanding of glial and adenosine-mediated modulation is likely to benefit both our basic understanding of motor control and ultimately the treatment of diseases which affect motor systems.

CHAPTER 2

PURINERGIC MODULATION OF SPINAL INTERNEURONS AND MOTONEURONS

This chapter is adapted from previously published work (Witts et al., 2015).

The 100nM DPCPX, SCH58261 and half of the TTX experiments were performed by Filipe Nascimento.

INTRODUCTION

Once the modulatory role of purines on the whole spinal locomotor network had been established, focus was shifted to understanding the cellular mechanisms underlying the effects of adenosine. To study these mechanisms, it was necessary to record from individual cells within the ventral horn of the spinal cord. This chapter therefore describes whole-cell patch-clamp recordings from motoneurons and ventral horn interneurons in the spinal cord of mice, using pharmacological manipulations to investigate the role of adenosine and its mechanism of action.

The predominantly excitatory effects of ATP are mediated via P2 receptors throughout the CNS while the generally inhibitory actions of adenosine result from binding to a family of P1 receptors (Burnstock et al., 2011). Although ATP dominates purinergic signalling in many areas of the nervous system, there are also examples where adenosine acts as an important neuromodulator. For example, adenosine, rather than ATP, appears to dominate purinergic modulation in the hippocampus (Zhang et al., 2003, Pascual et al., 2005), retina (Newman, 2003), calyx of Held (Wong et al., 2006) and cortex (Fellin et al., 2009). At the behavioral level, adenosine is known to play a role in the control of sleep (Porkka-Heiskanen et al., 1997, Thakkar et al., 2003, Basheer et al., 2004, Bjorness et al., 2009, Halassa et al., 2009, Nadjar et al., 2013), in relaying information regarding stress and pain (Bagley et al., 1999) and in the regulation of motor control systems (Dale and Gilday, 1996, Brown and Dale, 2000, Huxtable et al., 2009). Adenosine can be directly released from astrocytes, but the primary source of extracellular adenosine is thought to be ATP release from glia before break down to adenosine via ectonucleotidases (Hamilton and Attwell, 2010).

Adenosine-mediated modulation has been well-studied in the brainstem circuitry controlling mammalian respiration. Within these motor control circuits, glial-derived adenosine produces tonic depressive effects in rodents, which are strongest at fetal stages (Kawai et al., 1995, Schmidt et al., 1995, Herlenius and Lagercrantz, 1999, Mironov et al., 1999, Lorier et al., 2007, Huxtable et al., 2010). In addition to modulating respiratory frequency, presumably via

actions on rhythm generating neurons of the medullary pre-Bötzinger complex, adenosine also modulates the intensity of respiratory-related output generated by motoneurons (Funk et al., 1997, Miles et al., 2002). Several cellular mechanisms are likely to underlie the depressive effects of adenosine on respiratory output including suppression of excitatory glutamatergic inputs to motoneurons (Bellingham and Berger, 1994) and modulation of L-type Ca^{2+} channels and ATP-sensitive K^{+} channels (Mironov et al., 1999).

Within the spinal cord, adenosine is also important for the modulation of locomotor control networks. Of the four types of adenosine receptors, A1 and A2 receptors are most common in the spinal cord and are located diffusely throughout the ventral horn (Geiger et al., 1984, Choca et al., 1987, Deuchars et al., 2001; Brooke et al., 2004; Burnstock et al., 2011). Behavioural effects of adenosine have been observed during swimming in frog tadpoles, where ATP first facilitates swimming by reducing voltage-activated K^{+} currents and increasing the excitability of neurons within the locomotor CPG. Ectonucleotidases then facilitate the breakdown of ATP to adenosine, which activates A_1-type receptors, reducing voltage-activated Ca^{2+} currents and CPG excitability, resulting in the cessation of locomotor activity (Dale and Gilday, 1996, Brown and Dale, 2000, Brown and Dale, 2002).

In the previous chapter, it was demonstrated that adenosine also modulates the frequency of motor output generated by the locomotor CPG of mice (Witts et al., 2012). This demonstrates that glial-derived adenosine reduces, or perhaps in a physiological context, limits the frequency of locomotor-related output during on-going network activity. However, the cellular mechanisms which underlie this spinal adenosine-mediated modulatory system remain unknown. This chapter, therefore, utilises whole-cell patch-clamp recordings of spinal motoneurons and ventral horn interneurons to investigate the consequences of adenosine receptor activation on the cellular components of spinal motor circuitry. Data presented in this chapter show that adenosine reduces the probability of transmitter release from pre-synaptic terminals and also hyperpolarises interneurons. In contrast, adenosine depolarises motoneurons and has no effect on the probability of transmitter release from last order

premotor interneurons. Thus, it appears that a general inhibitory effect of adenosine on higher order ventral horn interneurons leads to a reduced frequency of locomotor network output, while the simultaneous depolarisation of motoneurons may help to ensure the maintenance of motor output and therefore an appropriate intensity of muscle activation.

METHODS

In vitro spinal cord slice preparation

All methods required to obtain tissue for *in vitro* experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 under personal licence 60/12934. Spinal cord preparations were obtained from postnatal day (P)1-9 C57BL/6 mice using techniques similar to those described previously (Jiang et al., 1999). Animals were killed via cervical dislocation, decapitated and the front paws were used to pin the preparation ventral side up in a petri dish containing Sylgard resin. Ventral skin was removed and mice were eviscerated, taking care not to damage the spinal column. The preparation was then transferred to a dissecting chamber surrounded by ice and containing artificial cerebrospinal fluid (aCSF; equilibriated with 95% oxygen, 5% carbon dioxide, ~4°C). The mouse was pinned into Sylgard resin at the bottom of this chamber, ventral side up and using all four paws. Vertebrae were removed and then the spinal cord was isolated by cutting through both dorsal and ventral roots. Once isolated, the spinal cord was pinned at both ends into the Sylgard resin and dura mater, dorsal roots and ventral roots were removed. A 1% agar solution was heated to the point of becoming a clear liquid in the microwave and then left to cool to 37°C. The tissue was then placed in a petri dish containing the agar and left to harden. Once hard, a block of agar containing the spinal cord was cut from the dish. The agar blocked was mounted with the longitudinal axis perpendicular to the vibrating microtome blade (Leica VT1200) and this was used to obtain 300µm transverse slices of the lumbar spinal cord. Slices were then transferred to a beaker containing recovery solution (equilibrated with 95% O_2, 5% CO_2; ~34°C) for 30

mins-1 hour, before being secured in a recording chamber containing recording aCSF (equilibrated with 95% O_2, 5% CO_2; room temperature).

Whole-cell patch-clamp recordings

Whole-cell patch-clamp recordings were obtained from motoneurons and ventral horn interneurons visualized under infrared differential interference contrast microscopy. Recordings were taken from a heterogeneous population of interneurons throughout the ventral horn (average whole-cell capacitance 32.9±1.7 pF; average input resistance 366.8±34.3 MΩ; n=119). Interneurons were not readily classifiable into distinct subpopulations based on location, passive properties or their responses to adenosine. Patch-clamp electrodes (3–5 MΩ) were pulled on a horizontal puller (Sutter Instrument, Novato, CA) from borosilicate glass (World Precision Instruments, Sarasota, FL). Signals were amplified and filtered (4-kHz low-pass Bessel filter) with a MultiClamp 700B amplifier (Molecular Devices) and acquired at ≥10 kHz using a Digidata 1440A A/D board and pClamp software (Molecular Devices). Details of voltage and current-clamp protocols appear in Results. The liquid junction potential, which was calculated as 14.2 mV in the solutions used, was not corrected for (Clampex JPCalcW).

Data analysis

Whole cell patch-clamp recordings were analyzed using either Clampfit software (Molecular Devices) or, for analyses of synaptic events and miniature post-synaptic currents (mPSCs), the Mini Analysis Program (Synaptosoft, Fort Lee, NJ). One minute of recording from control, drug, and wash conditions was used for analysis of synaptic events and 5 minutes of recording from control, drug and wash was used for analysis of mPSCs. The threshold for detection of events was set at three times the noise level for synaptic events and two times the noise level for mPSCs. Data are reported as means ± standard error. Differences in means between control and drug were compared using Student's ***t***-test. ANOVAs were used to compare means where there was more than one drug condition. The Kolmogorov-Smirnov test was used

to test for differences in mPSC amplitude or inter-event interval. Values of $P < 0.05$ were considered significant.

Solution and drugs

The aCSF solution used for dissecting contained 25 mM NaCl, 188 mM sucrose, 1.9 mM KCl, 1.2 mM NaH_2PO_4, 10 mM $MgSO_4$, 1 mM $CaCl_2$, 26 mM $NaHCO_3$, 25 mM D-glucose and 1.5 mM kynurenic acid (equilibrated with 95% O_2-5% CO_2). The aCSF solution used for recovery contained 119 mM NaCl, 1.9 mM KCl, 1.2 mM NaH_2PO_4, 10 mM $MgSO_4$, 1 mM $CaCl_2$, 26 mM $NaHCO_3$, and 20 mM D-glucose, 1.5 mM kynurenic acid and 3% dextran added on the day of use (equilibrated with 95% O_2-5% CO_2). The aCSF solution used for recording contained 127 mM NaCl, 3 mM KCl, 1.3 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose (equilibrated with 95% O_2-5% CO_2). The standard patch-clamp pipette solution contained 140 mM potassium methane sulfonate, 10 mM NaCl, 1 mM $CaCl_2$, 10 mM HEPES, 1 mM EGTA, 3 mM Mg-ATP, and 0.4 mM GTP-Na_2 (pH 7.2–7.3, adjusted with KOH). Adenosine, strychnine, picrotoxin, SCH58261 and and 8-cyclopentyl-1,3-dipopylxanthine (DPCPX) were purchased from Sigma-Aldrich (St Louis, MO); tetrodotoxin (TTX) was purchased from Tocris Bioscience (Bristol, UK). All drugs were made up fresh using aCSF, apart from strychnine and tetrodotoxin, which were stored as frozen aliquots prior to their use, and DPCPX which was made up fresh using DMSO. Unless otherwise stated, concentrations of drugs used were as follows: adenosine, 75 μM; DPCPX, 50 μM; SCH58261, 50 μM TTX, 0.5 μM; strychnine, 1 μM; picrotoxin, 60 μM.

RESULTS

Adenosine hyperpolarises spinal interneurons

In order to investigate the potential modulatory effects of adenosine receptor activation on the properties of individual spinal interneurons, adenosine (75 μM; Witts et al, 2012) was

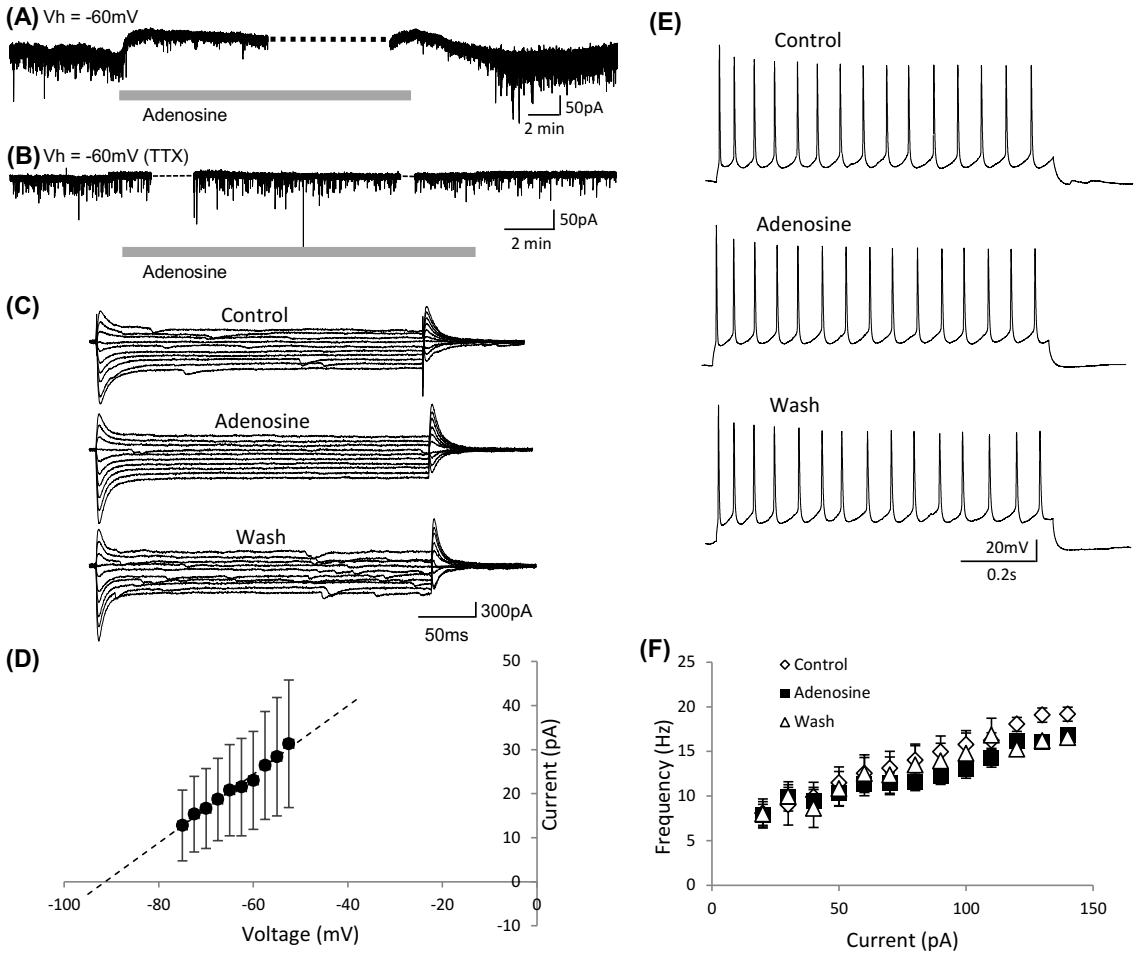

Figure 2.1: Adenosine hyperpolarises interneurons by opening leak potassium channels (A) Voltage-clamp recording from a ventral horn interneuron during a 15 minute bath application of 75 µM adenosine (Vh = -60 mV). Adenosine induced an outward current in ventral horn interneurons held at -60mV. **(B)** Voltage-clamp recording from a ventral horn interneuron in the presence of TTX during bath application of 75 µM adenosine (Vh = -60 mV). Adenosine failed to induce any current in ventral horn interneurons held at -60mV. **(C)** Example trace of 2.5 mV steps between -75 and -52.5 mV in control, adenosine and wash. Adenosine caused a reversible reduction in input resistance. **(D)** Averaged data (n=22) showing the *I-V* relationship of the adenosine-induced current revealed by subtracting control I-V relationships from those in the presence of adenosine. **(E)** Current-clamp recording showing repetitive action potential firing in a ventral horn interneuron in response to the injection of a square current pulse (110 pA) in control, adenosine and wash. **(F)** Averaged data (n=8) showing adenosine had no effect on frequency-current relationships in control, adenosine and wash (10 pA current steps from 20-140 pA).

bath applied to spinal cord slice preparations while performing whole-cell patch-clamp recordings of ventral horn interneurons. Despite being a heterogeneous population of interneurons, responses to adenosine were similar across interneuron recordings. It was first investigated whether adenosine receptor activation has any sub-threshold effects on interneurons that might, for example, modulate their resting membrane potential. Voltage-clamp recordings of ventral horn interneurons held at -60mV, revealed that bath application of adenosine induced an outward current (43.2±7.7 pA; n=20 cells; Figure 2.1A). This outward current was accompanied by a reduction in input resistance (control, 469.3±56.2 MΩ; adenosine, 118.2±72.0 MΩ), as calculated from current-voltage (I-V) relationships generated using a range of sub-threshold voltage steps (2.5 mV steps from -75 to -52.5 mV; Figure 2.1C). Current-voltage relationships in control conditions were subtracted from those obtained in the presence of adenosine in order to isolate the adenosine-induced current (Figure 2.1D). The I-V relationship of the adenosine-induced current revealed a reversal potential (-91 mV; Figure 2.1D), near the equilibrium potential for K^+ as calculated for our solutions using the Nernst equation (-98 mV). Thus, these data support that adenosine receptor activation leads to membrane hyperpolarisation of ventral horn interneurons due to the opening of leak potassium channels. Next, adenosine was applied during the blockade of synaptic transmission to determine whether this outward current reflected direct, postsynaptic actions of adenosine on interneurons. Interestingly, the adenosine-induced current was blocked by TTX (0.5μM; n=6 cells; Figure 2.1B).

Current-clamp mode was then utilised to investigate the effects of adenosine receptor activation on interneuron action potential firing. A series of square current pulses (20-140 pA; 10 pA increments; 1 s duration) were injected to induce firing in control conditions and during the bath application of adenosine (Figure 2.1E). Adenosine had no significant effect on average rheobase current (control, 41.3±2.5 pA; adenosine, 41.3±2.5 pA, n=8 interneurons) or the slope of frequency-current relationships of ventral horn interneurons (control, 0.095HzpA^{-1}; adenosine, 0.070HzpA^{-1}; Figure 2.1F, B; n=8 interneurons). Therefore, although adenosine

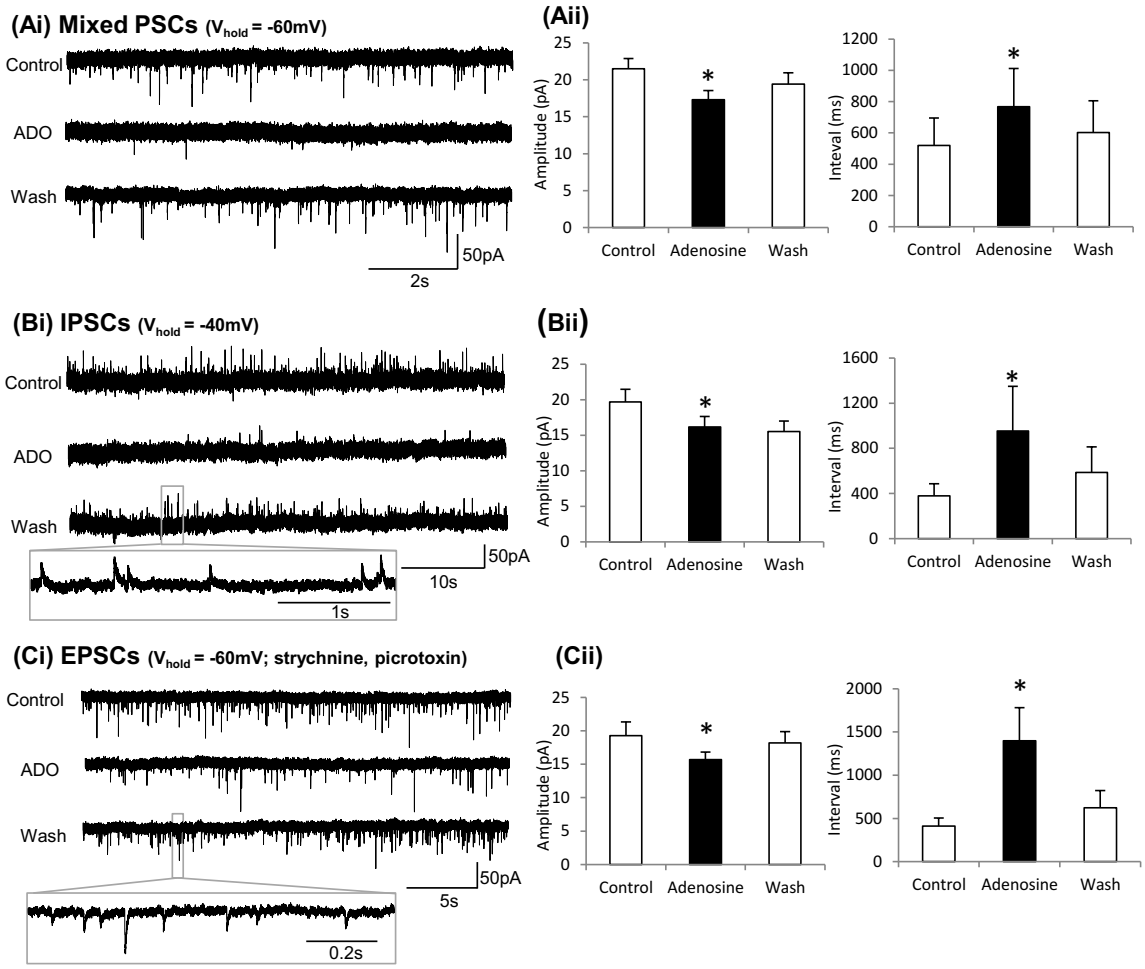

Figure 2.2: Adenosine reduces the frequency and amplitude of both excitatory and inhibitory inputs to interneurons (Ai) Voltage-clamp recordings from an interneuron held at -60 mV revealing spontaneous synaptic inputs in control, adenosine (ADO) and wash. **(Aii)** Averaged data showing that application of adenosine caused a reduction in the amplitude of synaptic events and an increase in the interval between events. Note: these synaptic events represent a mixture of excitatory and inhibitory post synaptic currents (both are depolarising at this potential in our recording solutions). **(Bi)** Voltage-clamp recordings taken from ventral horn interneurons held at -40 mV, a potential at which inhibitory post-synaptic currents (IPSCs) are hyperpolarising and therefore distinguishable from excitatory post-synaptic currents (EPSCs). **(Bii)** Averaged data showing that application of adenosine led to an irreversible reduction in IPSC amplitude and a reversible increase in interval between IPSCs. **(Ci)** Voltage-clamp recordings of EPSCs taken at -60 mV while inhibitory transmission was blocked using 1 µM strychnine (glycine receptor antagonist) and 60 µM picrotoxin (GABA receptor antagonist). **(Cii)** Averaged data showing that application of adenosine led to a reversible reduction in EPSC amplitude and reversible increase in interval between EPSCs.

receptor activation hyperpolarises ventral horn interneurons by mechanisms dependent on synaptic transmission, it does not lead to obvious changes in their input-output relationships as determined by stimulation using square current pulses.

Adenosine supresses synaptic inputs to interneurons

Given evidence of widespread involvement of adenosine receptor activation in the modulation of synaptic activity (Burnstock et al., 2011), it was next investigated whether adenosine affects synaptic inputs to ventral horn interneurons. Voltage-clamp recordings from interneurons held at -60mV revealed an adenosine-induced decrease in the amplitude of synaptic events (control 21.5±1.4pA, adenosine 17.3±1.2pA; n=20 cells) along with an increase in inter-event interval (control 519.1±176.0ms, adenosine 767.9±244.2ms; n=20 cells; Figure 2.2Ai and Aii).

Although these data clearly demonstrate modulation of synaptic activity, they do not allow separation of the effects on inhibitory and excitatory inputs. This is because the reversal potential of Cl^- in the recording solutions is such that both types of inputs are likely to be depolarising at a holding potential of -60mV. To dissect out the effects of adenosine receptor activation on excitatory and inhibitory post-synaptic currents, inhibitory post-synaptic currents (IPSCs) were isolated by using a holding potential of -40mV, at which IPSCs are hyperpolarising and therefore distinguishable from excitatory post-synaptic currents (EPSCs). It was found that adenosine caused a reduction in the amplitude of IPSCs (control 19.7±1.8pA, adenosine 16.2pA±1.5pA; n=10 cells), and an increase in the interval between IPSCs (control 379.3±107.2ms, adenosine 952.5±397.1ms; n=10 cells; Figure 2.2Bi, Bii).

EPSCs were then isolated by performing voltage-clamp recordings at -60mV while blocking inhibitory transmission using 1μM strychnine (glycine receptor antagonist) and 60μM picrotoxin (GABA receptor antagonist). Under these conditions application of adenosine caused a reduction in EPSC amplitude (control 19.3±2.1, adenosine 15.7±1.1pA; n=10 cells), and increased the interval between excitatory inputs (control 412.0±92.1ms, adenosine

1398.8±381.7ms; n=10 cells; Figure 2.2Ci, Cii). In addition, inward currents were still observed when adenosine was applied in the absence of inhibitory transmission (30.8±1.3pA; n=10 cells; data not shown). It is clear that adenosine reduces the frequency and amplitude of both inhibitory and excitatory synaptic events.

Taken together, these data therefore demonstrate that adenosine receptor activation has a general suppressive effect on synaptic input to ventral horn interneurons.

Reductions in synaptic activity could reflect diminished output from pre-synaptic neurons due to adenosine-induced hyperpolarisation or direct modulation of synaptic transmission via pre and/or post-synaptic mechanisms. To distinguish between these possibilities, the next experiments investigated the effects of adenosine application on action potential-independent miniature post-synaptic currents (mPSCs) recorded from ventral horn interneurons in the presence of TTX (0.5 μM). Cumulative frequency plots and average values measured in each condition were used to assess the effects of adenosine on mPSC amplitude and frequency (Figure 2.3). Adenosine was found to have no effect on mPSC amplitude (Figure 2.3B, C; control, 13.3±1.7 pA; adenosine, 13.6±2.2 pA; n=8 cells). In comparison, adenosine application significantly increased the interval between mPSCs (Figure 2.3D, E; control, 3278.1±1591.2 ms; adenosine, 4711.3±1824.1 ms; n=8 cells). These data therefore support that the primary effect of adenosine on synaptic transmission between spinal interneurons involves activation of pre-synaptic receptors which in turn reduce the probability of transmitter release.

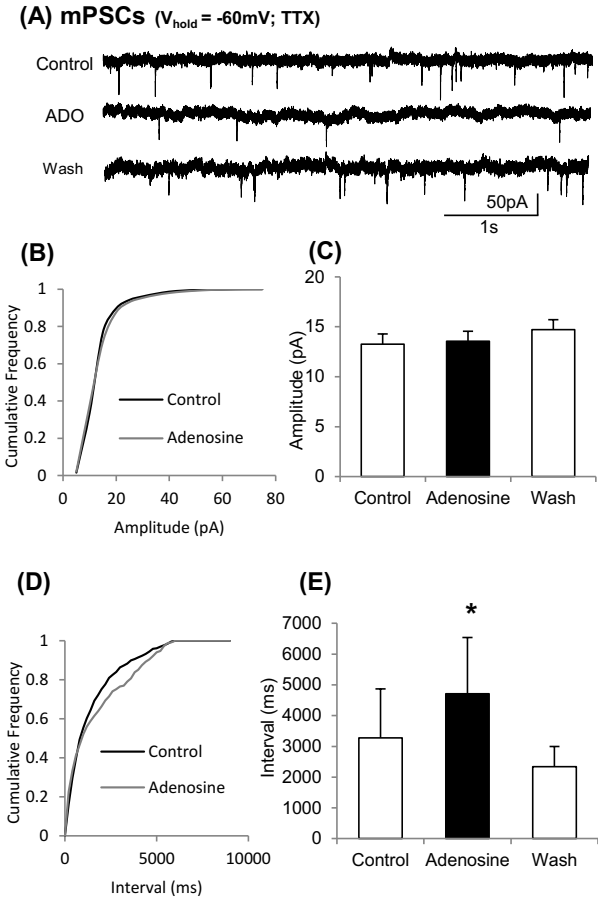

Figure 2.3: Adenosine (ADO) reduces the frequency of miniature post synaptic currents recorded from interneurons (A) Voltage-clamp recordings from an interneuron held at -60 mV in the presence of TTX, where sodium currents are blocked and so action potential-independent, miniature post-synaptic currents are isolated. **(B)** Averaged cumulative probability plot showing no change in amplitude of miniature post synaptic potentials (n=8 cells). **(C)** Averaged data showing that application of adenosine has no effect on the amplitude of miniature post-synaptic currents. **(D)** Averaged cumulative probability plot showing an increase in the interval between events in the presence of adenosine. **(E)** Averaged data showing that application of adenosine causes an increase in the interval between miniature post-synaptic currents. These data support involvement of pre-synaptic adenosine receptors in adenosine-mediated modulation of synaptic transmission.

Adenosine-mediated modulation of interneurons involves A_1 receptors

It was next investigated which receptor subtypes are involved in the modulatory effects of adenosine on ventral horn interneurons. Given widespread expression of A_1-type receptors in the rodent spinal cord (Deuchars et al., 2001) and previous findings demonstrating A_1 receptor-mediated modulation of locomotor network output (Witts et al., 2012), it was first tested whether the A_1 receptor antagonist DPCPX could block adenosine-mediated modulation. In the presence of DPCPX (100nM, n=11 or 50μM, n=8 interneurons), adenosine failed to induce an outward current and had no effect on mixed IPSCs and EPSCs recorded from interneurons held at -60 mV (Figure 2.4). DPCPX alone had no effect on synaptic inputs nor did it induce any sub-threshold currents indicating a lack of tonic activation of A_1 receptors in our slice preparations. It was next investigated whether adenosine acted exclusively via A1 receptors by applying adenosine in the presence of the adenosine A2A receptor antagonist SCH58261 (50μM). In the presence of SCH58261, adenosine induced an inward current (25.9±1.4 pA; n=5 cells). Adenosine also reduced the amplitude of PSCs (control, 10.1±2.0 pA; adenosine, 8.9±1.9 pA; n=5 cells) and increased the average interval between PSCs (control, 6552.1±1571.9 ms; adenosine, 8940.2±1700.5 ms; n=5 cells; data not shown). It therefore appears that the effects of adenosine in interneurons are predominantly mediated by A1 receptors.

Adenosine depolarises motoneurons

Having observed the effects of adenosine on interneurons, it was next assessed whether adenosine receptor activation also modulates the properties of motoneurons or the synaptic

inputs they receive. Whole-cell patch-clamp recordings of motoneurons were first utilised to investigate whether adenosine receptor activation induces sub-threshold responses in motoneurons. In contrast to ventral horn interneurons, when motoneurons were held at -60mV in voltage-clamp mode adenosine induced an inward current (47.6±8.7 pA; n=10; Figure 2.5A). This inward current was accompanied by an increase in input resistance (control, 90.6±33.5 MΩ; adenosine, 110.5±27.2 MΩ), as revealed by analysis of I-V relationships

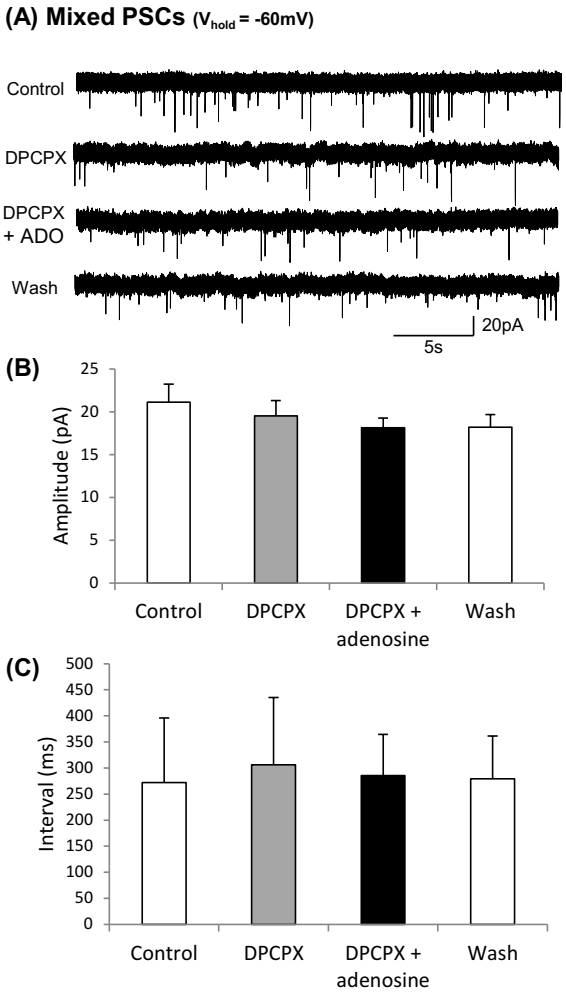

Figure 2.4: An A_1 receptor antagonist blocks the effect of adenosine on synaptic inputs to interneurons **(A)** Voltage-clamp recordings from an interneuron held at -60mV revealing spontaneous synaptic inputs in control, with the A_1 adenosine receptor antagonist DPCPX, in DPCPX and adenosine (ADO), and wash. **(B)** Averaged data showing that there was no change in the amplitude of synaptic inputs in the presence of DPCPX, and DPCPX blocked the previously observed effects of adenosine (n=10). **(C)** Averaged data showing that there was no change in the interval between synaptic inputs in the presence of DPCPX, and DPCPX blocked the previously observed effects of adenosine (n=10).

generated from sub-threshold voltage steps (2.5 mV increments, ranging from -75 to -52.5 mV; Figure 2.5B, C). I-V relationships obtained in control conditions were subtracted from those obtained during adenosine application in order to isolate the adenosine-induced current.The I-V relationship of the adenosine-induced current had a reversal potential of -71 mV (Figure 2.5C). Adenosine was also applied in the presence of TTX (0.5μM) and it was found that adenosine no longer induced an inward current (data not shown). Given that this current appeared to be dependent on synaptic transmission and had a reversal potential near that calculated for chloride in our solutions (-62mV), we hypothesised that it may reflect the blockade of a tonic inhibitory input to motoneurons. However, adenosine applied in the presence of strychnine and picrotoxin still induced an inward current in motoneurons (42.4±15.9 pA; n=5; data not shown). Next, motoneuron recordings were performed in current-clamp mode to investigate the effects of adenosine receptor activation on motoneuron input-output relationships. Square current pulses (ranging from 10-1210 pA; 50 pA increments; 1s duration) were injected to induce repetitive firing in control conditions and during the bath application of adenosine (Figure 2.5D). Adenosine had no significant effect on rheobase current (control, 353.8±34.5 pA; adenosine, 353.8±36.3 pA, n=8 motoneurons) or the slope of frequency-current relationships of ventral horn interneurons (control, 0.013 $HzpA^{-1}$; adenosine 0.012 $HzpA^{-1}$; Figure 2.5D, E; n=8 motoneurons). Therefore, although adenosine receptor activation depolarises motoneurons, it does not lead to obvious changes in their input-output relationships as determined by stimulation using square current pulses.

Adenosine modulates synaptic input but not mPSCs recorded from motoneurons

Given the effects of adenosine receptor activation on synaptic activity recorded from interneurons it was also assessed whether adenosine modulates synaptic inputs received by motoneurons. Voltage-clamp recordings of motoneurons held at -60mV once again demonstrated suppressive effects of adenosine on synaptic input with a reduction in amplitude (control 16.6±1.9pA, adenosine 13.1±1.0pA; n=10; Figure 2.6Ai,ii) and increase in inter-event

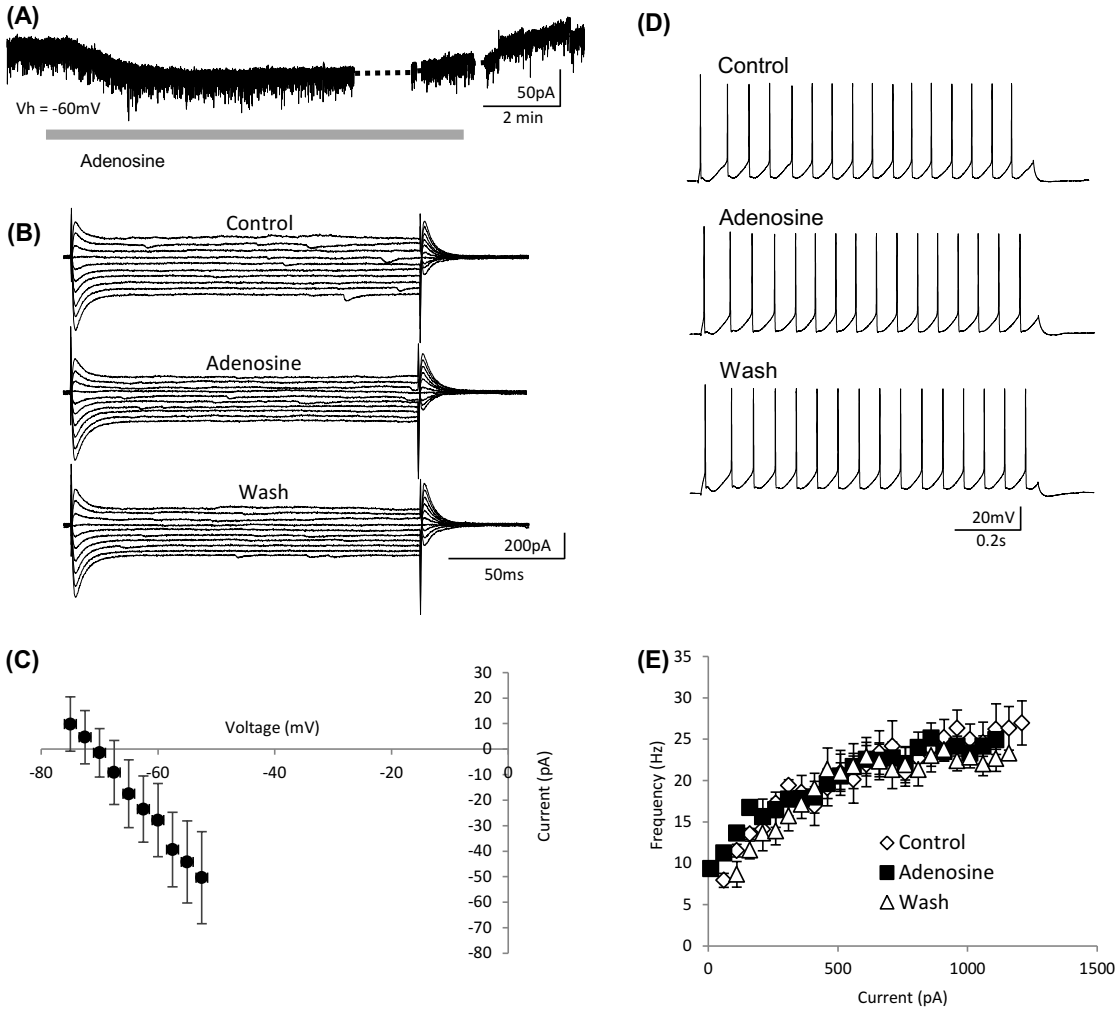

Figure 2.5: Adenosine depolarises spinal motoneurons (A) Voltage-clamp recording from a spinal motoneuron during bath application of 75 µM adenosine (Vh = -60mV). Adenosine induced an inward current in motoneurons held at -60mV. **(B)** Example trace of 2.5 mV steps between -75 and -52.5 mV in control, adenosine and wash. Adenosine caused an increase in input resistance. **(C)** Averaged data (n=11) showing the *I-V* relationship of the adenosine-induced current revealed by subtracting control I-V relationships from those in the presence of adenosine. **(D)** Current-clamp recording showing repetitive action potential firing in a ventral horn interneuron in response to the injection of a square current pulse in control, adenosine and wash. **(E)** Averaged data (n=8) of control, adenosine and wash showing adenosine had no effect on frequency-current relationships.

interval of synaptic inputs received by motoneurons (control 222.6±47.9ms, adenosine 516.6±151.5ms; n=10; Figure 2.6Ai,iii). However, in contrast to analyses of interneurons,recordings of mPSCs from motoneurons held at -60mV in the presence of TTX revealed that adenosine had no effect on the amplitude (Figure 2.6B, C, & D) or inter-event interval of mPSCs (Figure 2.6B, E & F; n=8). These data suggest that adenosine-mediated reductions in synaptic activity recorded from motoneurons most likely involves reductions in the excitability and synaptic activity in premotor networks rather than direct modulation of last-order synapses on motoneurons.

Adenosine-mediated modulation of motoneurons involves A_1 receptors

Finally, it was assessed whether adenosine mediated modulation of motoneurons and the inputs they receive reflects activation of A_1 receptors. In the presence of the A_1 receptor antagonist DPCPX (100nM, n=7 or 50μM, n=8 motoneurons), adenosine no longer induced an inward current in motoneurons and synaptic activity was unchanged (Figure 2.7). DPCPX again had no effect on its own, indicating no endogenous activation of A_1 receptors in these slice preparations. It was next investigated whether adenosine acted exclusively via A1 receptors by applying adenosine in the presence of the adenosine A2A receptor antagonist SCH58261 (50μM). In the presence of SCH58261, adenosine still induced an inward current (22.7±3.9 pA; n=5 cells), reduced the amplitude of PSCs (control, 11.5±1.1 pA; adenosine, 8.9±0.9 pA; n=7 cells) and increased the average interval between PSCs (control, 3412.7±414.8 ms; adenosine, 9540.1±1048.8 ms; n=7 cells). These data suggest that the effects of adenosine observed in motoneurons are predominantly mediated by A1 receptors.

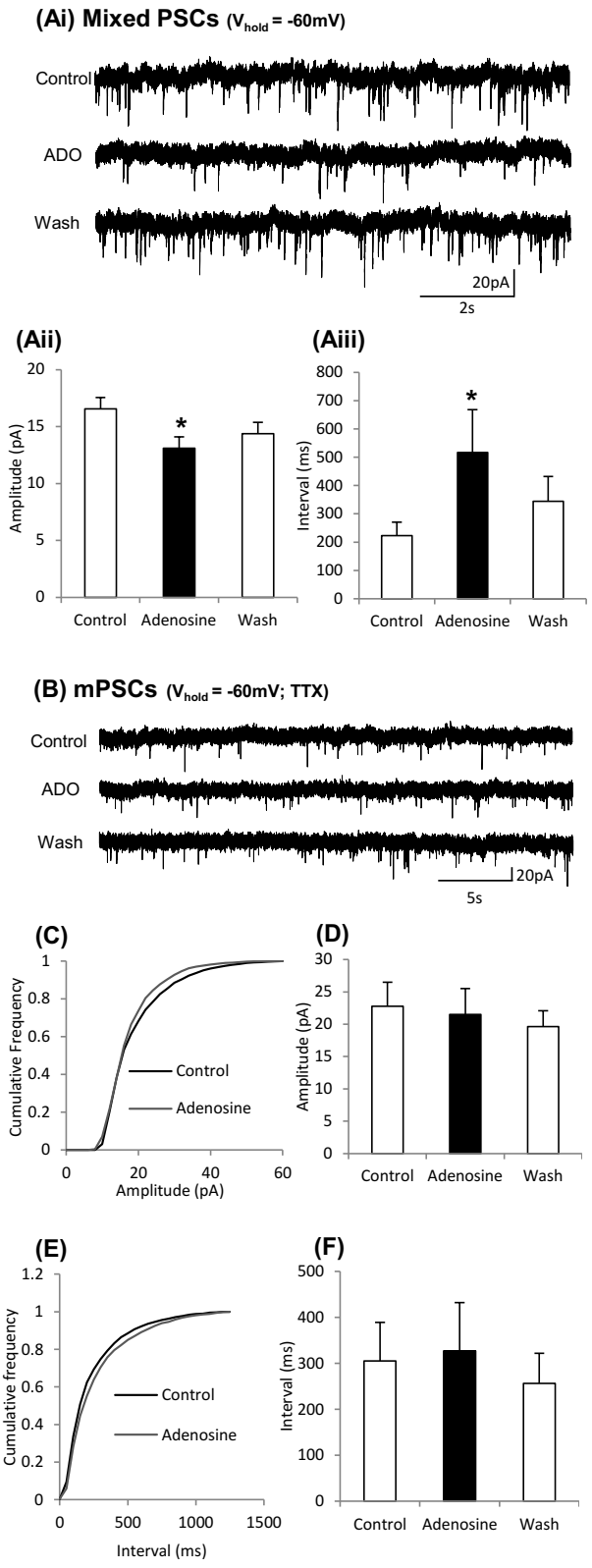

Figure 2.6: Adenosine reduces the frequency and amplitude of synaptic inputs to motoneurons but does not affect miniature post synaptic currents (Ai) Voltage-clamp recordings from a motoneuron held at -60 mV revealing spontaneous synaptic inputs in control, adenosine (ADO) and wash. **(Aii)** Averaged data showing that application of adenosine caused a reduction in the amplitude of synaptic events (n=12 cells). **(Aiii)** Averaged data showing that application of adenosine caused an increase in the interval between events (n=12). **(B)** Voltage-clamp recordings of miniature postsynaptic currents in a motoneuron held at -60 mV in the presence of TTX. **(C)** Averaged cumulative probability plot showing no change in amplitude of miniature post synaptic potentials. **(D)** Averaged data showing that application of adenosine does not affect the amplitude of miniature post-synaptic currents. **(E)** Averaged cumulative probability plot showing no change in the interval between events. **(F)** Averaged data showing that application of adenosine does not affect the interval between miniature post-synaptic currents. These data suggest that adenosine does not directly affect synaptic transmission onto motoneurons.

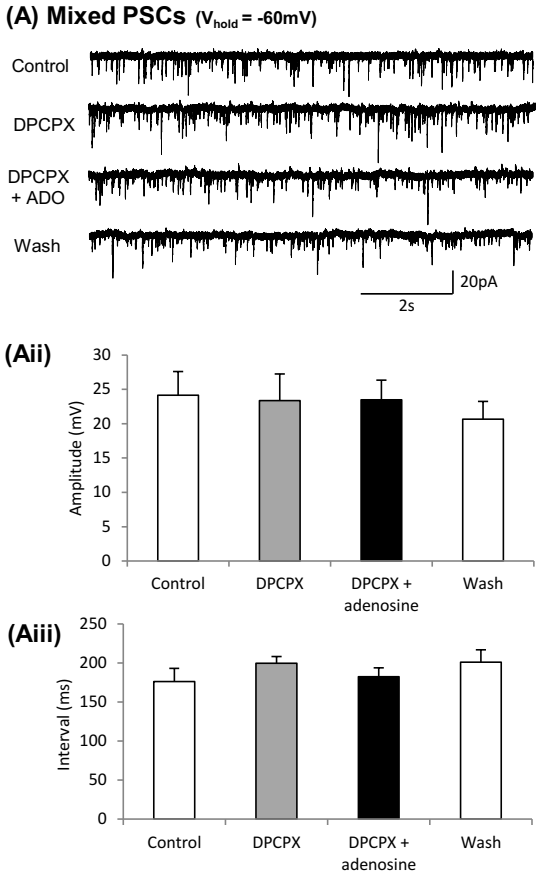

Figure 2.7: DPCPX blocks the effect of adenosine on synaptic inputs to motoneurons (A) Voltage-clamp recordings from a motoneuron held at -60mV revealing spontaneous synaptic inputs in control, DPCPX, DPCPX and adenosine (ADO), and wash. **(Aii)** Averaged data showing that there was no change in the amplitude of synaptic inputs in the presence of DPCPX, and DPCPX blocked the previously observed effects of adenosine (n=8). **(Aiii)** Averaged data showing that there was no change in the interval between synaptic inputs in the presence of DPCPX, and DPCPX blocked the previously observed effects of adenosine (n=8).

DISCUSSION

Adenosine has been shown to modulate spinal circuits responsible for the generation of tadpole swimming, mammalian respiration and, in the previous chapter, locomotor-related activity in neonatal mice (Dale and Gilday, 1996, Lorier et al., 2007, Taccola et al., 2012, Witts et al., 2012, Acton and Miles, 2015). This chapter reports potential cellular mechanisms for purinergic modulation of mammalian locomotor control circuitry. It was found that the effects of adenosine receptor activation differ between ventral horn interneurons and spinal motoneurons despite acting via A1 receptors in both cases. Adenosine hyperpolarises ventral horn interneurons and modulates their synaptic transmission via actions on pre-synaptic A_1 receptors. In contrast, A_1 receptor activation depolarises motoneurons, but does not directly affect fast synaptic transmission between last-order interneurons and motoneurons. Interestingly, currents induced in interneurons and motoneurons were blocked by TTX, implying a role for synaptic transmission in these responses.

In the previous chapter, it was shown that bath application of adenosine to *in vitro* preparations of the whole spinal cord reduces the frequency of locomotor output generated by the spinal locomotor CPG (Witts et al., 2012). This was proposed to be caused by a general inhibition of CPG neurons. This chapter has shown that ventral horn interneurons are indeed inhibited by A_1 adenosine receptor activation. Application of adenosine led to the hyperpolarisation of interneurons and a general reduction in synaptic activity, likely due to pre-synaptic inhibition of transmitter release. Both of these effects were blocked by the A_1 receptor antagonist DPCPX (100nM). Although adenosine-induced currents remained in the presence of the A2A receptor antagonist SCH58261, they were reduced in magnitude suggesting that there may also be some involvement of A_{2A} receptors. However, given the complete block by 100nM DPCPX, the reduction in current magnitude in the presence of SCH58261 (50μM) may reflect non-specific actions on A_1 receptors. Our evidence of a primary role for A_1 receptors in the modulation of ventral horn interneurons and locomotor output is consistent with reports of extensive expression of A_1 receptors throughout the rodent spinal cord (Deuchars et al., 2001)

and A_1 mediated-modulation of rat spinal neurons (Miyazaki et al., 2008). Furthermore, our findings in the mouse spinal cord and previous reports investigating tadpole swimming and rodent respiration (Dale and Gilday, 1996, Lorier et al., 2007), are consistent with a general inhibitory role for A_1 receptors in neuronal circuits throughout the CNS (Burnstock et al., 2011).

Adenosine has often been shown to have global modulatory effects on neuronal networks throughout the CNS (Burnstock et al., 2011). This likely explains why, despite the heterogeneous nature of ventral horn interneurons (Arber, 2012), the effects of adenosine receptor activation were consistent across the different interneurons from which we recorded. Although a large undertaking, it would be interesting in future work to investigate the effects of adenosine on the wide range of specific interneuron populations defined using genetic markers (Kiehn, 2011). This would elucidate whether adenosine truly has the same effects on all ventral horn interneurons and, if there is variation in responses, may provide insight into which interneurons are critical components of the locomotor CPG circuitry.

Despite the apparent homogeneity of responses to adenosine among ventral horn interneurons, a very clear difference was observed between the responses of motoneurons and interneurons. Motoneurons showed a clear, reversible depolarisation, rather than a hyperpolarisation, in response to bath application of adenosine. Although previous work supports a postsynaptic A_1 receptor-mediated modulation of potassium currents in rat spinal neurons (Miyazaki et al., 2008), here it was found that currents induced in interneurons and motoneurons were blocked by TTX, implying a role for synaptic transmission in these responses. This is somewhat surprising as the properties of these currents appear to point to intrinsic membrane changes and would therefore be expected to persist in the absence of action potentials when sodium channels are blocked using TTX. Although perhaps the simplest explanation in motoneurons was that adenosine reduced tonic inhibitory inputs, the finding that adenosine-mediated currents persisted in the presence of strychnine and picrotoxin did not support this mechanism. It is therefore difficult to fully explain the hyperpolarising current induced in interneurons and depolarising current induced in motoneurons, and further probing

of the exact nature of these currents will be needed for a complete understanding the mechanism of adenosine modulation in the spinal cord. One possible explanation that may be uncovered is that in both motoneurons and interneurons, adenosine acts as a metamodulator, modulating the effects of other neuromodulatory inputs, which in turn regulate currents involved in setting the resting membrane potential. This phenomenon of metamodulation has already been reported in locomotor networks of the tadpole spinal cord where nitric oxide modulates the actions of noradrenaline (McLean and Sillar, 2004b). In addition, adenosine has been shown to act as a metamodulator in other systems (Ribeiro and Sebastiao, 2010).

The differential effect of adenosine on motoneurons and interneurons may relate to the importance of maintaining motor output and hence muscle contraction during locomotion. Adenosine-mediated depolarisation along with associated increases in input resistance may help to ensure that motoneurons remain responsive to synaptic inputs despite a generalised inhibition of other components of spinal motor circuitry. Although no obvious changes in the frequency-current relationships of motoneurons upon adenosine receptor activation was observed, one would still expect motoneurons to be more excitable due to their depolarisation and higher resistance. Lack of detection of clear changes in rheobase or frequency-current relationships may reflect the limited resolution of graduated steps of current injection, particularly when dealing with relatively small depolarising currents. It would be interesting to investigate whether effects could be detected using current ramps.

The dual, opposing effects of adenosine on interneurons and motoneurons reported here provide further evidence that single neuromodulators can have multiple effects within distinct neuronal networks (Harris-Warrick, 2011; Brooke et al., 2004). Modulators can act differently according to the state of a neuronal circuit (Doi and Ramirez, 2010), receptor subtype activated (Duarte-Araujo et al., 2004), or downstream pathway engaged (Nanou and El Manira, 2010, Iwagaki and Miles, 2011). Given the large number of modulators identified to date, neuromodulation therefore has the potential to facilitate the production of commands for a wide range of movements. Here we show differential effects of adenosine receptor activation,

presumably due to the engagement of different pathways linking A_1 receptors to spinal motoneurons versus interneurons. Previous work has also shown that adenosine can have opposing actions within the rat myenteric plexus depending upon its source and the exact receptor type activated (Duarte-Araujo et al., 2004; Brooke et al., 2004). Thus, like other single modulators, adenosine has the potential in its own right to facilitate the production of a variety of outputs from motor networks.

The previous chapter demonstrated that endogenous adenosine released from within isolated spinal cord preparations modulates locomotor circuitry (Witts et al., 2012). Furthermore, this endogenous adenosine is derived from the breakdown of ATP following its release from glial cells. In this chapter, application of the A_1 receptor antagonist DPCPX alone had no effect on synaptic transmission or the intrinsic properties of interneurons or motoneurons, suggesting a lack of endogenous adenosine in our *in vitro* slice preparations. Thus, it seems likely that whole network activity is needed to stimulate purine release from glia. Further support of a role for glial-derived adenosine in the mouse spinal cord has recently been provided by a study showing that stimulation of astrocytes leads to an adenosine-mediated inhibition of synaptic transmission between ventral horn neurons (Carlsen and Perrier, 2014). In agreement with findings presented here, this study also concluded that this inhibition involved pre-synaptic A_1 receptors. It remains unclear, however, what physiological signals normally stimulate the release of gliotransmitters, such as purines, during network activity.

The release of adenosine has been associated with a range of pathological conditions including spinal cord injury (McAdoo et al., 2000, Burnstock et al., 2011). Subsequent activation of adenosine receptors is most often thought to be neuroprotective (Burnstock et al., 2011). However, there remains controversy regarding the potential neuroprotective role of adenosine receptors in the spinal cord (Rivera-Oliver and Diaz-Rios, 2014). Neuroprotective effects of A_1 receptor activation in the brain are thought to involve concurrent pre-synaptic inhibition of transmitter release and hyperpolarisation of post-synaptic neurons (Burnstock et al., 2011). Given that data presented here shows similar effects of A_1 receptor activation on ventral horn

interneurons, our data identify A_1 receptors as a potential target for the treatment of pathological conditions affecting the spinal cord. This might include neurodegenerative diseases such as Amyotrophic Lateral Sclerosis, since recent work has shown that blockade of adenosine receptors shortens survival in ALS model mice (Potenza et al., 2013). Further analysis of the effects of purinergic signalling in the mammalian spinal cord is therefore likely to be important not only for advancing our understanding of the neural control of movement, but also for the treatment of injury and disease affecting the spinal cord.

CHAPTER 3

C-BOUTON MEDIATED MODULATION OF MOTONEURONS

Anatomical experiments in this chapter were performed in conjunction with Dr Laskaro Zagoraiou and Ms Ismini Rozani, Academy of Athens, Athens, Greece. Tissue was prepared in St Andrews before being sent to Athens for processing.

Behavioural experiments in this chapter were performed in conjunction with Dr Eiman Azim, Columbia University, New York, USA. All experiments were performed jointly in New York and methods for this section are adapted from Azim et al., 2014.

INTRODUCTION

The focus of the previous two chapters has been the role of purines in modulating motor networks in the neonatal mouse spinal cord. Another potentially important modulator of neuronal networks within the spinal cord is acetylcholine (ACh). Although there are relatively few sources of ACh in the spinal cord, it is known that there are prominent cholinergic inputs on motoneurons, known as C bouton synapses. Some efforts have been made to study C bouton structure and function, but there are still large gaps in our understanding of this synpase. A method to directly study this synapse, its neuromodulatory effect on motoneuron activity, and the behavioural implications of this modulation, was therefore designed and implemented.

C bouton inputs to motoneurons are large synaptic inputs characterised by flattened membrane disks positioned just below the postsynaptic cleft, typically extending the entire length of the synapse and known as sub-surface cisternae (Conradi, 1969, Conradi and Skoglund, 1969). Due to the cisternae, these large, distinctive synapses are known as C-type to distinguish them from other types of synapse. They are around 2-6μm in diameter and are found on the soma and proximal dendrites of motoneurons in a wide range of mammals (Conradi and Skoglund, 1969, Bodian, 1975, Bernstein and Bernstein, 1976, Hamos and King, 1980, Pullen et al., 1992, Wilson et al., 2004). Anatomical experiments have shown that m2-type muscarinic ACh receptors (Hellstrom et al., 2003), Kv2.1 channels, SK channels (Deardorff et al., 2013) and N-type calcium channels (Wilson et al., 2004) all cluster postsynaptically at the C bouton synapse.

Data obtained from whole-cell patch-clamp recordings of motoneurons in neonatal mouse spinal cord slice preparations has revealed that activation of m2 receptors increases motoneuron excitability (Miles et al., 2007). This increase in excitability was postulated to be caused by a reduction in AHP amplitude, as m2 activation also reduced AHP. Analysis of the mechanisms underlying reductions in AHP amplitude revealed a blockade of SK-type calcium-dependent potassium channels.

As well as understanding the mechanisms and effects of synaptic transmission at C boutons, efforts have been made to ascertain the source cells of C boutons and their circuitry. Research utilising knowledge of the molecular identity of C boutons revealed that the source of C boutons is a medially positioned population of partition cells (Miles et al., 2007) which is known to be defined by the paired-like homeodomain transcription factor Pitx2 (Zagoraiou et al., 2009). Pitx2 expression defines a small cluster of V0 ($Dbx1^{+}$) interneurons which form a longitudinal column near the central canal of the spinal cord and can be further subdivided into cholinergic ($V0_C$) and glutamatergic ($V0_G$) subtypes. Remarkably, molecular genetic techniques have demonstrated that this small cluster of $V0_C$ interneurons, which is outnumbered by motoneurons by a factor of ~10:1, represent the sole source of C bouton inputs to all spinal motoneurons (Zagoraiou et al., 2009).

In addition, a functional role for the C bouton system in whole animal behaviour has been demonstated (Zagoraiou et al., 2009). This work utilised mice in which the cholinergic output of V0c interneurons is inactivated due to the conditional knockout of choline acetyltransferase. The motor performance of mutant and control animals was assessed during locomotor behavioural assays designed to uncover task-dependent modulation in the activation of hind limb muscles. The degree of muscle activation was monitored via recordings of electromyographic (EMG) activity while animals were subjected to sequential walking and swimming tasks. In rodents, swimming elicits greater activation of some hind limb muscles compared to walking. However, in mutant animals in which C boutons were inactivated the enhancement of muscle activation during swimming was significantly diminished compared to controls (Zagoraiou et al., 2009). This suggests that C boutons modulate motoneuron activity during locomotion to match the intensity of muscle activation to the biomechanical demands of different motor tasks.

Despite a reasonably thorough understanding of the anatomy of the C bouton synapse, some research into its effect on motoneuron output, and theories suggested for its mechanism of action, the synapse has never been studied directly. Therefore, an attempt was made to firstly

develop a method by which the C bouton synapse and its circuitry could be directly studied, and secondly, to build on previous research to further investigate the behavioural role of this unique synapse.

Firstly, although current research suggests that Pitx2+ cells modulate motoneuron excitability via the C bouton synapse, evidence for this theory is indirect. Specifically, the theory is that Pitx2+ cells release acetylcholine at the C bouton, which binds to m2 receptors on motoneurons, reducing AHP size via inhibition of SK channels, and therefore increasing motoneuron excitability. It has been shown that Pitx2+ cells are the sole source of the cholinergic C bouton, that m2 acetylcholine receptors are clustered postsynaptically at the C bouton synapse, and that m2 receptor activation alters motoneuron excitability. However, as the relationship between Pitx2+ cells and motoneurons has not been studied directly, it is not possible to unequivocally link these findings. An attempt was therefore made to design an experimental method to directly investigate the synaptic mechanism underlying C bouton inputs to motoneurons. To directly study the effect of Pitx2+ cell activation on motoneuron excitability, the method needed to enable stimulation of Pitx2+ cells and simultaneous recording of motoneuron activity. A method of this type would provide important new information about C bouton circuitry and function.

Secondly, the role of Pitx2+ cells in controlling behavioural output is largely unknown. It has been shown that Pitx2+ cells are necessary to produce the high level muscle contractions observed in mouse swimming, but beyond this very little research has been performed in this area. It is not known, for example, whether Pitx2+ cells are exclusively involved in swimming, or whether they are also important in other rhythmic outputs. It is also not known whether C boutons only influence rhythmic motor activity or whether they also have a role in controlling more intricate movements. Further investigation of the behavioural changes that occur when Pitx2+ cells are manipulated within the spinal cord is required. A behavioural study was therefore designed to investigate whether mice lacking C boutons could successfully perform a reaching task requiring fine control of forelimb movements, and a ladder walking task requiring

limb coordination and fast corrective movements. This would further knowledge about the role of the C bouton synapse in motor output, an area of research where very little work has been carried out.

METHODS

Genetic tools

Cre-Lox recombination was used for the targeted expression of proteins of interest in Pitx2+ interneurons; Pitx2::Cre mice (Liu et al., 2003) were used to target Pitx2+ cells, and ROSA-loxP-STOP-loxP-TDTomato (Madisen et al., 2010) mice were used as a reporter. Pitx2+ cells displayed red fluorescence in Pitx2::Cre ROSA-loxP-STOP-loxP-TDTomato mice.

In order to stimulate Pitx2+ cells, the light-sensitive protein channelrhodopsin was used. This protein is a mixed cation channel that can be expressed in the membrane of mammalian cells, causing cells to depolarise in the presence of blue light. A Cre-dependent channelrhodopsin virus (AAV2/1.EF1a.DIO.hChR2(H134R)-eYFP.WPRE.hGH) was purchased from the University of Pennsylvania (K. Deisseroth; Addgene plasmid 20298). This virus contained channelrhodopsin conjugated to a YFP reporter sequence, inserted into Cre-dependent adenosine-associated virus (AAV). By combining this virus with Pitx2::Cre mice channelrhodopsin could be expressed exclusively in Pitx2+ cells.

Surgical procedures

Surgical procedures were used to insert the Cre-dependent channelrhodopsin virus into the spinal cord of Pitx2::Cre mice. Surgery was performed in accordance with the UK Animals (Scientific Procedures) Act 1986, under project licence 60/4369 and personal licence 60/12934. Methods were similar to those described previously (Fink et al., 2014). Neonatal mice (P0-P3) were removed from their home cage and placed in a container lined with soft material. The container was then positioned on a heat pad. One pup was removed and anesthetised using isoflurane, before being secured to the surgery table. An incision in the skin

was made above the spinal cord, and a glass capillary attached to a syringe via thin tubing was used for injections. Glass capillaries (1.0mm outer diameter, 0.58mm inner diameter; Harvard Apparatus) were pulled to a point using a vertical puller and broken back to approximately 100μm inner diameter using a glass block. The glass tip was changed for each animal. Virus was injected in three to five segments of the lumbar spinal cord, in between the vertebrae. This ensured that the virus was distributed across the rostral-caudal axis. The first insertion site was slightly to the left of the midline, the second was slightly right of the midline, and alternation continued for all insertion sites. This ensured that the virus was evenly distributed on both sides of the spinal cord. The needle was inserted into the first site, and 50nl of virus was ejected. The needle was then raised approximately 200μm and a further 50nl of virus was ejected. Three to five ejections were performed per insertion site. This ensured the virus was evenly distributed across the ventral-dorsal axis of the spinal cord (Figure 3.1A). Approximately 800nl of virus was injected into the cord in total. The wound was closed using inside-out sutures (non-absorbable 6/0; Braun), and the mouse removed from the anaesthetic and returned to the heat pad. They were given an injection of the analgesic buprenorphine at a concentration of 0.2μg/g. Mice were left to recover until they were freely moving, which took approximately 20 minutes. They were then returned to their home cage and left for 5-9 days. All equipment was cleaned using Virkon.

In vitro spinal cord preparations

All methods required to obtain tissue for *in vitro* experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. Spinal cord preparations were obtained from P6-P12 C57BL/6 mice using techniques similar to those described previously (Jiang et al., 1999). Briefly, animals were killed via cervical dislocation, decapitated and eviscerated before spinal cords were isolated from the mid-cervical to upper sacral segments in a chamber containing dissecting artificial cerebrospinal fluid (aCSF; equilibrated with 95% O_2-5% CO_2, ~4^{o}C). Dorsal and ventral roots were trimmed and the tissue was laid in 1% agar.

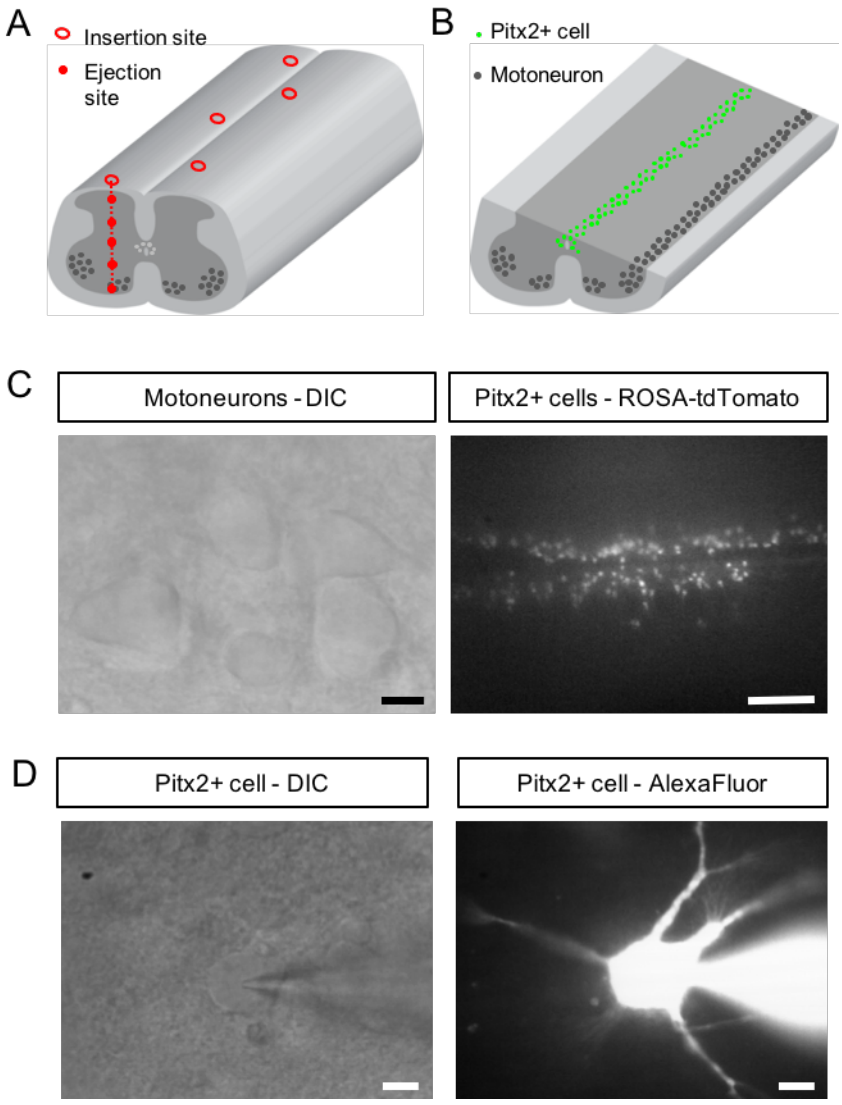

Figure 3.1: A spinal cord preparation allowing access to Pitx2 cells and motoneurons simultaneously. **(A)** Injections of virus into the spinal cord were made either side of the midline and ejections of virus were made at several points on the injection tract to ensure that the virus was spread evenly throughout the spinal cord. **(B)** Spinal cords from neonatal mice were sliced diagonally such that Pitx2+ cells and motoneurons were both visible on the surface of the tissue. **(C)** DIC image of motoneurons and texas red fluorescence image of Pitx2 cells around the central canal in a diagonal slice preparation. **(D)** Whole-cell patch-clamp recordings were taken from Pitx2+ cells. Scale bars = 20µM, 300µM (C), 5µM (D).

A preparation enabling access to Pitx2+ interneurons and motoneurons

Although methods to obtain spinal cord tissue from neonatal mice are well described, they are normally used to perform experiments using the whole cord or simple transverse slices. In order to directly assess the relationship between Pitx2+ cell stimulation and motoneuron response, it was necessary to develop a new preparation in which both Pitx2+ cells and motoneurons could be accessed from the surface of the tissue. This means that light could be directed at the surface of the tissue to excite Pitx2+ cells, while a patch clamp electrode could be attached to motoneurons to measure any changes resulting from Pitx2+ cell excitation.

To achieve this arrangement, the cord needed to be sliced diagonally in the longitudinal plane (Figure 3.1B). The slicing was performed using a vibratome (VT1200, Leica), to ensure precision. However, the vibratome used was not able to slice diagonally, so a sloping platform was created. To do this, Sylgard was poured into a petri dish which was raised up at one side, creating an angle of approximately 15°. The isolated spinal cord was laid on the sloping Sylgard, and then covered with 0.6% agar, and the dish was surrounded with ice. Once solid, a section of the agar containing the spinal cord was cut from the dish. This agar block was turned over and mounted in the vibratome, the flat bottom flush with the vibratome platform, resulting in the sloped part of the agar facing upwards and the spinal cord sitting at a slight angle. The flat blade, therefore, sliced the tissue diagonally in the longitudinal plane, such that both Pitx2 cells and motoneurons in the ventral horn are accessible from the surface of the tissue (Figure 3.1C). Preparations were removed from the agar and pinned onto clear Sylgard that was glued into a recording chamber containing recording aCSF (equilibriated with 95% O_2-5% CO_2, ~20°C).

Patch clamp recordings

Whole cell patch-clamp recordings were performed on fluorescently labelled interneurons and motoneurons visualized under infrared differential interference contrast microscopy (Figure 3.1D). Patch electrodes (3–5 MΩ) were pulled on a horizontal puller (Sutter Instrument, Novato, CA) from borosilicate glass (World Precision Instruments, Sarasota, FL).

Patch-clamp signals were amplified and filtered (4-kHz low-pass Bessel filter) with a MultiClamp 700B amplifier (Molecular Devices) and acquired at ≥10 kHz using a Digidata 1440A A/D board and pClamp software (Molecular Devices). Details of voltage and current-clamp protocols appear in Results.

Solution and drugs

The aCSF solution used for dissecting contained 25 mM NaCl, 188 mM sucrose, 1.9 mM KCl, 1.2 mM NaH_2PO_4, 10 mM $MgSO_4$, 1 mM $CaCl_2$, 26 mM $NaHCO_3$, 25 mM D-glucose and 1.5 mM kynurenic acid (equilibriated with 95% O_2-5% CO_2). The aCSF solution used for recording contained 127 mM NaCl, 3 mM KCl, 1.3 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose (equilibriated with 95% O_2-5% CO_2). The standard patch-clamp pipette solution contained 140 mM potassium methane sulfonate, 10 mM NaCl, 1 mM $CaCl_2$, 10 mM HEPES, 1 mM EGTA, 3 mM Mg-ATP, and 0.4 mM GTP-Na_2 (pH 7.2–7.3, adjusted with KOH).

Data analysis

Whole cell patch-clamp recordings were analyzed using Clampfit software (Molecular Devices). Data are reported as means ± standard error. Differences in means were compared using Student's ***t***-test. Values of $P < 0.05$ were considered significant.

Anatomy

Anatomy was performed in order to estimate the proportion of Pitx2+ cells infected with virus. Animals were killed by cervical dislocation and the spinal cord was isolated and sliced diagonally as described above. Anatomical experiments were performed in conjunction with colleagues at the Biomedical Research Foundation, Academy of Athens. Diagonally sliced spinal cord tissue was fixed overnight in 4% paraformaldehyde, transferred to phosphate buffer solution (PBS) and then sent to the University of Athens. Here, colleagues incubated tissue in 1ml solutions of primary antibody diluted in 1% bovine serum albumen, 0.1% Triton X PBS

solution. Tissue was then washed every 20 minutes for four hours with PBS before being incubated with secondary antibodies for 24 hours at 4°C. It was once again washed every 20 minutes for four hours with PBS and then mounted on slides using Vectashield. Primary antibodies: Rabbit dsred (1:2000), Clonetech 632496; Sheep GFP (1:500), Biogenesis 4745-1051; Chicken GFP (1:1000), Abcam ab13970; Goat ChAT (1:200), Chemicon AB144AP; Guinea pig Hb9 (1:500), Jessell lab. Secondary antibodies (1:500): Anti rabbit CY3, FITC; Anti chicken FITC; Anti sheep FITC; Anti goat CY3, FITC; Anti guinea pig CY3, all Jackson Immunoresearch.

Behaviour

It is known that Pitx2+ cells are involved in producing high-level muscle contractions during swimming, but little else is known about their role in motor control. In order to further investigate the role of Pitx2+ cells in behaviour, animals with and without Pitx2+ cells were subjected to a reaching task, performed with colleagues at Columbia University, New York. Because performance in this task is known to be variable across different mice, it was necessary to use the same mice before and after Pitx2+ cell ablation. Surgical procedures were therefore required to inject a Cre-dependent virus containing diptheria toxin receptor directly into the spinal cord of Pitx2::Cre mice. Diptheria toxin receptors were therefore exclusively expressed in Pitx2+ cells and diptheria toxin could be used to selectively ablate these cells. This meant that the performance of these animals in the reaching task could be compared before and after diptheria toxin administration, first when Pitx2 cells were intact and then when they were ablated. Loss of Pitx2+ cells was confirmed by Dr Eiman Azim, who performed a set of anatomical experiments to image spinal cord tissue of Pitx2::Cre after Pitx2+ cell ablation. Dr Azim reported that no TDTomato fluorescence was observed in the cervical horn, while still being clearly visible in the lumbar region, suggesting that Pitx2+ cells had been successfully ablated in the rostral spinal cord in a selective manner. Some TDTomato+ projections were still visible in the ventral horn of the cervical spinal cord, which could be long distance projections or projections from local Pitx2+ cells which persist even after cell death. Despite this, no C

boutons were visible on motoneurons of the T1-C3 segments so it is likely that most if not all functional connections between Pitx2+ cells and motoneurons had been ablated in these areas controlling forelimb movement.

Behavioural and surgical techniques were similar to those described previously (Azim et al., 2014), and were performed jointly with Dr Azim and other colleagues at Columbia University. Briefly, adult Pitx2::Cre mice were food deprived to ~80–90% of their original body weight and trained to reach for a food pellet. This was done by placing them in a clear acrylic box with an acrylic tray in front of a narrow opening at one end of the box. Food pellets were placed on a tray in front of the opening, and mice learnt to reach out and pick them up. After training for two weeks, surgery was performed. Adult mice were anaesthetized with tribromoethanol (Sigma-Aldrich) via intraperitoneal injection. Hair was removed from the back and neck with a shaver and the mouse was placed in a stereotaxic frame (David Kopf Instruments). Eye lubricant was applied (Puralube Vet Ointment; Dechra), an incision was made over the cervical spinal cord and the muscles overlying the cord were separated along the midline using forceps. A Cre-dependent adenosine-associated virus containing a diptheria toxin receptor (AAV-FLEX-DTR-GFP; Azim et al. 2014) was injected into the spinal cord in 23nl increments using a Nanoject II (Drummond Scientific). Three injection tracks were made on the right side of the cord, evenly distributed across the dorsoventral extent of the segment and the mediolateral extent of the grey matter. Injections were made in all segments from C3 to T1. Following injection, the skin was sutured and the mouse was allowed to recover in a postoperative chamber before being housed individually. Postoperative buprenorphine (Henry Schein Medical) was given at 0.032 mg kg^{-1} body weight as analgesic.

After recovery from surgery (5-7 days), reach success was quantified once a day for 9 days, by counting the number of successful retrievals of the pellet and attempted retrievals where the pellet was missed over a period of 20 min or 20 hits, whichever came first. After 9 days, diptheria toxin (DT) was administered using an intraperitoneal injection. Reach success

was then quantified again in the same way for 10 days. Percentage success rate was calculated for each mouse on each day after recovery from surgery.

For the horizontal ladder task, two standing platforms measuring 10 cm long and 4 cm wide were placed at the ends of a clear acrylic box 68 cm long and 4 cm wide. At the inner edge of each platform were 10 rungs, each 1 cm apart, serving as starting rungs. In between the two groups of 10 rungs were 13 rungs, each 2 cm apart. Only steps on these 13 rungs were considered for quantification. Below the walkway was an angled mirror to provide a bottom-up view of the paws. Mice were placed in the box and videotaped with a high-definition camcorder (Canon VIXIA HF11) as they walked across the ladder 10–15 times; a walk was only considered valid if the mouse did not stop for an extended period of time and did not reverse direction. Pre-DT walks were recorded following recovery from viral injection, and post-DT walks were recorded 1 week after DT administration. Mistakes were recorded and mean values were calculated within each mouse and across all mice pre- and post-DT administration. T-tests were used to compare the means.

RESULTS

Optogenetic control of Pitx2+ cell activity

It was first necessary to test whether viral transfection methods led to the selective expression of channelrhodopsin-YFP fusion protein in Pitx2+ neurons. Electrophysiological recordings were used to ensure the channel functioned correctly and could be used to control the activity of Pitx2+ interneurons. Whole-cell patch-clamp recordings were obtained from Pitx2+ cells, identified in Pitx2Cre:ROSATDTom mice by their TDTomato expression (Figure 3.1D). Blue light (λ=470nm; pE100, CoolLED) directed through the objective onto *in vitro* spinal cord preparations infected with Cre-dependent channelrhodopsin AAV induced a depolarising current in some Pitx2+ neurons. Pitx2+ cell recordings were made in a total of 15 diagonal preparations. In 4 of these preparations all Pitx2+ cells depolarised in response to light, in 9 preparations no light response was observed in any Pitx2+ cells, and in 2 preparations some

Pitx2+ cells responded to light and some did not. A depolarising current was induced by blue light directed through the objective in 11 out of 36 Pitx2+ cells recorded in total.

These light-evoked currents tended to have two distinct components: a fast, desensitising component and a slow, steady-state component (Figure 3.2B). Such currents are consistent with previous studies investigating channelrhodopsin-mediated currents (Boyden et al., 2005). In order to investigate the magnitude and consistency of light-evoked currents, measurements were obtained using the steady-state component of the current. Directing blue light through the objective onto Pitx2+ cells induced an average current of -60.1±5.8pA (n=11 cells). The magnitude of response varied both within and between cells. The response to light tended to decrease over time in any given cell. The largest variation within a single cell was -143 to -442 pA. The mean response across cells ranged from -10pA to -315pA.

Next, the effect of channelrhodopsin-mediated currents on the firing output of Pitx2+ cells was investigated using current-clamp mode recordings. Out of 11 Pitx2+ cells in which a depolarising current was induced, 7 were tested in current clamp mode. Blue light was able to induce firing in all 6 silent Pitx2+ cells (Figure 3.2C), and very short pulses of blue light could induce single action potentials in all of 6 these cells (Figure 3.2D). Blue light also increased the frequency of firing in one tonically active cell (Figure 3.2E).

Channelrhodopsin expression in Pitx2+ cells

In order to quantify the number of Pitx2+ cells expressing the channelrhodopsin-YFP fusion protein, anatomical experiments were performed. Spinal cords that had been used for electrophysiology were fixed overnight using 4% paraformaldehyde solution and then transferred to PBS. A vibratome (V1200) was then used to slice the tissue into 300nm transverse sections, and an anti-GFP antibody was used to maximise the fluorescence of the YFP conjugated to the channelrhodopsin. No antibodies were needed for TDTomato labelling as the fluorescence is bright enough to see clearly in fixed as well as fresh tissue. In Pitx2Cre::TDTomato animals, many Pitx2+ cells showed clear co-labelling of TDtomato and YFP

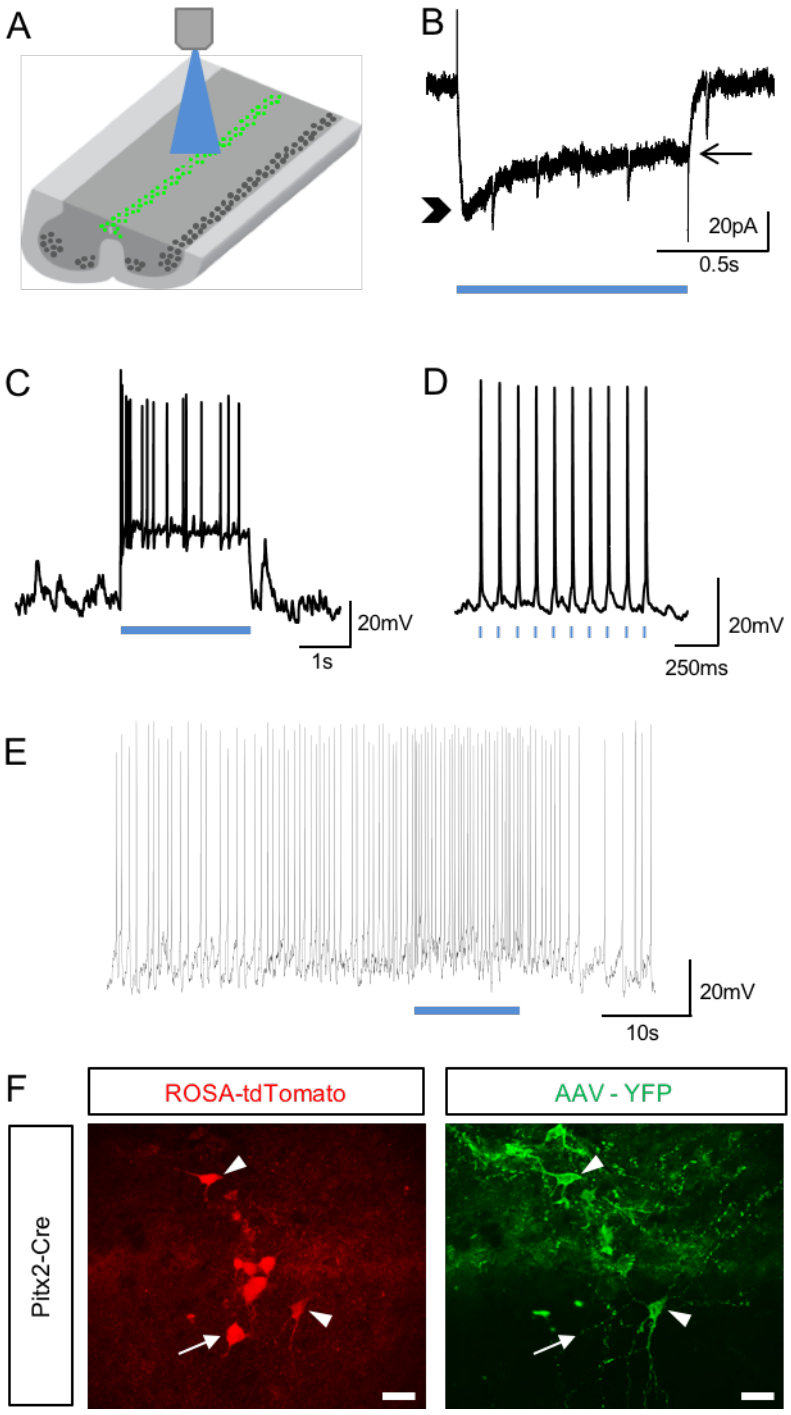

Figure 3.2: Optogenetic control of Pitx2 cell activity. (A) Blue light was directed at Pitx2+ cells expressing channelrhodopsin through the objective. **(B)** Example of the current induced in response to blue light in a Pitx2+ cell held at -60mV in voltage clamp mode. Two distinct components are visible; a fast transient component (arrowhead) and slow steady state component (arrow). **(C)** An example of blue light inducing firing in a Pitx2 cell which is silent at rest. **(D)** An example trace of a recording of a Pitx2+ cell in current clamp mode showing that blue light can induce single spikes. **(E)** An example of blue light causing an increase in the rate of firing in a tonically active Pitx2+ cell in current clamp mode. **(F)** Anti-GFP immunohistochemistry showed that some (arrowheads), but not all (arrows), Pitx2 cells expressed channelrhodopsin. Scale bars = 50µM.

(arrowheads in figure 3.2F). However, there were also Pitx2+ cells labelled with TDtomato that did not appear to express YFP (arrow in Figure 3.2F) and therefore, presumably, were not infected with the channelrhodopsin-containing virus.

To try to estimate the proportion of Pitx2+ cells expressing the channelrhodopsin-YFP fusion protein, some post-electrophysiology tissue was also sent to collaborators at the University of Athens for analysis. Here, immunohistochemical experiments were performed on fixed diagonal spinal cord preparations that had been used for electrophysiology. However, it was not possible to successfully image Pitx2+ cells in this post-physiology tissue, and therefore the exact proportion of Pitx2+ cells expressing the channelrhodopsin-YFP fusion protein in any one diagonally sliced spinal cord remains unknown.

Optogenetic control of Pitx2+ cell activity using different light sources

Although it was not possible to quantify the exact proportion of Pitx2+ cells expressing channelrhodopsin, it is clear that individual Pitx2+ cells can be optogenetically manipulated in this preparation. However, very little is known about the projection pattern of Pitx2+ cells to motoneurons and so it is important to activate as many Pitx2+ cells as possible to maximise the chances of seeing effects in any single motoneuron during patch clamp recordings. To test the area of tissue affected by light from the objective and therefore what proportion of the total population of Pitx2+ cells could be excited, the objective through which light is delivered was moved away from individual Pitx2+ cells during recordings. Moving the objective 50μm away from the patched cell did not substantially diminish the response. However, when the objective was moved 150μm away, such that the cell was no longer in the field of view, the response was severely attenuated (Figure 3.3A). This showed that blue light directed through the objective only excited a small portion of tissue and therefore a small number of the Pitx2+ cell population. In order to ensure that as many Pitx2+ cells as possible were excited, to maximise the chances of revealing subsequent effects in individual motoneurons, the method of light delivery was altered. Instead of light being focussed through the objective onto a small section of the spinal

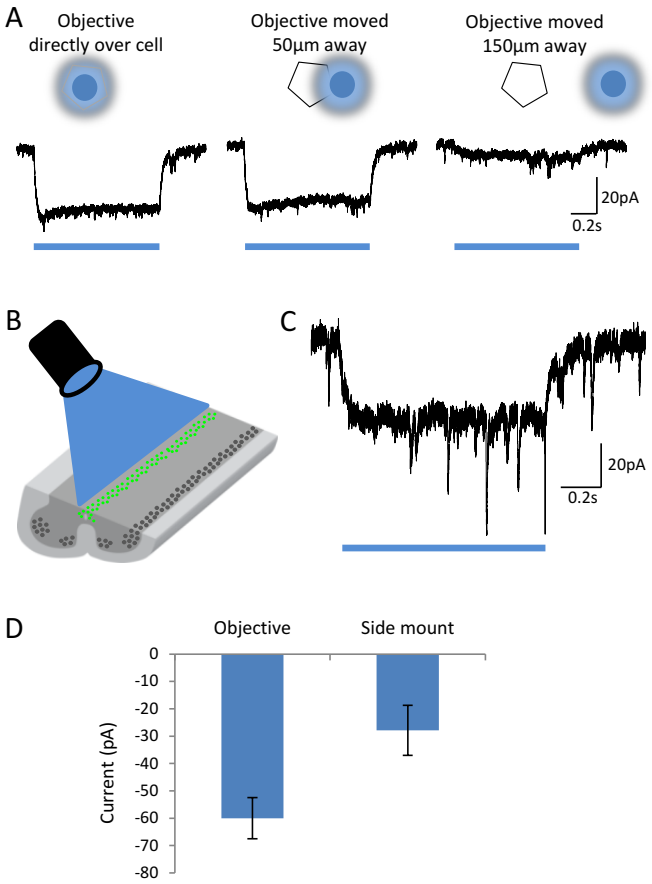

Figure 3.3: Response to blue light from objective compared to side-mounted light. (A) Whole-cell patch-clamp recording of a Pitx2+ cell with light from the objective directly over (left panel), slightly to one side of (middle panel) and further to one side of (right panel) the cell being patched. **(B)** Altered set up with a much broader cone of light directed at the whole Pitx2+ cell population using a side-mounted LED light source. **(C)** Whole-cell patch-clamp recording in voltage clamp mode showing that depolarising currents are still induced in Pitx2+ cells when the side-mounted light source is used to illuminate the preparation. **(D)** Comparison of the average response of Pitx2+ cells to blue light from the objective and side-mounted light source.

cord, a large, side-mounted light source was used to flood the whole preparation with light (Figure 3.3B). This method produced an attenuated light response, the average steady-state current response being 60.1±5.8pA through the objective compared to 28.6±7.1pA from the side-mounted light (Figure 3.3D). However, this method was still able to induce depolarising currents in Pitx2+ cells (Figure 3.3C) and had the advantage of producing a light response in a more Pitx2+ cells simultaneously and was therefore utilised for subsequent experiments to investigate the effects of Pitx2+ cell excitation on individual motoneurons.

Changes in motoneuron excitability with optogenetic manipulation of Pitx2+ cell activity

Although the number of Pitx2+ cells expressing the channelrhodopsin-YFP fusion protein was unknown, the side-mounted light source caused a clear depolarisation in a greater proportion of Pitx2+ cells than light directed through the objective. The focus of patch-clamp recordings was therefore switched to motoneurons to directly assess the effect of C bouton activation on motoneuron properties and output. Based on previous work we predicted that activation of Pitx2+ cells and therefore C boutons would lead to changes in the excitability of motoneurons.

Activation of m2 receptors has been shown to reduce AHP amplitude via inhibition of SK channels, causing an increase in the frequency of motoneuron firing (Miles et al., 2007). To investigate whether the size of the motoneuron AHP is affected by activation of Pitx2+ cells, single action potentials were induced using short pulses of current at a level just above the minimum needed to induce firing in any given cell. The size of the AHP was measured before and during Pitx2+ cell activation using blue light. No change in AHP amplitude was observed in any motoneurons (mean AHP amplitude control -4.79 ± 0.19 pA; light -5.04 ± 0.21 pA; n=16).

Although no change in AHP amplitude could be detected, it is possible that Pitx2+ cells modulate motoneuron excitability via another mechanism. Motoneuron excitability was therefore investigated by measuring the minimum current needed to induce action potential firing (rheobase). To test the rheobase of motoneurons before and during the application of blue

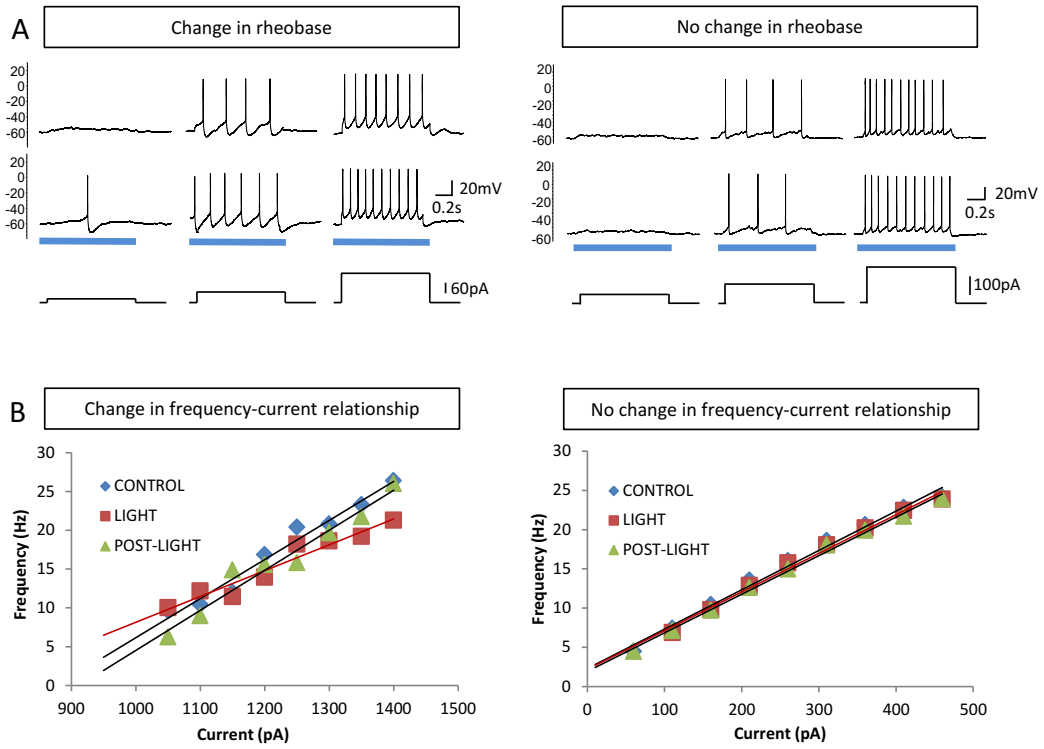

Figure 3.4: Electrical stimulation of Pitx2 cells caused changes in rheobase and frequency current relationships in motoneurons. (A) Blue light was found to reversibly reduce the current needed to induce action potential firing (rheobase) in 5 of of 16 motoneurons recorded from Pitx2::Cre mice infected with a Cre dependent virus containing channelrhodopsin. Shown here is an example of blue light causing a reduction in rheobase (left panel) and having no effect on rheobase (right panel). **(B)** An example of blue light reversibly reducing (left panel), and having no effect on (right panel), the slope of frequency-current relationships in an animal in which channelrhodopsin is expressed in Pitx2 cells. The slope of the frequency-current relationship was reversibly altered in 2 of 16 motoneurons.

light to preparations, a series of square current steps (1s duration, 10pA increments) were injected into motoneurons and the level of current needed to induce action potential firing was assessed. Of 16 motoneurons recorded in diagonally sliced spinal cord preparations, 5 showed a 50-100pA reduction in the current needed to induce firing in the presence of blue light (Figure 3.4A).

Another measure of excitability is the frequency of action potential firing generated in response to the level of current injected during recordings from motoneurons. This input-output relationship of motoneurons was investigated using a series of current steps (50pA increments; duration 1 second), ranging from just below rheobase to the current needed to trigger maximum firing rate.

Of 16 motoneurons recorded from diagonally sliced spinal cord preparations, 14 showed no change and 2 showed a small reduction in the slope of frequency-current relationships during the excitation of Pitx2+ cells with blue light (Figure 3.4B). Interestingly, the 2 cells in which the frequency-current relationships changed were a subset of the 5 that showed a reduction in rheobase.

It is possible that further effects of Pitx2+ cell activation on motoneurons could be uncovered by increasing acetylcholine at the synapse. To test this, the cholinesterase inhibitor eserine (1mM, Sigma) was bath applied to 4 motoneurons, 1 of which had previously shown a reduction in rheobase in response to Pitx2+ cell activation, and 3 of which had previously shown no effect. The same tests of excitability were performed. Eserine was not able to uncover any additional effects in any of these 4 motoneurons.

To ensure changes in motoneuron output properties were not caused by the light directly influencing motoneurons, control experiments were performed. Firstly, surgical insertion of virus was performed on wildtype mice, where there was no Cre recombinase in any cells and therefore no expression of channelrhodopsin. In these animals, shining blue light on Pitx2+ cells had no effect on either Pitx2+ cell or motoneuron activity (n=6; 4 Pitx2+ cells, 2 motoneurons). Secondly, recordings were taken from non- Pitx2+ cells in Pitx2::Tom mice

which had been injected with virus. These cells did not respond to light either directed through the objective or from the side-mounted light source (n=10 cells).

Taken together, these results show that excitation of Pitx2+ cells increases the excitability of 5 out of 16 motoneurons. In 2 of these 5 motoneurons, Pitx2+ cell excitation increases the frequency of firing during low current input, but reduces the frequency of firing when there is a greater current input. This effect can be seen clearly in Figure 3.4B and shows that Pitx2+ cell activation reduces the range of firing frequencies in some motoneurons.

No overt change in motor behaviour following ablation of cervical Pitx2+ cells

In collaboration with colleagues at Columbia University, the role of Pitx2+ cells and C boutons in motor behaviour was also investigated. C boutons have previously been shown to be involved in modulating muscle activation during the transition from walking to swimming (Zagoraiou et al., 2009). To further investigate the role of Pitx2+ cells and C boutons in motor behaviour, in particular goal-directed movements, mice were subjected to a reaching task before and after Pitx2+ cell ablation. This task involves the use of muscles involved in balance, strength, and fine control, so provides the opportunity to study the role of C boutons in broader aspects of motor control than has been studied previously.

Adult Pitx2::CreTDTomato mice were trained to reach for a food pellet from a small platform (Figure 3.5A). Following this initial training Cre-dependent adeno-associated virus containing diptheria toxin receptor was injected directly into the segments T1-C3 of the spinal cord of these mice. After recovery from surgery, the success of these mice in the reaching task was measured on 8 consecutive days. Diptheria toxin was then administed, causing ablation of Pitx2+ cells in the rostral spinal cord. The success of these mice in the reaching task was then once again measured over 10 consecutive days. There was no significant difference in the success of the mice in reaching with the right forepaw before (mean = 33.7±9.3% success) versus after (mean = 36.8±9.5% success) diptheria toxin administation (Figure 3.5B). There was

also no qualitative difference in the overall kinematics of reaches before and after Pitx2+ cell ablation.

Although this reaching task tested goal-directed movements requiring fine control, it is also possible that C boutons play a role in correcting limb movements after error, or preventing error. This is because Pitx2+ cells are known to receive sensory input and have been shown here to limit the range of firing in some motoneurons, which provides a potential circuit for error prevention and correction. The performance of mice on a ladder task was therefore also tested before and after diphtheria toxin administration. This task involves both fine limb control and support of body weight, and so may give an insight into the role of Pitx2+ cells and C boutons in gross as well as fine motor control.

In this task, mice were placed on one of the two solid standing platforms at either end of an enclosed ladder with 36 rungs. To encourage the mouse to start walking, the 10 rungs nearest the standing platform at each end were spaced 1cm apart, while the middle 13 rungs were spaced 2cm apart (Figure 3.5C). Mice were videoed as they walked along the ladder 10-15 times and only performance on the middle 13 rungs was used for analysis. The number of mistakes with the right forepaw was recorded before and after diphtheria toxin administration (Figure 3.5D). No difference was observed in number of mistakes with the right forepaw for any of the mice between pre and post DT performance (n=3 mice). The mean number of mistakes with the right forepaw across all mice before DT administration was 10.3±2.9 and after DT administration was 8.3±2.5.

Behavioural testing using these two tasks did not reveal any difference in the motor performance before and after Pitx2+ cell ablation. Pitx2+ cells may therefore have other roles in motor control that were not evident in these two behavioural tasks. It is also possible that ablation of Pitx2+ cells was insufficient to reveal their roles in the motor behaviours tested.

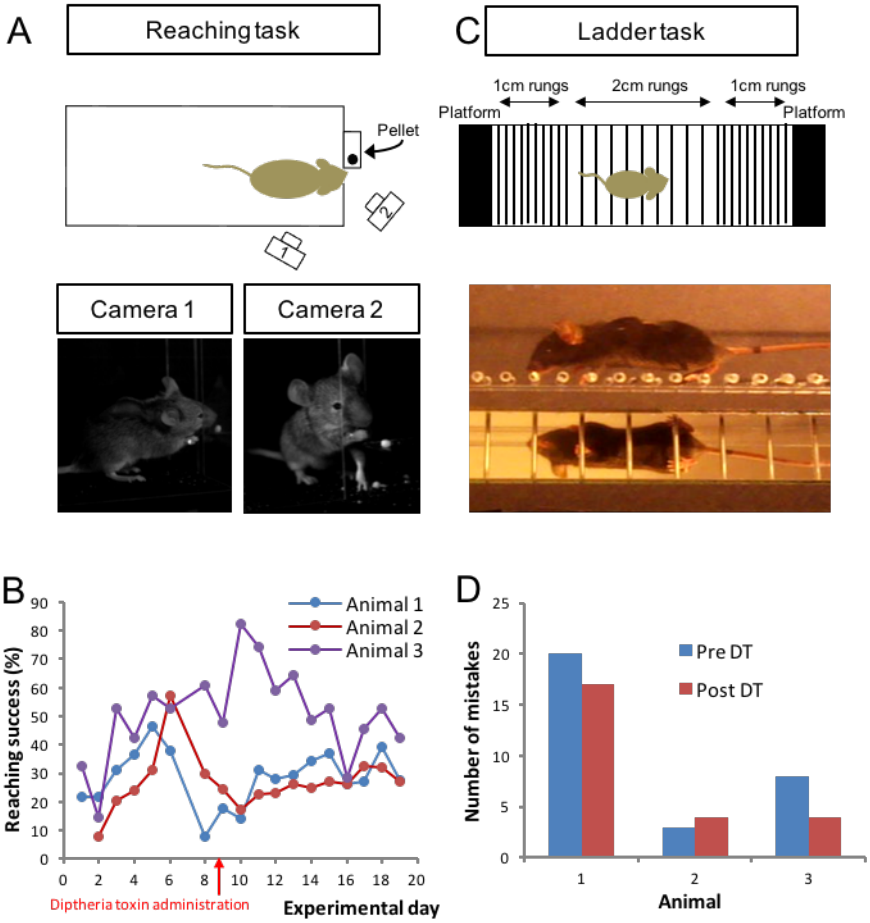

Figure 3.5: No overt change in motor behaviour following ablation of cervical Pitx2 cells. Pitx2+ cells were ablated in the area of the spinal cord controlling right forepaw movement by injecting a Cre-dependent adeno-associated virus containing diptheria toxin receptor, directly into segments T1-C3 of the spinal cord of Pitx2::Cre adult mice. The receptor is activated and Pitx2 cells ablated using diptheria toxin, so the same mice could be tested before and after Pitx2 cell ablation. There was no significant difference in the success of the mice in reaching **(A, B)** or ladder walking **(C, D)** with the right forepaw after diptheria toxin administation.

DISCUSSION

Much is known about the anatomy of the C bouton synapse and several theories have been proposed to explain the mechanisms underlying C bouton signalling. However, the evidence supporting these proposed mechanisms is all indirect. The aim of the experiments presented here, therefore, was to develop a method to directly investigate the C bouton synapse, and to study the behavioural impact of cholinergic transmission at these synapses in the mouse spinal cord.

Taken together, these results show that selective expression of channelrhodopsin in Pitx2+ interneurons can be achieved using surgical procedures to insert a Cre-dependent virus containing channelrhodopsin directly into the spinal cord of neonatal Pitx2::Cre mice. Blue light can then be used to selectively depolarise Pitx2+ cells in isolated spinal cord preparations, and induce, or increase the frequency of, action potential firing. In the new *in vitro* spinal cord preparation established in this chapter, it is then possible to perform whole-cell patch clamp recordings from motoneurons while blue light is directed at the whole Pitx2 cell population.

Depolarising Pitx2+ cells in this way caused a 50-100pA reduction in the current needed to induce firing in 5 out of 16 motoneurons studied. A change in frequency current relationships was observed in 2 out of 16 motoneurons. Overall, in motoneurons in which changes in output were observed, depolarisation of Pitx2+ cells caused an increase in the frequency of motoneuron firing at low current input and a decrease in the frequency of firing during high levels of current input. There was no change in AHP amplitude in any of the 16 motoneurons.

Based on previous research it was hypothesised that firing of Pitx2+ cells leads to an increase in motoneuron output by activating m2 receptors at C bouton synapses. It has been shown that activation of m2 receptors on motoneurons closes SK channels and causes a reduction in the size of the AHP, which increases the frequency of motoneuron firing (Miles et al., 2007). This regulation in motoneuron excitability is thought to contribute to increases in muscle activation required when shifting from activities involving lower level muscle

contraction to those requiring strong contractions, e.g. walking to swimming (Zagoraiou et al., 2009).

In this study, a reduction in rheobase and an increase in firing frequency at low levels of current input during activation of Pitx2+ cells were observed in a small proportion of motoneurons (Figure 3.4). These results support an increase in excitability in some motoneurons caused by Pitx2+ cell activity. However, no change in the amplitude of motoneuron AHP was observed during activation of Pitx2+ cells. We also saw no response to Pitx2+ cell activation in 11 out of 16 motoneurons. It may be that the mechanism of Pitx2+ cell communication to motoneurons is different to the previously proposed model, but it may be that problems with the technically challenging experimental methodology employed did not allow the full extent of Pitx2+ cell modulation of motoneurons to be revealed.

Firstly, the surgical methods used to infect Pitx2+ cells involved precise injections in mice one to two days old being performed by hand, so it is difficult to be certain of consistency in both the location and volume of virus injected. Responses to light did vary between cells, and it is difficult to test how much of this variation may come from small differences in surgical procedures. The success of light activation of neurons varied from preparation to preparation. In some preparations all Pitx2+ interneurons tested responded to light, in others only a subset of Pitx2+ interneurons responded, while in some preparations no response to light was observed in Pitx2+ cells or motoneurons. This may be due to the virus infecting none, some or many Pitx2+ cells in different preparations even when the same protocol is used. Similarly, Pitx2 is known to induce Cre somewhat unreliably (Zagoraiou et al., 2009), so even with successful injections, the virus may not be expressed in all Pitx2+ cells. The rate of expression of virally-delivered channelrhodopsin-YFP fusion protein in Pitx2+ interneurons could be investigated using anatomical experiments. Tissue from Pitx2Cre::ROSATDTom mice could be treated using anti-GFP antibodies to visualise the expression of fluorescent protein derived from genes delivered by the virus. This type of experiment was attempted on tissue that had been used for electrophysiological recordings but it was not possible to get high quality images

in preparations that had been used for experiments for a full day, so fresh tissue fixed soon after dissection and slicing would need to be used. Going forward, detailed analysis of the consistency of surgery would be useful, as well as more information about the spread of the virus and the proportion of Pitx2+ cells successfully transfected.

Secondly, even with good viral uptake, there may not be enough ChR expressed on the plasma membrane in all Pitx2+ cells to depolarise them enough to activate C boutons sufficiently to have an effect on motoneurons. It is likely that each Pitx2+ cell connects to more than one motoneuron and each motoneuron receives connections from more than one Pitx2+ cell. It is known that motoneurons outnumber Pitx2+ cells by a factor of roughly 10:1 in the lumbar spinal cord of mice (Zagoraiou et al., 2009) and Pitx2+ cell projections are likely to extend through multiple sections of the spinal cord (Stepien et al., 2010). Therefore, the few motoneurons that were seen to respond to light may be the ones that happened to be contacted by sufficient C boutons from those Pitx2+ cells with good ChR expression.

Thirdly, even with good ChR expression, the light source used to stimulate the entire population of Pitx2+ cells may not have been sufficient to excite the necessary number of cells. Many Pitx2+ cells responded to light from the wide light source, but the depolarisation produced by lighting the whole preparation was smaller than that produced through the objective when focussed on a single cell. Light from the wide light source may be insufficient to induce firing in the majority of silent Pitx2+ cells, and so insufficient to effect changes in motoneurons. In order to understand how the two light sources differ in their effect on the tissue, it would be useful to measure the intensity of each light source and how well they penetrate the tissue. Light directed through the objective may saturate ChR receptors on Pitx2+ cells, resulting in a large depolarisation. Conversely, the side-mounted light source may be more subject to scattering of light and so ChR may not be maximally activated, resulting in a reduced depolarisation of cells. It is known that in nervous system tissue, blue light is more prone to scattering than light with longer wavelengths (Jacques, 2013), and so a lower intensity starting source could make a big difference to the amount of activation of ChR receptors. If the side-

mounted light source had low starting intensity, this could have been the cause of the failure to induce Pitx2+ cell depolarisation sufficient to affect motoneuron activity.

Fourthly, it is possible that connections between Pitx2+ cells and motoneurons are disrupted or lost in the diagonally sliced spinal cord preparation. Little is known about the path taken by projections from Pitx2+ cells, but it has been postulated that 50% project ipsilaterally and 50% project bilaterally (Stepien et al., 2010). It is also thought that some Pitx2+ cell projections extend several spinal segments in the rostral-causal axis, but the anatomical position of these projections is unknown (Stepien et al., 2010). Other V0 interneurons are known to exhibit commissural axon trajectories (Lanuza et al., 2004) and are thought to travel in the ventral funiculus (Goulding, 2009). If this is also true of Pitx2+ cell axons, they should be unaffected by diagonal slicing, but because the exact position is not known, it could be that some connections between Pitx2+ cells and motoneurons were severed in diagonally-sliced preparations. It was not possible to standardise the amount of tissue removed from diagonally-sliced preparations, so there was some variation between spinal preparations. The preparations in which no motoneurons showed any response to light could be those where a large amount of tissue had been removed, cutting the connections between Pitx2+ cells and motoneurons in the process. This was not tested in these experiments but the amount of tissue remaining could be measured and compared across preparations using post-hoc fixing and imaging of transverse slices of tissue from diagonal preparations. Less tissue remaining in preparations where no light response was observed would support a loss of connections in these preparations. Ultimately it will be important to trace the axons of Pitx2+ cells all the way to motoneurons in order to give insight into the usefulness of the diagonal preparation and the margin for error when slicing and to reach a more complete understanding of the C bouton system. An attempt was made to trace Pitx2+ cell axons by attaching a pipette, filled with Alexa Fluor dye, to Pitx2+ cells and leaving in whole-cell patch clamp-mode for around 1 hour. The cell bodies of these cells were clearly visible both during filling and after fixing and imaging, but it was not possible to visualise Pitx2+ cell axons using this method. This may be because Pitx2+ cell axons are exceptionally

narrow, and a potentially better method would be to fill cells using horseradish peroxidase as it is a small molecule (44,174 Daltons compared to 66,000 Daltons for AlexaFluor avadin). Low titre virus injections could also be used so that only a small number of cells would be visible, potentially making it easier to trace the axons belonging to these cells. Similarly, Brainbow neuroimaging (Livet et al., 2007) could be used to mark individual Pitx2+ cells with a distinctive colour, again potentially making it easier to trace the axon patterns of single cells. If the axons were found to follow a path that may be interrupted by the diagonal slicing, the preparation could be changed to a hemisect laid ventral side up, with a side mounted light to activate Pitx2+ cells, and with the ventral white matter removed or disrupted to access motoneurons.

Lastly, depolarisation of Pitx2+ cells using ChR may not affect motoneuron activity because Pitx2+ cells are already depolarised and active and further depolarisation may not lead to large enough changes in their activity or could in fact reduce their activity due to a depolarising block. It is known that Pitx2+ cells are active at rest, and even though it is known that they can be pharmacologically driven to increase tonic firing rate, they may, as a population, be close to maximum activity at rest (Zagoraiou et al., 2009). This would mean that depolarisation would not substantially increase the output of the Pitx2+ cell population as a whole, and so any effect on motoneuron activity might be small and hard to reveal. It may be that turning down Pitx2+ cell activity is how they exert a modulatory effect. Recordings from Pitx2+ cells presented in this work appear to show that blue light clearly increases action potential firing in Pitx2+ cells, but patch-clamp recording is an invasive technique and the mechanical stress could cause some cells which normally fire action potentials at rest to become silent. Similarly, depolarisation could actually inhibit Pitx2+ cell activity. When cells depolarise, sodium channel inactivation gates will be closed. If cells do not hyperpolarise enough between spikes for inactivation to be removed, there may be insufficient sodium current available to sustain action potential firing. Continuous light application could therefore cause cells to be depolarised such that they cease to fire, particularly in cells with high levels of virus and therefore ChR expression. Depending on the number of cells with high ChR expression, some

Pitx2+ cells could be being inhibited rather than excited by blue light, which could explain the differing responses seen in motoneurons. Again, Pitx2+ cell recordings in this study do not appear to support this theory, as no loss of firing was observed during light application. Pitx2+ cells were observed during sustained light applications of several minutes (much longer than any protocol performed while recording from motoneurons), and no desensitisation of response could be detected. In addition, pulses of blue light were used to excite Pitx2+ cells in some cases. If Pitx2+ cells were subject to a depolarising block, pulses of blue light should ensure that sufficient hyperpolarisation was induced to remove inactivation from sodium channels and therefore sustain action potential firing in Pitx2+ cells. No obvious difference between the effects of this method and sustained light were observed, suggesting that the activation of Pitx2+ cells using continuous light does not cause a depolarising block.

Despite these caveats, it may be that the results presented here do show the full extent of motoneuron responses to Pitx2+ cell activation. The reason a response is not observed in all motoneurons could be that only a subpopulation is affected by Pitx2+ cell depolarisation. It was found that stimulation of Pitx2+ cells caused a reduction in the current needed to induce firing in 5 out of 16 motoneurons. It is known that m2 receptors cluster postsynaptically at C bouton synapses and it has previously been shown that activation of muscarinic receptors on motoneurons causes an increase in the excitability of motoneurons by reducing AHP amplitude (Miles et al., 2007). However, in this case no change in AHP amplitude was observed in any motoneurons, and so other potential mechanisms need to be explored. Kv2.1 channels are known to be clustered postsynaptically at the C bouton synapse, but as yet their role is unknown. It has been demonstrated that phosphorylation of Kv2.1 channels causes a rightward shift in their activation curve (Murakoshi et al., 1997) which has been postulated to increase excitability of motoneurons (Wilson et al., 2004). In the experiments presented here, depolarisation of Pitx2+ cells and subsequent acetylcholine release leading to m2 receptor activation may cause phosphorylation of Kv2.1 channels. Delayed opening of Kv2.1 channels

would in turn lead to reduced outward potassium current, resulting in a more depolarised resting membrane potential in motoneurons and therefore increasing their excitability.

It was also found that 2 out of 16 motoneurons showed a reduction in the slope of frequency current relationships in response to Pitx2+ cell activation. Kv2.1 channels have been shown to play a role in regulating excitability of hippocampal neurons, where action potential repolarisation is prolonged during high frequency stimulation (Du et al., 2000). If m2 receptor activation phosphorylates Kv2.1 channels, this could suppress their opening and extend the time it takes for repolarisation after action potential firing. This would therefore reduce frequency of firing at high levels of current input. The involvement of Kv2.1 channels could therefore explain both the increased excitability at low level stimulation and reduced excitability at high level stimulation. This reduction in slope of the frequency current relationship and therefore reduced range of firing of motoneurons may be a protective mechanism. Increasing range of firing is normally thought to be a good thing because it allows for a greater range of outputs. However, it is known that hyperexcitability is a factor in neurodegenerative diseases such as motoneuron disease (Boillee et al., 2006, Wainger et al., 2014). Single cell recordings also show that motoneurons which are forced to sustain firing often become unhealthy very rapidly (de Carvalho et al., 2014). Pitx2+ cells may therefore play a role in controlling motoneuron activity so that action potential firing is prevented from reaching harmful frequencies.

In addition, no difference was observed in the success of reaching or ladder walking in mice in which Pitx2+ cells had been ablated. Previous work suggests that Pitx2+ cells are involved in ramping up muscle activation when moving from walking to swimming (Zagoraiou et al., 2009). It therefore seems likely that Pitx2+ cells are involved in upregulating motoneuron activity in situations where maximal muscle contractions are required, rather than fine-tuning intricate movements. It would be interesting to use this information to develop tasks that could specifically test involuntary movements requiring high level motoneuron activity, and then assess the performance of mice on these tasks before and after Pitx2+ cell ablation. There are at

least two interesting lines of work that could be pursued. Firstly, to test whether Pitx2+ cells are involved in maximally contracting muscles, performance could be tested in tasks that require strength, such as a hanging or weight moving tasks. Secondly, to test whether Pitx2+ cells are involved in escape behaviour rather than goal directed movements, performance could be tested in tasks that require unexpected muscle contractions, such as a balance beam that could be perturbed. This would require mice to increase motoneuron activity suddenly, to correct for the unexpected change. These tasks would help to provide insight into the specific role of Pitx2+ cells, known to be involved in upregulating motoneuron activity for swimming (Zagoraiou et al., 2009) but, as shown in this chapter, clearly not necessary for all motor functions.

Overall, the work in this chapter contributes to understanding the specialised nature of Pitx2+ cells, and highlights their specific role in task-dependent modulation of motoneuron excitability. There is much that remains uncertain about Pitx2+ cells and the C bouton synapse, but this work shows that Pitx2+ cell activity can affect the excitability of motoneurons, and has developed a methodology that can be used to investigate this further. It has also provided a platform for more informed studies into the behavioural role of Pitx2+ cells.

CONCLUDING REMARKS

The work described in this thesis has explored how purinergic and cholinergic modulation affects the output of spinal motor networks in neonatal mice. Firstly, the effects of purinergic signalling on spinal motor networks has been investigated using recordings taken from ventral roots of the mouse spinal cord as well as from individual cells within the cord. Secondly, the role of a small population of cholinergic interneurons in controlling motoneuron output has been investigated using whole-cell patch-clamp recordings coupled with channelrhodopsin-mediated activation of cholinergic Pitx2+ cells which synapse on motoneurons. The behavioural role of cholinergic inputs provided by Pitx2+ cells has also been studied by testing the performance of mice in reaching and ladder walking tests before and after Pitx2+ cell ablation.

Application of adenosine to *in vitro* isolated spinal cord preparations in which locomotor-like rhythmic activity was induced pharmacologically caused a reduction in the frequency of bursts of activity recorded from ventral roots, without affecting burst amplitude. Application of the adenosine receptor antagonist theophylline caused an increase in burst frequency, suggesting that endogenous adenosine slows locomotor output. A similar increase in burst frequency was observed upon application of the adenosine A_1 receptor antagonist DPCPX but not during application of the A_{2A} adenosine receptor agonist SCH58261, indicating that the effects of adenosine on locomotor-related output are mediated by A_1 receptors. Endogenous adenosine appears to be glial-derived as no effect of theophylline was observed when glia function was perturbed using the glial toxins fluoroacetate or methionine sulfoximine. Application of ATP showed similar effects on network output to those observed upon application of adenosine, but no effect of ATP was observed when A_1 receptors were blocked, even when ATP breakdown was inhibited. It therefore seems likely that ATP is released from glia cells before being broken down to adenosine which binds to A_1 receptors and produces the depressive effect on locomotor network output.

In order to reveal the mechanisms underlying this A_1 receptor-mediated effect of adenosine, single cell recordings were performed in *in vitro* spinal cord slice preparations.

Whole-cell patch-clamp recordings showed that motoneurons and ventral horn interneurons in the spinal cord respond differently to bath application of adenosine. Adenosine hyperpolarised ventral horn interneurons and caused a reversible reduction in input resistance, suggesting the opening of a channel. The reversal potential of the adenosine-induced current was near to that of potassium and so the hyperpolarisation of ventral horn interneurons by adenosine was attributed to the opening of leak potassium channels. In addition, adenosine reduces the frequency and amplitude of both inhibitory and excitatory synaptic inputs to ventral horn interneurons. This reduction in synaptic inputs was blocked by the A_1 receptor antagonist DPCPX but not by the A_{2A} receptor antagonist SCH58261 suggesting that reduction of synaptic inputs is mediated by A_1 receptors. Recordings performed in the presence of TTX showed that adenosine also reduces the frequency but does not affect the amplitude of miniature postsynaptic currents in ventral horn interneurons. Motoneurons, on the other hand, show a reversible depolarisation in response to bath application of adenosine, accompanied by an increase in input resistance. Adenosine causes a reduction in the frequency and amplitude of synaptic inputs to motoneurons that is similar to that observed in ventral horn interneurons. This reduction in synaptic inputs was again blocked by DPCPX but not SCH58261, suggesting that it is mediated by A_1 adenosine receptors. However, adenosine does not change miniature postsynaptic currents in motoneurons, and so does not appear to affect fast synaptic transmission between last-order interneurons and motoneurons. It is therefore proposed that as well as modulating the intrinsic properties of ventral horn interneurons and motoneurons, adenosine also reduces the probability of transmitter release from interneurons via pre-synaptic A_1 receptors.

Cholinergic modulation of the neonatal mouse spinal cord was also investigated. Pitx2+ cells, known to be the source of cholinergic C-bouton inputs to motoneurons, were transfected with channelrhodopsin and blue light was used to activate the Pitx2+ cell population. Pitx2+ cell activation was found to reduce the current needed to induce firing in a small proportion of spinal motoneurons, making them more excitable. Increasing Pitx2+ cell activity also caused a

reduction in the slope of frequency-current relationships in a small number of motoneurons, reducing the overall range of firing in these cells. Activation of Pitx2+ cells therefore appears to modulate the activity of some motoneurons via C bouton synapses. The role of Pit2+ cells in behaviour was also investigated. The performance of mice in reaching and ladder walking tasks was measured before and after Pitx2+ cell ablation. No difference was observed in the success of, or pattern of movement involved in, reaching for and picking up a small food pellet, nor in the number of mistakes recorded when walking across a ladder, before and after Pitx2+ cell ablation. These results suggest that although Pitx2+ cell activity can affect motoneuron output, this may not be physiologically significant in tasks that involve fine motor control requiring precise movements.

Taken together, these results extend our knowledge about modulation of spinal motor networks. The results from ventral root recordings and whole-cell patch-clamp experiments suggest that adenosine hyperpolarises interneurons and reduces synaptic activity to slow the frequency of whole network output. However, it is also clear that adenosine acts directly on motoneurons, causing them to depolarise and potentially allowing a greater strength of muscle contraction. Adenosine therefore appears to be involved in controlling locomotor output, possibly to ensure the underlying locomotor rhythm remains within a healthy frequency range. In normal conditions, this may simply mean altering locomotor output in response to environmental stimuli such as terrain. However, it may become particularly important in situations requiring unusual or particularly high levels of motor output, such as escaping predators or catching prey. In these situations, it is important that locomotor output is kept within a healthy range, no matter what external, sensory, or hormonal stimuli are being received. Similarly, if there is a lack of sensory feedback due to damage or malfunction, it is important to have a method of moderating output within networks generating motion. Over-excitation is known to be a cause of damage to the nervous system, and adenosine may play a role in ensuring that whatever the situation, locomotor activity is maintained at a healthy level.

The results from the electrophysiological and behavioural experiments investigating the role of Pitx2+ cells show that Pitx2+ cell activity can affect motoneuron output, although this is not physiologically relevant during all behaviours. Pitx2+ cells are likely to have task-specific roles in modulating motoneuron output. It is known that in the absence of Pitx2+ cells, muscle contractions when changing from walking to swimming are not as greatly increased (Zagoraiou et al., 2009). Pitx2+ cells may therefore play a role in movements involving gross motor movements rather than intricate movements requiring fine motor control. This would fit with the observation that C bouton numbers appear to be greater on motoneurons innervating large proximal muscles than those innervating small distal muscles (Hellstrom et al., 2003). There are many interneuron subtypes in the spinal cord and so information about functionally distinct neuronal subtypes is a valuable in trying to understand locomotor networks as a whole. The new data provided by the research presented in this thesis about the role of Pitx2+ cells contributes to an overall understanding of the networks controlling mammalian locomotion.

Overall, this thesis provides novel insight into the role of purinergic and cholinergic modulation in the neonatal mouse spinal cord. It contributes towards our understanding of basic motor control by offering new information about the roles of adenosine and Pitx2+ cells, a small part of the hugely complicated and dynamic effects of modulation of basic networks. Because modulation is now known to be a fundamental aspect of producing normal motor output, this work may also have important implications in the unhealthy nervous system. It is hoped that the advances in understanding of purinergic and cholinergic modulation that this thesis delivers may provide a platform for the development of new strategies to combat diseases of the nervous system that affect motor control.

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