Background.

The hypothalamus contains several neurone populations involved with the control of the cardiovascular system. Some of these neurones project directly to sympathetic centres of the spinal cord. Jansen *et al.* [28] identified these neurones as central command neurones of the stress response, first hypothesised by Walter B. Cannon, as long ago as 1912 (see [12]). Studying how these neurones are regulated by neurochemicals released during stress will facilitate a more thorough understanding of the central mechanisms underlying cardiovascular control.

The cardiovascular response to stress: "Fight or Flight" (Canon 1929)

Traditionally, cardiovascular disease is thought of as a disease of peripheral organs, such as the heart, kidneys and blood vessels. However, since the entire cardiovascular system exists under complex central control [66], it is likely that stress-related cardiovascular diseases also involve autonomic control centres of the brain. The classical response to psychological stress, be it fear, annoyance or pressure of work [12;63], has three principal elements; first, direct action of the sympathetic nervous system innervating the heart and blood vessels. Secondly, release of adrenaline via sympathetic activation of the adrenal medulla, and finally, HPA-axis activation of the adrenal cortex (and release of corticosteroids) [11;24;63]. The cardiovascular component of this stress response can be quite dramatic, cardiac output increasing by up to three or four fold within seconds [24]. The magnitude of this stress response, in a given individual, is a familial trait [24]. In a susceptible individual with, for example, a history of heart disease [18;22], this can prove fatal, and recently, it has become evident that sudden excessive activation of the stress response can even prove dangerous in the *absence* of any pre-existing cardiovascular disease [22].

The paraventricular nucleus: "Autonomic Master Controller" (Loewy 1991).

Many of the physiological responses to stress are mediated by the paraventricular nucleus of the hypothalamus (PVN), a region of the brain that regulates both the release of HPA (hypothalamus-pituitary-adrenal cortex) hormones and the activity of the sympathetic nervous system [61]. Regulation of the sympathetic nervous system is thought to be, in turn, mediated by efferent neurones projecting from the PVN to the medulla and spinal cord (see below and [1;2;13;36]). Any modulation of the activity of these neurones (by, for example, stress) is likely to be important to cardiovascular function.

Central origins of the stress response: The "hypothalamic defence area".

Experiments in the 19th century located the centre of primary cardiovascular control to the medulla [21]. In the first half of the 20th century, however, it became evident that stimulation of certain areas of the hypothalamus elicited an almost full "defence reaction" [4], including tachycardia and increased blood pressure. Furthermore, destruction of the hypothalamus largely abolished the stress response in experimental animals [5], and lesion of the brain immediately above the level of the hypothalamus rendered the animals in a permanent state of cardiovascular excitement, so called "sham-rage". This implied that extra-hypothalamic areas of the limbic system, in general, restrain the inherent pro-sympathetic drive of the hypothalamus [4].

Stimulation of the PVN itself increases both heart rate and blood pressure [40;41;43], however, Hilton and co-workers suggested that a more complete defence reaction, including, for example, skeletal muscle vasodilatation and pilo-erection, are elicited by electrical stimulation of the more caudal and lateral areas of the hypothalamus [26]. These areas are now termed the hypothalamic “defence areas”, and include the dorsomedial hypothalamus (DMH) and lateral hypothalamic area (LHA). In humans, the physiological stress response, or defence reaction, can be evoked by psychological factors, e.g., fear, excitement or even intense mental arithmetic [24]. In experimental animals, equivalent stimuli increase neuronal activity throughout the hypothalamus [25]. There is then, evidently a significant descending afferent pathway from the "higher centres" of the brain to the hypothalamus. Whilst the PVN is richly invested with efferent projections to the sympathetic centres of the medulla and spinal cord, it in fact receives few descending afferent inputs directly from other limbic structures [70]. Instead, it appears that most descending limbic inputs to the periventricular regions of the hypothalamus synapse first with neurones of either the bed nucleus of the stria terminalis [14], or the more lateral hypothalamic areas [57]. From there they connect to the PVN via shorter, mostly intra-hypothalamic projections [70]. One possibility is that the hypothalamic "defence areas" (including the DMH and LHA) mediate some of the cardiovascular stress response via connections to the PVN. Certainly, anatomical evidence shows that the densest efferent projections from the DMH terminate in the PVN in both

rodents and humans [16] [73], and injection of the $GABA_A$ receptor agonist, muscimol, into the DMH reduces stress induced neuronal activity in the PVN [45]. It appears that sympathetic neurones of the PVN are normally inhibited by dominant, inhibitory, GABA tone [41;43]. Physiological excitation of efferent sympathetic PVN neurones could then result either directly from an excitatory input or indirectly from disinhibition of this basal GABAergic tone.

A number of neurones projecting to the PVN from the hypothalamic "defence areas" (particularly the DMH and LHA) contain the tachykinin peptide neurotransmitter substance P [9]. The PVN also contains a very high density of tachykinin receptors [39]. Substance P itself has been shown to inhibit $GABA_A$ currents [76] and so it seems possible that the hypothalamic "defence areas" may excite PVN sympathetic neurones via intra-hypothalamic projections and disinhibition of GABA inhibitory tone.

The tachykinin hypothesis:

Tachykinins are a family of small neuropeptide transmitters sharing a common C-terminal sequence (-Phe-X-Gly-Leu-Met-NH_2). In the mammalian central nervous system there are at least three tachykinin peptides (substance P, neurokinin B and neurokinin A) and three tachykinin receptors (NK1, NK2 and NK3). It is generally accepted that substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) are the preferential agonists for NK1, NK2 and NK3 receptors, respectively [37]. It should be noted, however, that there are numerous examples, where, *in vivo*, tachykinins appear to act via their *non-preferential* agonist [23;59;74].

SP, the first tachykinin to be discovered, was first extracted from horse brain, but characterised as a *depressor* substance [75]. Applied systemically, SP powerfully decreases blood pressure, and it became evident in the 1980's that this action is mediated, principally, via endothelium dependent peripheral vasodilatation [52]. More recently, however, it has become evident that the central cardiovascular actions of tachykinins are very much more diverse. Microinjection of SP or NKA into the nucleus tractus solitarii, for example, induces hypotension and bradycardia, whereas, microinjection of NKB into the same region increases blood pressure and elicits tachycardia [49]. Intracerebroventricular or periventricular injection of SP, NKA or NKB all, however, elicit increases of heart rate and blood pressure [15]. Several pieces of evidence suggest that the paraventricular, dorsomedial and lateral hypothalamic nuclei are involved in these cardiovascular actions of the tachykinins and may be involved with the cardiovascular stress response: (i) There is dense staining of SP immunoreactive perikarya in the "hypothalamic defence" regions, including the DMH & LHA [35]. These neurones project to the parvocellular PVN [9]. The SP content of the DMH and LHA becomes depleted during stress [65], suggesting high activity of the tachykinin system. (ii) All three principal tachykinin receptors are present in the PVN [39;64], with NK3 being particularly abundant (in rats; preliminary data and [17;31], and in humans: [31;32]). Hypothalamic NK1 receptors are down regulated during stress (in rats; [72]), again consistent with high tachykininergic activity. (iv) Moreover, microinjection of tachykinins directly into the PVN induces a similar *pressor* response to that obtained by intracerebroventricular injection of tachykinins [71]. (v) Finally, our own preliminary data suggest that spinally projecting neurones express tachykinin receptors, activation of which inhibits GABA responses *in vitro*, and may thus relieve GABA inhibitory tone *in vivo*. There is no published electrophysiology on tachykinin actions in the PVN, and no direct evidence showing activity of the tachykinin projections to the PVN. In this project, we propose to tackle these problems, with particular regard to spinally projecting parvocellular PVN neurones.

The identity of retrogradely labelled spinally projecting PVN neurones:
"Central command neurones of the sympathetic nervous system" (Jansen et al. 1996).

Jansen *et al.* [28] included *spinally projecting* neurones of the PVN in the set of so called "central command neurones", arguing that these neurones project co-laterals to numerous cardiovascular targets and are central to execution of Cannon's 1929 "fight or-flight" stress response [12]. There is however, little published data on the identity of receptors expressed by, or the neurochemicals acting directly on these spinally projecting neurones. One reason for this is that it is quite difficult to be certain of the identity and rôle of a particular neurone studied *in vitro.* However, by the use of retrograde labelling/voltage-clamp methods (see below), we can be confident that visually *identified* spinally projecting neurones are autonomic pre-ganglionic neurones. There are also several lines of evidence that strongly suggest that this population of neurones includes those whose final (post-ganglionic) targets are the heart and splanchnic blood vessels:

(i) The injection site for retrograde labelling (the spinal cord intermediolateralis, level T2) is now firmly

established to be coincident with the point of origin for pre-ganglionic sympathetic neurones innervating the stellate (SG) and superior cervical ganglia (SCG) [27;48;67;68]. Post-ganglionic neurones originating at the SG and SCG in turn innervate several autonomic targets in the upper body - including blood vessels and the heart.
(ii) Direct stimulation of the PVN *in vivo* by glutamate [40], electrical current [42] or bicuculline (presumably by disinhibition of basal GABAergic tone) [30;41], increase sympathetic outflow, vasomotor tone and heart rate. Our own data show that spinally projecting neurones themselves express GABA receptors [8]. (Interestingly, during heart failure, the well known increase in basal sympathetic tone [20;53] is associated with increased activity of neurones in the PVN [78], which reflects a reduction of basal inhibitory GABAergic input to cardiovascular control neurones in the PVN [78]).
(iii) PVN neurones projecting to the spinal cord have been shown to contain vasopressin [60], this is released into the spinal cord on stimulation of the PVN [54], and intrathecal application of vasopressin increases blood pressure and heart rate [58], whereas block of spinal vasopressin receptors by a V_1-antagonist, reduces the sympathetically mediated cardiovascular effects of PVN stimulation [38].
(iv) Finally, procedures inducing a PVN dependent increase in blood pressure and heart rate (stress or haemorrhage) also induce FOS expression in PVN spinally projecting neurones, indicating the likely activation of these neurones [3;62].

Experimental Design

The following information gives is guide to details of the types of experiments the successful candidate will be able to undertake; specific training will be given as and when necessary.

The project will use retrograde labelling, patch-clamp recording and immunohistochemical analysis to study tachykinin - GABA interactions with *identified* spinally projecting neurones of the PVN. In the later stages of the project we will also investigate afferent tachykinin projections *to* the PVN and, specifically, to spinally projecting parvocellular neurones.

Central to completion of this project will be *whole-cell recording of identified spinally projecting neurones:*
Using suitable epifluorescent microscopy, the labelled soma of the spinally projecting neurones of the PVN can be identified and recorded from (Axopatch amplifier, DigiData interface; Burleigh piezo-electric PCS 5000 series micromanipulator). The patch-clamp recording conditions are similar to that used elsewhere [6], however, we also include a fluorescent label in the patch pipette, to contrast the retrograde label and allow us to confirm the identity of the recorded cell.

***Section 1*: Tachykinin Interaction with GABA currents. (Electrophysiology, 3 to 6 months):**
1(a) Inhibition of whole-cell GABA currents by bath applied substance P.
We have preliminary data showing substance P (SP) modulates $GABA_A$ receptor currents in spinally projecting neurones. The first 3 to 6 months will extend and strengthen this work. The first of these experiments will involve similar procedures to those used to obtain the preliminary data [7] and in previous experiments [77].

1(b) Does SP modulate the frequency of spontaneous action currents? Is this action GABA dependent?
In the final part of this section we will examine the sensitivity of parvocellular action current frequency to bath applied SP. *In vivo*, spinally projecting neurones are tonically inhibited by GABA [41;43]. Despite this, approximately 10% of spinally projecting parvocellular neurones fire action potentials spontaneously in the slice preparation. Spontaneous action potential activity is abolished by synaptic inhibition with either GABA, low Ca^{++}/high Mg^{++} or addition of the excitatory amino acid antagonists 5-APV and CNQX (unpublished observations and [77]). The frequency of these action potentials can be estimated by use of the cell-attached action current technique ([19;77]).

***Section 2*: Which tachykinin receptors are expressed by identified spinally projecting parvocellular neurones tachykinin receptors? (Immunohistochemistry; 8 to 10 months):**
Immunohistochemistry: For the majority of immunohistochemical experiments, spinally projecting neurones are first labelled with a retrograde tracer.
The results from this immunohistochemical study will provide strong evidence of exactly which tachykinin

receptors are expressed by spinally projecting PVN neurones.

***Section 3*: Do these tachykinin receptors have any other apparent electrophysiological actions? (If time allows):**

Section 4: What are the intracellular mechanisms of action of the tachykinins on spinally projecting neurones? (Electrophysiology, 8 to 10 months):

4(a) Intracellular pathway:
NK1, NK2 and NK3 are all G-protein coupled receptors and can activate both phospholipase C (PLC) and adenylyl cyclase [50;52]. Thus tachykinins can potentially stimulate protein kinase C (PKC) and protein kinase A (PKA) dependent phosphorylation. $GABA_A$ receptors are themselves substrates for phosphorylation by both PKA and PKC [10;44;46;55]. This gives rise to a wide range of potential intracellular pathways. Furthermore, phosphorylation of $GABA_A$ receptors can result in either activation or inhibition of GABA activity, depending on the identity of the expressed subunits and the mode of kinase activation [29;33;47;51;55;56;76]. Yamada *et al.* [76] showed $GABA_A$ currents of bullfrog DRG neurones to be inhibited by SP via a PKC dependent mechanism.
Experiments will use selective activators of PKC (PMA and the catalytic subunit of PKC) and also examine the sensitivity of the SP action to selective inhibitors of the PKC pathway (for example H7 and PKC 19-36).

Next, it will be important to investigate whether the $GABA_A$ currents of spinally projecting neurones are also modulated by the adenylyl cyclase- PKA pathway. We will test the SP modulation of $GABA_A$ currents for resistance to inhibitors of PKA such as Rp-cAMP, and investigate the possible activity of membrane permeant PKA stimulators such as dibutyryl-cyclic AMP.
Ion channels are often subject to tonic "background" modulation by protein kinases, i.e., they may already be phosphorylated, before the application of experimental protocols. This is important in its own right, because it provides additional mechanisms for possible physiological modulation (*dephosphorylation*). However, tonic background phosphorylation could also mask responses to exogenously applied neurotransmitters. To address the possibility of tonic activity of G-protein coupled protein kinases (via stimulation of adenylyl cyclase or PLC) we will use conventional whole-cell patch-clamp experiments. These will be performed with GDPβS (a non-selective inhibitor of G-protein activity) included in the patch pipette. Under these conditions, addition of membrane permeable protein kinase stimulators will be increased.
4(b) Mode of action of tachykinins:
The final part of the mechanistic work will be at the single channel level, to determine whether tachykinins inhibit $GABA_A$ receptors by reducing their unitary conductance (*i*), reducing the number of active channels (*n*), or by reducing the channel open probability.
It is likely that the tachykinin modulation of $GABA_A$ currents is lost on patch excision (due to the loss of intracellular phosphorylation components, membrane soluble kinases etc.,), and so we will record $GABA_A$ currents in cell attached mode. GABA will be included in the patch pipette at a low concentration (1 to 3μM, to reduce desensitisation), dissolved in extracellular physiological saline solution, and selective tachykinin agonists will be applied to the neurone by bath superperfusion. Cell attached patch recording does not reduce the efficacy of G-protein dependent agonists added to the neurone, even when they are added to the outside of the patch pipette (eg., [34;69]).

Section 5: What is the role of tachykinin projections from the dorsomedial and lateral hypothalamic nuclei to the PVN? (Approximately 12 months):
Hypothalamic nuclei will be stimulated by local application of a microdrop of the excitatory amino acid glutamate, released from the pipette tip (5-10μm) of a Picospritzer device. This will activate only neurones originating these areas (rather than "fibres of passage").
An important question is whether, as one would anticipate, endogenous GABA currents can be modulated by applied tachykinin agonists.

Expected Value of Results
Evidence suggests that stress related heart disease, and the elevated sympathetic outflow associated with heart

failure both involve neurones projecting directly from the paraventricular nucleus of the hypothalamus to the spinal cord. Over recent years, a great deal of research has investigated the effects of electrical and neurochemical stimulation of these spinally projecting neurones on heart rate, blood pressure and sympathetic outflow. Such a pathway is important to several aspects of autonomic control, including the rapid, sympathoneuronal portion of the "fight or flight" response. In this study, we propose to complement this work with a predominantly *in vitro* study of the regulation of these neurones by the family of tachykinin neurotransmitters. Understanding this pharmacology will be important for the future development of treatments for heart failure and stress-related heart disease.

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