

**THE AREA POSTREMA: A POTENTIAL SITE FOR CIRCADIAN REGULATION BY  
PROKINETICIN 2**

by

Matthew Victor Ingves

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## Abstract

Little is known regarding the neurophysiological mechanisms by which the neuropeptide prokineticin 2 (PK2) regulates circadian rhythms. Using whole-cell electrophysiology, we have investigated a potential role for regulation of neuronal excitability by PK2 on neurons of the area postrema (AP), a medullary structure known to influence autonomic processes in the central nervous system.

In current-clamp recordings, focal application of 1 $\mu$ M PK2 reversibly influenced the excitability of the majority of dissociated AP cells tested, producing both depolarizations (38%) and hyperpolarizations (28%) in a concentration-dependent manner. Slow voltage ramps and ion substitution experiments revealed a PK2-induced Cl<sup>-</sup> current was responsible for membrane depolarization, while hyperpolarizations were the result of inhibition of an inwardly rectifying non-selective cation current. In contrast to these differential effects on membrane potential, nearly all neurons that displayed spontaneous activity responded to PK2 with a decrease in spike frequency. These observations are in accordance with voltage-clamp experiments showing that PK2 caused a leftward shift in Na<sup>+</sup> channel activation and inactivation gating.

Lastly, using *post hoc* single cell RT-PCR technology, we have shown that 7 out of 10 AP neurons depolarized by PK2 were enkephalin-expressing cells. The observed actions on enkephalin neurons indicate PK2 may have specific inhibitory actions on this population of neurons in the AP acting to reduce their sensitivity to incoming signals. These data suggest that PK2 regulates the level of AP neuronal excitability and may impart a circadian influence on AP autonomic control.

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## List of Abbreviations

3V.....	Third Ventricle
4V.....	Fourth Ventricle
aCSF.....	Artificial Cerebrospinal Fluid
AdipoR.....	Adiponectin Receptor
AP.....	Area Postrema
BBB.....	Blood Brain Barrier
CART.....	Cocaine- and Amphetamine-Related Transcript
CC.....	Central Canal
CCK.....	Cholecystokinin
CNS.....	Central Nervous System
Cry.....	Cryptochrome Gene
CVO.....	Circumventricular Organ
DMH.....	Dorsomedial Hypothalamus
DMNV.....	Dorsal Motor Nucleus of the Vagus
DTN.....	Dorsal Tegmental Nucleus
$E_{Cl^-}$ .....	Chloride Equilibrium Potential
ENK.....	Enkephalin
GABA.....	$\gamma$ -Aminobutyric Acid
GAD67.....	Glutamate Decarboxylase 67
GAPDH.....	Glyceraldehyde 3-Phosphate Dehydrogenase
GI.....	Gastrointestinal
GnRH.....	Gonadotropin-Releasing Hormone
IL-1.....	Interleukin-1
LPBN.....	Lateral Parabrachial Nucleus
MAPK.....	Mitogen Activated Protein Kinase
NA.....	Nucleus Ambiguus
NSCC.....	Non-Selective Cation Current
NTS.....	Nucleus Tractus Solitarius
OVLT.....	Organum Vasculosum of the Lamina Terminalis
Per.....	Period Gene
PK.....	Prokineticin
PKR.....	Prokineticin Receptor
PVN.....	Paraventricular Nucleus of the Hypothalamus
SCN.....	Suprachiasmatic Nucleus of the Hypothalamus
scRT-PCR.....	Single Cell Reverse Transcriptase Polymerase Chain Reaction
SFO.....	Subfornical Organ
TH.....	Tyrosine Hydroxylase
VGLUT2.....	Vesicular Glutamate Transporter 2

## **Chapter 1: Introduction**

### **1.1 Circadian regulation by the suprachiasmatic nucleus**

Many physiological and behavioural processes follow daily and seasonal rhythms that permit the organism to function in a circadian manner in preparation to respond to environmental challenges that are critical to survival. These include oscillations in neuroendocrine and autonomic activity controlling the regulation of body temperature, metabolism, locomotor activity, and cardiovascular function. Circadian rhythms are governed by a master pacemaker in the anterior hypothalamus of the mammalian brain known as the suprachiasmatic nucleus (SCN), such that light cues synchronize neuronal electrical activity and gene expression to coordinate circadian output (Reppert & Weaver, 2002). An endogenous master clock was first proposed following the finding that animals maintained persistent 24 hour physiological rhythms in the absence of environmental cues. Anatomical evidence describing retinal projections to the SCN was a breakthrough finding that implicated this hypothalamic region as responsible for circadian timekeeping (Hendrickson *et al.*, 1972). Furthermore, damage to the SCN results in disruption of circadian function, confirming a role for this nucleus as the central clock.

Intrinsically, individual neurons within the SCN generate their own autonomous rhythms in electrical activity, cellular metabolism, and gene expression independent of environmental cues (Reppert & Weaver, 2002). As a result, researchers identified endogenous molecular machinery in the form of circadian clock genes using circadian mutants in fruit fly and rodent models (Reppert & Weaver, 2002;Hastings *et al.*, 2003). The molecular mechanism involves a 24

hour self-sustaining autoregulatory delayed feedback loop in which the protein products of three *period (Per)* genes and two *cryptochrome (Cry)* genes negatively regulate their own transcription. Transcription of the *Per* and *Cry* genes is driven by heteromeric complexes formed by Clock and Bmal1 proteins that act on their target genes at E-box regulatory DNA-binding sequences. Protein products of *Per* and *Cry* peak at the end of circadian day, accumulate in SCN neurons, and suppress their own expression. This cycle of clock gene expression therefore repeats itself daily in an inexhaustible loop as an internal framework for SCN circadian regulation.

The SCN relies on environmental light cues to synchronize circadian timekeeping of electrical activity, gene expression, and synaptic output. This is accomplished by photoreceptive retinal ganglion cells that contain the photosensitive pigment, melanopsin, and direct glutamatergic projections that increase neuronal electrical firing and induce clock gene expression in the core region of the SCN (Qiu *et al.*, 2005;Hastings & Herzog, 2004). Neuronal activation spreads to the outer shell region of the SCN, leading to further clock gene expression likely via synaptic transmission from GABA, vasoactive intestinal polypeptide, and gastrin-releasing peptide containing neurons (Hastings & Herzog, 2004). The main SCN efferent fibres driving circadian signalling pass through the subparaventricular zone and dorsomedial hypothalamus, major relay centres in the brain, to synapse with nuclei controlling sleep, arousal, and autonomic function (Saper *et al.*, 2005).

A major limitation to our current understanding of the proposed circadian communication by synaptic neurotransmission from the SCN arises from our still minimal knowledge of the neurotransmitters/neuromodulators through which SCN neurons

communicate this information to the multitude of brain centres critical to such circadian regulation. The question thus remains, how does the SCN, a relatively small nucleus in the hypothalamus, organize circadian physiology and behaviour at central and peripheral sites? Studies demonstrating that transplantation of the SCN to the brain restores circadian activity independent of physical connectivity between SCN axonal projections and target nuclei, suggested the alternative explanation that circadian output of behavioural and physiological processes can also be regulated by diffusible paracrine factors produced by the SCN (Silver *et al.*, 1996). Prokineticin 2 has been suggested to be one such factor secreted by the SCN that has been shown to play important roles in the transmission of circadian signals (Cheng *et al.*, 2002).

## **1.2 Prokineticins**

### **Discovery**

The description of the prokineticins dates back to 1980 and the identification of a snake venom protein from the black mamba, later named mamba intestinal toxin 1, that together with the frog skin protein homologue Bv8, stimulates gastrointestinal (GI) smooth muscle contraction (Schweitz *et al.*, 1999; Mollay *et al.*, 1999; Joubert & Strydom, 1980). The mammalian homologue prokineticins consist of two cysteine-rich secreted proteins, prokineticin 1 (PK1) and prokineticin 2 (PK2), that are 86 and 81 amino acids, respectively (Li *et al.*, 2001; LeCouter *et al.*, 2001). The prokineticins share conserved amino acid sequences with their non-mammalian homologues, including an N-terminal hexapeptide sequence (AVITGA) and five disulfide bonds that were demonstrated by mutagenesis to be critical for protein structure and function (Bullock *et al.*, 2004).

Three independent groups reported the molecular identification of two G protein-coupled receptors, PKR1 and PKR2, that share 87% sequence homology and bind prokineticins non-selectively with high affinity, and although PKR1 and PKR2 share a high degree of sequence conservation, their genes are present on different chromosomes (Lin *et al.*, 2002a; Masuda *et al.*, 2002; Soga *et al.*, 2002). Prokineticins activate receptors in a concentration-dependent manner, leading to G<sub>q</sub>-coupled mobilization of intracellular Ca<sup>2+</sup> through phosphoinositide turnover pathways, phosphorylation of p44/42 mitogen activated protein kinase (MAPK), and activation of protein kinase C $\epsilon$  (Lin *et al.*, 2002a; Masuda *et al.*, 2002; Vellani *et al.*, 2006). Both prokineticin receptors are distributed in peripheral tissues; however, PKR1 is the most abundantly expressed and is present in the GI tract, lungs, cardiovascular system, and endocrine tissues. Within the central nervous system (CNS), PKR2 is expressed on neurons throughout the brain, with *in situ* hybridization showing prominent expression in primary SCN output targets, including autonomic nuclei in the hypothalamus and medulla, as well as two circumventricular organs, the area postrema and subfornical organ (Cheng *et al.*, 2002; Cheng *et al.*, 2006).

### **Noncircadian functions of prokineticins**

In addition to the initial description of prokineticin expression in the GI tract and stimulation of smooth muscle contractility, a number of studies have examined non-circadian functions of prokineticins in mammals, with the suggestion of important roles in angiogenesis, pain perception, the function and development of blood cells, and neuronal development (Zhou & Meidan, 2008).

An important role for prokineticins in angiogenesis has been established in tissue growth and survival. PK1 is also known as endocrine gland-derived vascular endothelial growth

factor for its ability to function as an angiogenic mitogen for endothelial cells derived in endocrine organs (LeCouter *et al.*, 2003b;LeCouter *et al.*, 2001). The prokineticins and their receptors are expressed on endothelial cells of vascular tissues; more specifically, PKR1 likely acts to enhance cell proliferation and survival, whereas PKR2 is implicated in regulating endothelial cell permeability (LeCouter *et al.*, 2003a;Lin *et al.*, 2002b;Kisliouk *et al.*, 2003;Podlovni *et al.*, 2006). In the disease state, PK1 is highly expressed in many malignant tumours and is thought to be partly responsible for neoplastic angiogenesis (Zhang *et al.*, 2003;Pasquali *et al.*, 2006;Ferrara *et al.*, 2003;Monnier *et al.*, 2008a;Morales *et al.*, 2008;Shojaei *et al.*, 2007). In addition, transgenic PKR2 overexpression in cardiomyocytes indicates that PKR2 signalling contributes to hypertrophy and impaired vascular integrity (Urayama *et al.*, 2009). Contrastingly, PKR1 activation protects the heart against myocardial infarction and may promote neovasculogenesis, indicating the prokineticin receptors are emerging as potentially important regulators of myocardial repair and vessel growth (Urayama *et al.*, 2008).

With regard to its involvement in pain perception, systemic injection of PK2 into the whole animal results in hyperalgesia by enhancing the activity of the transient receptor potential vanilloid 1 channel, a non-selective cation conductance that is activated by painful chemical and thermal stimuli (Vellani *et al.*, 2006;Mollay *et al.*, 1999). This action is mediated by mobilization of intracellular  $Ca^{2+}$  in dorsal root ganglion neurons where prokineticin receptors are colocalized with transient receptor potential vanilloid 1 channels (Negri *et al.*, 2006;Negri *et al.*, 2002). Prokineticins and their receptors are also expressed in developing and mature leukocytes, as well as at inflammatory sites. Because PK2 stimulates cytokine production and functions as a chemoattractant for monocyte recruitment, the peptide is also implicated in

inflammatory nociception (LeCouter *et al.*, 2004;Dorsch *et al.*, 2005;Martucci *et al.*, 2006;Monnier *et al.*, 2008b).

Prokineticin signalling has been indirectly linked to activation of the reproductive axis via a contribution to neuronal development. The olfactory bulb is a unique area in the mammalian brain where adult neurogenesis persists and where PK2 is thought to act as a chemoattractant for migrating progenitor cells originating in the subventricular zone (Luskin, 1993;Puverel *et al.*, 2009). Proper olfactory bulb development is necessary for normal gonadotropin-releasing hormone (GnRH) neuronal migration and homozygous loss of function mutations in PK2 signalling result in dysfunctional olfactory bulb neurogenesis, a lack of GnRH due to the absence of hypothalamic GnRH neurons, and severe atrophy of the reproductive system (Leroy *et al.*, 2008;Abreu *et al.*, 2008;Matsumoto *et al.*, 2006;Dode *et al.*, 2006;Ng *et al.*, 2005;Prosser *et al.*, 2007a). The resulting human condition is Kallmann syndrome, characterized by hypogonadotropic hypogonadism and anosmia (Pitteloud *et al.*, 2007). It is thus clear that in addition to their roles in circadian signalling, prokineticins have a broad array of functions associated with the regulation of additional physiological systems.

### **Circadian functions of prokineticins**

The SCN in the anterior hypothalamus is the master pacemaker that controls circadian changes in physiology and behaviour in mammals (Reppert & Weaver, 2002). An understanding of circadian clock gene expression has developed; however, elucidating the mechanisms through which the SCN influences activity in other brain regions to control circadian behaviour is essential for an integrated understanding of how the SCN regulates different circadian patterns in a variety of hypothalamic and medullary autonomic control centres. The available literature

suggests that PK2 is an ideal circadian output molecule that may play critical roles as a mediator utilised by the SCN to control daily behaviour and neuroendocrine function. Within the brain, PK2 mRNA is expressed in the olfactory bulb, nucleus accumbens, islands of Calleja, medial amygdala, Edinger-Westphal nucleus, and nucleus tractus solitarius (Negri *et al.*, 2004; Cheng *et al.*, 2006). The hypothalamic arcuate nucleus and medial preoptic area also contain PK2 mRNA, while levels are highly expressed in the SCN. PK2 mRNA expression within the SCN oscillates over 24 hours such that daytime levels are 50-fold higher than those measured at night (Cheng *et al.*, 2002; Masumoto *et al.*, 2006). Rhythmic PK2 expression is under the control of the endogenous SCN clock genes, *Clock* and *Bmal1*, and is maintained in light entrained and free-running animals (Cheng *et al.*, 2002). Furthermore, PK2 oscillation is abolished in mutant mice lacking the clock genes, *Clock* and *Cry* (Cheng *et al.*, 2002). A low amplitude PK2 oscillation is detectable in *Cry* knockout mice; however, this rhythm only occurs under light/dark conditions, suggesting light can modulate PK2 expression independent of the functional circadian clock (Cheng *et al.*, 2005). Because PKR2 is expressed in the SCN, PK2 also acts as a positive feedback signal on its own transcription within the SCN, but has no influence on clock gene expression (Cheng *et al.*, 2002; Li *et al.*, 2006a). Therefore, in addition to light entrainment, PK2 may act locally within the SCN to synchronize circadian output.

The PK2 system has been studied in diurnal mammals to determine if differences occur in either peptide or receptor expression in comparison to nocturnal species. The African grass rat, *Arvicanthis niloticus*, and Syrian hamster, *Mesocricetus auratus*, are two diurnal rodents that have been shown to have rhythmical PK2 mRNA expression in the SCN similar to nocturnal mammals, with peak levels evident during the day (Lambert *et al.*, 2005; Ji & Li, 2009). The amino

acid sequences are also homologous to both the rat and mouse PK2 peptide sequence (Lambert *et al.*, 2005; Ji & Li, 2009). Finally, PKR2 is expressed in the SCN throughout Syrian hamster development; however, the expression of PK2 is undetectable until postnatal day 3 and may represent the maturation process of the molecular SCN circadian clock (Ji & Li, 2009). According to these findings, neither PK2 nor its receptor expression seem to be the difference in determining daily activity patterns between diurnal and nocturnal species, rather the endogenous control over diurnality lies downstream of the SCN in these two species.

The effects of PK2 on behaviour have been investigated *in vivo* through delivery of PK2 into the brain, as well as using PK2 and PKR2 knockout mice. Inhibition of nocturnal locomotor activity and feeding behaviour occurs in rats following intracerebroventricular delivery of recombinant PK2 at times when endogenous PK2 levels are minimal (Cheng *et al.*, 2002; Negri *et al.*, 2004). Intracerebroventricular administration of PK2 also increases anxiety and depression-like behaviour and may have an additional role in the molecular link between circadian rhythms and mood regulation (Li *et al.*, 2009). Furthermore, mice lacking the PK2 gene show a reduction in these behaviours, and show impaired responses to new environments (Li *et al.*, 2009). The importance of PK2 in maintaining circadian rhythmicity is evident in the daily regulation of locomotor activity, sleep-wake cycles, body temperature, and glucocorticoid and glucose levels. All of these factors are compromised under light/dark and constant dark conditions in PK2 deficient animals, confirming this SCN output molecule is essential for maintaining robust circadian rhythms in key physiological processes (Li *et al.*, 2006a; Hu *et al.*, 2007). Similarly, the coordination of circadian behaviour and homeostasis is affected in PKR2 null mice, as sporadic bouts of torpor are observed and precise timing of nocturnal locomotor activity onset and

thermoregulation is disrupted (Prosser *et al.*, 2007b;Jethwa *et al.*, 2008). Interestingly, neither light entrainment nor cellular SCN timekeeping is dependent on PKR2.

The control of locomotor rhythms by the SCN can be overridden by food-entrained oscillators in the brain using sudden large changes in food availability (Gooley *et al.*, 2006). Consequently, rodents will feed at unusual periods in response to daytime restricted feeding, and gradually become more active in anticipation of food availability, known as food-anticipatory activity. Restricted feeding in PK2 knockout mice increases food-anticipatory activity, thus indicating that SCN control over locomotor activity is weakened in the absence of PK2 (Li *et al.*, 2006a). Lastly, disrupted circadian sleep and activity patterns are important pathological features of Huntington's disease as reductions in PK2 in the SCN have been reported in a transgenic model of Huntington's disease, an animal which interestingly also shows a complete disintegration of daily locomotor activity (Morton *et al.*, 2005). Thus there is a considerable body of evidence supporting the perspective that PK2 may be an important SCN-secreted peptide acting to convey circadian signalling to CNS nuclei.

The hypothalamus and medulla contain autonomic nuclei important in regulating circadian neuroendocrine and homeostatic processes. The paraventricular nucleus of the hypothalamus (PVN) and subfornical organ (SFO) are two such structures with important actions in cardiovascular function, the stress response, feeding and drinking behaviour, and reproduction (Swanson & Sawchenko, 1980;Cottrell & Ferguson, 2004;Ferguson *et al.*, 2008). The demonstrated expression of PKR2 in the PVN and SFO suggests that these structures likely play pivotal roles in the integration of circadian PK2 signalling (Cheng *et al.*, 2006).

*In vitro* electrophysiology has been used to investigate the neurophysiological mechanisms of PK2 signalling at the cellular level. Primary SCN projections to the PVN provide a potential direct synaptic source of PK2 for regulating endocrine and autonomic rhythms. PK2 does in fact increase the excitability of PVN neurons, as the majority of magnocellular and parvocellular neurons in a brain slice preparation depolarize in response to peptide application (Yuill *et al.*, 2007). More importantly, application of a PK2 antagonist during the light phase (when PK2 expression amplitude is highest in the SCN) was able to decrease the basal excitability of parvocellular neurons in hypothalamic slices containing the SCN (Yuill *et al.*, 2007).

The SFO is a circumventricular organ responsible for integrating and relaying peripheral signals to autonomic centres, such as its role in angiotensin-induced drinking (Simpson & Routenberg, 1973; Ferguson & Renaud, 1986; McKinley *et al.*, 1998). A similar role for PK2 actions in the SFO in the regulation of fluid homeostasis has emerged from studies showing that microinjection of the PK2 homologue, Bv8, into the SFO stimulates water intake (Negri *et al.*, 2004). In addition, when applied to dissociated SFO neurons in culture, PK2 primarily depolarized and increased spontaneous action potential firing (Cottrell *et al.*, 2004). Thus, these effects on neuronal excitability provide important links between oscillating PK2 expression in the SCN and neuronal firing patterns that contribute to controlling autonomic function.

### **1.3 Sensory circumventricular organs**

In order to maintain physiologic homeostasis, peripheral sensory information must reach the brain and be processed in hypothalamic and medullary structures for autonomic output. The circulation contains many important signals which provide information regarding the status of the internal environment. Neurons within the CNS however, are protected from

potentially harmful substances in the circulation by the blood brain barrier (BBB), a boundary created in the cerebral vasculature by endothelial tight junctions and astrocyte end-feet processes (Abbott *et al.*, 2006; Wolburg & Lippoldt, 2002). Although many hydrophobic/lipophilic substances can readily diffuse across the BBB, many impermeable circulating peptides theoretically require alternative means of CNS access. Alternative routes of access which have been suggested include afferent neural pathways (e.g. vagus), transendothelial signalling (angiotensin stimulation of nitric oxide release), and peptide transport systems (leptin transporter - although these may be limited or saturable, and many peptides currently do not have identified transporters) as possible routes for communication between the periphery and CNS (Kastin *et al.*, 1999).

An alternative suggestion has proposed a dynamic role for specialized structures within the brain, known as sensory circumventricular organs (CVOs), as sensors of circulating signals which do not cross the BBB. The sensory CVOs are midline structures located on the walls of the third and fourth ventricles and include the subfornical organ, organum vasculosum of the lamina terminalis, and area postrema. These brain sites are unique in that they are highly vascularised compared to other brain regions and lack a BBB because the capillary system supplying the CVOs contains fenestrated endothelial cells (Gross, 1991). Because of their location and vascular architecture, sensory CVOs have privileged access to homeostatic signals in both the cerebrospinal fluid and blood. Sensory CVOs express many different peptides and receptors which play key roles in the regulation of a variety of physiological functions, and these structures have been shown to play important roles in detecting and transmitting homeostatic information to autonomic brain nuclei (Cottrell & Ferguson, 2004). The remainder of this

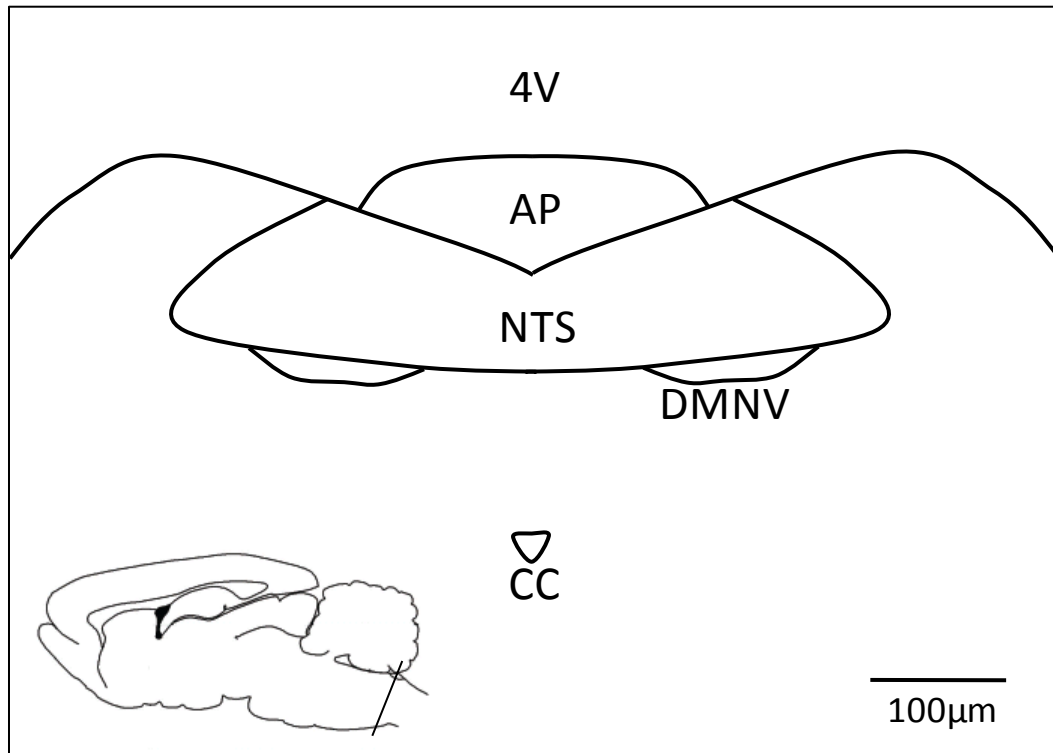
introduction will focus on one of these CVOs, the area postrema (AP), and its role in maintaining autonomic homeostasis.

## **1.4 The area postrema**

### **Anatomy**

The AP, initially described in the early 20<sup>th</sup> century, is the most caudal of the sensory CVOs and is situated in the dorsal surface of the medulla at the base of the fourth ventricle (Figure 1) (Wilson, 1906). The AP sits adjacent to the nucleus tractus solitarius (NTS) and morphologically consists of three specific regions: the central and mantle zones rich with neuronal cell bodies and axons, and the ventral zone that contains mainly glia (McKinley *et al.*, 2003). Between the ventral zone and adjacent NTS exists a border zone of astrocytes and a row of helically arranged tanocytes joined together by tight junctions, creating a distinct diffusion barrier (McKinley *et al.*, 2003; Wang *et al.*, 2008).

Uptake of intravenously injected dyes into the AP, and not surrounding brainstem structures, first suggested a unique ability of CVOs to access signals within the circulation (Wislocki & Leduc, 1952). A closer look at microcirculatory architecture indicates the AP vasculature contains a high blood volume, large surface area, and lacks a traditional BBB. Accordingly, due to an absence of tight junctions, there are fenestrations between the endothelial cells that allow blood and its constituents to permeate through and collect in sinuses

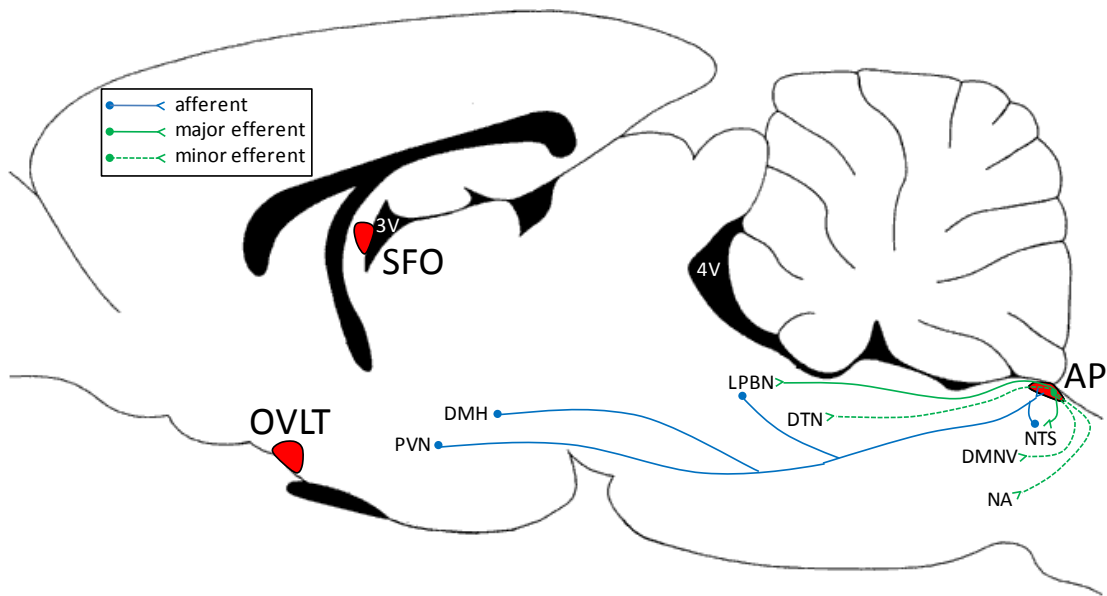


**Figure 1: Anatomy of the AP in relation to autonomic structures in the brainstem**

Schematic representation of a coronal brainstem section at the level of the AP (lower inset), showing its location with respect to the nucleus tractus solitarius (NTS), dorsal motor nucleus of the vagus (DMNV), fourth ventricle (4V), and central canal (CC).

known as Virchow-Robin spaces (Gross, 1991; Dempsey, 1973; Krisch *et al.*, 1978). Virchow-Robin spaces thus slow blood flow, creating pools of interstitial fluid in the perivascular spaces that allow for optimal interaction of circulating chemical messengers with receptors/sensors located on AP neurons and glia. Structurally, AP neurons are bipolar in shape, with basal ventral facing dendrites that receive neuronal inputs and short apical dendrites that extend dorsally into the external basal lamina of capillaries (Morita & Finger, 1987). The apical dendrites receive few synaptic inputs and appear ideally positioned to detect blood constituents.

Neuroanatomical labelling studies using anterograde and retrograde tracers have examined the neural connections of the AP (Figure 2) (van der Kooy & Koda, 1983; Shapiro & Miselis, 1985). Axonal projections are sent to autonomic medullary structures, including major efferents to the NTS and lateral parabrachial nucleus. Minor efferent projections are also identified in the dorsal motor nucleus of the vagus, the nucleus ambiguus, and dorsal regions of the tegmental nucleus. The AP receives afferent neuronal connections from autonomic hypothalamic and medullary nuclei. Notably, major afferent inputs arise from functionally distinct regions of the PVN and dorsomedial nucleus of the hypothalamus, while reciprocal connections exist with the NTS and lateral parabrachial nucleus. Vagal afferents also relay visceral information to the AP, including peripheral chemoreceptor inputs from the aortic depressor nerve (Contreras *et al.*, 1982; Kalia & Welles, 1980). These neural networks suggest the AP has the capacity to monitor humoral signals, integrate this information with neural inputs, and transmit integrated neural output signals to multifunctional central autonomic control centres.



**Figure 2: Location of the sensory CVOs and anatomical connections of the AP**

Schematic representation of a mid-sagittal section through a rat brain illustrating the sensory CVOs (red), including the afferent (blue) and efferent (green) connections of the AP with nuclei in the medulla and hypothalamus. OVLT, organum vasculosum of the lamina terminalis; SFO, subfornical organ; 3V, third ventricle; 4V, fourth ventricle; PVN, paraventricular nucleus of the hypothalamus; DMH, dorsomedial hypothalamus; LPBN, lateral parabrachial nucleus; DTN, dorsal tegmental nucleus; DMNV, dorsal motor nucleus of the vagus; NA, nucleus ambiguus; NTS, nucleus tractus solitarius.

The neurochemistry of the AP has been studied in an attempt to understand the functional mechanisms in relaying peripheral sensory information to autonomic nuclei. In addition to the classical neurotransmitters glutamate and GABA, Newton and colleagues (1985;1985a;1985b;1985c;1987) have shown the AP synthesizes multiple neuropeptides including substance P, neurotensin, somatostatin, and cholecystokinin. These are thought to act as neurotransmitters at axonal projection sites, although further work is required to determine their exact function and efferent targets. Tracing and immunohistochemical studies have, however, provided a link between the projection site and potential action for some chemical phenotypes of AP neurons. These include catecholaminergic inputs to the NTS and serotonergic inputs to the lateral parabrachial nucleus, two major efferent projection sites in the medulla controlling autonomic and homeostatic function (Hermann *et al.*, 2005; Lanca & van der Kooy, 1985). The AP also contains a high density of cocaine- and amphetamine-related transcript and enkephalin, two peptides shown to have important actions in regulating feeding behaviour and cardiovascular function (Koylu *et al.*, 1998; Fallon & Leslie, 1986).

### **Function**

Traditionally, the AP was established to be the chemoreceptor zone in the control of emesis, indicating this CVO is positioned anatomically to detect and respond to noxious chemical stimuli in the cerebrospinal fluid and circulation (Borison & Brizzee, 1951; Carpenter *et al.*, 1983; Miller & Leslie, 1994). Additional functions for the AP have also implicated this CVO in regulating immune function, cerebrospinal fluid balance, cardiovascular function, metabolism, and food intake (Borison, 1974). Moreover, expression of numerous receptors and sensors on AP neurons has outlined a prominent role for sampling blood-borne constituents and relaying

homeostatic signals to medullary structures situated behind the BBB (Cottrell & Ferguson, 2004).

A number of groups have described the electrical membrane properties of AP neurons using both *in vitro* and *in vivo* preparations. Given that AP neurons receive very few synaptic inputs, individual neurons have relatively high input resistances ( $\sim 3\text{G}\Omega$ ) (Yang & Ferguson, 2003; Ferguson & Bains, 1996). AP neurons also exhibit relatively low spontaneous activity of between 1 and 5Hz, which may be attributable to regulation by the cAMP-dependent hyperpolarization-activated cation current (Brooks *et al.*, 1983; Lowes *et al.*, 1995; Funahashi *et al.*, 2003). Additionally, the biophysical properties of voltage-dependent conductances for  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Ca}^{2+}$  have been characterized with respect to voltage and time dependence, as well as sensitivity to antagonists (Hay & Lindsley, 1995; Hay *et al.*, 1996). Modulation of many of these ionic currents by various peptides, including voltage-gated  $\text{Ca}^{2+}$  and  $\text{K}^+$ , as well as non-selective cationic conductances, have been shown to influence AP membrane excitability (Hay *et al.*, 1996; Yang & Ferguson, 2002; Fry & Ferguson, 2009).

Successful defense against pathogens during the immune response requires communication between the immune system and the brain. Following lipopolysaccharide treatment, anatomical evidence describes interleukin-1 (IL-1)-expressing immune cell membrane apposition with AP dendrites and terminals, and implies the AP possesses the ability to access peripheral immune signals, thereby providing an interface for immune cell signalling to the CNS (Goehler *et al.*, 2006). In accordance with this, IL-1 receptors are present in the AP, and activation of these receptors induces expression of *c-fos*, an indicator of neuronal activation (Ericsson *et al.*, 1995). Additionally, administration of immune stimuli, such as IL-1, to AP

microcultures results in transient increases in intracellular  $\text{Ca}^{2+}$  and the release of pro-inflammatory cytokines (Wuchert *et al.*, 2008;Wuchert *et al.*, 2009). The observation that NTS and PVN *c-fos* activation, as well as increases in plasma adrenocorticotrophic hormone and corticosterone concentrations, in response to peripheral immune signals are dependent on an intact AP (Brady *et al.*, 1994;Lee *et al.*, 1998), also argues strongly for a critical role of this CVO in mediating these responses.

Initially described as a sensor for noxious and toxic stimuli, the AP has also been suggested to sense circulating hormones that regulate feeding behaviour and energy homeostasis. During feeding, *c-fos* activation is greatly elevated in the AP, indicating that many GI-derived signals transmitting satiety-related information are received by the AP (Johnstone *et al.*, 2006). Receptors for anorexigenic hormones derived from the GI tract including amylin, cholecystokinin (CCK), glucagon-like peptide 1, oxyntomodulin, and peptide YY (Barth *et al.*, 2004;Mercer & Beart, 2004;Merchenthaler *et al.*, 1999;Parker & Herzog, 1999;Baggio *et al.*, 2004;Price *et al.*, 2007) are present on AP neurons. Receptor activation for the aforementioned peptides has been described *in vitro* and *in vivo* in the AP, leading to activation of *c-fos* expression and changes in membrane excitability (Mollet *et al.*, 2004;van der Kooy, 1984;Yamamoto *et al.*, 2003;Bonaz *et al.*, 1993). Specifically, amylin-induced *c-fos* expression and anorectic effects are significantly blunted using receptor antagonists and by AP lesions (Mollet *et al.*, 2004;Riediger *et al.*, 2004). In addition to the ability of CCK to reduce food intake in rats, an intact AP is also required for peptide YY to inhibit CCK-stimulated pancreatic exocrine secretion (van der Kooy, 1984;Deng *et al.*, 2001). Extracellular recordings in slices show amylin and CCK to be excitatory in roughly half of AP neurons tested. Intriguingly, the majority of

amylin- and CCK-sensitive neurons have also been suggested to be glucose sensitive (Sun & Ferguson, 1997; Riediger *et al.*, 2002; Funahashi & Adachi, 1993).

In comparison, ghrelin is an orexigenic peptide originally isolated from the stomach that also has actions in the AP, presumably via binding with the growth hormone secretagogue receptor which has been shown to be expressed in the AP (Zigman *et al.*, 2006; Fry & Ferguson, 2009). Peripheral injection of ghrelin increases feeding in animals and increases the number of *c-fos*-expressing neurons in the AP and NTS, while selective ablation of the AP abolishes these effects (Gilg & Lutz, 2006; Li *et al.*, 2006b). In contrast, electrophysiology studies demonstrated that ghrelin has direct excitatory and inhibitory actions on separate subpopulations of dissociated AP cells, which supports the notion that the AP provides a conduit for ghrelin to gain access to autonomic feeding centres behind the BBB (Fry & Ferguson, 2009). Similar work has shown that the insulin sensitizing adipocyte hormone, adiponectin also influences the excitability of AP neurons (Fry *et al.*, 2006). A large proportion of AP neurons respond to adiponectin with either excitation or inhibition, and responsive AP neurons appear to express both adiponectin receptors (AdipoR1 and AdipoR2). Lastly, adiponectin microinjected into the AP also caused increases in blood pressure, providing a potential link between the control of energy homeostasis and cardiovascular function.

Circulatory demands fluctuate daily and the cardiovascular system compensates with circadian changes in blood pressure, heart rate, and vascular tone (Millar-Craig *et al.*, 1978; Krantz *et al.*, 1996). Tissues must receive a sufficient perfusion of blood for the supply of oxygen and metabolic fuels, as well as the removal of metabolic waste. The AP is vital for normal cardiovascular function. Initial reports demonstrated clear hypertensive effects in animals as a

result of AP destruction (Ylitalo *et al.*, 1974). Because of its location outside the BBB, the AP has been extensively studied and described as a CVO important in gathering peripheral information related to cardiovascular function, including mediating the central effects of circulating peptides involved in cardiovascular control. Neurons within the AP are also responsive to blood pressure changes and receive excitatory baroreceptor inputs (Papas & Ferguson, 1991). In response to increased blood pressure, the baroreceptor reflex compensates with a decrease in heart rate. Angiotensin II and vasopressin are circulating peptides that act to control the sensitivity of the baroreceptor reflex and these regulatory actions require an intact AP (Xue *et al.*, 2003; Bishop & Sanderford, 2000; Stebbins *et al.*, 1998; Cox *et al.*, 1990).

In support of these observations, the expression of angiotensin and vasopressin receptors has been reported in the AP, suggesting direct actions at this site (Gerstberger & Fahrenholz, 1989; Lenkei *et al.*, 1997). Electrophysiological experiments *in vitro* and *in vivo* show angiotensin II to have excitatory effects in approximately half of AP cells tested, while vasopressin produced both excitatory and inhibitory responses in subpopulations of neurons (Ferguson & Bains, 1996; Smith *et al.*, 1994). These actions on neuronal firing therefore may explain how microinjection of angiotensin II and vasopressin into the AP act to decrease and increase NTS activity, respectively (Cai *et al.*, 1994). Furthermore, increases in blood pressure are observed following administration of angiotensin II into the AP, whereas studies show mixed effects occur due to vasopressin injection (Lowe *et al.*, 1993; Yang *et al.*, 2006). Estrogen also plays a neuromodulatory role in the AP, as 17 $\beta$ -estradiol decreases angiotensin II-activated intracellular Ca<sup>2+</sup> responses in cultured neurons (Pamidimukkala & Hay, 2003).

The AP is also responsive to vasoactive peptide hormones secreted from vascular endothelial cells, such as adrenomedullin and endothelin, that result in neuronal excitation (Ferguson & Smith, 1991; Allen & Ferguson, 1996). Endothelin receptors are present in the AP and microinjection of endothelin into the AP has dose-dependent biphasic effects on blood pressure (Ferguson & Smith, 1990; Kurokawa *et al.*, 1997). Although it functions as a vasodilator in the periphery, direct administration of adrenomedullin into the AP acts to increase blood pressure and heart rate, in accordance with reports that intracerebroventricular adrenomedullin administration causes induction of *c-fos* in the AP (Allen *et al.*, 1997; Ueta *et al.*, 2001). These studies have, therefore, shown the AP as an important structure in sensing circulating factors involved in cardiovascular modulation.

The AP thus plays crucial roles in regulating autonomic processes such as energy homeostasis and cardiovascular function. This caudal CVO responds to, and conveys neural and humoral homeostatic signals to autonomic control centres within the CNS. Metabolic and cardiovascular requirements fluctuate throughout the day such that the regulation of these systems is under circadian control by the SCN (Ruiter *et al.*, 2006; Scheer *et al.*, 2003). Importantly, the initiation of feeding depends on the time of day, and many circulating satiety hormones have a diurnal variation in their ability to limit food intake (Kraly *et al.*, 1983; Kraly, 1981). Moreover, blood pressure and heart rate also follow a 24 hour rhythm, indicated by an early morning peak in cardiovascular activity that is coincident with peak PK2 expression levels (Guo & Stein, 2003). Therefore, the AP is an ideal site at which PK2 may act to play a circadian modulatory role on the ability of the AP to respond to homeostatic signals.

## 1.5 Research aims

Although significant research has established PK2 to be an important signalling peptide regulating circadian rhythms in the CNS, less is known regarding the specific neuronal substrates and cellular signalling mechanisms mediating these actions. The PKR2 is distributed throughout several brain regions, including prominent mRNA expression in the AP (Cheng *et al.*, 2006), a sensory CVO important in central autonomic function. The primary objective of the studies described in this thesis was to elucidate the role of PK2 in controlling the excitability of AP neurons. We have used whole-cell electrophysiology and RT-PCR techniques to test the following hypotheses:

- i. PK2 modulates the membrane excitability of AP neurons
- ii. The actions of PK2 are mediated through MAPK signalling
- iii. PK2 has excitatory and inhibitory effects in the AP which are due to differential ion channel regulation
- iv. Specific excitatory or inhibitory effects occur in chemically identified subpopulations of neurons within the AP

## Chapter 2: Materials and Methods

### 2.1 Cell culture

All animal protocols were in accordance with guidelines of the Canadian Council on Animal Care and were approved by the Queen's University Animal Care Committee. Male Sprague-Dawley rats (100-200g, Charles River, QC) were decapitated, the brainstem quickly removed, and placed in cold artificial cerebrospinal fluid (1-4°C) containing (in mM): 124 NaCl, 2.5 KCl, 1.3 MgSO<sub>4</sub>, 1.24 KH<sub>2</sub>PO<sub>4</sub>, 20 NaHCO<sub>3</sub>, 2.27 CaCl<sub>2</sub>, and 10 glucose. The brainstem was mounted on a stage and 300µm coronal slices containing the AP were cut using a vibratome (Leica, Nussloch, Germany) and placed in Hibernate media (Brain Bits, Springfield, IL) supplemented with B27 (Gibco, Invitrogen, Burlington, ON). AP was microdissected from brainstem slices and incubated in Hibernate media containing 2mg/ml of papain (Worthington, Lakewood, NJ) at 30°C for 30 min. Following incubation, AP tissue was washed and triturated in Hibernate/B27 media and dissociated cells were centrifuged at 500 x g for 8 min. The supernatant was removed and the pellet resuspended in Neurobasal A/B27 media (Invitrogen) supplemented with 5mM glucose, 100U/ml penicillin/streptomycin, and 0.5mM L-glutamine (Invitrogen). Dissociated cells were plated on 35mm uncoated glass bottom culture dishes (MatTek, Ashland, MA) at a low density (~10 cells/mm<sup>2</sup>) to ensure synaptic contacts did not form, and incubated at 37°C in 5% CO<sub>2</sub>. Electrophysiological experiments were performed on neurons maintained for 1-5 days in culture, at which time no synaptic contacts were observed.

## 2.2 Electrophysiology

Whole-cell recordings from dissociated AP neurons were made using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Palo Alto, CA). Data were collected using Signal (voltage-clamp) and Spike2 (current-clamp) software packages (Cambridge Electronics Design, Cambridge, United Kingdom). Signals were filtered at 2kHz and digitized at 5kHz using a Micro 1401 MKII interface (Cambridge Electronics Design). Voltage measurements were corrected for liquid junction potential. Unless otherwise noted, recordings were obtained using external recording solution containing (in mM): 140 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 10 HEPES, and 5 glucose, pH 7.2 (adjusted with NaOH). Patch electrodes were made from borosilicate glass (World Precision Instruments, Sarasota, FL) on a Flaming Brown micropipette puller (P87, Sutter Instrument Co, Novato, CA), heat polished, and had resistances of 3-6MΩ. Unless noted, electrodes were filled with intracellular recording solution that contained (in mM): 130 K-gluconate, 10 KCl, 1 MgCl<sub>2</sub>, 0.1 CaCl<sub>2</sub>, 10 HEPES, 10 EGTA, 2 NaATP, pH 7.2 (adjusted with KOH).

AP cells were perfused with external recording solution (37°C) using a gravity fed perfusion system at a rate of 1-2ml/min. Cells in whole-cell recording configuration were defined as neurons by the presence of voltage-gated Na<sup>+</sup> currents in voltage-clamp and >60mV action potentials in current-clamp. PK2 was filled into puffer pipettes for pneumatic application (10s, 3-8psi) using a Multichannel Picospritzer (General Valve Corporation, Fairfield, NJ) under visual guidance approximately 10-50μm from the neuron, once a stable baseline membrane potential of at least 100s was achieved. Changes in membrane potential were calculated from the maximal difference between the average membrane potential in 50s segments prior to and following peptide application. AP neurons were considered responsive if this difference was at

least 3 standard deviations of the mean baseline membrane potential and the cell showed recovery towards baseline. Changes in action potential frequency were assessed by comparing the difference between the mean action potential frequency 100s immediately prior to and following PK2 application. Comparison of action potential height was analyzed in neurons that did not respond with a change in membrane potential by comparing the difference in mean action potential amplitude during the control segment of the recording and the segment containing the peak effect on action potential height.

A voltage ramp protocol (12.5mV/s) was used to assess the effects of bath-applied PK2 on whole-cell steady-state current. AP neurons were clamped at -75mV and ramp currents were determined from an average of 3 ramps between -100 and -20mV before (control) and following 3 min PK2 application. During the experiment the access resistance did not vary by more than 25%. The current-voltage relationship was plotted and difference current (PK2-induced current) calculated by subtracting the control current from the current obtained after peptide treatment. Linear regression analysis was used to determine conductance (slope of the PK2-induced current) and reversal potential. Voltage ramps performed under high internal  $\text{Cl}^-$  utilized an intracellular solution similar to above, but substituted KCl for K-gluconate.

Experiments analyzing changes in  $\text{Na}^+$  channel gating used external and intracellular recording solutions that blocked all other voltage-gated currents. The external solution contained the following (in mM): 25 NaCl, 130 TEA, 1  $\text{MgCl}_2$ , 2  $\text{CaCl}_2$ , 1 CsCl, 1  $\text{BaCl}_2$ , 0.3  $\text{CdCl}_2$ , 10 HEPES, 5 glucose, pH 7.2 (adjusted with NaOH). Intracellular solution contained (in mM): 125 CsMeSO<sub>4</sub>, 2  $\text{MgCl}_2$ , 5.5 EGTA, 10 CsCl, 0.1 CaCl, 2 NaATP, pH 7.2 (adjusted with KOH). Both activation and steady-state inactivation protocols were performed in each cell before and

following 3 min PK2 application. Na<sup>+</sup> channel activation was tested using a voltage step protocol between -80 and -20mV in 10mV increments from -90mV. Normalized conductance was plotted against test potential and points fitted with a Boltzmann function. Steady-state inactivation was assessed using 200ms prepulse steps between -110 and -20mV in 10mV increments, followed by a test pulse to -10mV. Normalized currents were plotted against prepulse potential and points fitted with a Boltzmann function. The half maximal activation and inactivation voltages and slope factor *k* were determined for control and PK2 treatment.

### **2.3 Single cell RT-PCR**

Whole-cell current-clamp recordings were performed using bath-applied PK2 and electrodes that had been sterilized at 200°C for at least 6h and filled with 12μl of RNase-free intracellular recording solution. After completion of the experiment and recovery toward baseline during the washout period, suction was applied to the pipette interior and the cell collected. Immediately following cytoplasm collection, the contents of the cell were expelled into a 0.5ml centrifuge tube containing DNase (1μl) and DNase buffer (1μl) (Fermentas, Burlington, ON). The tube was incubated for 30 min at 37°C, after which EDTA (10mM) was added and the tube heated at 65°C for an additional 10 min. To synthesize cDNA the following were added: dithiothreitol (26mM), dNTPs (3mM), random hexamer primers (3μM), MgCl<sub>2</sub> (4mM), RNase inhibitor (20U), and Superscript II reverse transcriptase (100U) (all from Invitrogen). The cDNA synthesis reaction was incubated overnight at 37°C and cDNA stored at -80°C until PCR was performed. A two step multiplex PCR protocol was used to detect the presence of mRNA encoding genes of interest (see Table 1 for primer sets) using reagents provided in the Qiagen Multiplex Kit (Qiagen, Mississauga, ON). The first amplification step

**Table 1: List of primers used in the multiplex PCR reactions**

Primer		Outside	Inside	Size (bp)
GAPDH	Sense:	gatggtgaaggcgggtgtg	taccaggctgccttctct	360
	Antisense:	gggctaagcagttggtggt	ctcgtggtcacacccatc	
ENK	Sense:	gccaaactcctccgacctgct	tgggaacaggagacaaccgtg	276
	Antisense:	gcctccgatctttttccatctc	agtaacttgccttcttcatccg	
CART	Sense:	tggacatctactctgccgtg	gctgtgtgcagattgaagc	213
	Antisense:	aatatgggaaccgaaggagg	gtcaccctcacaagcact	
GAD67	Sense:	cacaaactcagcggcataga	cacaaactcagcggcataga	149
	Antisense:	gagatgaccatccggaagaa	ctggaagaggtagcctgcac	
CCK	Sense:	cgcactgctagcccgataca	atacatccagcaggccgca	151
	Antisense:	ttctcattccgctcctcc	tgggtattcgtagctcctcagcac	
TH	Sense:	cacctggagtattttgtgcg	tcgaccagtatatccgcca	376
	Antisense:	cctgtgggtggtaccctatg	tcggacacaaagtacacagg	
VGLUT2	Sense:	aggttggctaccacctctt	cccgcaaagcatccaacca	164
	Antisense:	tgagagtagccaacaaccagaagca	cctgcagaagtttgcaaca	

Primers are listed in the 5' to 3' direction.

consisted of a multiplex reaction in 100 $\mu$ l volume with the synthesized cDNA and 'outside' primers (0.2 $\mu$ M each) for all the genes of interest. The reaction was denatured at 95°C for 15 min and cycled 20 times through a temperature protocol consisting of 30s at 94°C, 90s at 60°C, and 90s at 72°C. In the second nested reaction, 'inside' primers were used in individual 50 $\mu$ l reactions for each gene of interest using 2 $\mu$ l of first round product as the template and 0.2 $\mu$ M of each primer. The reaction mixture was cycled 35 times using the same temperature protocol described above. Finally, PCR products were run on a 2% (w/v) agarose gel containing ethidium bromide and periodically sequenced to confirm their identity (Robarts Institute, London, ON).

## **2.4 Chemicals and peptides**

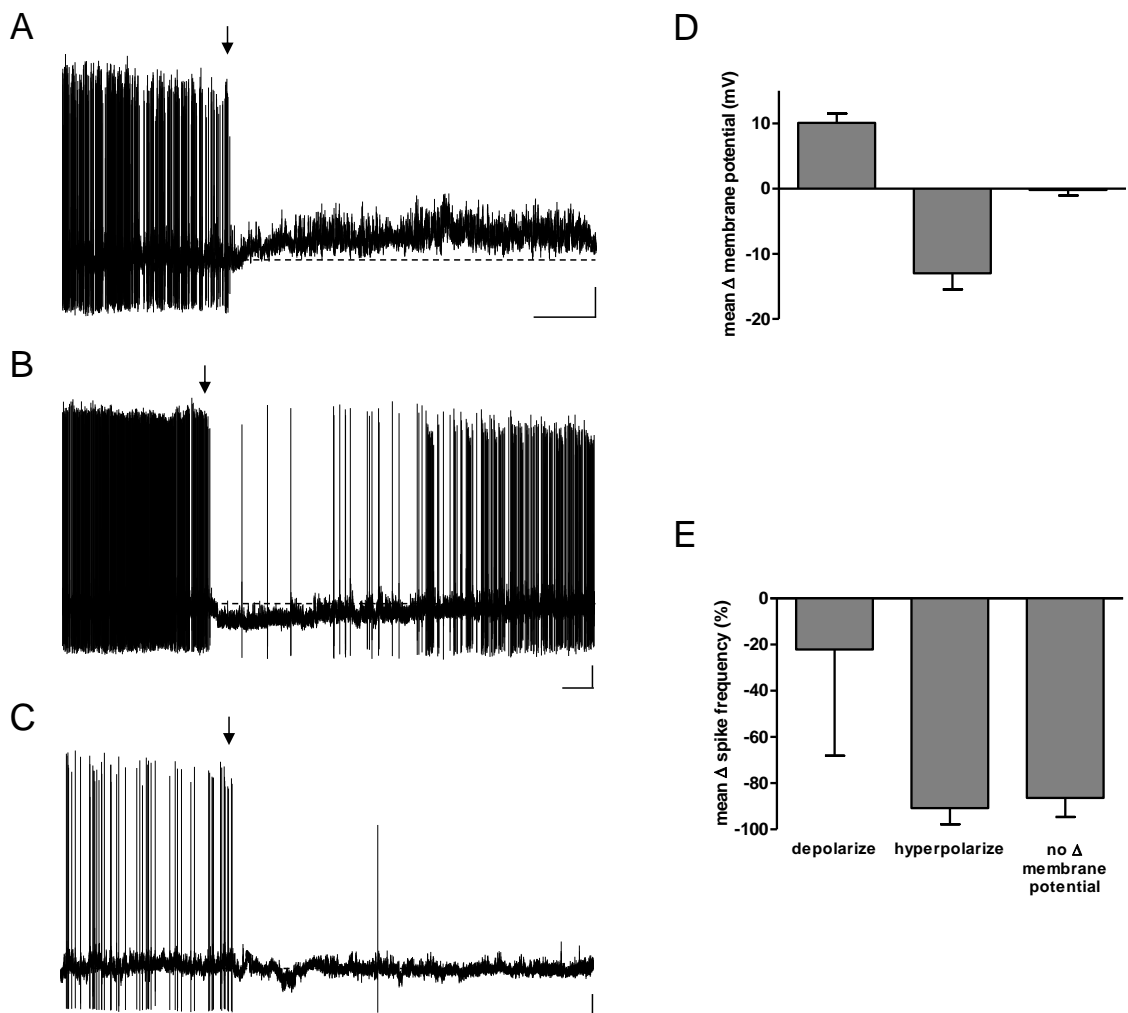
All chemicals used to make solutions were purchased from Sigma (Oakville, ON). RNase free intracellular recording solution was made using molecular biology grade chemicals. The specific MAPK inhibitor, PD 98059, was purchased from Sigma (St. Louis, MO). PK2 was generously provided by Dr Qun-Yong Zhou, University of California at Irvine, synthesized using recombinant techniques (Li *et al.*, 2001) and reconstituted in external recording solution to working concentrations.

## Chapter 3: Results

### 3.1 PK2 influences the excitability of AP neurons

We initially used whole-cell current-clamp recordings to examine the effects of focal application of PK2 on the excitability of dissociated AP neurons. Long term stable recordings were obtained from 86 dissociated AP neurons maintained in culture for 1-5 days. Following the development of a stable control baseline membrane potential for a minimum of 100s, PK2 was rapidly applied by pressure ejection under visual guidance for 10s in the immediate vicinity of the recorded neuron. Local application of 1 $\mu$ M PK2 influenced the membrane potential of 66% of AP neurons tested (n=29), producing either membrane depolarization (Figure 3A) or hyperpolarization (Figure 3B) in 38% and 28% of cells, respectively. These effects were reversible upon washout and significantly different from control cells treated with aCSF (n=10, unpaired *t*-test,  $p < 0.001$ ), zero of which met imposed criteria to be considered responsive. The remaining AP neurons tested did not respond to PK2 with significant changes in membrane potential (Figure 3C). The mean change in membrane potential is summarized for each group in Figure 3D.

In many cases AP neurons that displayed spontaneous activity responded to 1 $\mu$ M PK2 treatment with decreases in action potential frequency (14 out of 16 neurons). The mean change in spike frequency of all cells tested was  $-71.1 \pm 13.5\%$ , a value significantly different (unpaired *t*-test,  $p < 0.05$ ) from aCSF treated control cells (n=6). Changes in action potential frequency did not correspond with changes in membrane potential, as AP neurons that depolarized showed no significant change (unpaired *t*-test,  $p > 0.05$ ) in spike frequency (Figure 3E, n=4). Interestingly, decreases in spike frequency were observed in all cells that either



**Figure 3: PK2 has direct effects on membrane potential and firing frequency in dissociated AP neurons**

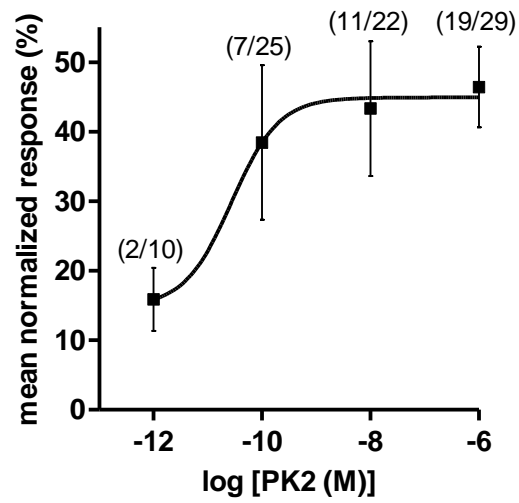
Representative current-clamp recordings showing AP neurons (A) depolarize (n=11), (B) hyperpolarize (n=8), or (C) show no change in membrane potential (n=10) in response to 1 μM PK2 application. The majority of spontaneously active neurons showed a decrease in firing frequency. Arrows indicate time of PK2 application. Scale bars: 10mV, 20s. Bar graphs showing (D) the mean change in membrane potential and (E) mean change in spike frequency for cells tested with 1 μM PK2.

hyperpolarized (n=3) or did not show a membrane potential change (n=9), effects which were significantly different from aCSF treated controls (unpaired *t*-test,  $p < 0.05$ ). As illustrated in Figure 3A-C, the resulting inhibition of spike frequency was often profound, as 9 out of 12 neurons responded with a >94% reduction.

Concentrations of PK2 ranging from 1pM to 1 $\mu$ M produced concentration-dependent changes in membrane potential, with the highest proportion of responsive neurons and largest effects observed at 1 $\mu$ M (Figure 4). For experiments performed at 1pM only 2 responsive cells were observed and therefore mean responses were characterized using all cells tested at this concentration. The responses were normalized to the peak effect observed at each PK2 concentration, averaged, and fitted with a Hill equation to yield an EC<sub>50</sub> value of 27.5pM. Collectively, these results show PK2 directly caused either depolarizing or hyperpolarizing effects on AP neurons, both of which were found to be concentration dependent, while effects on spike frequency were more homogenous with only inhibitory effects observed.

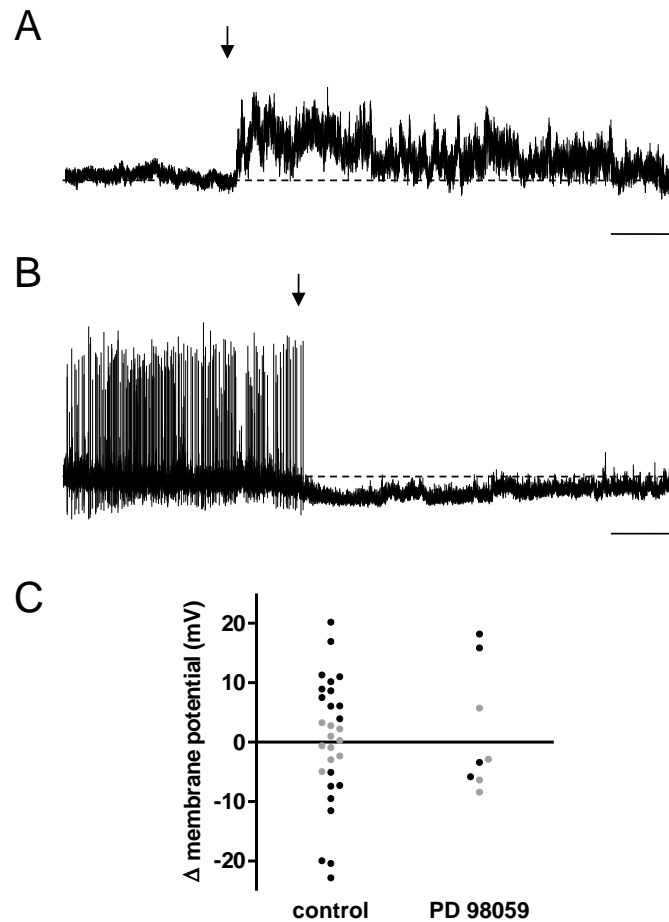
### **3.2 The effects of PK2 are not mediated by a MAPK cascade**

Previous studies showing that PK2 increases PVN and SFO neuronal excitability through activation of a MAPK signalling cascade (Yuill *et al.*, 2007; Fry *et al.*, 2008), led us to next examine whether the effects seen in AP neurons also require MAPK signalling. The specific MAPK inhibitor PD 98059 (10 $\mu$ M) was used to determine if the effects of PK2 on membrane potential and spike frequency could be abolished. Out of 8 cells tested, pre-treatment with PD 98059 for a minimum of 300s prior to 1 $\mu$ M PK2 application did not abolish the effects on membrane potential, as both depolarizations (Figure 5A, n=2) and hyperpolarizations (Figure 5B, n=2) were observed. This proportion of responses was not significantly different (Fisher's exact test,



**Figure 4: The effects of PK2 on membrane potential are concentration dependent**

Mean changes in membrane potential normalized to the maximal response observed at each concentration for depolarizations and hyperpolarizations were fitted with a Hill equation to give an  $EC_{50}$  of 27.5pM. Fractions indicate the proportion of neurons responding at each concentration.



**Figure 5: Inhibition of MAPK signalling does not attenuate the effects of PK2 on AP excitability**

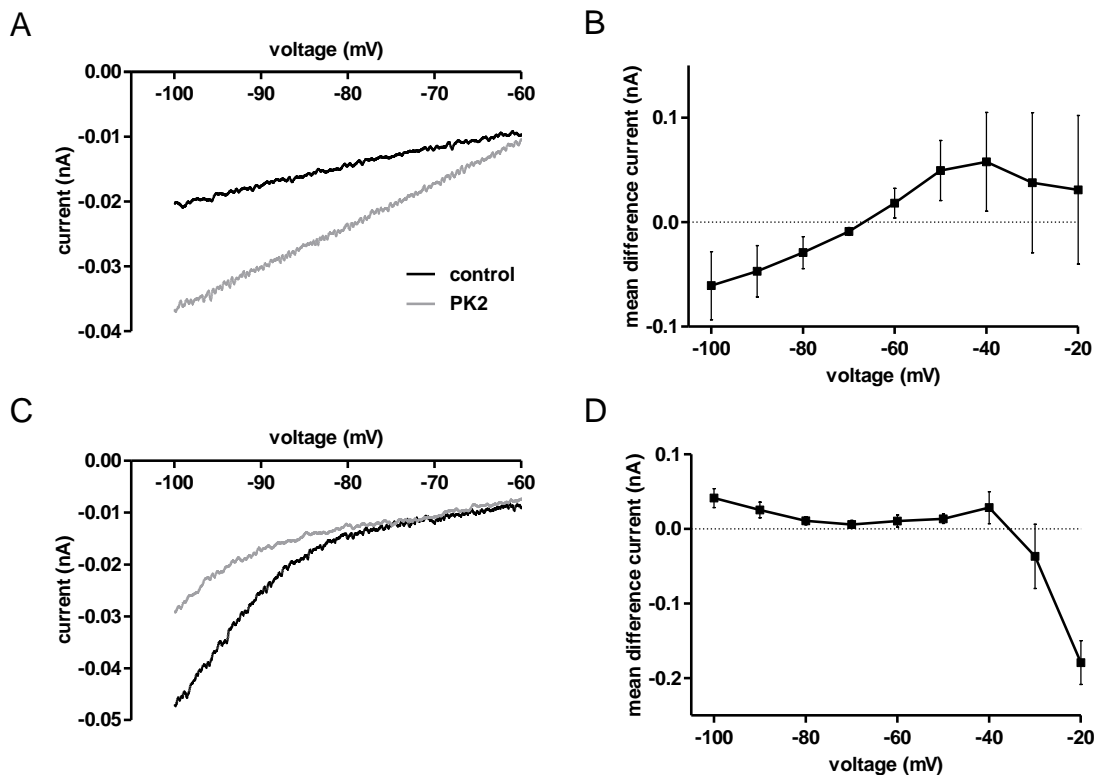
Current-clamp records show 1 $\mu$ M PK2-induced (A) depolarizations and (B) hyperpolarizations, including inhibition of firing frequency, were still observed following pre-application of 10 $\mu$ M PD 98059. Arrows indicate time of PK2 application. Scale bars: 10mV, 50s. (C) Scatter plot illustrating the responses to 1 $\mu$ M PK2 in control conditions and in the presence of PD 98059. Dark circles represent AP neurons considered responsive based on imposed criteria, while grey circles are AP neurons that did not meet criteria following PK2 exposure.

$p > 0.05$ ) when compared to control conditions (Figure 5C). In addition, inhibition of spontaneous action potential frequency was still observed in the presence of PD 98059 as a result of peptide application (Figure 5B,  $n=2$ ).

### **3.3 Ion channel mechanisms mediating PK2-induced changes in membrane potential**

We next attempted to examine the ion channels influenced in AP neurons by PK2 using voltage-clamp techniques to measure currents evoked by slow voltage ramps (12.5mV/s) run from -100 to -20mV both in the presence of aCSF and following 10nM PK2 application. Out of 12 cells tested, current-voltage relationships in control conditions generated a voltage-independent current and following PK2 treatment an inward whole-cell current shift was observed in 5 cells from a holding potential of -75mV (mean  $-17.4 \pm 7.9$ pA, Figure 6A). The mean change in conductance was  $4.0 \pm 2.7$ nS and the difference current that was obtained by subtracting the control current from the current obtained in PK2 elicited a mean reversal potential of  $-63.6 \pm 3.1$ mV (Figure 6B). Based on the ionic concentrations of the bath and pipette recording solutions, these findings suggest that PK2 activated a current that reversed near the calculated equilibrium potential for  $\text{Cl}^-$  ( $E_{\text{Cl}^-} = -64$ mV) and leads to membrane depolarization.

In a separate group of AP neurons, PK2 produced outward whole-cell currents (mean  $7.6 \pm 5.8$ pA,  $n=5$ ) that were associated with an inwardly rectifying control ramp current at hyperpolarized potentials (Figure 6C). Peptide administration in this phenotype of cells resulted in a mean decreased conductance of  $1.1 \pm 0.6$ nS and a PK2-induced current that reversed at a mean membrane potential of  $-34.9 \pm 3.5$ mV (Figure 6D), suggesting that inhibition of a voltage-dependent non-selective cation current (NSCC) leads to membrane hyperpolarization. The proportion of responding neurons in voltage-clamp configuration closely resembles the



**Figure 6: Current-voltage relationships in subpopulations of AP neurons in response to PK2 application**

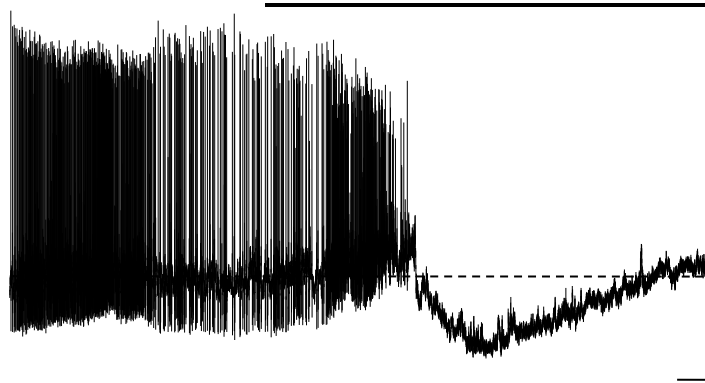
(A) Whole-cell current response of an AP neuron to a voltage ramp that responded to 10nM PK2 with an inward shift in current. PK2-induced inward whole-cell current shifts typically occur in AP cells possessing non-rectifying currents between -100 and -60mV and are accompanied by increased conductance. (B) The mean difference in whole-cell current between PK2 treatment period and control period plotted at 10mV intervals for cells that responded with inward current shifts (n=5). The mean difference current reversed at  $-64 \pm 3\text{mV}$ . (C) Decreased conductance of an inwardly rectifying current in an AP neuron is representative of a second subpopulation of AP neurons that respond to PK2 with outward shifts in whole-cell current. (D) The mean difference current plotted in AP cells that responded with outward currents reversed at  $-34.9 \pm 3.5\text{mV}$  (n=5).

proportion of depolarizing and hyperpolarizing responses on membrane potential (Fisher's exact test,  $p > 0.05$ ). In light of previous work demonstrating peptidergic modulation of NSCC in the AP (Yang & Ferguson, 2002; Fry & Ferguson, 2009), current-clamp experiments were next carried out in an attempt to mimic PK2-induced hyperpolarization through inhibition of a NSCC. Accordingly, application of  $200\mu\text{M Gd}^{3+}$ , a NSCC blocker, mimicked PK2-induced hyperpolarizations in 6 out of 9 AP cells tested (Figure 7). These findings suggest that a population of AP neurons expressing a NSCC may be hyperpolarized by PK2.

To determine whether activation of a  $\text{Cl}^-$  current is responsible for membrane depolarization, slow voltage ramps were again performed using a pipette solution containing  $139\text{mM Cl}^-$ . Under high  $\text{Cl}^-$  conditions, the PK2-activated current reversal would be expected to shift towards the set  $E_{\text{Cl}^-}$  of  $-2\text{mV}$ . As represented in Figure 8A, the resultant inward currents activated by PK2 treatment converged with control ramp currents at a mean reversal potential of  $-24.0 \pm 8.3\text{mV}$  ( $n=6$ ). These results are in accordance with a predicted depolarizing shift of the  $\text{Cl}^-$  reversal potential in these recording conditions. Furthermore, due to a greater  $\text{Cl}^-$  driving force at resting membrane potential, depolarizations (mean  $17.3 \pm 4.8\text{mV}$ ,  $n=5$ ) but not hyperpolarizations (mean  $-16.2 \pm 6.5\text{mV}$ ,  $n=2$ ) were significantly larger in response to  $1\mu\text{M PK2}$  (Figure 8B and C, unpaired  $t$ -test,  $p < 0.05$ ), indicating activation of a  $\text{Cl}^-$  conductance contributes to membrane depolarization.

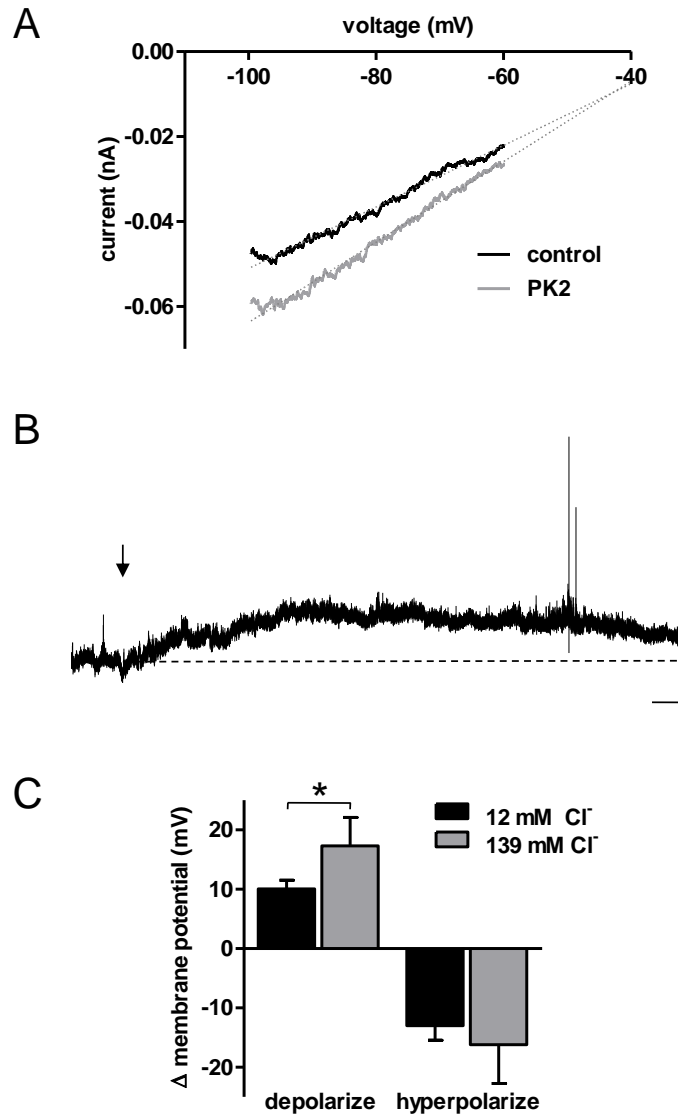
### **3.4 Inhibition of action potential firing is mediated through modulation of $\text{Na}^+$ channel gating**

Although different effects of PK2 were observed on the membrane potential of different groups of AP neurons, as outlined above the majority of AP neurons treated with PK2 responded



**Figure 7: Gd<sup>3+</sup> mimics PK2-induced membrane hyperpolarization**

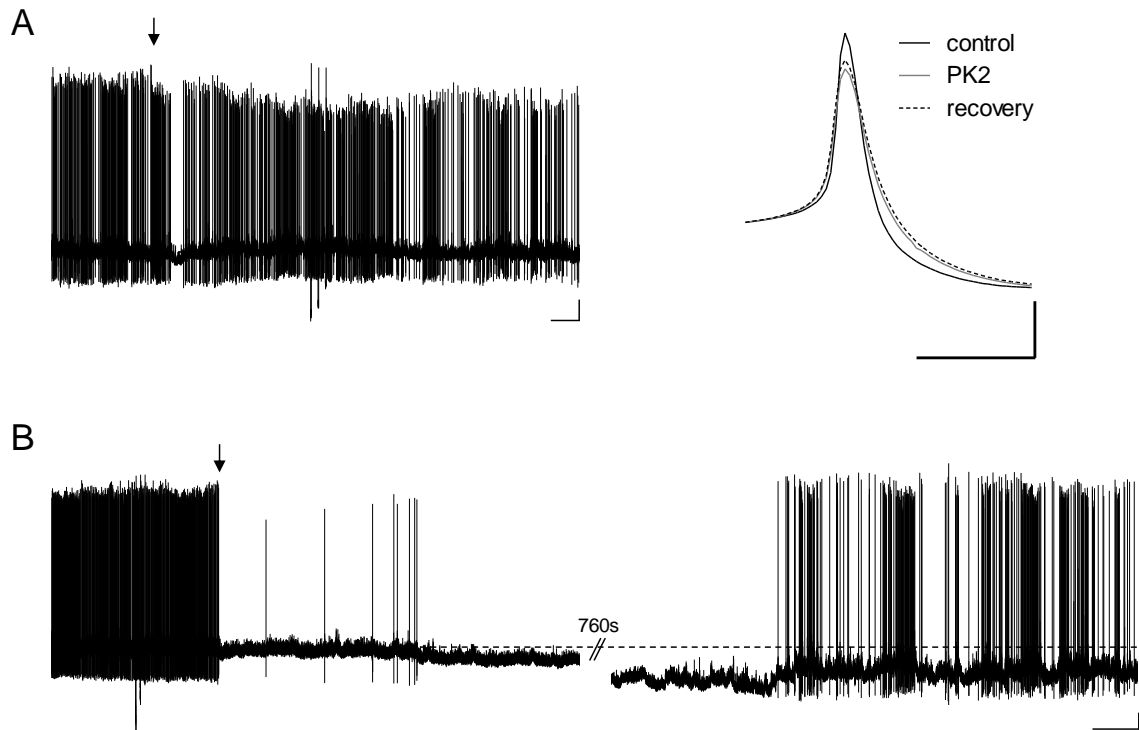
Current-clamp trace showing that application of 200 μM Gd<sup>3+</sup>, a non-selective cation channel blocker, hyperpolarizes an AP neuron. This effect occurred in 67% of AP cells tested, indicating that inhibition of this conductance in a phenotypic group of neurons may underlie hyperpolarizations induced by PK2. Bar above trace indicates the duration of Gd<sup>3+</sup> application. Scale bars: 10mV, 10s.



**Figure 8: PK2 depolarizes AP neurons through activation of a Cl<sup>-</sup> carrying current**

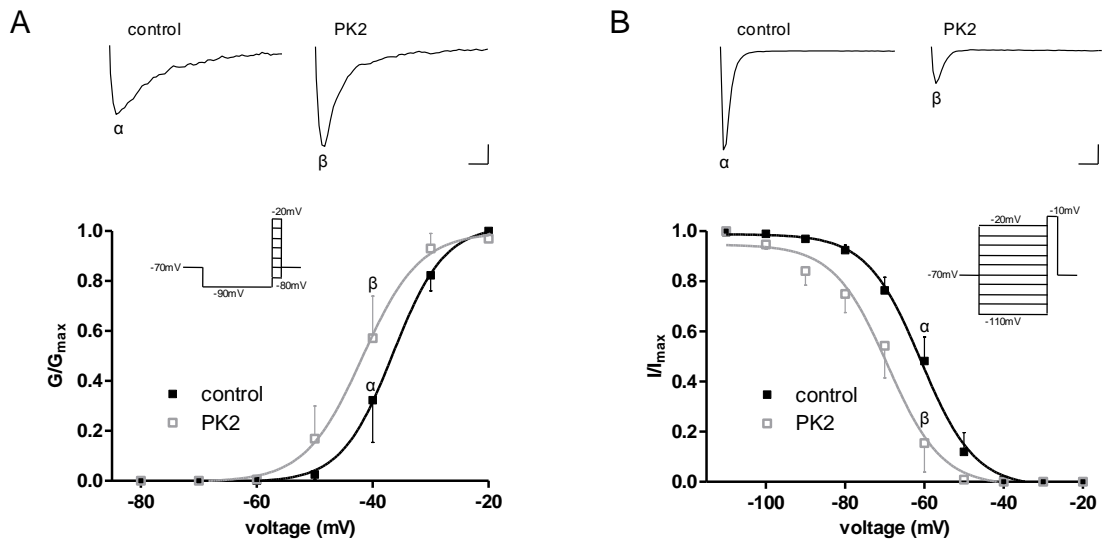
(A) Current-voltage relationship in an AP neuron under high internal Cl<sup>-</sup> conditions (139mM) that responded to 10nM PK2 with an inward whole-cell current shift. The extrapolated reversal potential of the PK2-induced current (n=6) shifted towards the set E<sub>Cl<sup>-</sup></sub> (-2mV). (B) Current-clamp trace showing a large depolarization under high internal Cl<sup>-</sup> following 1 $\mu$ M PK2 application, as a result of a greater driving force for Cl<sup>-</sup> to leave the cell at the resting membrane potential. Arrow indicates time of PK2 application. Scale bars: 10mV, 100s. (C) Summary graph showing mean depolarizations but not hyperpolarizations were significantly larger in 139mM internal Cl<sup>-</sup> in response to 1 $\mu$ M PK2 (\* p<0.05, unpaired t-test).

with decreases in spike frequency. These observations suggested potential additional actions of PK2 on Na<sup>+</sup> channels, effects which would be in accordance with our own recent report of PK2 effects on Na<sup>+</sup> channels in SFO neurons (Fry *et al.*, 2008). We therefore undertook an analysis of mean action potential height in 5 cells that demonstrated decreased spike frequency but no change in membrane potential in response to PK2, an analysis that revealed PK2 induced a significant decrease in spike amplitude (paired *t*-test,  $p < 0.05$ ) from  $71.2 \pm 7.5$  to  $52.8 \pm 7.8$  mV (Figure 9A). In addition, we often observed that recovery from spike inhibition occurred at hyperpolarized membrane potentials below baseline (Figure 9B). We therefore hypothesized that PK2 inhibits AP action potential firing through modulation of voltage-gated Na<sup>+</sup> channels and undertook whole-cell voltage-clamp experiments to investigate the effect of 10 nM PK2 on Na<sup>+</sup> channel gating. Na<sup>+</sup> current activation and steady-state inactivation were studied in 6 AP neurons before and following PK2 application. Activation curves were generated by applying voltage steps between -80 and -20 mV in 10 mV increments from a -90 mV prepulse potential, and the conductance corrected for changes in driving force and normalized (Figure 10A). After fitting the resulting activation curves with a Boltzmann function, 4 cells out of 6 showed a significant leftward shift in the half activation potential (extra sum-of-squares *F*-test,  $p < 0.05$ ) to more hyperpolarized values (control  $-36.5 \pm 1.6$  mV, PK2  $-41.8 \pm 1.9$  mV), while there was no significant change in the slope factor *k* (control  $4.5 \pm 1.3$  mV, PK2  $4.9 \pm 1.7$  mV). To assess steady-state inactivation, AP neurons were subjected to 200 ms prepulse potentials between -110 and -20 mV in 10 mV increments, followed by a test pulse to -10 mV. A total of 6 normalized steady-state inactivation plots revealed a significant PK2-mediated hyperpolarizing shift in the half inactivation potential in 4 neurons (extra sum-of-squares *F*-test,  $p < 0.05$ ). The mean half



**Figure 9: PK2 decreases action potential amplitude in AP neurons**

(A) Left panel: Current-clamp record showing application of  $1\mu\text{M}$  PK2 decreased spike frequency and height in an AP neuron. Scale bars: 10mV, 20s. Right panel: Mean change in action potential height in response to PK2 application. Scale bars: 20mV, 5ms. (B) Current-clamp trace showing recovery from spike inhibition occurred at hyperpolarized membrane potentials in an AP neuron that hyperpolarized following PK2 administration, suggesting a change in  $\text{Na}^+$  channel voltage dependence. Scale bars: 10mV, 50s. Arrows indicate time of PK2 application.



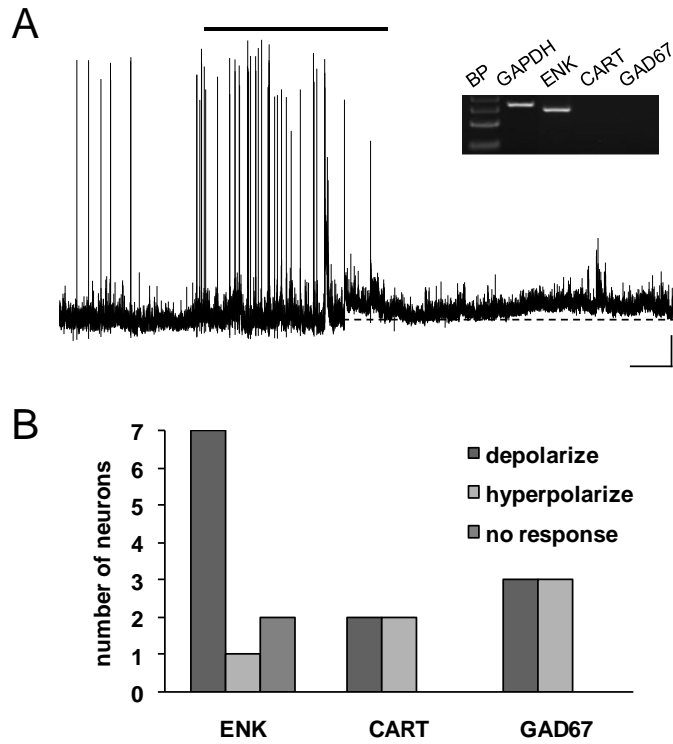
**Figure 10: PK2 induces a leftward shift in Na<sup>+</sup> channel activation and inactivation voltage dependence**

(A) Upper panel: Na<sup>+</sup> current activation from a test pulse of -40mV before and following 10nM PK2 application. Scale bars: 50pA, 1ms. Lower panel: Graph illustrating the mean normalized conductance plotted as a function of test potential in AP neurons showing a shift in activation gating induced by PK2 (n=4). Data points were fitted with a Boltzmann function. Inset shows the activation voltage step protocol. (B) Upper panel: Na<sup>+</sup> current inactivation following a prepulse step to -60mV before and following 10nM PK2 application. Scale bars: 200pA, 1ms. Lower panel: Graph illustrating the mean normalized current plotted versus prepulse potential in AP neurons showing a shift in inactivation gating caused by PK2 (n=4). Data points were fitted with a Boltzmann function. Inset shows the inactivation voltage step protocol.

inactivation potential was  $-59.4 \pm 1.0\text{mV}$  in control and  $-68.6 \pm 1.7\text{mV}$  following PK2 application (Figure 10B). Again, the slope factor  $k$  remained unchanged (control  $-6.6 \pm 0.9\text{mV}$ , PK2  $-7.1 \pm 1.5\text{mV}$ ). These data suggest that a shift to hyperpolarized potentials in the activation and steady-state inactivation of  $\text{Na}^+$  currents underlies the effect of PK2 on spontaneous firing in AP neurons.

### **3.5 Molecular identification of AP neurons influenced by PK2**

Many autonomic processes the AP regulates have a circadian component to them such as feeding behaviour and cardiovascular function. A number of neurotransmitters and neuropeptides implicated in the control of feeding behaviour and cardiovascular function are expressed in the AP (for review see Price *et al.*, 2007), and may therefore be involved in circadian autonomic output from this nucleus. We sought to identify whether specific chemical phenotypes of AP neurons are influenced by PK2 using a combination of electrophysiology and single cell RT-PCR (scRT-PCR). This allowed us not only to characterize the membrane potential response of single AP neurons but also to identify the specific peptide mRNAs expressed by individual neurons using *post hoc* molecular processing techniques. Nested primer sets were designed to detect the following mRNA: enkephalin (ENK), cocaine- and amphetamine-related transcript (CART), glutamate decarboxylase 67 (GAD67), cholecystokinin (CCK), tyrosine hydroxylase (TH), and vesicular glutamate transporter 2 (VGLUT2) (Table 1) and the response to 10nM PK2 was evaluated in 30 neurons that expressed GAPDH, a 'housekeeping' gene and positive control marker. ENK mRNA was expressed in 10 of these neurons, and as illustrated in Figure 11, the vast majority of responsive ENK-positive neurons (88%) demonstrated membrane depolarization as a consequence of peptide application. Additionally, 5 out of 6 ENK neurons



**Figure 11: PK2 predominantly depolarizes ENK neurons of the AP**

(A) Current-clamp recording demonstrating a 10nM PK2-induced depolarization accompanied by a decrease in spike frequency. This AP cell was identified to express ENK mRNA *post hoc* using scRT-PCR (inset). Bar above trace indicates the duration of PK2 application. Scale bars: 10mV, 50s. (B) Graph summarizing the frequency distribution of responses to PK2 in AP neurons expressing mRNA for ENK, CART, and GAD67.

displaying spontaneous activity responded to PK2 with decreased firing frequency (mean  $-99.3 \pm 0.7\%$ ). We also identified 2 depolarizing ENK neurons that co-expressed GAD67; however, GAD67 expressing neurons were not homogenously influenced (n=6). GAPDH-positive cells that hyperpolarized following PK2 exposure showed no distinguishing phenotype. Because there was only a small population of cells expressing CART (n=4) and CCK (n=1), it is difficult to make any conclusions regarding the effect of PK2 on these neurons. Lastly, there were no neurons identified to express TH.

## Chapter 4: Discussion

In this report we demonstrate that PK2, a circadian messenger produced in the SCN and NTS, has direct actions on the membrane properties of dissociated AP neurons. Using whole-cell electrophysiology, we have shown that nearly all spontaneously active AP cells respond to focal PK2 application with significant decreases in action potential frequency, an effect characterized by a leftward shift of voltage-dependent Na<sup>+</sup> channel activation and inactivation gating to hyperpolarized potentials. The primary effect of PK2 on membrane potential was depolarization due to the activation of a voltage-independent Cl<sup>-</sup> current, while inhibition of an inwardly rectifying NSCC led to hyperpolarization in a smaller proportion of neurons. Using scRT-PCR technology, we also identified a population of ENK-expressing neurons, the majority of which depolarized with a similar decrease in spike frequency in response to PK2, indicating the potential for specific circadian modulation of excitability on this phenotype of AP neurons.

### 4.1 Use of dissociated AP neurons

This study was performed using whole-cell patch clamp electrophysiology in combination with RT-PCR techniques to assess the effects of PK2 on dissociated AP neurons maintained in culture for 1 to 5 days. The dissociation protocol and timeframe used in this study allowed us to record from viable cultured neurons that possessed similar properties to those *in vivo* (Ferguson *et al.*, 1997). Using a dissociated cell culture approach allowed us to characterize responses to PK2 that were direct and independent of synaptic influence. This *in vitro* cell preparation is quite suitable for studying the responses of AP neurons to acute exposure of peptide hormones, given the sensory CVOs are readily accessible to blood-borne signals, receive

a high volume of blood, and are influenced by relatively few synaptic inputs (Morita & Finger, 1987;Gross, 1991;McKinley *et al.*, 2003). Neurons in this study displayed similar electrophysiological membrane properties to AP cells recorded by others using dissociated cultures and acute brain slices (Hay & Lindsley, 1995;Funahashi *et al.*, 2003). In addition, when compared to acute brain slices, dissociated AP neurons respond to peptide application in a similar fashion (Fry *et al.*, 2006).

#### **4.2 Use of scRT-PCR**

Experiments using scRT-PCR allowed us to characterize a change in excitability as a result of PK2 application with the peptide mRNA expression of individual neurons. Because small quantities of template were used in the RT-PCR reactions, we undertook a number of steps to minimize the chance of false positive results due to the amplification of contaminating nucleic acids. Immediately following the cell collection process, a DNase reaction was performed (see Materials and Methods) to eliminate genomic DNA contamination. In addition, a negative control reaction was performed by omitting the cDNA template from the PCR reactions. As a positive control reaction, the primer set for the 'housekeeping' gene, GAPDH, was also used in order to be confident that cytoplasm collection and cDNA synthesis were successful and that an absence of signal reflected the mRNA expression of the neuron at the time of experimentation, and not a faulty RT-PCR reaction.

#### **4.3 PK2-mediated effects on membrane excitability**

Earlier studies examining the effects of PK2 on forebrain neurons in the SFO and PVN identified only excitatory effects on membrane activity in these nuclei (Yuill *et al.*, 2007;Cottrell

*et al.*, 2004). In contrast, in the current study we have shown PK2 produced both depolarizing and hyperpolarizing actions on AP neurons, in addition to inhibitory effects on spontaneous action potential frequency, findings suggesting for the first time that PK2 has inhibitory effects on neurons in this region of the brain. Similarly, other peptides have been shown to influence AP neuronal activity, and AP cells respond to these peptides with both excitation and inhibition (Yang & Ferguson, 2002; Yang & Ferguson, 2003; Fry *et al.*, 2006; Fry & Ferguson, 2009). The above findings indicate there are separate populations of neurons present in the AP that may be unique in terms of function or output site.

PK2 was also shown to influence AP cells in a concentration-dependent manner with an apparent EC<sub>50</sub> of 27.5pM. This value falls between previously identified EC<sub>50</sub> values for PK2 of 2.3 and 63pM, and is in agreement with the concentration-response relationships found in the PVN and SFO (Yuill *et al.*, 2007; Cottrell *et al.*, 2004). Neurons within the AP appear to be highly sensitive to small quantities of PK2 given that application of approximately  $2-5 \times 10^{-19}$  moles (0.2-0.5 $\mu$ l of 1pM) still elicited demonstrable effects. These findings indicate that PK2 inhibits neuronal firing in the AP, with additional effects on membrane potential in subpopulations of neurons that are receptor-mediated.

#### **4.4 PK2 signalling and ion channel mechanisms mediating the effects on AP excitability**

Activation of the PKR2 is known to stimulate an intracellular signalling cascade involving the phosphorylation of p44/42 MAPK (Lin *et al.*, 2002a; Masuda *et al.*, 2002). Both Yuill *et al.* (2007) and Fry *et al.* (2008) showed that the PK2-mediated effects on membrane excitability in the brain are abolished using a specific MAPK inhibitor. However, our findings indicated that

MAPK signalling is not necessary for the effects of PK2 on membrane potential and spike frequency in the AP, and suggest a different intracellular signalling mechanism is responsible for these effects; perhaps inositol phosphate-mediated mobilization of intracellular  $\text{Ca}^{2+}$  or activation of protein kinase C (Lin *et al.*, 2002a; Masuda *et al.*, 2002; Vellani *et al.*, 2006).

Our observations indicated that action potential firing was consistently affected by PK2, as the majority of spontaneously active AP cells displayed a reduction in firing frequency accompanied by decreases in spike amplitude. It is well known that the voltage-gated  $\text{Na}^+$  channel is a critical component for the initiation and upstroke of the action potential in neurons (Hodgkin & Huxley, 1952). In support of the hypothesis that PK2 decreases spike frequency and amplitude through modulation of  $\text{Na}^+$  channel gating, we found that  $\text{Na}^+$  channel activation and inactivation gating was shifted to more hyperpolarized potentials. Similarly, PK2 has also been shown to influence  $\text{Na}^+$  channel activity in cultured SFO neurons, although an increase in  $\text{Na}^+$  current was found to be responsible for increased neuronal excitability (Fry *et al.*, 2008). These differences between the modulatory actions of PK2 on  $\text{Na}^+$  currents in the SFO versus the AP may be the result of differential  $\text{Na}^+$  channel subunit expression in these nuclei or as demonstrated in this study, different intracellular PK2 signalling pathways.

Near a resting membrane potential of -60mV, the observed hyperpolarizing shift in  $\text{Na}^+$  channel voltage sensitivity would result in a decrease of the  $\text{Na}^+$  current mediating the action potential and most likely underlies the effect of PK2 on decreased spike height. In addition, these findings indicate that AP cells would have a lower probability of firing an action potential in the presence of PK2 given that a greater proportion of  $\text{Na}^+$  channels would be in the

inactivated state. Thus at a constant resting membrane potential, fewer channels would be available to elicit a spike and a lower spontaneous firing rate would result (Jung *et al.*, 1997).

Whole-cell recordings using slow voltage ramps suggested that differential ion channel modulation by PK2 was a determinant of the effects of this peptide on membrane potential in the AP. AP cells that showed voltage-independent currents between -100 and -60mV responded to PK2 with depolarizing shifts in whole-cell current. In contrast, cells expressing an inwardly rectifying current responded with hyperpolarizing shifts in whole-cell current. These data indicate a difference in whole-cell properties between two populations of AP neurons and are supported by the fact that 67% of cells were observed to express a  $Gd^{3+}$ -sensitive current that mimicked PK2-mediated hyperpolarization. The observed reduction in inward current may represent PK2-mediated inhibition of the hyperpolarization-activated cation current that is present in roughly 60% of AP neurons and possesses a similar reversal potential (-36mV) as neurons in this study (Funahashi *et al.*, 2003). Therefore, inhibition of inward current may be the result of PK2 causing a decrease in the conductance of a  $Gd^{3+}$ -sensitive NSCC, and this would contribute to hyperpolarization (Oliet & Bourque, 1996). Similarly, orexin-A and ghrelin are two other peptides with actions in the brain that have been shown to modulate NSCCs in the AP, although these two neuropeptides depolarized AP neurons through activation of the NSCCs (Yang & Ferguson, 2002; Fry & Ferguson, 2009). In combination with a hyperpolarizing shift in  $Na^+$  channel voltage dependence, PK2-mediated membrane hyperpolarization and increased input resistance creates an ideal situation in which the responsiveness of an AP neuron to input signals, such as excitatory postsynaptic potentials, would result in an increased probability of

firing. Therefore PK2-mediated hyperpolarization likely maintains an optimal membrane potential necessary for Na<sup>+</sup> channel function, and consequently, action potential firing.

In neurons, Cl<sup>-</sup> channel activity is critical for controlling membrane excitability. Previous work has demonstrated that regulation of Cl<sup>-</sup> channel gating affects neuronal activity, the most well known mechanisms of gating being changes in voltage, pH, intracellular Ca<sup>2+</sup>, cell volume, phosphorylation, ATP, and osmolarity (Suzuki *et al.*, 2006). Furthermore, metabolic signals such as fatty acids and glucose are thought to regulate membrane excitability through modulation of Cl<sup>-</sup> channel activity; more specifically, closing of Cl<sup>-</sup> channels has been proposed as a mechanism of excitation in glucose-sensitive neurons of the ventromedial hypothalamic nucleus (Tewari *et al.*, 2000; Song *et al.*, 2001). We have demonstrated that depolarizing shifts in whole-cell current in the AP were the result of a PK2-activated conductance that reversed at the Nernst E<sub>Cl<sup>-</sup></sub>. Furthermore, recordings under high internal Cl<sup>-</sup> (creating a greater driving force for Cl<sup>-</sup> to leave the cell at resting membrane potentials) resulted in significantly larger depolarizations following peptide administration. Depolarizations often occurred when the resting membrane potential of the neuron was less negative than E<sub>Cl<sup>-</sup></sub>, suggesting it is possible that another conductance is influenced in addition to Cl<sup>-</sup> channel activation. This is supported by the fact that the mean reversal potential of the PK2-activated depolarizing current did not fully shift to the calculated E<sub>Cl<sup>-</sup></sub> (-2mV) when AP cells were loaded with high internal Cl<sup>-</sup>, indicating the possible involvement of a second ionic conductance in mediating PK2-induced depolarization. It is difficult to speculate the type of Cl<sup>-</sup> channel that is influenced by PK2 as the expression of Cl<sup>-</sup> currents and transporters in the AP have not yet been described.

Because whole-cell patch electrodes were used in our experiments, we assume the contents of the pipette solution fully dialysed into the internal compartment of the cell and the  $E_{Cl^-}$  was accurate (Kawa, 2007). According to our findings, two factors would determine whether the effect of PK2-mediated  $Cl^-$  channel opening in the AP would be depolarizing or hyperpolarizing: the resting membrane potential of AP neurons in physiological conditions, and the electrochemical gradient of  $Cl^-$  i.e. the  $E_{Cl^-}$ . A resting membrane potential less negative than the  $E_{Cl^-}$  would result in hyperpolarization and inhibition due to  $Cl^-$  channel activation, whereas a cell resting more negative than the  $E_{Cl^-}$  would depolarize. Depolarizations were observed in our experiments likely because the resting membrane potentials of the cells tested were normally more negative than  $E_{Cl^-}$ . Finally,  $Cl^-$  channel activation likely shunts the membrane potential of AP neurons, an effect that would decrease the responsiveness of these neurons to humoral or neural signals. In addition to shunting by  $Cl^-$  channel activation, a leftward shift in  $Na^+$  channel gating would likely decrease firing activity and further decrease the excitability of the cell. Therefore, these results suggest PK2 plays a modulatory role in controlling AP neuronal excitability that may regulate the efficacy of peripheral homeostatic signals, ultimately controlling AP autonomic function.

#### **4.5 PK2 inhibits enkephalin-expressing AP neurons**

As a sensory CVO, the AP has an important role in detecting and relaying neural and blood-borne signals to nuclei in the hypothalamus and medulla (Cottrell & Ferguson, 2004). AP neurons express a number of different neuropeptides which are presumably used by these cells as peptidergic neurotransmitters which would be released from nerve terminals in brain centres to which these cells project (Price *et al.*, 2007). In our experiments scRT-PCR was used to identify

the chemical phenotype of AP neurons and we have obtained data indicating that PK2 primarily caused membrane depolarization combined with suppression of action potential firing in ENK-expressing neurons of the AP. These data suggest that PK2 has the ability to suppress neuropeptide release from this population of cells and potentially impact autonomic output from the AP. Although ENKs are highly expressed in the AP (Fallon & Leslie, 1986), neither the axonal projections, nor the physiological relevance of ENK output have been described for this CVO. However, the NTS is a major autonomic integration centre in the medulla, receives direct inputs from the AP, and represents a possible site where ENK-neurons may project.

In accordance with such a proposal, ENKs have been shown to induce changes in cardiovascular function when administered into the brain ventricular system (Schaz *et al.*, 1980;Yukimura *et al.*, 1981). Direct injection of opioids into the NTS, a major cardiovascular control centre, also has receptor subtype specific effects on cardiovascular function (Hassen *et al.*, 1982). ENKs are endogenous agonists for the  $\delta$  opioid receptors, expression and activation of which has been described in the NTS (Atweh & Kuhar, 1977;Reid & Rubin, 1987). Microinjection of ENKs into the NTS of rats has been demonstrated to serve a pressor function by increasing mean arterial pressure that is accompanied by tachycardia, perhaps through attenuation of the baroreceptor reflex (Petty & De, 1983). Intriguingly, neurons of the AP are implicated in regulating cardiovascular function through excitatory synaptic connections with the NTS, including interactions with barosensitive NTS neurons (Cai *et al.*, 1996;Hay & Bishop, 1991). Furthermore, alteration of arterial baroreflex sensitivity by angiotensin II and vasopressin is thought to occur as a direct consequence of actions of these peptides on AP neurons and subsequent signalling through neuronal connections between the AP and NTS (Cai *et al.*, 1994).

It is therefore possible that ENK-expressing neurons of the AP may project to the NTS and contribute to cardiovascular regulation in the brainstem; however, retrograde tracing from the NTS combined with ENK immunolabelling in the AP would be required to confirm such a hypothesis.

The mechanism for PK2-induced depolarization is suggested to occur through enhancement of a Cl<sup>-</sup> carrying conductance in the AP and indicates a shunting effect may render ENK neurons less responsive to humoral or neural signals, such as regulation of baroreflex sensitivity by angiotensin II and vasopressin. Double immunostaining also reveals ENK-expressing AP neurons receive synapses from neurons that express dopamine, angiotensin II, catecholamines, and GABA (Li *et al.*, 2001; Guan *et al.*, 1995a; Guan *et al.*, 1995b; Guan *et al.*, 1996). In addition, this effect on membrane conductance would likely inhibit hyperpolarizing inputs and their ability to remove inactivation, leading to an exacerbation of action potential inhibition. Ultimately, decreased spike activity would reduce ENK release at AP projection sites. Since some GAD67-expressing cells hyperpolarized following PK2 application, it is possible that PK2 has the potential to increase the excitability of a population of GABAergic interneurons that synapse onto ENK neurons within the AP (Newton & Maley, 1987). Increased GABA release onto AP neurons would further inhibit ENK output. Dendrites of ENK neurons are also found in close proximity to the basal laminae of blood vessels in the AP, indicating the likelihood that ENK-expressing neurons of the AP sample from the circulation as well (Guan *et al.*, 1995a).

Previous work suggests PK2-mediated increases in excitability in SFO neurons may be responsible for its role in stimulating drinking in rats, while the PK2 homologue, Bv8, acts to suppress circadian feeding through actions in the arcuate nucleus of the hypothalamus (Negri *et*

*al.*, 2004;Cottrell *et al.*, 2004;Fry *et al.*, 2008). In addition, PK2 depolarizes magnocellular and parvocellular neurons of the paraventricular nucleus (Yuill *et al.*, 2007), a hypothalamic nucleus that receives direct SCN efferent projections, and may be a site where SCN-derived PK2 controls endocrine rhythms because PK2-deficient mice show reduced rhythms of circulating corticosterone levels (Li *et al.*, 2006a). To our knowledge, there has been no description of the actions of PK2 on cardiovascular function. Our description of specific actions of PK2 on ENK neurons in the AP indicates a potential mechanism for circadian modulation of autonomic output from the AP.

Although CART and GAD67 neurons were encountered at a much lower frequency in this study, these preliminary data indicate an ability of PK2 to modulate the release of CART and GABA from AP neurons, influencing the physiological actions of these signalling molecules at AP projection sites. The effect of PK2 on these neurons will need further testing to elucidate the potential for specific effects on membrane excitability. The chemical identification of neurons that hyperpolarize in response to PK2 also warrants further investigation and may represent a different and functionally distinct population of AP neurons. Lastly, neurons that expressed mRNA for CCK, VGLUT2, or TH were rarely encountered. This may be the result of a limitation in the detection sensitivity of our scRT-PCR technique and not necessarily due to a lack of mRNA expression. Taken together, these findings indicate that PK2 modulates AP neuronal excitability with specific inhibitory (depolarization and decreased spike frequency) actions on ENK neurons.

#### **4.6 Physiological significance of PK2 signalling in the AP**

A final question arises regarding the source and expression pattern of PK2 with respect to its ability to function as a circadian signalling molecule in the AP. To date, there are no known

direct axonal projections from the SCN to the AP. Furthermore, PK2 has not been detected within the cerebrospinal fluid, although humoral release from the SCN into the cerebrospinal fluid cannot be ruled out. Similar to other peptides produced in the GI tract, PK2 may also signal to the AP as a circadian hormone in the circulation. However, neither the presence nor any circadian profile of PK2 expression has presently been described in the bloodstream. The ongoing difficulty in identifying PK2 protein expression arises from its amino acid sequence and complex folding structure. Currently an antibody for PK2 has not been reported.

The molecular clock genes are critical cellular components of circadian timekeeping and output from the SCN that drive rhythmic expression of PK2 in the SCN. Similar to their expression in the SCN, the clock genes also clearly show a 24 hour rhythmic expression pattern in the NTS (Herichova *et al.*, 2007; Kaneko *et al.*, 2009). Interestingly, mRNA for PK2 and the PKR2 is found in the NTS (Negri *et al.*, 2004; Cheng *et al.*, 2006), indicating this major brainstem autonomic integration centre is a potential source for PK2 influence on the AP. However, Negri *et al.* (2004) did not show PK2 levels being significantly different between the light and dark phases in the NTS of rats, although the mRNA expression did increase at night. The circadian expression of the core clock genes are altered in the NTS in models of obesity and hypertension, suggesting that circadian regulation of metabolic and cardiovascular function may occur via output from the NTS (Herichova *et al.*, 2007; Kaneko *et al.*, 2009). Furthermore, the risk for cardiovascular incidents also follows a daily rhythm, being maximal during the early morning when blood pressure and heart rate are highest (Krantz *et al.*, 1996). The NTS represents a likely source of PK2 expression and possible circadian regulation of AP excitability and autonomic output. This possibility may even extend to reciprocal influence or feedback mechanism from

the AP to the NTS. Whether the AP is exposed to levels of PK2 that follow a circadian pattern and what the source of PK2 is remains to be investigated. The remarkable daily fluctuation in PK2 from the SCN, from daytime levels that are 50-fold higher than at night, indicates that the above actions on rat AP neurons by this circadian signalling molecule may suppress AP activity during circadian day when behavioural, metabolic, and cardiovascular activity is reduced in nocturnal species (Ikonomov *et al.*, 1998;Scheer *et al.*, 2003). In support of previous work showing high expression of PKR2 mRNA in the AP (Cheng *et al.*, 2006), we have shown clear effects demonstrating PK2 modulation of AP excitability in the majority of cells tested. Not all neurons responded to PK2 with shifts in Na<sup>+</sup> channel gating, depolarizations, or hyperpolarizations, and this suggests that variability in ion channel subunit expression within AP neurons may be a determinant of whether a neuron responds to PK2. Alternatively, PKR2 expression is likely also a factor in neuronal sensitivity to PK2.

We can conclude from this study that PK2 inhibits spike frequency through actions on Na<sup>+</sup> channel voltage dependence. Additionally, the membrane potential of native AP neurons *in vivo*, in combination with the effects of PK2 on membrane resistance and specific ionic conductances, suggest that the level of AP neuronal excitability may be regulated by the circadian cycle of PK2 expression. Therefore, PK2 may influence the ability of the AP to respond to homeostatic signals, thus impacting autonomic output within the CNS.

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