Neurobiological Consequences of Stress: Tyrosine Hydroxylase Phosphorylation in Response to Stress

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Statement of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

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Acknowledgment of Authorship

I hereby certify that the work embodied in this thesis contains a published paper/s/scholarly work of which I am a joint author. I have included as part of the thesis a written statement, endorsed by my supervisor(s), attesting to my contribution to the joint publication/s/scholarly work.

Lin Kooi Ong

Endorsement of Authorship by Supervisors

*We attest that Research Higher Degree candidate **Lin Kooi Ong** contributed to 1) the conception and design of the research, 2) collection, analysis and interpretation of research data and 3) drafting and revision of significant parts of the work to contribute to the interpretation of the publications entitled:*

The sustained phase of tyrosine hydroxylase activation in vivo

Ong L.K., Sominsky L., Dickson P.W., Hodgson D.M. and Dunkley P.R. Neurochem Res. (2012) DOI: 10.1007/s11064-012-0812-3

The effects of footshock and immobilization stress on tyrosine hydroxylase phosphorylation in the rat locus coeruleus and adrenal gland

Ong L.K., Guan L., Stutz B., Dickson P.W., Dunkley P.R. and Bobrovskaya L. Neuroscience, 192, 20-27 (2011)

The effect of social defeat on tyrosine hydroxylase phosphorylation in the rat brain and adrenal gland

Ong L.K., Bobrovskaya L., Walker F.R., Day T.A., Dickson P.W. and Dunkley P.R. Neurochemical Research, 36(1), 27-33 (2011)

Signal transduction pathways and tyrosine hydroxylase regulation in the adrenal medulla following glucoprivation: an in vivo analysis

Bobrovskaya L., Damanhuri H.A., Ong L.K., Schneider J.J., Dickson P.W., Dunkley P.R. and Goodchild A.K. Neurochemistry International, 57(2), 162-167. (2010)

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E/Prof. Peter Dunkley	A/Prof. Phillip Dickson	Dr Larisa Bobrovskaya

Thesis by Publication

I hereby certify that this thesis is in the form of a series of published papers of which I am a joint author. I have included as part of the thesis written statement from each co-author, endorsed by the Faculty Assistant Dean (Research Training), attesting to my contribution to the joint publications.

Lin Kooi Ong

Prof. John Rostas

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Contents

Thesis Abstract

Stress is part of our daily life. One of the major cell types involved in the stress response are the catecholaminergic cells in the brain, the peripheral nervous system and the adrenal medulla. These cells, which produce adrenaline, noradrenaline and dopamine, are subject to a range of controls each of which is involved in the stress response. The major subject of this thesis is the effect of stress on one of these controls namely biosynthesis of the catecholamines. Tyrosine hydroxylase (TH) is the rate-limiting enzyme in catecholamine biosynthesis. TH is itself subject to a range of regulatory mechanisms, including feedback inhibition by the catecholamines, phosphorylation of serine residues (Ser19, Ser31 and Ser40) which can contribute directly or indirectly to enzyme activation, as well as mRNA expression and protein synthesis which determine the availability of TH. In response to stress catecholaminergic cells are depolarized and extracellular calcium enters leading to the release of catecholamines from these cells and also to the activation of signal transduction pathways that lead to an increase in TH phosphorylation and TH activity. When catecholamines are released from cells during the stress response it has been shown that the concomitant increase in TH activity and catecholamine synthesis maintains catecholamine levels in the cells at a constant level. The phosphorylation of each serine residue does not affect TH activity equally. Ser19 phosphorylation does not increase TH activity directly, Ser31 phosphorylation increases TH activity modestly and Ser40 phosphorylation, which relives the feedback inhibition by catecholamines, increases TH activity substantially. Three phases of TH activation (acute, sustained and chronic) have been identified and the regulatory mechanisms for each phase have been

extensively characterized *in vitro* and *in situ*. The acute phase involves TH phosphorylation which occurs and is mostly reversed over the first hour after exposure to stress. The sustained phase also involves TH phosphorylation via different mechanisms but it occurs from 1 to 24 h after exposure to stress. The chronic phase involves mRNA synthesis and TH protein synthesis and this occurs from 4 to 72 h after exposure to stress. To date, there have been only limited studies that have investigated the acute phase of TH activation in response to stress and no studies that have investigated the sustained phase *in vivo*. Only the chronic phase of TH activation in response to stress has been extensively investigated *in vivo*.

The work presented in this thesis aimed to systematically investigate the different phases of TH activation, especially the acute and sustained phases, by measuring TH phosphorylation and TH protein at different time points in response to a range of stressors *in vivo*. The adrenal medulla and the locus coeruleus (LC) where chosen as representative catecholaminergic cells for these studies. We have compared the profile of TH phosphorylation and TH protein elicited by two stressors tentatively classified as physical (footshock or glucoprivation stress) and two stressors tentatively classified as psychological (immobilization or social defeat stress) in the adrenal medulla and the LC over a 1 h period. We found that the different stressors all induce the acute phase of TH activation, but provide different temporal profiles of TH phosphorylation at Ser19, Ser31 and Ser40, without TH protein synthesis in the adrenal medulla and the LC over the first hour *in vivo*. The physical stressors both activated the catecholaminergic cells to a greater extent when compared to the psychological stressors. We have also compared the profile of TH phosphorylation and TH protein elicited by three different stressors social defeat, glucoprivation or LPS stress in the

adrenal medulla at 24 h. It should be noted that the LPS studies were undertaken with neonatal rats. We found that social defeat or glucoprivation stress do not induce sustained phosphorylation. However, LPS stress induces the sustained phase of TH activation by inducing sustained TH phosphorylation at Ser31 and Ser40 without TH protein synthesis being increased in neonatal rats' adrenal medulla at 24 h. The reason for the difference is unknown, but it is possible that sustained phosphorylation only occurs in neonatal animals or perhaps LPS stress activates the adrenal via a different set of intracellular messengers to the other stressors. Whatever the mechanism this is the first study to demonstrate that the sustained phase of TH activation occurs *in vivo*.

Overall we provided evidence that different catecholaminergic cells respond differently in term of the temporal profiles of TH phosphorylation at Ser19, Ser31 and Ser40, presumably due to differences in the frequency of cell firing and/or the nature of the neurotransmitters released onto these cells, which in turn led to differential activation of signal transduction pathways. In addition, we demonstrated that the activation of TH is associated with the enzymes phosphorylation at Ser31 and Ser40 *in vivo*, an effect that had previously been demonstrated mainly in cultured cells. This thesis has substantially improved our understanding of the mechanism of action of the catecholaminergic cells in mediating stress responses *in vivo*.

Abbreviations list

2DG	2-deoxy-D-glucose
AAAD	aromatic L-amino acid decarboxylase
ACTH	adrenocorticotropic hormone
AM	adrenal medulla
ANOVA	analysis of variance
APG	anterior pituitary gland
CaMKII	Ca^{2+}/ calmodulin-dependent protein kinase II
CDK	cyclin-dependent kinase
CRH	corticotrophin releasing hormone
COMT	catechol-O-methyltransferase
DBH	dopamine-β-hydroxylase
ERK1/2	extracellular signal-regulated kinases 1/2
FS	footshock
HCC	home cage control
HPA	hypothalamic-pituitary-adrenocortical
IMO	immobilization
LC	locus coeruleus
LPS	lipopolysaccharide
MAO	monoamine oxidase
PAGE	polyacrylamine gel electrophoresis
PFC	prefrontal cortex
PKA	cAMP-dependant protein kinase

PND	postnatal day
PNMT	phenylethanolamine-N-methyl transferase
PVN	paraventricular nucleus
SDS	sodium dodecyl sulfate
Ser	serine
SN	substantia nigra
TBST	tris-buffered saline with Tween
TH	tyrosine hydroxylase
VMAT	vesicular monoamine transpoters
VTA	ventral tegmental area

Chapter 1: Introduction

1.1 Stress and catecholamines

Stress is part of our daily life and for decades researchers have been trying to define stress in scientific terms. In this thesis, stress is defined as per the definition by Goldstein and Kopin, 2007 "*a condition in which expectations, whether genetically programmed, established by prior learning, or deduced from circumstances, do not match the current or anticipated perceptions of the internal or external environment, and this discrepancy between what is observed or sensed and what is expected or programmed elicits patterned, compensatory responses*" (Goldstein, 2003; Goldstein & Kopin, 2007).

A stressor is a stimulus, whether it is real or perceived which potentially can have detrimental outcomes to the homeostasis and the well being of an individual. The physiological and behavioural responses evoked by stressors involve multiple organ systems. First, the sensory systems detect information contained in the potential stress and relay this information to the processing systems in the brain including the locus coeruleus (LC), hippocampus, amygdala and pre-frontal cortex (de Kloet et al., 2005). Then, the sensory and processing systems mediate a variety of responses by the effector systems such as the autonomic nervous system and the endocrine system (Carrasco & Van de Kar, 2003; Charmandari et al., 2005). One of the major cell types involved in the stress response are the catecholaminergic cells. Catecholamines (dopamine, noradrenaline and adrenaline) have many functions and influence almost every tissue (Goldstein, 2003). Dopamine acts as a neurotransmitter in the central catecholaminergic neurons (Bjorklund & Dunnett, 2007). It also has some autocrine and/or paracrine roles in the peripheral catecholaminergic system (Goldstein et al., 1995). Noradrenaline and

adrenaline act as neurotransmitters in the central catecholaminergic neurons (Moore & Bloom, 1979). Noradrenaline also acts as neurotransmitter in the sympathetic division of the autonomic nervous system. Both noradrenaline and adrenaline act as hormones when released into the circulatory system (Goldstein, 2003; de Diego et al., 2008).

The catecholaminergic systems are activated rapidly in response to stressors (Figure 1.1) (Sabban & Kvetnansky, 2001; Sabban et al., 2004; de Diego, et al., 2008; Kvetnansky et al., 2009). One of the central catecholaminergic neurons that are very responsive to stress is the ascending noradrenergic neurons of the LC. Stress triggers the activation of the LC and the release of noradrenaline onto target sites such as the ventral tegmental area (VTA), striatum, thalamus and prefrontal cortex (PFC) (Stanford, 1995). The LC influences the hypothalamic-pituitary-adrenocortical (HPA) axis by the activation of paraventricular nucleus (PVN) of the hypothalamus, although its contribution is minor compared with other catecholaminergic neurons (Young et al., 2005). The activation of the PVN triggers the release of corticotrophin-releasing hormone which, in turn, stimulates the anterior pituitary gland (APG) to release adrenocorticotropic hormone (ACTH). ACTH stimulates the adrenal cortex to produce and release the stress hormone, cortisol (corticosterone in rodent) into the circulatory system (Douglas, 2005). Other stress hormones include adrenaline and noradrenaline. The sympathetic ganglion cells, the stellate ganglia, synthesize and release noradrenaline and this is the major source of plasma noradenaline. Stress triggers the activation of the splanchnic nerve and stimulates the adrenal medulla to produce and release adrenaline and noradrenaline into the circulatory system (Wakade, 1981). The adrenal medulla chromaffin cells contribute the majority of plasma adrenaline and about 30 % of plasma noradrenaline (Kvetnansky, et al., 2009). Once released into the

circulatory system, some of the major functions of these stress hormones are to increase heart rate, blood pressure, ventilation rate and depth of respiration as well as production of plasma glucose for rapid energy and heat generation (Charmandari, et al., 2005). Through these actions, these stress hormones mediate the "fight or flight response" contributing to the maintenance of homeostasis.

There are two possible outcomes in response to stressors. First, short-term stressors trigger an adaptive response which promotes survival and heath by enabling individuals to cope with emergencies. Second, long-term (or repetitive) stressors cause continual release of the stress hormones which can contribute to the development of diseases in susceptible individuals. In fact, two-thirds of the diseases that are reported are either related to or induced by stress (Sabban & Kvetnansky, 2001). **This thesis will focus on the effects of a range of short-term stressors on parts of the catecholaminergic systems especially the adrenal medulla and the LC. Although the stressors will be short-term we will focus on the time course of the responses after exposure to the stressor and for up to 48 h after exposure to the stressor.**

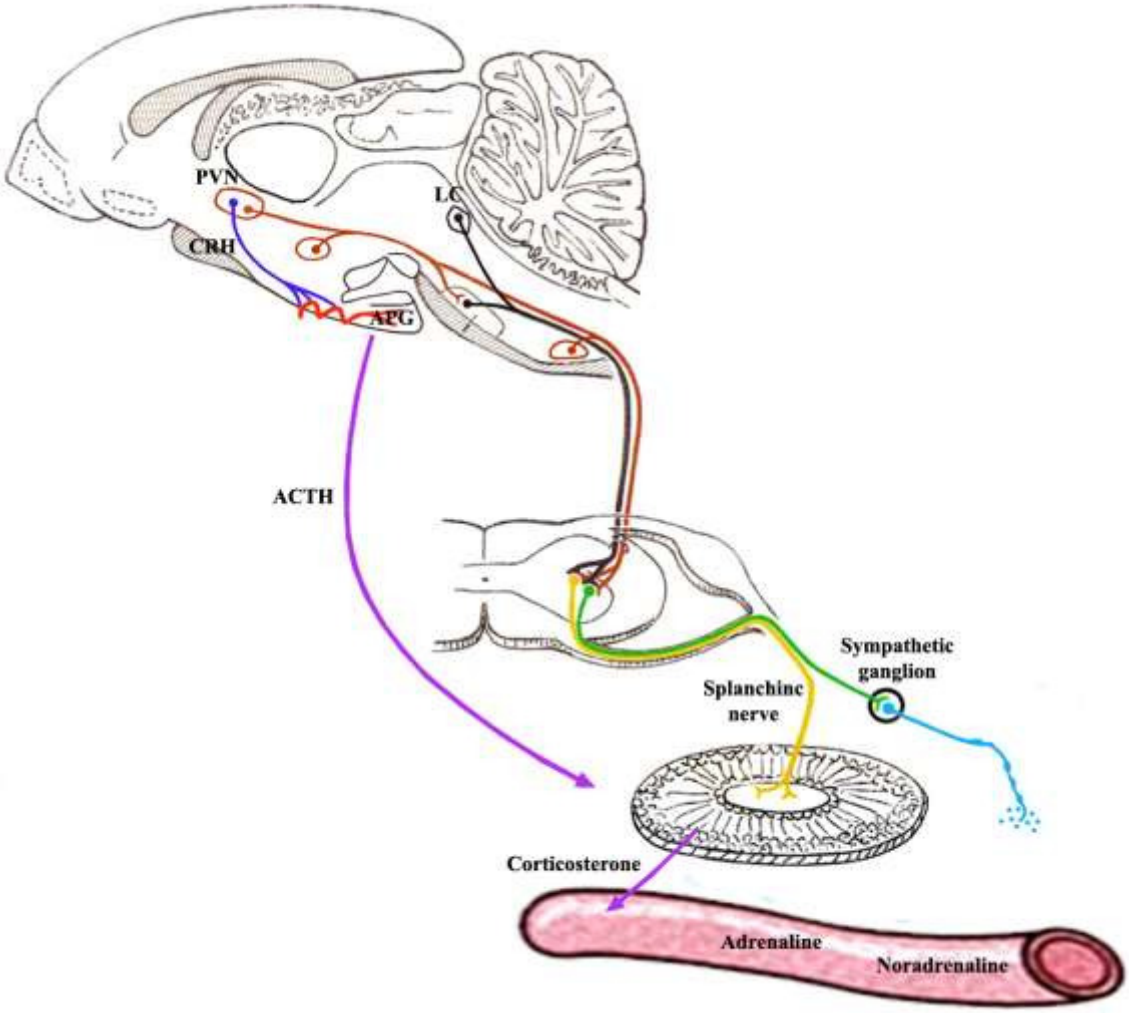

Figure 1.1: The adaptive stress responses. Stress induces 1) the release of corticosterone from the adrenal cortex via activation of HPA axis, 2) the release of noradrenaline from the sympathetic ganglion and 3) the release of adrenaline and noradrenaline from the adrenal medulla via activation of splanchnic nerve. Modified from Kvetnansky, et al., 2009.

1.2 Control of catecholaminergic cells

Intracellular catecholamines exist either stored inside vesicles or free in the cytosol. The intracellular catecholamine levels vary between different cell types (adrenal medulla chromaffin cells vs. catecholaminergic neurons). The cytosolic catecholamine levels in the adrenal medulla chromaffin cells were determined to be between 2 to 50 μM and in the catecholaminergic neurons (PC12 cells and cultured midbrain dopaminergic neurons) were determined to be less than 100nM (Mosharov et al., 2006). The intracellular catecholamine levels are dependent on a range of control mechanisms including vesicular packaging of catecholamines, reuptake of catecholamines from the extracellular milieu, breakdown and biosynthesis of the catecholamines (Figure 1.2).

Cytosolic catecholamines are packed into vesicles by specific carrier proteins called vesicular monoamine transporters 1/2 (VMAT1/2) (Henry et al., 1994). Adrenal medulla chromaffin cells express both isoforms with VMAT1 predominating in rodents and VMAT2 predominating in humans. In contrast, catecholaminergic neurons express only VMAT2. As the vesicular catecholamine levels (approximately 550 mM) are higher than the cytosolic catecholamine levels, VMAT transports cytosolic catecholamines into vesicles using an active transport mechanism. Vesicular packaging of catecholamines is not a static process as a substantial amount of vesicular catecholamines leak from the vesicles back into the cytosol (Eisenhofer et al., 2004).

Reuptake of catecholamines from the extracellular milieu can contribute to intracellular catecholamine levels in neurons. Released dopamine and noradrenaline are

able to be taken back into the neurons by the dopamine and noradrenaline transporters (Giros & Caron, 1993; Mandela & Ordway, 2006). In adrenal medulla chromaffin cells, released adrenaline and noradrenaline are less likely to be taken back into the cells although monoamine transporters are present (Wakade et al., 1996). This is due to adrenaline and noradrenaline rapidly diffusing into the circulatory system and therefore having limited access to the monoamine transporters. This represents a major difference between the two cell types, with reuptake of catecholamines from the extracellular milieu being a significant contributor to intracellular catecholamine levels in neurons, while having a less important role in adrenal medulla chromaffin cells.

Cytosolic catecholamines are subject to oxidative deamination by monoamine oxidase (MAO), degradation by catechol-O-methyltransferase (COMT) and auto-oxidation (Eisenhofer, et al., 2004). MAO catalyses the breakdown (deamination) of catecholamines leading to the production of aldehydes, which are then further metabolized to carboxylic acids or alcohols. MOA inhibitors were one of the first antidepressant drugs. Catecholaminergic cells also contain COMT which breaks down adrenaline and noradrenaline into metanephrine and normetanephrine. Catecholamines can also undergo auto-oxidation with production of toxic catechol-quinones along with hydrogen peroxide (Eisenhofer, et al., 2004). Breakdown of catecholamines is an important additional mechanism for the control of intracellular catecholamine levels.

The final mechanism for control of intracellular catecholamine levels is the biosynthesis of the catecholamines. This is very tightly regulated 1) to ensure that there are sufficient catecholamines for vesicular packaging following catecholamine release via exocytosis, 2) to maintain sufficient basal catecholamine biosynthesis to compensate

for breakdown and 3) to ensure that catecholamines do not accumulate in the cells because of their potential toxicity. In response to stress, the catecholaminergic cells are activated by depolarization of the cells, leading to an influx of extracellular calcium via voltage-sensitive calcium channels. The influx of extracellular calcium causes the secretion of catecholamines from the cells (de Diego, et al., 2008). When the catecholamines are released, it has been shown that there is no significant change in their levels within the catecholaminergic cells. This is because in parallel to catecholamine secretion there is also a concomitant increase in the rate of catecholamine biosynthesis (Wakade et al., 1988; Zigmond et al., 1989). **This thesis will focus only on the biosynthesis of catecholamines.**

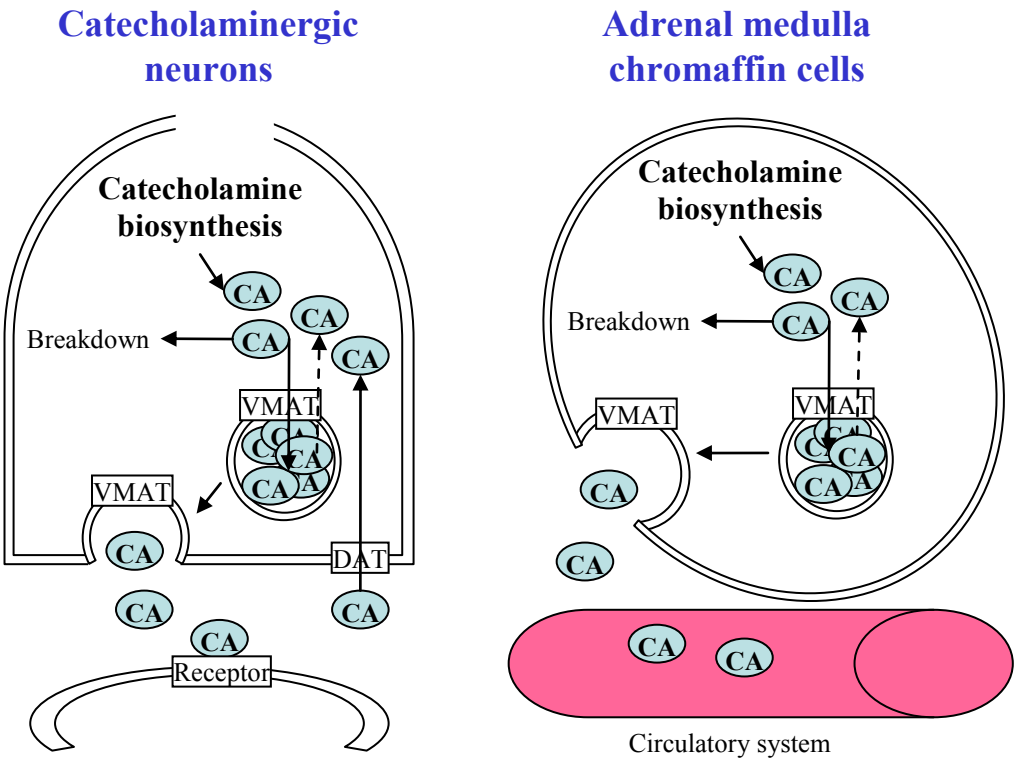

Figure 1.2: Catecholaminergic cells controls. The intracellular catecholamine levels are dependent on a range of control mechanisms, including vesicular packaging of catecholamines by vesicular monoamine transporters (VMAT), reuptake of catecholamines from the extracellular milieu by dopamine or noradrenaline transporters (e.g. DAT), breakdown by oxidative deamination, chemical degradation and auto-oxidation and biosynthesis of catecholamines.

1.3 Biosynthesis of catecholamines

The biosynthesis of catecholamines requires a number of enzymes. Catecholamines are synthesised from the precursor amino acid L-tyrosine in a common biosynthetic pathway (Figure 1.3). First, L-tyrosine is hydroxylated to dihydrooxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH) in the cytoplasm (Nagatsu et al., 1964). The hydroxylation of L-tyrosine to L-DOPA is catalysed by TH together with tetrahydrobiopterin (BH_4) and oxygen (O_2). The ferrous ion (Fe^{2+}) bound to TH is oxidized to ferric ion (Fe^{3+}) resulting in the formation of dihydrobiopterin (BH_2) and water (H_2O) (Fitzpatrick, 1999).

Then, L-DOPA is decarboxylated to dopamine by aromatic L-amino acid decarboxylase (AAAD) in the cytoplasm. Dopamine is transported into vesicles where it is hydroxylated to noradrenaline by dopamine-β-hydroxylase (DBH). Then, noradrenaline is transported back in the cytoplasm and is methylated to adrenaline by phenylethanolamine N-methyl transferase (PNMT). Catecholamines are stored in vesicles and are released when the central catecholaminergic neurons and the adrenal medulla chromaffin cells are activated (Kumer & Vrana, 1996; Tank et al., 2008; Kvetnansky, et al., 2009). When the catecholamines are released, there is a concomitant increase in TH activity and the biosynthesis of catecholamines (Wakade, et al., 1988; Zigmond, et al., 1989). **This thesis will focus on TH, the rate-limiting enzyme in the biosynthesis of catecholamines.**

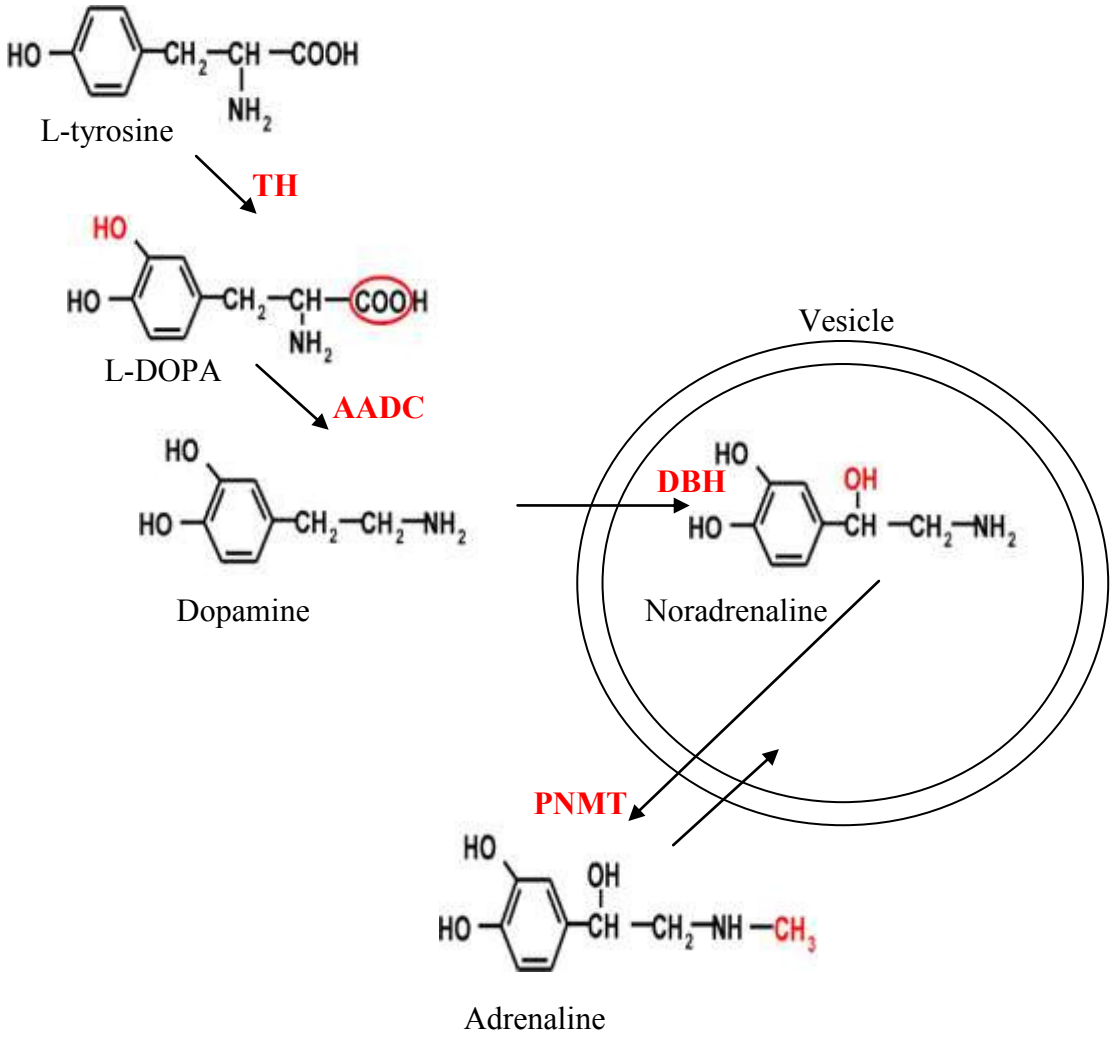

Figure 1.3: Biosynthetic pathway of catecholamines. Dopamine, noradrenaline and adrenaline are synthesised from L-tyrosine by the enzymes, tyrosine hydroxylase (TH), aromatic amino acid decarboxylase (AADC), dopamine β-hydroxylase (DBH) and phenylethanolamine N-methyl transferase (PNMT) (shown in red). Dopamine and adrenaline are synthesised in the cytoplasm whereas noradrenaline is synthesised in the vesicle. The catecholamines that are present in a particular cell are dependent on the enzymes that are expressed in the cell.

1.4 Tyrosine hydroxylase

TH is the rate-limiting enzyme in catecholamine biosynthesis (Nagatsu, et al., 1964). For details on TH structure, see review articles (Nagatsu, 1995; Nakashima et al., 2009; Daubner et al., 2011). Briefly, TH is found in the central catecholaminergic neurons, the sympathetic neurons and the adrenomedullary cells. This iron-containing enzyme belongs to a family of biopterin-dependent amino acid hydroxylases which includes tryptophan hydroxylase and phenylalanine hydroxylase (Goodwill et al., 1998). TH contains four subunits with a molar mass of approximately 240 kDa (Kumer & Vrana, 1996). In rats, each TH subunit has 498 amino acids with a molar mass of 55904 Da (Grima et al., 1985). Each TH subunit has a central catalytic domain, a C-terminal association domain, and an N-terminal regulatory domain containing the phosphorylation sites. In all species, TH is coded for by a single gene. In the adrenal medulla, protein analysis showed that in humans there were four TH isoforms and in most other species including rats (which are the focus of this thesis) there was only one isoform. The multiple isoforms in humans are formed by generation of splice variants from a single gene to form multiple mRNAs (Haycock, 2002b).

As the rate limiting enzyme in the biosynthesis of catecholamines, TH activity is primarily responsible for determining the cytosolic catecholamine levels. It is regulated by a range of different mechanisms.

TH activity is regulated primarily by feedback inhibition. TH contains two distinct catecholamine binding sites which are regulated by different mechanisms. First, catecholamine binding to the low affinity site competes with BH_4 for binding to the

active site. Evidence suggests that TH is subsaturated with respect to BH_4 *in vitro* (Mosharov, et al., 2006) and *in situ* (Gordon et al., 2008; Gordon et al., 2009). Therefore, inhibition of BH_4 binding will have a direct effect on TH activity. The low-affinity binding site is thought to maintain equilibrium of cytosolic catecholamine levels within a narrow range (Gordon, et al., 2008; Gordon, et al., 2009) and is not regulated by phosphorylation. In addition, TH contains a high-affinity binding site in which the catecholamines bind almost irreversibly (Ramsey & Fitzpatrick, 1998) (Figure 1.4). When catecholamines bind covalently to the ferric ion (Fe^{3+}) at the high affinity site, TH structure is stabilized and TH activity is inhibited by decreasing V_{max} and increasing the K_M for BH_4. The feedback inhibition cannot be reversed by changes in cytosolic catecholamines, but can be reversed by TH phosphorylation at the N-terminal regulatory domain. TH phosphorylation by protein kinases causes a conformational change in TH structure which leads to dissociation of the inhibitory catecholamines (Kumer & Vrana, 1996) and an increase in TH activity. Decrease of TH activity occurs when the protein phosphatases, phosphatase PP2A (and PP2C to a lesser extent) dephosphorylate TH (Dunkley, et al., 2004) and catecholamines rebind to the enzyme. In response to acute stressors, TH phosphorylation becomes a major regulatory mechanism for TH activity as it controls the amount of TH available for catecholamine synthesis without having to change the amount of TH protein present in the cell.

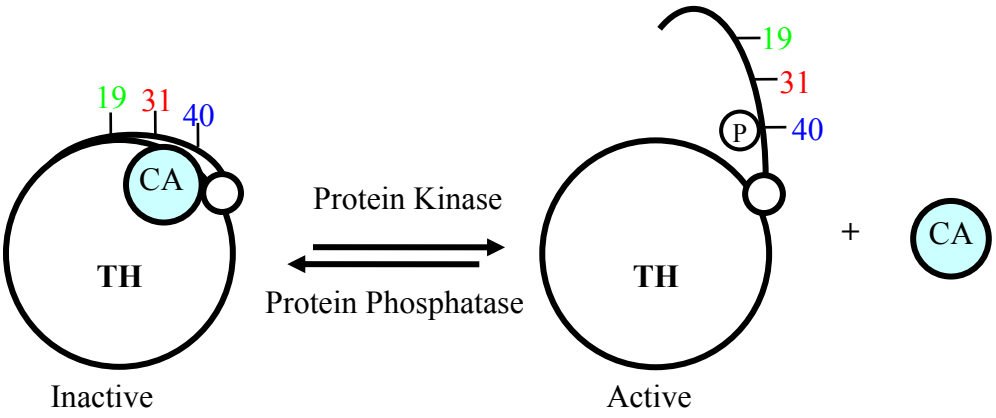

Figure 1.4: Feedback inhibition and phosphorylation of TH. TH activity is primarily regulated by feedback inhibition by catecholamines. When catecholamines bind to the catalytic site, TH structure is stabilized leading to inactivation of the enzyme. Phosphorylation by protein kinase(s) allows the dissociation of catecholamine from the high-affinity binding site, which returns TH to its active form.

TH activity is also regulated by the amount of TH protein present in the cells. This is primarily controlled by activation of transcription factors, which over time increases TH mRNA expression and TH protein synthesis (Kumer & Vrana, 1996). However, TH protein levels can also be regulated by enzyme stability, transcriptional regulation, RNA stability, alternative RNA splicing and translational activity. Changes in TH gene expression has been the focus of extensive research and a comprehensive review has been undertaken (Kvetnansky, et al., 2009). Therefore, this will not be discussed in depth in this thesis. **This thesis will focus primarily on the short-term regulatory mechanisms of TH activity and especially TH phosphorylation.**

1.5 TH phosphorylation

TH can be phosphorylated by a variety of kinases at several serine residues (Ser8, Ser19, Ser31 and Ser40) in the N-terminal regulatory domain. TH phosphorylation has been a topic of extensive research *in vitro* and *in situ*. A comprehensive review of TH phosphorylation, TH activity and the biosynthesis of catecholamines has been undertaken (Dunkley, et al., 2004).

Briefly, no stimuli has been identified that can reproducibly increase TH phosphorylation at Ser8 *in vitro* or *in situ*. Ser8 is phosphorylated to a very low stoichiometry and has not been shown to effect TH activity *in vivo* (Dunkley, et al., 2004). Therefore, this site will not be further discussed in this thesis. Much of the focus in TH research has gone into determining the functional roles of TH phosphorylation at the remaining three serine residues (Figure 1.5).

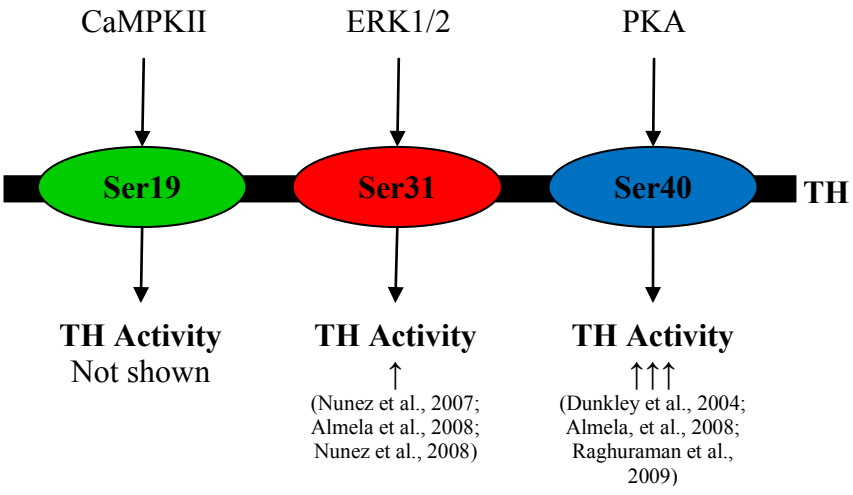

Figure 1.5: The effects of the activation of different protein kinases on TH phosphorylation and TH activation. The activation of CaMPKII mediates Ser19 phosphorylation but does not lead to TH activation (not shown *in vivo*). The activation of ERK1/2 mediates Ser31 phosphorylation and leads to TH activation (shown in the brain and the heart *in vivo*). The activation of PKA mediates Ser40 phosphorylation and leads to TH activation (strong evidence *in vivo*).

Ser19 can be phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II (CaMPKII) *in vitro* (Tsutsui et al., 1994). Depolarizing stimuli lead to increases in intracellular Ca^{2+} have been shown to activate CaMPKII and TH phosphorylation at Ser19 *in situ* (Padmanabhan & Prasad, 2009). Ser19 phosphorylation alone does not increase TH activity *in vitro* and *in situ* (Haycock et al., 1998; Salvatore et al., 2001). There is evidence that with the presence of the 14-3-3 protein, CaMPKII phosphorylates TH at Ser19 which results in an increase in TH activity *in vitro* (Toska et al., 2002). It remains unknown whether this phenomenon actually occurs *in vivo*, and if it has any functional relevance. Although Ser19 is known to be controlled under regulatory mechanisms *in vitro* and *in situ* and is phosphorylated to high stoichiometry under basal conditions in the adrenal medulla and the brain *in vivo* (Salvatore et al., 2000; Saraf et al., 2007), the functional roles of TH phosphorylation at Ser19 remain unclear. Further discussion of the possible functional roles of TH phosphorylation at Ser19 is provided (below).

Ser31 can be phosphorylated by extracellular signal-regulated protein kinases 1/2 (ERK1/2) (Haycock et al., 1992; Haycock, 2002a) and cyclin-dependent kinase (CDK) *in vitro* and *in situ* (Moy & Tsai, 2004). Ser31 phosphorylation alone increases TH activity (about 2 fold) *in vitro* (Haycock, et al., 1992; Sutherland et al., 1993). TH phosphorylation at Ser31 does not lead to dissociation of inhibitory catecholamines (Haycock, et al., 1992), but leads to a decrease in K_M value for BH_4 *in situ* (Gordon, et al., 2009). Depolarizing stimuli have been shown to activate ERK1/2 and TH phosphorylation at Ser31. When depolarizing stimuli are used, Ser31 phosphorylation only appears to play a role in increasing TH activity and the biosynthesis of

catecholamines at times after that due to Ser40 phosphorylation *in situ* (Dunkley, et al., 2004). Ser31 is phosphorylated to variable stoichiometry levels under basal conditions in the adrenal medulla and the brain *in vivo* (Salvatore, et al., 2000; Saraf, et al., 2007). TH activity is activated 1.5 to 3-fold by phosphorylation via ERK *in vivo*. There is strong evidence that the activation of ERK1/2 mediates TH phosphorylation at Ser31 and leads to TH activation and the biosynthesis of catecholamines in the brain (Nunez, et al., 2007; Nunez, et al., 2008) and the heart (Almela, et al., 2008) *in vivo*. TH phosphorylation at Ser31 is important in the regulation of TH activation and the biosynthesis of catecholamines *in vivo*. Further discussion of the other functional roles of TH phosphorylation at Ser31 is provided (below).

Ser40 can be phosphorylated by cAMP-dependant protein kinase (PKA) *in vitro* and *in situ* (Dunkley, et al., 2004). Ser40 can also be phosphorylated many other protein kinases *in vitro* and *in situ* (Dunkley, et al., 2004). Ser40 phosphorylation alone increases TH activity (about 40 fold) and the biosynthesis of catecholamines *in situ*, especially in response to the activation of the PKA pathway (Dunkley, et al., 2004). TH phosphorylation at Ser40 leads to a conformational change in TH structure and dissociation of inhibitory catecholamines *in vitro* (Figure 1.4). In catecholamine-free TH, Ser40 phosphorylation has minimal effects on TH activity. However in catecholamine-bound TH, Ser40 phosphorylation increases TH activity by dissociation of inhibitory catecholamines (Ramsey & Fitzpatrick, 1998). Ser40 is phosphorylated to quite low stoichiometry levels compared to Ser19 and Ser31 under basal conditions in the adrenal medulla and the brain *in vivo* (Salvatore, et al., 2000; Saraf, et al., 2007). TH activity is activated perhaps 20-fold by phosphorylation by PKA *in vivo* (Daubner, et al., 2011). There is strong evidence that the activation of PKA mediates TH

phosphorylation at Ser40 and leads to TH activation and the biosynthesis of catecholamines *in vivo* (Dunkley, et al., 2004; Almela, et al., 2008; Raghuraman, et al., 2009).

While Ser19 phosphorylation does not directly increase TH activity and Ser31 phosphorylation modestly increases TH activity, Ser19 and Ser31 phosphorylation also have indirect effects on TH activity. TH phosphorylation at Ser19 increases the rate of TH phosphorylation at Ser40 (about 3 fold) *in vitro* and *in situ* (Bevilaqua et al., 2001; Toska, et al., 2002; Bobrovskaya et al., 2004). TH phosphorylation at Ser31 increases the rate of TH phosphorylation at Ser40 (9 fold) *in vitro* and *in situ* in a similar way to that described for Ser19 (Lehmann et al., 2006). TH phosphorylation at Ser40 has been shown to have no effect on the rate of TH phosphorylation at Ser19 or Ser31. The hierarchical effects of TH phosphorylation at Ser19 and Ser31 are accomplished by the conformational changes in TH structure (to a more open conformation) and increase the rate of TH phosphorylation at Ser40 and TH activity (Lehmann, et al., 2006). It should be noted that the hierarchical effects of TH phosphorylation at Ser19 and Ser31 occurred with catecholamine-free TH and have not been tested with catecholamine-bound TH *in vitro*. There is strong evidence that Ser19 phosphorylation may facilitate Ser40 phosphorylation in the brain *in vivo* (Yu et al., 2011; Salvatore & Pruett, 2012). However, the functional roles of the hierarchical effects of TH phosphorylation at Ser19 and Ser31 remain unclear *in vivo*. Therefore, this will not be discussed in depth in this thesis.

Ser19, Ser31 and Ser40 can be dephosphorylated by phosphatase PP2A (Haavik et al., 1989). Only Ser19 and Ser40 can be dephosphorylated by phosphatase PP2C

(Leal et al., 2002). Generally, depolarizing stimuli and increases in intracellular Ca^{2+} cause activation of protein kinases. Subsequently, the continually increasing levels of Ca^{2+} cause the activation of protein phosphatases (Robinson & Dunkley, 1985; Padmanabhan & Prasad, 2009). There is strong evidence that the activation of protein phosphatases mediates TH dephosphorylation of the three serine residues and lead to inactivation of TH by catecholamines binding and feedback inhibition of the enzyme *in vivo* (Saraf, et al., 2007; Padmanabhan & Prasad, 2009).

The major focus of this thesis will be on the measurement of the phosphorylation of TH at Ser19, Ser31 and Ser40 in response to stress. If there is a change in the phosphorylation (or dephosphorylation) of any of these sites it will confirm that the cells must have been altered in some way by the stressors. The only way that phosphorylation of Ser19, Ser31 and Ser40 can be altered is by the action of protein kinases or protein phosphatases acting on these sites, assuming total TH protein levels are unchanged. Therefore any observed change will provide some insight into the possible signal transduction processes going on within the catecholaminergic cells in response to the stressors. Any changes in TH phosphorylation, especially at Ser40 will also provide insight into the activation state of TH. As TH is the rate limiting enzyme TH phosphorylation is also likely to correlate with the rate of catecholamine biosynthesis, assuming cofactor levels and the activity of other enzymes in the pathway are not substantially altered. **Overall, measurement of TH phosphorylation at Ser19, Ser31 and Ser40 will provide an indication of 1) the state of activation of catecholaminergic cells, 2) the possible signal transduction pathways activated within these cells and 3) the state of TH activation.**

1.6 The three phases of TH activation

Three phases of TH activation have been identified (acute, sustained and chronic) (Figure 1.6). In each phase, TH is subject to different regulatory mechanisms each of which can lead to altered TH activity. The regulatory mechanisms of the three phases of TH activation have been extensively studied *in vitro* and *in situ* but they have not all been extensively studied *in vivo*.

1.6.1 The acute phase of TH activation

Briefly, the acute phase of TH activation is mediated by TH phosphorylation at Ser19, Ser31 and Ser40 (Kumer & Vrana, 1996). In adrenal medulla chromaffin cell cultures *in vitro*, incubation (<1 h) with nicotine leads to TH phosphorylation which follows a pattern: Ser19 phosphorylation is rapidly increased to maximal levels within 1 min and then dephosphorylation occurs; Ser40 phosphorylation begins more slowly than Ser19 reaching a plateau by 4 min, without any subsequent dephosphorylation; Ser31 phosphorylation is delayed until 4 min but rapidly increased up to 10 min. Ser19, Ser31 and Ser40 phosphorylation is then returned to basal levels by 1 h (Haycock, 1993). During the acute phase, TH phosphorylation at Ser31 and Ser40 leads to TH activation and the biosynthesis of catecholamines (Dunkley, et al., 2004).

1.6.2 The sustained phase of TH activation

While TH phosphorylation is considered to be a short-term regulator of TH activity, it has been demonstrated that TH phosphorylation at Ser40 is able to extend

past the acute phase of TH activation to the sustained phase. The sustained phase of TH activation is mediated primarily by TH phosphorylation at Ser40. In adrenal medulla chromaffin cell cultures *in vitro*, sustained incubation (1 – 24 h) with nicotine leads to TH phosphorylation which follows a pattern: Ser19 and Ser31 phosphorylation are very low but above the unstimulated control levels at 24 h; Ser40 phosphorylation is decreased to below acute levels from 1 to 8 h (but still significantly above basal levels) and then is substantially increased from 8 to 24 h. Sustained activation of PKC together with inhibition of protein phosphatases mediate the sustained TH phosphorylation at Ser40 (Bobrovskaya et al., 2007a; Bobrovskaya et al., 2007b). During the sustained phase, TH phosphorylation at Ser40 leads to TH activation and the biosynthesis of catecholamines (Bobrovskaya, et al., 2007a; Bobrovskaya, et al., 2007b). This increase in Ser40 phosphorylation occurred in response to nicotine, angiotensin, histamine and PACAP but did not occur in response to bradykinin and muscarine. The sustained phase plays an important role in the regulation of TH activity between the acute and chronic phases. The hallmarks of this phase are 1) TH phosphorylation of Ser40 is increased, 2) TH protein is not altered and 3) the signal transduction pathways activation are different from those which occur in the acute phase.

1.6.3 The chronic phase of TH activation

The chronic phase of TH activation is mediated primarily by the activation of transcription factors, which over time increases TH mRNA expression and TH protein synthesis (Kumer & Vrana, 1996). The increased TH protein levels provide more enzymes for activation and can increase in the rate of catecholamine biosynthesis. In adrenal medulla chromaffin cell cultures *in vitro*, chronic incubation (48 h) with

nicotine leads to increased TH protein synthesis and TH activity (Craviso et al., 1992). During the chronic phase, TH mRNA expression and TH protein synthesis lead to TH activation and the biosynthesis of catecholamines (Kumer & Vrana, 1996).

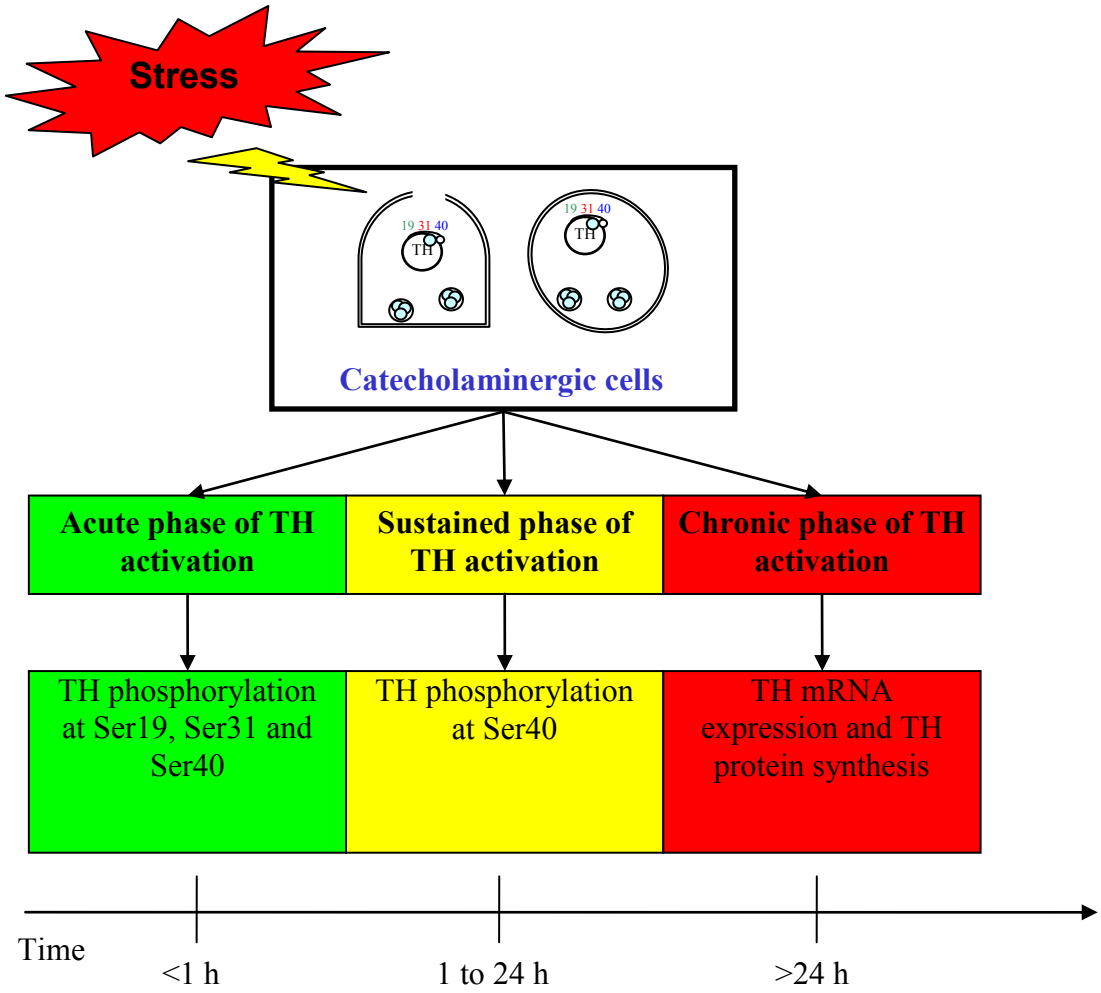

Figure 1.6: The three phases of TH activation. The acute phase of TH activation is characterised by TH phosphorylation at Ser31 and Ser40. The sustained phase of TH activation is characterised by TH phosphorylation only at Ser40. The chronic phase of TH activation is characterised by TH mRNA expression and TH protein synthesis.

As mentioned earlier, the regulatory mechanisms of the three phases of TH activation have been extensively studied *in vitro* and *in situ*. Only the chronic phase of TH activation has been extensively studied *in vivo*. The acute and sustained phases of TH activation have not been extensively investigated *in vivo*. **This thesis will investigate the different phases of TH activation, especially the acute and sustained phases, in response to short-term stress *in vivo*.**

1.7 The rodent stress models

The effects of a range of stressors on the regulation of TH have been studied *in vivo* in many different rodent stress models. We can classify these stressors into two categories:

1) **Physical stressor** is defined as real threat to health and well-being. This type of stressor causes actual disturbance of homeostasis. (e.g. pain, changes in metabolic or immunity status and drug effects)
2) **Psychological stressor** is defined as an emotional or perceived threat to health and well-being. This type of stressor is due to an individual's pre-programmed evolutionary goals being put at risk. In this case, the level of the stimulus and its impacts on the individual depend on the interpretation, perception and cognitive processing of the stressor (Dayas et al., 2001; Kvetnansky, et al., 2009). (e.g. social defeat and aversive environmental stimuli)

Stressors can be classified as either short-term or long-term (often repetitive). Stressors also differ in their intensity. Stressors induce different responses in different catecholaminergic cells, with responses varying according to the nature of the stressors and the time at which the responses are analysed.

This thesis will focus on 5 different rodent stress models (footshock, immobilization, glucoprivation, social defeat or LPS stress) under short-term conditions.

1) **Footshock stress** is employed by placing the rat in a footshock chamber, where an electrical current is passed through the shock grid of the chamber resulting in

the rat receiving this shock. The shock is 1 mA, 1 s pulse per min for the times specified.

2) **Immobilization stress** is produced by physically confining the rat inside a wire mesh. The mesh is used as it does not heat the animal as occurs in other protocols, but it effectively maintains the rat in a confined space.
3) **Glucoprivation stress** is induced by 2-deoxyglucose, which interferes with normal glucose metabolism and essentially initiates a hypoglycaemic-like response.
4) **Social defeat stress** is based on initial occupation by a male rat in a cage which is called the resident. Then, a smaller, naïve male rat, which is called the intruder, is introduced into the cage. Social defeat occurs when the resident attacks the intruder and establishes its dominancy. In this study we are investigating the intruder rats' response to social defeat.
5) **LPS stress** (protocol used in this thesis) when rat is challenged with LPS injection on postnatal days three and five. It is a commonly used model of early life bacterial driven immune response as the response to LPS mimics many of the responses to bacterial infection.

Therefore, these stressors can be tentatively classified into two categories; physical stressors (footshock, glucoprivation or LPS stress) and psychological stressors (immobilization or social defeat stress). The rodent stress models, footshock (Henn & Vollmayr, 2005) and social defeat stress (Rygula et al., 2006), have been shown to be useful models in the study of chronic stress and depression. Immobilization and glucoprivation stress have been used to study a range of neural and endocrine responses such as plasma ACTH, corticosterone, adrenaline and noradrenaline (Pacak & Palkovits,

2001). LPS stress has been used to study immediate and long-term physiological and behavioural alterations (Walker et al., 2009; Walker et al., 2011). These rodent stress models were used in many studies but information on the different phases of TH activation, particularly in the acute and sustained phases is limited.

To date, there have been limited studies that have investigated the acute phase of TH activation *in vivo*. Salvatore et al. (2000) investigated the effects of haloperidol, an antipsychotic drug, on TH phosphorylation in different rat brain regions *in vivo*. Haloperidol increased TH phosphorylation at Ser19, Ser31 and Ser40 (1.6 - 2 fold) at ±30 min (animals were decapitated 30 to 40 min) in the terminal field regions (striatum and accumbens) after intraperitoneal injection. The effects of haloperidol in the cell body regions (SN and VTA) differed from those in the terminal fields (striatum and accumbens). Although different rat brain regions were investigated, only one time point was measured and the changes in TH activity were not measured in the same study (Salvatore, et al., 2000). Jedynak et al. (2002) investigated the effect of cocaine (30 mg/kg) on different rat brain regions (amygdala, caudate, accumbens and VTA) over different time points *in vivo.* An acute administration of cocaine decreased TH phosphorylation at Ser19 and Ser40 at 15 and 40 min and Ser31 at 15, 40 and 120 min in the caudate and accumbens after injection. The decreases in TH phosphorylation were paralleled by decreases in TH activity in all locations (Jedynak et al., 2002). Both studies show that the acute phase of TH activation is mediated by different patterns in TH phosphorylation at Ser19, Ser31 and Ser40, without any changes in TH protein levels. However, the effects of a range of short-term stressors have not been investigated in the adrenal medulla and the LC *in vivo* using such an approach.

To date, there have been no studies that have specifically investigated the sustained phase of TH activation *in vivo*. However, Yu et al. (2011) investigated the effects of intracerebroventricular administration of ouabain, a Na/K-ATPase inhibitor on striatum over different time points (1, 2, 4 and 8 h) *in vivo*. Intracerebroventricular administration of ouabain (1 mM) increased and sustained TH phosphorylation at 19, 31 and 40, without any change in TH protein levels between 1 to 8 hr after the treatment (Yu, et al., 2011). It is possible that ouabain may have led to sustained phosphorylation at 24 h but this was not measured.

Extensive studies have investigated the changes in TH mRNA expression and TH protein synthesis in the chronic phase of TH activation *in vivo*. For details on TH mRNA and/or TH protein changes *in vivo*, see review articles (Sabban & Kvetnansky, 2001; Sabban & Serova, 2007; Kvetnansky, et al., 2009). Most studies were focussed on long-term (repetitive) stressors and measurements were made at later time points (> 48 h). This will not be discussed in depth in this thesis.

Inconsistencies have been reported in the correlation between TH mRNA, TH protein and TH activity levels especially in response to short-term stressors *in vivo*. This suggests that neither the changes in TH mRNA nor the changes in TH protein levels indicate the extent of TH activation. In response to stressors, TH mRNA increases rapidly. Changes in TH mRNA levels are due to the activation of transcription factors. However, due to the post-transcription regulation the changes in TH mRNA levels are often inconsistent with the changes in TH protein levels (Wong & Tank, 2007). During short-term stressors, increases in TH mRNA levels are transient, and do not lead to significant increases in TH protein levels (Wong & Tank, 2007). Only with long-term

(usually repetitive) stressors do increases in TH mRNA levels lead to significant increases in TH protein levels (Sabban & Kvetnansky, 2001; Wong & Tank, 2007; Tank, et al., 2008). Single immobilization stress (2 h) drastically increases TH mRNA levels in adrenal medulla at 3 to 24 h but TH protein and TH activity levels are not increased at that time (Nankova et al., 1994; Xu et al., 2007). Similar results were observed using a different rodent stress model. Glucoprivation stress (induced by 2-deoxy-D-glucose, 500 mg/kg) increases TH mRNA levels in adrenal medulla at 5 h. However, TH protein and TH activity levels are not increased (Rusnak et al., 1998). Therefore, it can be concluded from theses studies that TH mRNA expression is not always an accurate representation of subsequent TH protein expression *in vivo*.

In addition, TH protein levels do not always correlate with TH activity (Tank, et al., 2008). Glucoprivation stress (induced by 2-deoxy-D-glucose, 500 mg/kg) increases TH activity levels in adrenal medulla at 5 h but TH protein levels are not increased. Previous studies showed that chronic cold stress evokes significant increases in TH protein levels without inducing changes in TH activity (Fluharty et al., 1983; Baruchin et al., 1990). Therefore, it can be concluded from these studies that TH protein expression may not always represent the state of TH activation. This may be as a result of the newly synthesised TH protein being expressed in the absence of Ser40 phosphorylation, thus rendering them inactive a feedback inhibition mechanism by catecholamines.

Recent studies have investigated the effects of long-term stressors (morphine withdrawal and intermittent hypoxia rodent models) on catecholaminergic cells by measuring TH phosphorylation at Ser19, Ser31 and Ser40 and TH protein *in vivo*

(Nunez, et al., 2007; Almela, et al., 2008; Nunez et al., 2009; Raghuraman, et al., 2009). Presumably, the increased TH protein levels provide more TH for activation and the increased TH phosphorylation at Ser31 and Ser40 levels leads to TH activation and the biosynthesis of catecholamines.

Overall, TH activity, TH phosphorylation, TH mRNA and TH protein levels were used to investigate different regulatory mechanisms of TH in the catecholaminergic cells *in vivo*. **No studies have systematically investigated the effects of a range of short-term stressors on the catecholaminergic system especially the adrenal medulla and the LC by measuring TH phosphorylation at different time points *in vivo*. No studies have investigated the sustained phase of TH activation *in vivo*.**

1.8 Rationale and aims

The overall aim of this thesis is to systematically investigate the acute and sustained phases by measuring TH phosphorylation and TH protein in the adrenal medulla and different brain regions, especially the LC, at different time points (<48 h) in response to a range of short-term stressors *in vivo*. To do this we have proposed two hypotheses to test:

1. Different stressors will each induce the acute phase of TH activation
2. Some stressor(s) will induce the sustained phase of TH activation

1. Different stressors will induce different patterns of TH phosphorylation without any change in TH protein in the adrenal medulla and the LC over a 1 hour period as depicted in Figure 1.7.

Rationale: TH is the rate-limiting enzyme in the catecholamine biosynthesis and is subject to a range of regulatory mechanisms (such as feedback inhibition by catecholamines, phosphorylation of the three serine residues, mRNA expression and protein synthesis). These regulatory mechanisms have been extensively studied *in vitro* and *in situ*. The acute phase of TH activation has not been systematically investigated *in vivo* by using different stressors and then measuring TH phosphorylation at a range of time points for up to an hour.

The catecholaminergic neurons in the brain that have previously been shown to be involved in responses to most types of stressors are the noradrenergic neurons of the LC. The LC is involved in the transfer of stress signals from the periphery to specific forebrain areas and in the overall coordination and organization of the stress responses.

The chromaffin cells of the adrenal medulla receive direct input from the splanchnic nerve and as a consequence secrete the stress hormones (adrenaline and noradrenaline) into the blood.

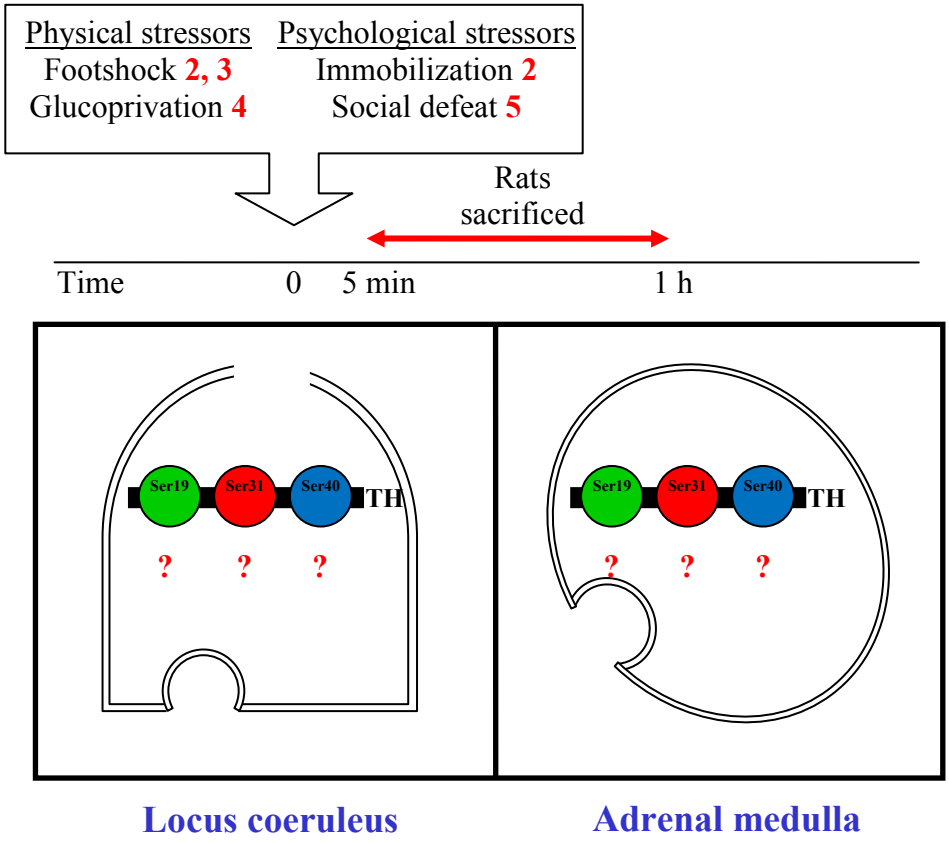

Figure 1.7: Different stressors will induce different patterns of TH phosphorylation without any change in TH protein in the adrenal medulla and the LC over 1 hr period. Numbers represent the chapters in this thesis when these stressors are investigated.

Therefore, we have compared the profile of TH phosphorylation at Ser19, Ser31 and Ser40 and TH protein elicited by two stressors tentatively classified as physical (footshock or glucoprivation stress) and two stressors tentatively classified as psychological (immobilization or social defeat stress) in the adrenal medulla and the LC over a 1 h period. The LC provides information on brain processing of the stress responses and the adrenal medulla provides information on the peripheral responses to the stressors.

2. Some stressor(s) will induce TH phosphorylation at Ser40 without any change in TH protein in the adrenal medulla at 24 h as depicted in Figure 1.8.

Rationale: We have shown that the sustained phase of TH activation exists but we have only measured *in vitro*. The sustained phase plays a potentially important role in the regulation of TH activity between the acute and chronic phases which are well established. The hallmarks of the sustained phase are 1) TH phosphorylation at Ser40 is increased, 2) TH protein is not altered and 3) the signal transduction pathways are different from those which occur in the acute phase. The sustained phase of TH activation has not been found *in vivo* and the types of stressors which could elicit it have not been investigated.

Therefore, we have compared TH phosphorylation at Ser40 and TH protein levels elicited by three different stressors (glucoprivation, social defeat or LPS stress) in the adrenal medulla at 24 h.

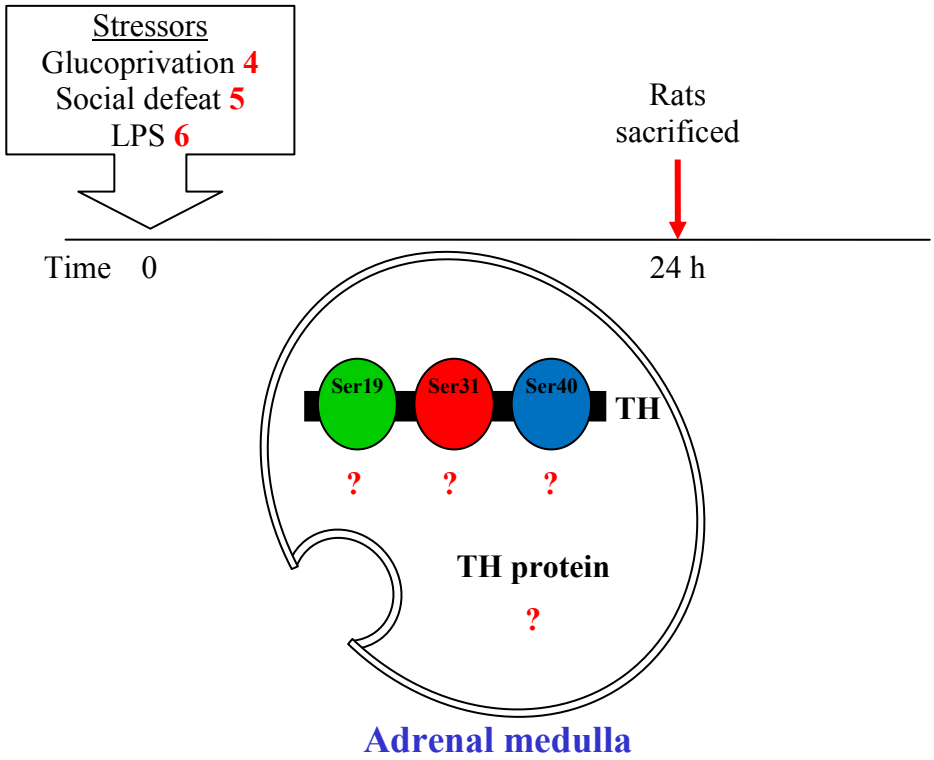

Figure 1.8: Some stressors will induce TH phosphorylation at Ser40 without any change in TH protein in the adrenal medulla at 24 h. Numbers represent the chapters in this thesis when these stressors are investigated.

Outcomes: By examining the changes in TH phosphorylation, activity and protein levels in the adrenal medulla and in different brain regions, this study will provide evidence of the biological changes that are common in the stress response and those that are specific to a particular stressor. This will put us in a better position to better understand the mechanism(s) of normal stress and potentially how in some instances this can lead to stress-related diseases. It may also provide insights into how to alleviate stress related pathologies.

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Chapter 2: The effects of footshock and immobilization stress on tyrosine hydroxylase phosphorylation in the rat locus coeruleus and adrenal gland.

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Acknowledgement of Collaboration

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers.

The work in the following chapter (Chapter 2) was submitted to Neuroscience in May 2011. I (Lin Kooi Ong) was the first author of this manuscript, and the work embodied in this chapter was primarily performed by myself, with the exception of the following:

Rat treatments were performed by myself with assistance from Liying Guan and Bernardo Stutz.

Sample preparation and Western Blotting for the footshock experiments which were performed by Liying Guan.

The work embodied by these experiments is inextricably tied to the overall findings of the manuscript, and therefore this work will be discussed in the final chapter (Chapter 7) of this thesis.

Lin Kooi Ong

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THE EFFECTS OF FOOTSHOCK AND IMMOBILIZATION STRESS ON TYROSINE HYDROXYLASE PHOSPHORYLATION IN THE RAT LOCUS COERULEUS AND ADRENAL GLAND

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Abstract—Tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis, is regulated acutely by protein phosphorylation. No studies have systematically investigated the time course of TH phosphorylation *in vivo* in response to different stressors. We therefore determined the extent of TH phosphorylation at Ser19, Ser31, and Ser40 over a 40-min period in response to footshock or immobilization stress in the rat locus coeruleus and adrenal medulla. There were significant changes in TH phosphorylation in both tissues and the responses to the two stressors differed markedly. With each of the phosphorylation sites immobilization stress caused a much smaller, or less sustained, response than footshock stress. With immobilization stress there was a transient increase in Ser31 phosphorylation in the locus coeruleus and in the adrenal medulla, but there were no effects on Ser19 or Ser40 phosphorylation. With footshock stress there was a substantial decrease in Ser19 phosphorylation over time, a substantial increase in Ser31 phosphorylation over time, but there were no effects on Ser40 phosphorylation. Measuring TH phosphorylation at Ser19, Ser31, and Ser40 over time can therefore be used as a sensitive index to differentiate the effects of different stressors on catecholaminergic cells.

Key words: tyrosine hydroxylase, serine residues, locus coeruleus, adrenal medulla, footshock, immobilization.

Stress induces activation of central catecholaminergic neurons, the peripheral sympathetic nervous system, and the adrenomedullary system (Kvetnansky et al., 2009). Exposure to stress leads to an increase in the release of catecholamines from these neurons and chromaffin cells. One of the major central catecholaminergic systems that is involved in the stress response is the ascending noradrenergic system, originating primarily in the A6 noradrenergic neurons of the locus coeruleus (LC) (Morilak et al., 2005). Stress stimulates electrical activity in the LC and the release of noradrenaline onto forebrain target sites (Smith et al., 1991; Chang et al., 2000). The firing rate of LC neurons has been shown to be increased by footshock and immobilization stress (Stanford, 1995). Likewise, chromaffin cells of the adrenal medulla (AM) produce noradrenaline and adrenaline which are released into the circulatory system in response to splanchnic nerve activation (Wakade, 1981). The firing rate of splanchnic nerve has been shown to be increased by various stressors (de Diego et al., 2008; Kvetnansky et al., 2009).

When catecholamines are released from the LC or the AM there is a compensatory increase in catecholamine synthesis. This maintains the cellular catecholamines in these tissues at a constant level (Wakade et al., 1988). The rate-limiting enzyme in catecholamine synthesis is tyrosine hydroxylase (TH) (Nagatsu et al., 1964; Dunkley et al., 2004). Stress increases TH activity (Kvetnansky et al., 1970) and many different stressors, including footshock (Melia and Duman, 1991; Chang et al., 2000) and immobilization (Nankova et al., 1994), have been shown to induce TH activation in the LC and the AM. TH activation leads to increased catecholamine synthesis to replace the released catecholamines. This occurs by two mechanisms. Firstly, the extracellular calcium influx into the cells that leads to the release of the catecholamines, also leads to activation of signal transduction pathways that acutely increase TH phosphorylation and activity (Kumer and Vrana, 1996). Secondly, the activation of signal transduction pathways also leads to phosphorylation of transcription factors, which over time increases TH mRNA and TH protein levels (Kumer and Vrana, 1996). TH phosphorylation, TH mRNA, and TH protein levels are therefore all indicators of catecholamine cell activation in response to stress.

TH phosphorylation at Ser19, Ser 31, and Ser 40 is involved in the acute activation of TH. TH phosphorylation follows a pattern over the first 10 min *in vitro*: Ser19 phosphorylation is rapidly increased to maximal levels within a minute and then dephosphorylation occurs; Ser40 phosphorylation begins more slowly than Ser19 reaching a plateau by 4 min, without any subsequent dephosphorylation; Ser31 phosphorylation is delayed until 4 min but rapidly increases up to 10 min (Haycock, 1993). TH phosphorylation at Ser19 does not increase TH activity directly *in vitro* (Sutherland et al., 1993). TH phosphorylation at Ser31 increases TH activity about two-fold *in vitro* (Haycock, 2002). TH phosphorylation at Ser40, which relieves the feedback inhibition by catecholamines (Kumer and Vrana, 1996), can increase TH activity up to 40-fold *in vitro*. TH phosphorylation has been shown to correlate with

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Abbreviations: AM, adrenal medulla; FS, footshock; HCC, home cage control; IMO, immobilization; LC, locus coeruleus; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Ser, serine residue; TBST, Tris-buffered saline with Tween; TH, tyrosine hydroxylase.

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TH activity in cell cultures (Haycock, 1993; Dunkley et al., 2004; Bobrovskaya et al., 2007a,b). The profile of TH phosphorylation at Ser19, Ser31, and Ser40 can therefore be used as an indicator of TH activation in response to acute stress. There are studies *in vivo* on changes in TH phosphorylation at Ser19, Ser31, and Ser40 (Jedynak et al., 2002; Dunkley et al., 2004; Bobrovskaya et al., 2010; Ong et al., 2011). However, no studies have systematically investigated the time course of phosphorylation of these sites *in vivo* in response to different stressors.

Many studies have investigated TH mRNA and/or TH protein changes in response to stressors (Watanabe et al., 1995; Rusnák et al., 2001; Tank et al., 2008), but these measures are only detectable many hours after exposure to the stressor. In the LC, TH mRNA levels were significantly increased 6 h after a 15-min period of footshock stress (Chang et al., 2000), while TH protein levels were significantly increased 24 h after a 2-h period of immobilization stress (Hebert et al., 2005). In the AM, TH mRNA levels were significantly increased only after 2 h of immobilization stress, whereas TH protein levels were significantly increased only 6 h after a 2-h period of immobilization stress (Nankova et al., 1994). TH mRNA cannot be used as an index of TH activation as the level does not necessarily correlate with the level of TH protein. Furthermore, the level of TH protein does not necessarily correlate with the level of TH activation as the enzyme may be synthesized, but be inactive due to catecholamine binding (Kumer and Vrana, 1996). The levels of TH mRNA or TH protein cannot therefore be used as a direct indicator of TH activation in response to acute stress.

We investigated the effects of two acute stressors on TH phosphorylation as an indicator of both catecholamine cell activation and TH activation *in vivo* in male rats. We therefore determined the level of TH phosphorylation at Ser19, Ser31, and Ser40 over a 40-min period in response to footshock and immobilization in the LC and AM.

EXPERIMENTAL PROCEDURES

Materials

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) reagents were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Molecular weight PAGE standards, nitrocellulose membrane (Hybond ECL), ECL plus kit, anti-rabbit immunoglobulin (horseradish peroxidase-linked whole antibody from donkey), and anti-mouse immunoglobulin (horseradish peroxidase-linked whole antibody from sheep) were obtained from GE Health Care (Little Chalfont, UK). EGTA, EDTA, Tween-20, bovine serum albumin, sodium azide, isopentane, and β-actin antibody were from Sigma Chemical Co. (St. Louis, MO, USA). Anti-sheep antibody (horseradish peroxidise-linked whole antibody from rabbit) was obtained from Pierce Biotechnology (Rockford, IL, USA). Total-TH antibody and phospho-specific TH antibodies (pSer19, pSer31, and pSer40) were generated and tested for specificity according to (Gordon et al., 2009).

Rats

All experiments were approved by the University of Newcastle Animal Care and Ethics Committee and performed in accordance with the New South Wales Animal Research Act and the "Australian code of practice and use of animals for scientific purposes." Adult male Sprague–Dawley rats (300–400 g) were obtained from Animal Resources Centre (ARC), Perth, Australia. Rats were maintained in group housing under standard laboratory conditions in temperature-controlled rooms (21±1 °C), reverse 12 h light cycle with darkness from 2:00 to 14:00 h, food and water *ad libitum*. Rats were habituated and handled for 7 days prior to experiments in order to reduce stress associated with handling.

Rat treatments

Footshock sham (FS−): rats were placed into a footshock chamber (San Diego Instruments, CA, USA) for 10 min (n=6), 20 min (n=6), or 40 min (n=6), but they did not receive any footshock. Footshock (FS+): rats were placed into a footshock chamber and received a series of footshocks (1 mA, 1 s pulse per minute) for 10 min (n=6), 20 min (n=6), or 40 min (n=6). Immobilization sham (IMO−): rats were placed into a holding box (30×20×20 cm^3) for 10 min (n=6), 20 min (n=6), or 40 min (n=6), but were not immobilized. Immobilization (IMO+): rats were physically immobilized with wire mesh so that they were unable to turn or move substantially and were placed into a holding box for 10 min (n=6), 20 min (n=6), or 40 min (n=6). Home cage control (HCC): rats remained in their home cages (n=6). Immediately after treatment, rats were administered intraperitoneally with sodium pentobarbital (Lethabarb, 80 mg/kg). Rats were decapitated by guillotine once the rats showed a lack of response to painful stimuli (foot pinch) (<5 min). Immediately, the brain and adrenals were dissected and frozen in −80 °C in isopentane. Tissues were kept at −80 °C until required.

Sample preparation

LC was identified by reference to the Paxinos and Watson stereotaxis brain atlas. Sections (Bregma −9.86 to −10.52 mm) were cut at −20 °C on a customized freezing microtome (SM2000R, Leica, Nussloch, Germany) with an attached digital micrometer and rapidly transferred to a refrigerated working bench held at 4 °C. The bilateral LC regions were punched out using a 5-mm bore punch. LC and adrenal tissues were processed as previously described (Ong et al., 2011). Briefly, the LC and adrenal samples were homogenized using a sonicator (Soniprep 150, MSE, London, UK) in homogenization buffer (2% SDS, 2 mM EDTA, 50 mM Tris, pH 6.8). Samples were then centrifuged at 15,000 rpm for 20 min at 4 °C. The clear supernatants were collected and were mixed with sample buffer (1% SDS, 10% glycerol, 0.5% DTT, and minimal Bromophenol blue).

SDS-PAGE and Western blotting

Samples were subjected then to SDS–polyacrylamide gel electrophoresis before being transferred to nitrocellulose (Jarvie and Dunkley, 1995). Membranes were then stained with Ponceau S (0.5% Ponceau in 1% acetic acid) to assess the efficacy of the transfer. Membranes then were washed in Tris-buffered saline with Tween (TBST) (150 mM NaCl, 10 mM Tris, 0.075% Tween-20, pH 7.5) and incubated with blocking solution (5% bovine serum albumin, 0.04% sodium azide in TBST) for 2 h at 25 °C. Membranes were washed in TBST and incubated with primary antibodies (total TH, phospho-specific TH, and β-actin) for 1 h at 25 °C. Membranes were washed in TBST and incubated with horse-radish peroxidase-linked anti-IgG secondary specific antibodies for 1 h at 25 °C. Membranes were visualized on Fujifilm Las-3000 imaging system (Fuji, Stamford, CT, USA) using ECL plus detection reagents. The density of total TH, phospho-specific TH, and β-actin bands were measured using a MultiGauge V3.0 (Fuji, Stamford, CT, USA). Total TH protein levels were expressed as the ratio of TH protein to β-actin. β-actin levels were used as house keeping protein. Site-specific TH phosphorylation at Ser19, Ser 31, and Ser40 was expressed as a ratio relative to total TH protein to account for variability in total TH between samples.

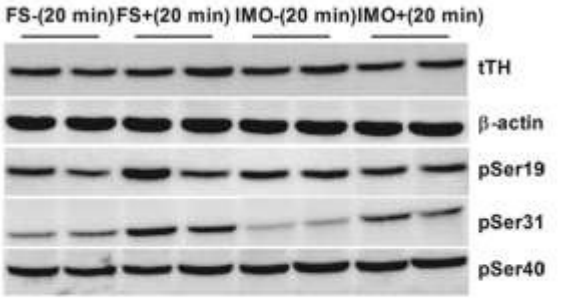

Fig. 1. Representative immunoblots show the effect of 20-min footshock stress (FS) or 20-min immobilization stress (IMO) on the levels of total-TH protein, β-actin, and phospho-TH (pSer19, pSer31, and pSer40) in the AM.

Statistical analysis

The data for FS−, FS+, IMO−, and IMO+ groups were expressed as a fold increase of the mean±SEM for each group relative to the mean of the HCC group. These data were analyzed by using PRISM V4.02 (GraphPad Software, Inc., CA, USA). Two-way ANOVA was used to examine whether there were any significant effects of treatment and/or time across the groups. Additionally, Bonferroni post tests were used to compare between FS− vs. FS+ and IMO− vs. IMO+ differences at each of the time points, where overall treatment effects were found. The significant differences shown on the graphs with asterisks refer to the post hoc tests for treatment effects. All differences were considered to be significant at $P<0.05$.

RESULTS

In the AM samples total-TH protein and phospho-TH (pSer19, pSer31, and pSer40) appeared as single bands corresponding to molecular masses of approximately 60 kDa (Fig. 1). Similar results were found with the LC (not shown). In this paper the results for TH protein levels were calculated relative to the levels of β-actin (Fig. 2A–D) and the results for TH phosphorylation were calculated relative to TH protein levels (Figs. 3–5). The results for the FS+, FS−, IMO+, and IMO− groups are presented relative to the HCC group data being set as equal to 1.0, in order to allow direct comparisons between the two stressors.

The effect of footshock or immobilization stress on total-TH protein in the locus coeruleus and the adrenal medulla

Rats were exposed to either footshock (Fig. 2A, C) or immobilization stress (Fig. 2B, D) for 10, 20, or 40 min. There was no effect of treatment or time on total TH protein expression in the LC or the AM.

The effect of footshock or immobilization stress on TH phosphorylation at Ser19 in the locus coeruleus and the adrenal medulla

Rats were exposed to either footshock (Fig. 3A, C) or immobilization stress (Fig. 3B, D) for 10, 20, or 40 min. There was a significant effect of treatment ($F=10.28$,

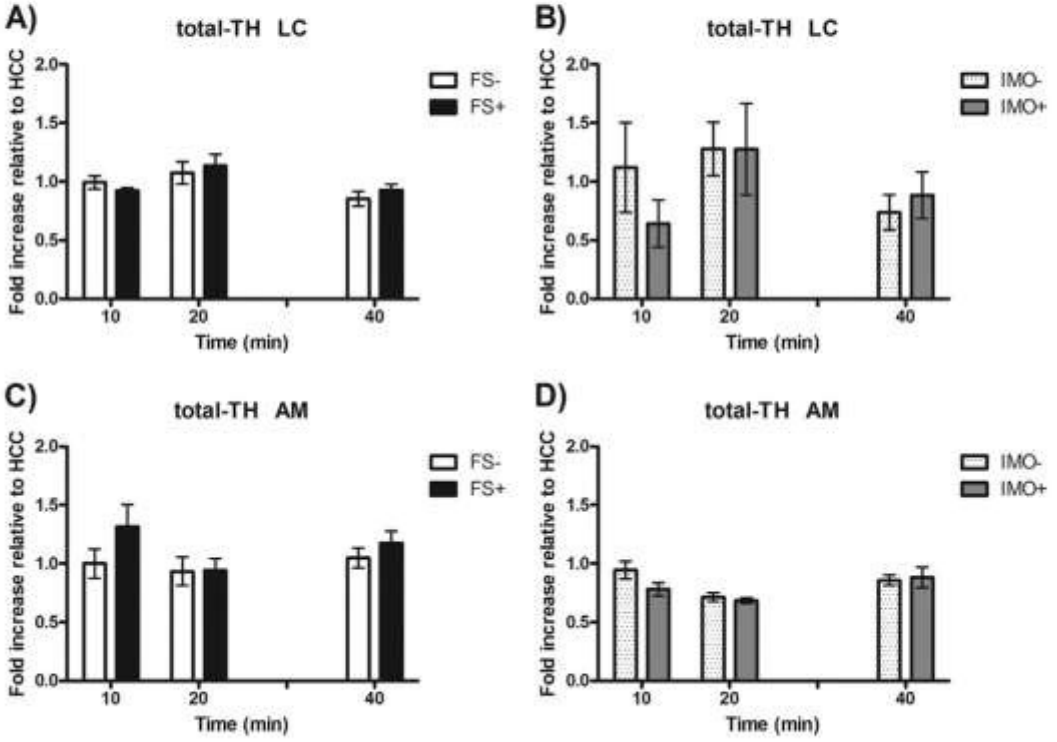

Fig. 2. The regulation of total-TH protein (relative to β-actin), in the locus coeruleus (LC; A, B) and the adrenal medulla (AM; C, D), 10, 20, and 40 min after the treatment with FS (A, C) ($n=6$ for each time point) or IMO (B, D) ($n=6$ for each time point). The results are presented relative to the home cage control (HCC) rats.

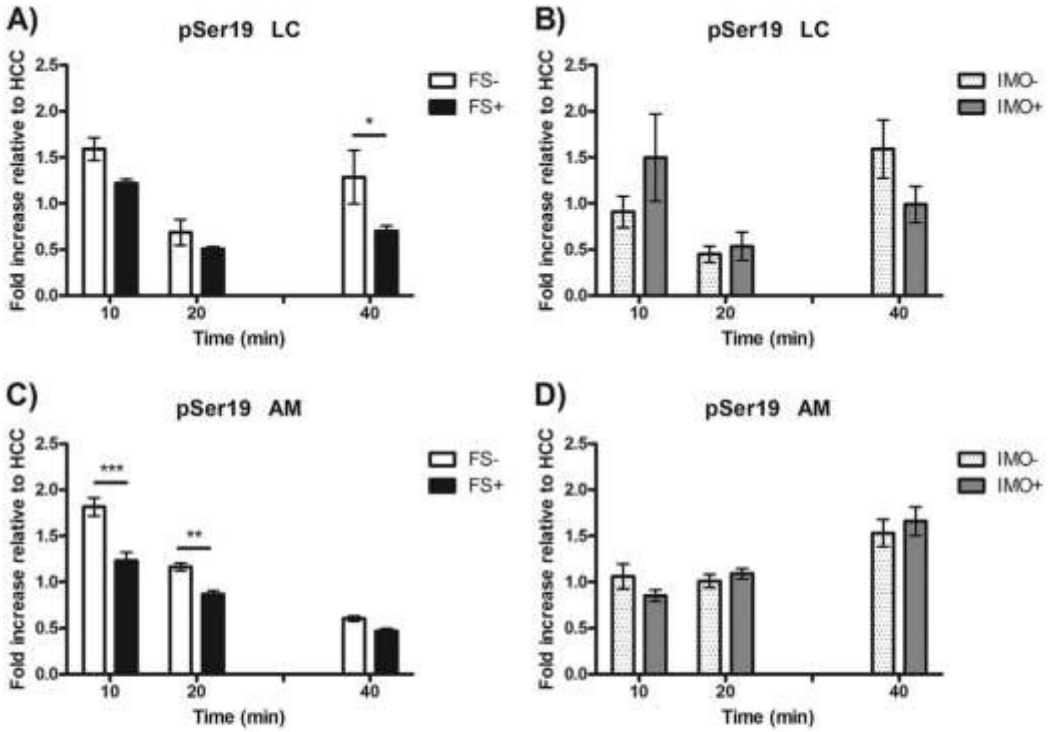

Fig. 3. The regulation of TH phosphorylation at Ser19 (relative to total-TH), in the LC (A, B) and the AM (C, D), 10, 20, and 40 min after the treatment with FS (A, C) (n=6 for each time point) or IMO (B, D) (n=6 for each time point). The results are presented relative to the HCC rats. * P<0.05, ** P<0.01, *** P<0.001.

DFn=1, P=0.0032) and time (F=15.56, DFn=2, P<0.0001) on pSer19 levels in the LC in rats exposed to footshock stress (Fig. 3A). Post hoc analysis indicated that relative to FS−, FS+ displayed a significant decrease in pSer19 at 40 min (P<0.05) (Fig. 3A). There was a significant effect of time (F=5.24, DFn=2, P=0.0098) on pSer19 levels in the LC in rats exposed to immobilization stress (Fig. 3B). There was a significant effect of treatment (F=45.66, DFn=1, P<0.0001) and time (F=129.00, DFn=2, P<0.0001) on pSer19 levels is the AM in rats exposed to footshock stress (Fig. 3C). Post hoc analysis indicated that relative to FS−, FS+ displayed a significant decrease in pSer19 at 10 min (P<0.001) and 20 min (P<0.01) (Fig. 3C). There was a significant effect of time (F=18.86, DFn=2, P<0.0001) on pSer19 levels in the AM in rats exposed to immobilization stress (Fig. 3D).

The effect of footshock or immobilization stress on TH phosphorylation at Ser31 in the locus coeruleus and the adrenal medulla

Rats were exposed to either footshock (Fig. 4A, C) or immobilization stress (Fig. 4B, D) for 10, 20, or 40 min. There was a significant effect of treatment (F=64.00, DFn=1, P<0.0001) and time (F=4.56, DFn=2, P=0.0175) on pSer31 levels in the LC in rats exposed to footshock stress (Fig. 4A). Post hoc analysis indicated that relative to FS−, FS+ displayed a significant increase in pSer31 at 20 min (P<0.001) and 40 min (P<0.001) (Fig. 4A). There was a significant effect of treatment (F=9.70, DFn=1, P=0.0040) and time (F=7.22, DFn=2, P=0.0028) on pSer31 levels in the LC in rats exposed to immobilization stress (Fig. 4B). Post hoc analysis indicated that relative to IMO−, IMO+ displayed a significant increase in pSer31 at 10 min (P<0.01) (Fig. 4B). There was a significant effect of treatment (F=27.54, DFn=1, P<0.0001) on pSer31 levels in the AM in rats exposed to footshock stress (Fig. 4C). Post hoc analysis indicated that relative to FS−, FS+ displayed a significant increase in pSer31 at 10 min (P<0.05), 20 min (P<0.05), and 40 min (P<0.01) (Fig. 4C). There was a significant effect of treatment (F=14.81, DFn=1, P=0.006) and time (F=9.77, DFn=2, P=0.0005) on pSer31 levels in the AM in rats exposed to immobilization stress (Fig. 4D). Post hoc analysis indicated that relative to IMO−, IMO+ displayed a significant increase in pSer31 at 20 min (P<0.01) (Fig. 4D).

The effect of footshock or immobilization stress on TH phosphorylation at Ser40 in the locus coeruleus and the adrenal medulla

Rats were exposed to either footshock (Fig. 5A, C) or immobilization stress (Fig. 5B, D) for 10, 20, or 40 min. There was no effect of treatment or time on pSer40 levels in the LC or the AM in any of the groups analyzed (Fig. 5A–D).

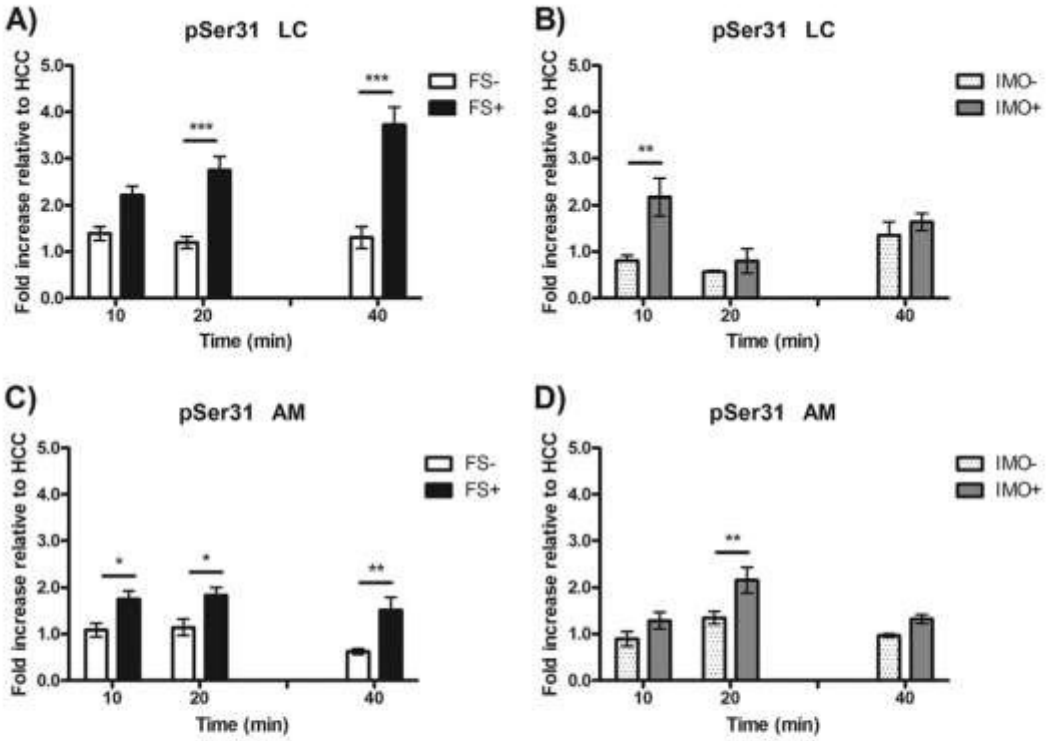

Fig. 4. The regulation of TH phosphorylation at Ser31 (relative to total-TH), in the LC (A, B) and the AM (C, D), 10, 20, and 40 min after the treatment of FS (A, C) (n=6 for each time point) or IMO (B, D) (n=6 for each time point). The results are presented relative to the HCC rats. * P<0.05, ** P<0.01, *** P<0.001.

DISCUSSION

We have investigated the effects of two different acute stressors on TH phosphorylation in rat LC and AM. The stressors used included footshock, which is primarily a physical stressor (pain) when used acutely and immobilization stress, which is primarily a psychological stressor (Dayas et al., 2001). We found that there were no changes in TH protein levels in the LC and AM over the first 40 min with both stressors. However, there were changes in TH phosphorylation at Ser19, Ser31, and Ser40 in both the LC and the AM and the responses to the two stressors were markedly different. Immobilization stress caused only transient increases in Ser31 phosphorylation, while footshock stress both decreased Ser19 phosphorylation and increased Ser31 phosphorylation in a sustained manner. These findings indicated that immobilization stress had significantly less impact than footshock on stimulating the LC and AM cells and activating TH.

Our data are in keeping with previous studies on the effects of immobilization and footshock stress on TH protein levels. In the LC, there was no change in TH protein levels within the 2 h of a single immobilization stress, while there was a significant upregulation in TH protein observed 24 h after a single immobilization stress for 2 h (Hebert et al., 2005). In the AM, a single 2-h immobilization stress caused a slight, but not statistically significant, rise in TH protein, while a significant upregulation in TH protein was observed 6 h after the immobilization stress (Nankova et al., 1994). TH mRNA, but not TH protein, has been investigated in the LC after a single 15-min footshock stress; there was a delayed increase in TH mRNA level 3 h following the end point of exposure that lasted 24 h (Chang et al., 2000). Therefore, the earliest time that TH mRNA or TH protein can be used to detect catecholaminergic cell activation in response to footshock or immobilization stress is 3 h and 6 h, respectively.

Our data showed that footshock stress evokes changes in TH phosphorylation at Ser19 at much earlier times than seen with any of the changes in TH mRNA or TH protein seen previously (Kumer and Vrana, 1996; Dunkley et al., 2004). In contrast with immobilization stress there were no treatment effects overall, nor were there any treatment differences seen at individual time points. With footshock stress the treatment significantly decreased Ser19 phosphorylation overall in both tissues; this was seen especially after 10 and 20 min in the AM and after 40 min in the LC. The decrease in Ser19 phosphorylation in response to footshock stress, relative to the FS− group, could be due to either less activation of protein kinase(s), or to a greater activation of protein phosphatases. In primary adrenal chromaffin cell cultures Ser19 is rapidly phosphorylated and then rapidly dephosphorylated (Haycock, 1993). This phosphorylation is likely to be due to the depolarization of the cells, leading to the entry of extracel-

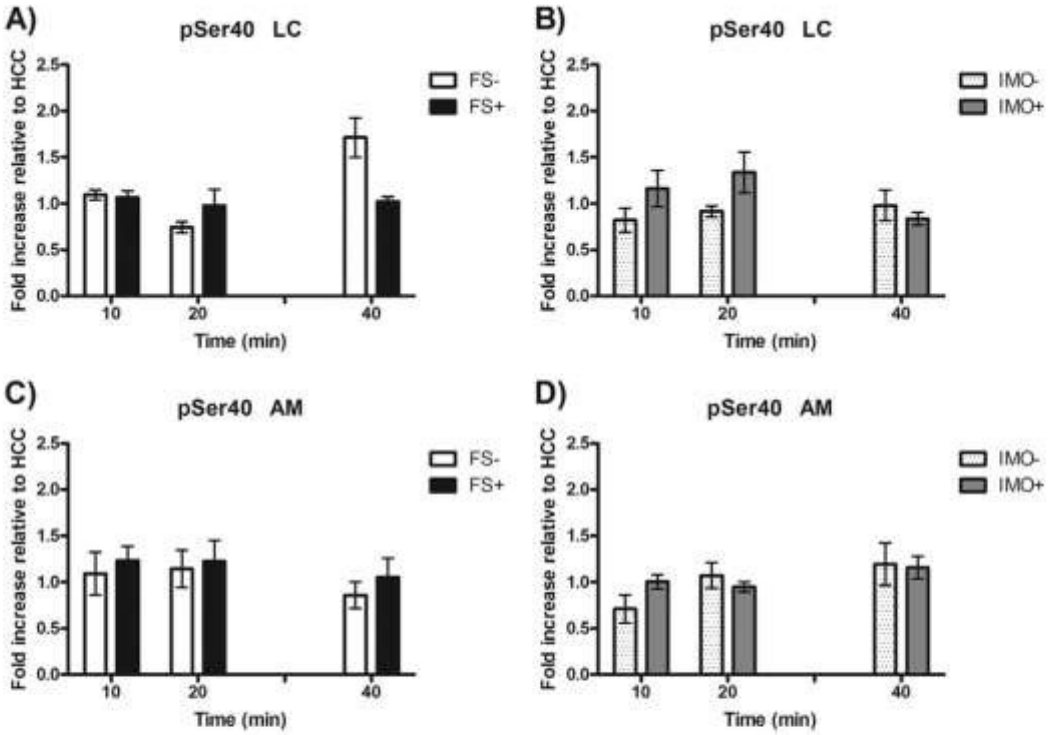

Fig. 5. The regulation of TH phosphorylation at Ser40 (with respect to total-TH), in the LC (A, B) and the AM (C, D), 10, 20, and 40 min after the treatment of FS (A, C) (n=6 for each time point) or IMO (B, D) (n=6 for each time point). The results are presented relative to the HCC rats.

lular calcium via voltage-sensitive calcium channels and then the direct activation of the calcium-dependent kinase CaMPKII (Dunkley et al., 2004). The subsequent dephosphorylation is likely to be due to activation of protein phosphatases as a result of the continually increasing levels of calcium (Robinson and Dunkley, 1985). If calcium levels rise rapidly, then rapid activation of the protein phosphatases leads to decreased phosphorylation of proteins without the initial increase being observed (Robinson and Dunkley, 1985). The fact that Ser19 phosphorylation progressively decreased in both tissues to well below that seen in the HCC suggests that greater phosphatase activation occurs in response to footshock, presumably due to greater activation of the cells and greater calcium entry. These data suggest that Ser19 phosphorylation is a sensitive index of LC and AM activation in response to footshock, but not to immobilization.

Our data showed that both footshock and immobilization stress evokes TH phosphorylation at Ser31. With footshock stress the treatment significantly increased Ser31 phosphorylation overall in both tissues; this was seen especially after 20 and 40 min in the LC and at all times investigated in the AM. With immobilization stress the treatment significantly increased Ser31 phosphorylation overall in both tissues; however, this was only seen at 10 min in the LC and 20 min in the AM. In primary adrenal chromaffin cell cultures Ser31 is always phosphorylated more slowly than Ser19 or Ser40 and Ser31 was not dephosphorylated over the first hour (Haycock, 1993; Bobrovskaya et al., 2007a,b). This is likely to be because the Ser31 kinases (ERK1/2) require a number of precursor kinases to be activated before they become activated and because the Ser31 phosphatases are not calcium sensitive and require inactivation of the kinases to allow dephosphorylation to occur (Dunkley et al., 2004). Immobilization stress increased Ser31 phosphorylation only transiently, while footshock stress increased Ser31 in a sustained manner and in the LC the increase continued to approximately four-fold by 40 min. A significant activation of ERK2 was observed after a single immobilization stress at 5 min in the LC consistent with our data (Hebert et al., 2005). These results suggest that the extent of activation of the LC and AM was much less in response to immobilization than in response to footshock stress and this is consistent with the results seen with Ser19.

Our data showed that footshock and immobilization stress has no significant effects on TH phosphorylation at Ser40. In primary adrenal chromaffin cell cultures Ser40 is phosphorylated at an intermediate time between Ser19 and Ser31 and then only very slowly dephosphorylated over the first hour (Haycock, 1993; Bobrovskaya et al., 2007a,b). A large number of protein kinases can phosphorylate Ser40 (Dunkley et al., 2004). Clearly footshock and immobilization stress did not activate either the LC or the AM cells in a manner which led to significant changes in Ser40 phosphorylation. Ser40 can be phosphorylated *in vivo* in response to stressors as we have recently shown that Ser40 phosphorylation is increased in the LC in re-

sponse to social defeat after 10 min (Ong et al., 2011) and in the AM after 20 min in response to 2-deoxy-D-glucose (2DG) treatment (Bobrovskaya et al., 2010). The reason for these differences in response must be related to the different frequency of cell firing and activation of different signaling pathways that occur with these different stressors.

TH is the rate-limiting enzyme in catecholamine synthesis and TH phosphorylation at Ser19, Ser31, and Ser40 can lead to TH activation and an increase in catecholamine synthesis. The catecholaminergic cells synthesize new catecholamines in order to replenish those released into the circulatory system in response to stressors. We have shown here that footshock and immobilization stress lead to quite different activation of the catecholaminergic cells in the LC and AM when using TH phosphorylation at Ser19, Ser31, and Ser40 as markers of cell activation. Our results show that footshock stress activates these cells to a greater extent than immobilization stress. The effects of footshock on Ser19 phosphorylation shed no light on whether TH is activated in these cells as Ser19 phosphorylation does not alter TH activity directly. However, the fact that Ser31 phosphorylation was markedly increased and sustained in response to footshock would suggest that TH activity is likely to be increased in these cells as Ser31 phosphorylation increases TH activity directly. However, as there was no increase in Ser40 phosphorylation in response to these stressors, the extent of any increase in TH activity would most likely be modest. Other stressors such as social defeat (Ong et al., 2011) and glucoprivation in response to 2DG clearly increase Ser40 phosphorylation (Bobrovskaya et al., 2010) and as a result they are also likely to increase TH activity in these tissues. Therefore, footshock stress and especially immobilization could be considered relatively mild stressors as they did not require substantial activation of TH to replace secreted catecholamines.

CONCLUSION

Measuring TH phosphorylation at Ser19, Ser31, and Ser40 over time can be used as a sensitive index to differentiate the effects of different stressors on catecholaminergic cells. This approach has been used to show that immobilization stress is a substantially milder stressor of catecholaminergic systems than footshock stress.

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Chapter 3: The effect of acute footshock on the adrenal *in vivo*.

Ong Lin Kooi, Guan L, Damanhuri H A, Goodchild A K, Bobrovskaya L, Dickson P W, Dunkley P R.

This manuscript has been prepared as an original article (February 2012).

Acknowledgement of Collaboration

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers.

The work in the following chapter (Chapter 3) was prepared as an original article for submission. I (Lin Kooi Ong) was the first author of this manuscript, and the work embodied in this chapter was primarily performed by myself, with the exception of the following:

Rat treatments were performed by myself with assistance from Liying Guan.

Figure 1C & D: Analysis of noradrenaline and adrenaline was performed by Hanafi Damanhuri under the supervision of Ann K. Goodchild.

The work embodied by these experiments is inextricably tied to the overall findings of the manuscript, and therefore this work will be discussed in the final chapter (Chapter 7) of this thesis.

Lin Kooi Ong

THE EFFECTS OF ACUTE FOOTSHOCK STRESS ON THE ADRENAL *IN VIVO*

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3.1 Abstract:

Stress activates the hypothalamo-pituitary-adrenalcortical (HPA) axis, the sympathetic-adrenomedullary system and downstream metabolic pathways. Our aim was to investigate the relationships between these stress responses over a 40 min period after exposure of rats to footshock stress. Here we explored the effects of footshock on; plasma adrenocorticotropic hormone (ACTH), corticosterone and glucose, body temperature, as well as adrenal medulla protein kinase activity and tyrosine hydroxylase (TH) synthesis, phosphorylation and activity. There were significant increases in plasma ACTH at 20 min and corticosterone at 20 and 40 min. There were significant increases in plasma glucose at 20 and 40 min and body temperature was increased between 10 and 40 min. Extracellular signal-regulated kinases 2 (Erk2) was activated between 10 and 40 min, whereas protein kinase A (PKA) was activated only at 40 min. TH protein and Ser19 phosphorylation levels were not altered. Ser31 phosphorylation was increased between 10 and 40 min, whereas Ser40 phosphorylation was increased only at 40 min. TH activity was increased at 20 and 40 min. These findings indicate that acute footshock led to HPA axis activation, causing the release of ACTH and corticosterone, sympathetic-adrenomedullary system activation, causing activation of protein kinases (Erk2 and PKA), Ser31 and Ser40 phosphorylation and an increase in TH activity. These effectors led to activation of downstream metabolic pathways leading to an increase in plasma glucose and an increase in body temperature.

Keywords: footshock, hypothalamo-pituitary-adrenalcortical axis, sympathetic-adrenomedullary system, tyrosine hydroxylase.

Abbreviations:

ACTH	adrenocorticotropic hormone
CaMPKII	Ca^{2+}/calmodulin-dependent protein kinase
CRH	corticotrophin releasing hormone
Erk	extracellular signal-regulated kinases
HPA	hypothalamo-pituitary-adrenalcortical
PAGE	polyacrylamine gel electrophoresis
PKA	protein kinase A
SDS	sodium dodecyl sulfate
Ser	serine residue
TBST	Tris-buffered saline with Tween
TH	tyrosine hydroxylase

3.2 INTRODUCTION

Stress induces activation of the hypothalamo-pituitary-adrenalcortical (HPA) axis, the sympathetic-adrenomedullary system and downstream metabolic pathways (Odio and Maickel, 1985, Marquez et al., 2002, Kvetnansky et al., 2009). The HPA axis is controlled by the activation of the locus coeruleus during the stress response (Young et al., 2005). Activation of the locus coeruleus results in release of noradrenaline which activates the paraventriclar nucleus in the hypothalamus to release vasopressin and corticotrophin releasing hormone (CRH) which, in turn, stimulate the anterior pituitary gland to produce pro-opiomelanocortin and release adrenocorticotropic hormone (ACTH) into plasma. Cortisol (corticosterone in rodents) is synthesised and released from the adrenal cortex into plasma in response to ACTH (Douglas, 2005). The firing rate of the locus coeruleus neurons has been shown to be increased by footshock stress (Stanford, 1995). Likewise, the sympathetic-adrenomedullary system is controlled by the activation of the splanchnic nerve. Activation of the splanchnic nerve results in release of adrenaline and noradrenaline from the adrenal medulla into plasma (Wakade, 1981). The firing rate of the splanchnic nerve has been shown to be increased by footshock stress as well (de Diego et al., 2008, Kvetnansky et al., 2009).
Once secreted into plasma, some of the major functions of glucocorticoids and catecholamines are to increase blood pressure, heart rate, ventilation rate and depth of respiration as well as glycogenolysis and gluconeogenesis, which leads to an increase in plasma glucose and oxygen use for rapid energy and heat generation during the stress response (Rhoades and Pflanzer, 2003, Charmandari et al., 2005).

During the stress response, catecholamines are secreted from the sympathetic-adrenomedullary system (Kvetnansky et al., 2004). Despite the fact that catecholamines

are released their cellular levels remain constant (Wakade et al., 1988). This suggests that the rate of catecholamine synthesis is coupled to cellular secretion. The catecholamine biosynthetic pathway is activated during stress to replenish the released catecholamines. Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the biosynthetic pathway of catecholamines (Nagatsu et al., 1964, Dunkley et al., 2004). During the stress response, TH activity is increased (Kvetnansky et al., 1970) and many stressors, including footshock stress have been shown to increase TH activity in the brain and adrenal medulla (Stone et al., 1978, Chang et al., 2000). TH regulation is distinctive at different time points. In response to stress, TH activity is regulated either by 1) acute activation via the influx of the extracellular calcium into the cells that leads to activation of protein kinases that increase TH phosphorylation and the dissociation of inhibitory catecholamine or 2) chronic activation via mRNA expression and protein synthesis (Kumer and Vrana, 1996). TH phosphorlyation at serine residues 19, 31 and 40 (Ser19, Ser31 and Ser40) is involved in acute TH activation. Ser19 can be phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase (CaMPKII), Ser31 can be phosphorylated by extracellular signal-regulated kinases 1/2 (Erk1/2) and Ser40 can be phosphorylated by a range of protein kinases including protein kinase A (PKA) (Dunkley et al., 2004). Ser19 phosphorylation does not increase TH activity directly (Sutherland et al., 1993), Ser31 phosphorylation increases TH activity about 2 fold (Haycock, 2002, Jedynak et al., 2002, Nunez et al., 2007) and Ser40 phosphorylation, which abolishes the feedback inhibition by catecholamines, increases TH activity up to 40 fold (Dunkley et al., 2004). Thus, TH activity is highly modulated during stress and can be used to investigate the stress response.

The aim of this study was to investigate the effects of acute footshock stress *in vivo* on the HPA axis, the sympathetic-adrenomedullary system and downstream metabolic pathways. Footshock stress which is primarily a physical stressor (pain) when used acutely (Dayas et al., 2001) activates the HPA axis (Odio and Maickel, 1985, Belda et al., 2004) and sympathetic-adrenomedullary system (Melia and Duman, 1991, Chang et al., 2000, Ong et al., 2011). Footshock stress also increases plasma glucose (Odio and Maickel, 1985), which can be mediated by plasma glucocorticoids and/or catecholamines. These changes can be utilized to measure the stress response in the animals. The relationship between these pathways in response to acute footshock stress has yet to be determined. We therefore investigated the effects of acute footshock stress on the HPA axis, the sympathetic-adrenomedullary system and downstream metabolic pathways *in vivo* in male rats by measuring plasma ACTH, corticosterone and glucose, body temperature, as well as adrenal medulla protein kinase activity and TH synthesis, phosphorylation and activity.

3.3 EXPERIMENTAL PROCEDURES

Materials

EGTA, reduced glutathione, dithiothreitol (DTT), dehydroxybenzylamine (DHBA), noradrenaline bitartrate salt, adrenaline hydrochloride, methanol, ammonium molybate, sodium pyrophosphate, sodium vanadate, β-glycerolphosphate, microcystin, sodium chloride, Tris, Tween-20, bovine serum albumin, sodium azide, β-actin antibody, catalase, β-mercaptoethanol and activated charcoal were from Sigma Chemical Co. (St Louis, MO, USA). Sodium 1-octanesulfonate was from Tokyo Chemical Industry (Tokyo, Japan). Activated alumina was from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Disodium EDTA was from Merck Pty Limited (Kilsyth, Victoria, Australia). Protease inhibitor cocktail tablets were from Roche Diagnostics Australia (Castle Hill, NSW, Australia). Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) reagents were from Bio-Rad Laboratories (Hercules, CA, USA). PageRuler Prestained Protein Ladder was from Thermo Fisher Scientific (Rockford, IL, USA). Anti-rabbit immunoglobulin (horseradish peroxidase-linked whole antibody from donkey) and anti-mouse immunoglobulin (horseradish peroxidase-linked whole antibody were from sheep) and 3,5-[^{3}H]-L-tyrosine were from GE Health Care (Little Chalfont, UK). Total-TH antibody and phospho-specific TH antibodies (pSer19, pSer31 and pSer40) were generated and were tested for specificity as described (Gordon et al., 2009). Total-p44/42 MAPK (Erk1/2) antibody, phospho-p44/42 MAPK (Erk1/2) antibody and phospho-PKA substrate antibody were from Cell Signalling technology (Beverly, MA, USA). Anti-sheep antibody (horseradish peroxidise-linked whole antibody from rabbit) was from Pierce Biotechnology (Rockford, IL, USA). L-tyrosine

was from DBH Biochemicals (Poole, UK). Tetrahydrobiopterin was supplied by Dr. Schirck's Laboratory (Jona, Switzerland). Optiphase HiSafe scintillation cocktail was from Perkin-Elmer (Waltham, MA, USA).

Animal protocols

All animal protocols were approved by the University of Newcastle Animal Care and Ethics Committee and performed in accordance with the New South Wales Animal Research Act and the "Australian code of practice and use of animals for scientific purposes". Adult male Sprague-Dawley rats (300-400 g) were obtained from Animal Resources Centre (ARC), Perth, Australia. Rats were maintained in group housing (n=4) under standard laboratory conditions in temperature controlled rooms (21±1°C), reverse 12 h light cycle with darkness from 02:00 to 14:00 h, food and water *ad libitum*. Rats were handled and habituated by placing them in a footshock chamber (San Diego Instruments, CA, USA) for 7 days prior to experiments, in order to reduce the stress associated with handling and exposure to a novel environment. Sham rats were placed into a programmable footshock chamber for 10 min (n=6), 20 min (n=6) or 40 min (n=6), but did not receive any footshocks. Footshock rats were placed into a programmable footshock chamber and received a series of footshocks (1 mA, 1 sec pulse per 1 min) for 10 min (n=6), 20 min (n=6) or 40 min (n=6). Immediately after the protocol, rats were administered intraperitoneally with sodium pentobarbital 80 mg/kg (Lethabarb Euthanasia Injection, Virbac Pty. Ltd, Milperra, NSW, Australia) and core temperatures were measured by insertion of a digital thermometer into the rat rectum (approximately 3.0 cm). Once the rats showed a lack of response to painful stimuli (foot pinch reflex) (<5 min), blood and tissue samples were collected.

Blood and tissue collection

Blood samples were collected by cardiac puncture and were mixed immediately into tubes containing 4 mM EGTA and 4 mM reduced glutathione. Blood samples were centrifuged at 1800 RPM for 10 min at 4°C. Resulting plasma samples were then centrifuged at 2700 RPM for 10 min at 4°C. Plasma samples were kept in dark container and frozen at -80°C until further analysis. Whole adrenal glands were dissected and adrenal cortex was removed using a surgical scalpel. The adrenal medulla samples were kept frozen at -80°C until further analysis.

Plasma samples analysis

Plasma glucose levels were measured using blood glucose meter (Accu-chek Performa, Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Plasma ACTH levels were determined using ACTH double antibody ^{125}I radioimmunoassay kit (MP Biomedicals Australia, Seven Hills, NSW, Australia) according to the manufacturer's instructions. The reported recovery of exogenous ACTH is 100 % and the intra- and inter-assay variability of 3.9 % and 6.8 %, respectively. Plasma corticosterone levels were determined using corticosterone double antibody ^{125}I radioimmunoassay kit (MP Biomedicals Australia) according to the manufacturer's instructions. The reported recovery of exogenous ACTH is 100 % and the intra- and inter-assay variability of 8 % and 10 %, respectively. Plasma catecholamine extractions were performed as previously described with some modifications (Anton and Sayre, 1962). Briefly, 500µl of plasma was placed into a 2

mL tube containing 30 mg of activated alumina, 1 mL of 1.5 M Tris buffer pH 8.6, 100 µL of 0.1 M disodium EDTA and 0.5 ng of DHBA. The DHBA was used as an internal standard. The mixtures were gently mixed for 10 min and were washed 3 times using 1 mL of de-ionized water. The catecholamines were extracted using 200 µL elution solution (2 % acetic acid containing 100 µM disodium EDTA). Plasma noradrenaline and adrenaline levels were measured using HTEC-500 Complete Stand-Alone HPLC-ECD systems (Eicom Corporation, Kyoto Japan). Analytical conditions were as follows: detector, +450 mV potential against a Ag/AgCl reference electrode; column, Eicompak CA-5ODS, 2.1 × 150 mm; mobile phase (0.1 M phosphate buffer pH 5.7 containing 50 mg/L disodium EDTA, 700 mg/L sodium 1-octanesulfonate and 12 % methanol) at a flow of 0.23 mL/min. The specific retention time for each compound was determined using noradrenaline and adrenaline standards. The catecholamines levels were calculated from the peak height ratio relative to DHBA using PowerChrom v2.6.3 software (eDAQ Pty Ltd, NSW Australia).

Adrenal medulla sample preparation

Adrenal medulla samples were sonicated in 100 µL of homogenizing buffer (2 mM potassium phosphate buffer pH 7.4, 1 mM EGTA, 1 x protease inhibitor cocktail tablet, 1 mM DTT, 80 µM ammonium molybate, 1 mM sodium pyrophosphate, 1 mM sodium vanadate, 5 mM β-glycerolphosphate, 2 µM microcystin, final concentration) with a microsonicator (UP50H, Hielscher Ultrasonics GmbH, Teltow, Germany) for 3 x 30 sec pulses at 4°C. Samples were centrifuged at 16000 RPM for 20 min at 4°C. The clear supernatants were collected and protein concentrations were determined by Pierce BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Samples were diluted with homogenizing buffer to equalize protein concentrations (5 mg/mL), were aliquoted and were kept frozen at -80°C for further analysis.

Western Blotting

Western blotting were performed as previously described with some modifications (Ong et al., 2011). Aliquots (one-half volume) of the adrenomedullary samples were mixed with sample buffer (1 % SDS, 10 % glycerol, 0.5 % DTT, minimal bromophenolblue, final concentration). 30 μg of each samples were subjected to SDS-PAGE gel electrophoresis and were transferred to nitrocellulose membranes (Hybond ECL, GE Health Care). Nitrocellulose membranes were stained with Ponceau S (0.5 % ponceau in 1 % acetic acid) to assess the efficacy of the transfer. Membranes were washed in Tris-buffered saline with Tween (TBST) (150 mM sodium chloride, 10 mM Tris, 0.075 % Tween-20, pH 7.5) and incubated with blocking solution (5 % bovine serum albumin, 0.04 % sodium azide in TBST) for 2 h at 25ºC. Membranes were washed in TBST and incubated with primary antibodies (total- and phospho-TH, β-actin, total- and phospho-p44/42 MAPK (Erk1/2) and phospho-PKA substrate) for 1 h at 25ºC. Membranes were washed in TBST and incubated with horse-radish peroxidase-linked anti-IgG secondary specific antibodies for 1 h at 25ºC. Membranes were visualized on Fugifilm Las-3000 imaging system (Fuji, Stamford, CT, USA) using detection reagents (Amersham ECL Plus Western Blotting Detection Reagents, GE Health Care). The density of the bands was measured using a MultiGauge V3.0 (Fuji, Stamford, CT, USA). Total-Erk1/2 protein, phospho-PKA and total-TH protein levels were normalized to β-actin. Phospho-Erk1/2 levels were normalized to total-Erk1/2 protein levels. Site-specific phospho-TH

at pSer19, pSer 31 and pSer40 levels were normalized to total-TH protein levels and were expressed as a fold increase relative to the sham samples.

TH activity assay

TH activity was measured using a method based on the tritiated water release assay with slight modification (Reinhard et al., 1986, Briggs et al., 2011). Briefly, aliquots of the adrenomedullary samples were mixed in the reaction mixture (50 µg sample, 2 mM potassium phosphate pH 7.4, 36 µg catalase, 0.008 % β-mercaptoethanol, 24 µM L-tyrosine, 1 µCi 3,5-[^{3}H]-L-tyrosine, final volume 50 µL). The 50 µL reactions were initiated with the addition of 100 µM tetrahydrobiopterin in 5 mM HCl. Control representing background reactions were added with 5 mM HCl but did not contain tetrahydrobiopterin. Assays were performed for 20 min at 30°C and were stopped by addition of 700 µl charcoal slurry (7.5 % activated charcoal in 1 M HCl). Mixtures were vortexed for 1 min and were centrifuged at 16000 RPM for 10 min at 30°C. 350 µl supernatants were added to 3 mL scintillation cocktail and were vortexed for 10 sec. Mixtures were assayed by scintillation spectrometry for 20 min per sample. TH activity assays which were performed under these conditions were linear. The changes in TH activity were normalized to total-TH protein levels and expressed as a fold increase relative to the sham samples.

Statistical analysis

The data for sham and footshock groups were expressed as a fold increase of the mean (M) ± SEM for each group relative to the mean of the sham group. These data were

analysed by using Prism 5 for Windows (Version 5.04, GraphPad Software, Inc., CA, USA). Two-Way ANOVA was used to determine whether there were any significant effects of footshock treatment and/or time across the groups. Additional Bonferroni post tests were used to analyse differences between sham and footshock groups at each of the time points (10, 20 and 40 min), where an overall footshock treatment or time effects was found. The significant differences shown on the graphs with asterisks (*) refer to the post hoc tests for footshock treatment effects. All differences were considered to be significant at $p<0.05$.

3.4 RESULTS

The effect of footshock on plasma ACTH, corticosterone, noradrenaline, adrenaline, glucose levels and body temperature

The plasma samples were analysed for ACTH, corticosterone, noradrenaline, adrenaline and glucose levels. There was a significant effect of footshock ($F_{(1,27)}$=22.4, p<0.001) and time ($F_{(2,27)}$=4.7, p<0.05) on plasma ACTH levels (Fig. 1A). Post hoc analysis indicated that footshock caused a significant increase in ACTH levels relative to sham treatment at 20 min (5.2 fold, p<0.001) (Fig. 1A). There was a significant effect of footshock ($F_{(1,27)}$=15.0, p<0.001) and time ($F_{(2,27)}$=8.6, p<0.01) on plasma corticosterone levels (Fig. 1B). Post hoc analysis indicated that footshock caused a significant increase in corticosterone levels relative to sham treatment at 20 min (1.4 fold, p<0.05) and 40 min (1.5 fold, p<0.05) (Fig. 1B). There was no effect of footshock or time on plasma noradrenaline (Fig. 1C) or adrenaline (Fig. 1D) levels. There was a significant effect of footshock ($F_{(1,27)}$=40.0, p<0.001) and time ($F_{(2,27)}$=26.0, p<0.001) on plasma glucose levels (Fig. 1F). Post hoc analysis indicated that footshock caused a significant increase in glucose levels relative to sham treatment at 20 min (1.6 fold, p<0.001) and 40 min (1.2 fold, p<0.05) (Fig. 1F). There was a significant effect of footshock ($F_{(1,27)}$=61.0, p<0.001) but not time on body temperature (Fig. 1E). Post hoc analysis indicated that footshock caused a significant increase in body temperature relative to sham treatment at 10 min (sham, 37.8°C to footshock, 38.6°C, p<0.001), 20 min (sham, 37.7°C to footshock, 38.3°C , p<0.01) and 40 min (sham, 37.5°C to footshock, 38.6°C, p<0.001) (Fig. 1E).

The effect of footshock on protein kinase activation in the adrenal medulla

The adrenal medulla samples were analysed by immunoblotting with antibodies that recognise total-Erk1/2 protein, phospho-Erk1/2 and phospho-protein kinase A (PKA) substrates. Erk1 (p44) and Erk2 (p42) protein appeared as two bands corresponding to molecular masses of 44 and 42 kDa respectively (Fig. 2A). The results for total-Erk1 and total-Erk2 levels were calculated relative to β-actin levels (Fig. 2B & 2C) and the results of phospho-Erk1 and phospho-Erk2 levels were calculated relative to total-Erk1 and total-Erk2 levels respectively (Fig. 2D & 2E). Phospho-PKA substrates appeared as several bands (Fig. 3A). The results for phospho-PKA substrates levels (quantified as optical density of all bands for each lane) were calculated relative to β-actin levels (Fig. 3B). There was no effect of footshock or time on total-Erk1 (Fig. 2B) and total-Erk2 (Fig. 2C) levels. There was a significant effect of footshock ($F_{(1,27)}$=13.4, p<0.01) but not time on phospho-Erk1 levels (Fig. 2D). Post hoc analysis indicated that footshock caused a significant increase in phospho-Erk1 levels relative to sham treatment at 10 min (1.9 fold, p<0.05) (Fig. 2D). There was a significant effect of footshock ($F_{(1,27)}$=26.4, p<0.001) but not time on phospho-Erk2 levels (Fig. 2E). Post hoc analysis indicated that footshock caused a significant increase in phospho-Erk2 levels relative to sham treatment at 10 min (1.5 fold, p<0.05), 20 min (1.5 fold, p<0.05) and 40 min (1.5 fold, p<0.05) (Fig. 2E). There was a significant effect of footshock ($F_{(1,27)}$=5.9, p<0.05) but not time on phospho-PKA substrates levels (Fig. 3B). Post hoc analysis indicated that footshock caused a significant increase in phospho-PKA substrates levels relative to sham treatment at 40 min (1.8 fold, p<0.05) (Fig. 3B).

The effect of footshock on total-TH protein, site-specific phospho-TH and TH activity in the adrenal medulla

The adrenal medulla samples were analysed by immunoblotting with antibodies that recognise total-TH and site-specific phospho-TH at Ser19, Ser31 or Ser40. Total-TH protein and phospho-TH appeared as single bands corresponding to molecular masses of approximately 60 kDa (Fig. 4A). The results for total-TH levels were calculated relative to β-actin levels (Fig. 4B) and the results of phospho-TH levels were calculated relative to total-TH levels because the ratios more accurately represent phosphorylation states and account for variability in total-TH among samples (Fig. 4C, 4D & 4E). There was no effect of footshock or time on total-TH levels (Fig. 4B). There was also no effect of footshock or time on pSer19 levels (Fig. 4C). There was a significant effect of footshock ($F_{(1,27)}$=29.5, p<0.001) but not time on pSer31 levels (Fig. 4D). Post hoc analysis indicated that footshock caused a significant increase in pSer31 levels relative to sham treatment at 10 min (1.7 fold, p<0.01), 20 min (1.5 fold, p<0.05) and 40 min (1.5 fold, p<0.05) (Fig. 4D). There was a significant effect of time ($F_{(2,27)}$=4.9, p<0.05) but not footshock on pSer40 levels (Fig. 4E). Post hoc analysis indicated that footshock caused a significant increase in pSer40 levels relative to sham treatment at 40 min (1.5 fold, p<0.01) (Fig. 4E).

TH activity was assayed in the same adrenal medulla samples. The results for TH activity levels were calculated relative to total-TH levels. There was a significant effect of footshock ($F_{(1,27)}$=45.2, p<0.001) on TH activity levels (Fig. 5). Post hoc analysis indicated that footshock caused a significant increase in TH activity levels

relative to sham treatment at 20 min (2.1 fold, $p<0.01$) and 40 min (2.8 fold, $p<0.001$) (Fig. 5).

3.5 DISCUSSION

In this study, we demonstrated that acute footshock stress can alter the HPA axis and the sympathetic nervous system leading to: 1) the release of ACTH from the anterior pituitary gland and corticosterone from the adrenal cortex, 2) splanchnic nerve activation of the adrenomedullary catecholaminergic cells, including activation of protein kinases (Erk1/2 and PKA), TH phosphorylation at Ser31 and Ser40 and an increase in TH activity, 3) activation of metabolic pathways including an increase in plasma glucose and an increase in body temperature. Taken together, our results suggest that acute footshock triggers adaptive responses which assist the animals to cope with the stressor.

Footshock stress (1 mA, 1 sec pulse per 1 min) evokes the release of ACTH and corticosterone into plasma consistent with previous studies (Odio and Maickel, 1985, Smith et al., 1991, Marquez et al., 2002, Belda et al., 2004). The HPA axis driven stress response starts when the paraventricular nucleus of the hypothalamus produces CRH and vasopressin, which stimulate the anterior pituitary gland to synthesize pro-opiomelanocortin and release ACTH into plasma which initiates the synthesis and release of corticosterone from the adrenal cortex into plasma (Douglas, 2005). Our data are in general agreement with previous studies which showed that footshock stress evokes significant increases in plasma ACTH levels by 20 min and corticosterone levels between 20 min and 40 min. However, the sham animals in this study have higher baseline when compared to “basal or control” groups from previous studies (Belda et al., 2004). The reason for the difference is plasma samples were collected by cardiac puncture in this study whereas, a tail-nick procedure was used in the studies mentioned

above. Plasma ACTH and corticosterone levels were significantly increased at 30 min after footshock stress (0.5 mA, 6 sec pulse per 90 sec) was initiated (Belda et al., 2004) and plasma corticosterone levels peaked at 30 min after footshock stress (0.5 mA, 2 sec pulse, 1 pulse per 10 min) was initiated and was sustained up to 4 h (Odio and Maickel, 1985). Therefore, the activation of the HPA axis is critical in response to the stress of acute footshock.

Footshock stress evokes an increase in plasma glucose and body temperature. Two distinct systems are activated rapidly in order to maintain the stress response including the HPA axis and the sympathetic adrenomedullary catecholaminergic system. Activation of the HPA axis results in the secretion of the glucocorticoids from the adrenal cortex, whereas activation of the adrenomedullary catecholaminergic system results in the secretion of the catecholamines from the adrenal medulla. Once they are secreted into the circulation, both glucocorticoids and catecholamines mediate glycogenolysis and gluconeogenesis, which leads to an increase in blood glucose and oxygen use for rapid provision of energy and this contributes to heat generation during the stress response (Rhoades and Pflanzer, 2003, Charmandari et al., 2005). The time course of footshock stress (0.5 mA, 2 sec pulse, 1 pulse per 10 min) on plasma glucose has been investigated; there was a slight, but not statistically significant, increase at 30 min footshock stress, while a significant increase was observed 1 and 2 h after initiation of the footshock stress (Odio and Maickel, 1985). Plasma adrenaline and noradrenaline levels were significantly increased at 5 min immediately after footshock stress (2.5 mA, 0.4 sec pulse per 5 sec) (McCarty and Kopin, 1978). We found an increase in plasma glucocorticoids (ACTH and corticosterone) but not in plasma catecholamines (adrenaline and noradrenaline). This finding suggested that the increase in plasma

glucose and body temperature is most likely mediated by plasma glucocorticoids, although the catecholamines could have been increased before the earliest time we measured at 10 min.

Our data are consistent with our previous studies which showed that footshock stress has no significant effects on TH protein levels in the adrenal medulla over the first 40 min (Ong et al., 2011). TH is regulated acutely by protein phosphorylation and chronically by protein synthesis (Kumer and Vrana, 1996). Previous studies showed that TH protein changes are only detectable in the adrenal gland many hours after exposure to stressors (Watanabe et al., 1995, Rusnak et al., 2001, Tank et al., 2008), with the earliest detectable change being 6 h after a 2 h immobilization stress. Therefore, TH protein levels cannot be used to detect acute changes in adrenomedullary catecholaminergic cells.

Footshock stress has no significant effects on Ser19 phosphorylation in the adrenal medulla over the first 40 min. Previous studies showed that changes in Ser19 phosphorylation are detectable sec to min after exposure to receptor activators in bovine adrenal chromaffin cell cultures; Ser19 was rapidly phosphorylated due to the depolarization of the cells leading to the entry of extracellular calcium via voltage sensitive calcium channels and activation of the CaMPKII (Haycock, 1993, Dunkley et al., 2004). Ser19 is then rapidly dephosphorylated (Haycock, 1993, Dunkley et al., 2004), presumably due to the continually increasing levels of calcium and activation of protein phosphatases (Robinson and Dunkley, 1985, Haycock, 1993). We recently showed that Ser19 phosphorylation was significantly decreased *in vivo* after 10 and 20 min of footshock stress (Ong et al., 2011). The fact that in the earlier study the rats were

not habituated to the footshock chamber prior to the footshock protocols suggests that there is a significant effect of exposure to a novel environment on Ser19 phosphorylation. We have also shown that in adrenal medulla Ser19 phosphorylation was able to be increased in response to a different stressor namely hypotension elicited by injection of hydralazine (10 mg/kg) after 5 min (Unpublished data). In the current study we did not find any changes in Ser19 phosphorylation in adrenal medulla suggesting that footshock stress itself does not alter Ser19 phosphorylation levels.

Footshock stress evokes an increase in Erk1/2 and Ser31 phosphorylation in the adrenal medulla between 10 min and 40 min. In bovine adrenal chromaffin cell cultures Ser31 is always phosphorylated more slowly than both Ser19 and Ser40, presumably because Erk1/2 require a number of precursor kinases to be activated before they become activated (Haycock et al., 1992, Haycock, 1993, 2002, Dunkley et al., 2004). Ser31 is not readily dephosphorylated over the next hour as the Ser31 phosphatases are not calcium sensitive and dephosphorylation requires inactivation of the protein kinases before dephosphorylation can occur under basal Ser31 phosphatase activity (Dunkley et al., 2004). Recently, we have showed that both Erk1/2 and Ser31 phosphorylation are significantly increased at 20 and 60 min in the adrenal medulla in response to another stressor namely glucoprivation elicited by 2-deoxy-D-glucose (2DG) (400mg/kg) (Bobrovskaya et al., 2010). In this study we did not find any changes in total-Erk1/2 protein, but there were increases in Erk1 phosphorylation in response to footshock stress at 10 min and Erk2 phosphorylation at 10, 20 and 40 min. Our data are consistent with our previous studies which showed that Ser31 phosphorylation was increased in response to footshock stress at 10, 20 and 40 min (Ong et al., 2011) and they confirm that these changes were not due to exposure to a novel environment. These findings

suggested that footshock stress has a rapid and long lasting effect on Erk2 activation and Ser31 phosphorylation in the adrenal medulla.

Our data showed that footshock stress evokes an increase in PKA substrates and Ser40 phosphorylation in the adrenal medulla, but only after 40 min. In bovine adrenal chromaffin cell cultures Ser40 is phosphorylated by a large number of protein kinases, including PKA, at an intermediate time between Ser19 and Ser31 (Haycock, 1993, Dunkley et al., 2004, Bobrovskaya et al., 2007). Ser40 is then very slowly dephosphorylated over 1 h (Dunkley et al., 2004). We did not find any changes in Ser40 phosphorylation in response to footshock stress in the adrenal medulla in our previous study (Ong et al., 2011). Ser40 can be phosphorylated *in vivo* as we have recently shown that both PKA and Ser40 phosphorylation are significantly increased at 20 and 60 min in response to another stressor glucoprivation elicited by 2-deoxy-D-glucose (2DG) in the adrenal medulla (Bobrovskaya et al., 2010). In addition, Ser40 phosphorylation is significantly increased in response to immunity challenge elicited by lipopolysaccharide (LPS) at 4 h (Sominsky et al., 2012). These findings suggested that footshock stress has a delayed effect on PKA activation and that PKA is likely to mediate Ser40 phosphorylation.

Our data showed that footshock stress evokes an increase in TH activity at both 20 and 40 min. Erk1 and Erk2 are known to mediate Ser31 phosphorylation which increases TH activity *in vitro* and *in vivo* (Haycock, 2002, Jedynak et al., 2002, Nunez et al., 2007). Ser40 phosphorylation plays a major role in modulating TH activity as it is known to abolish the feedback inhibition of TH caused by catecholamine binding and this results in a 40-fold increase in TH activity *in vitro* and there is evidence that Ser40

phosphorylation *in vivo* increases TH activity (Dunkley et al., 2004). Our results suggest that both Ser31 and Ser40 phosphorylation of TH contribute to the increases in TH activity, as Ser31 phosphorylation increases activity at 20 min when Ser40 is not increased, while Ser40 further increases TH activity at 40 min when there is no further increase in Ser31 phosphorylation. These increases in TH activity are presumably required to provide more catecholamines as a result of increased secretion. Plasma catecholamines levels were significantly increased at 10 min immediately after footshock stress (1.0 mA, 0.5 sec pulse per 5 sec) (Konarska et al., 1989). However, we did not find an increase in plasma catecholamines at 10, 20 and 40 min as had been observed in previous studies. This could be due to either footshock stress used here do not lead to any changes in catecholamine secretion or lead to an increase in catecholamine secretion earlier than the time points investigated here.

In conclusion our study provides evidence that acute footshock stress leads to the activation of the HPA axis as measured by an increase in plasma ACTH and corticosterone levels. Footshock stress also leads to activation of the adrenomedullary catecholaminergic cells as measured by an increase in Erk1/2 and PKA signalling pathways, which in turn contribute to an increase in TH activity via Ser31 and Ser40 phosphorylation. Footshock stress also leads to an increase in plasma glucose and body temperature. Identification of these pathways is of major importance in understanding the adaptive responses to the footshock stress.

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3.7 FIGURES

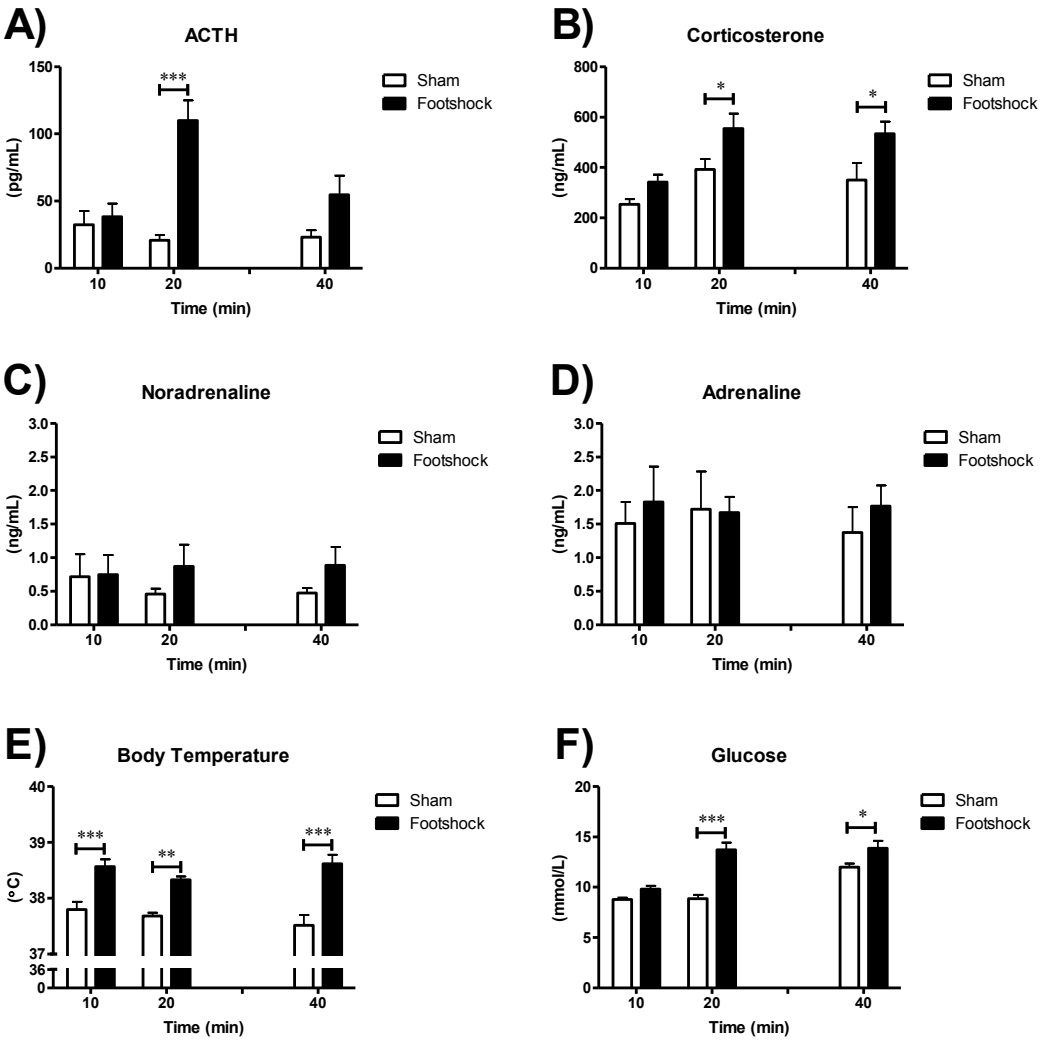

Fig. 1

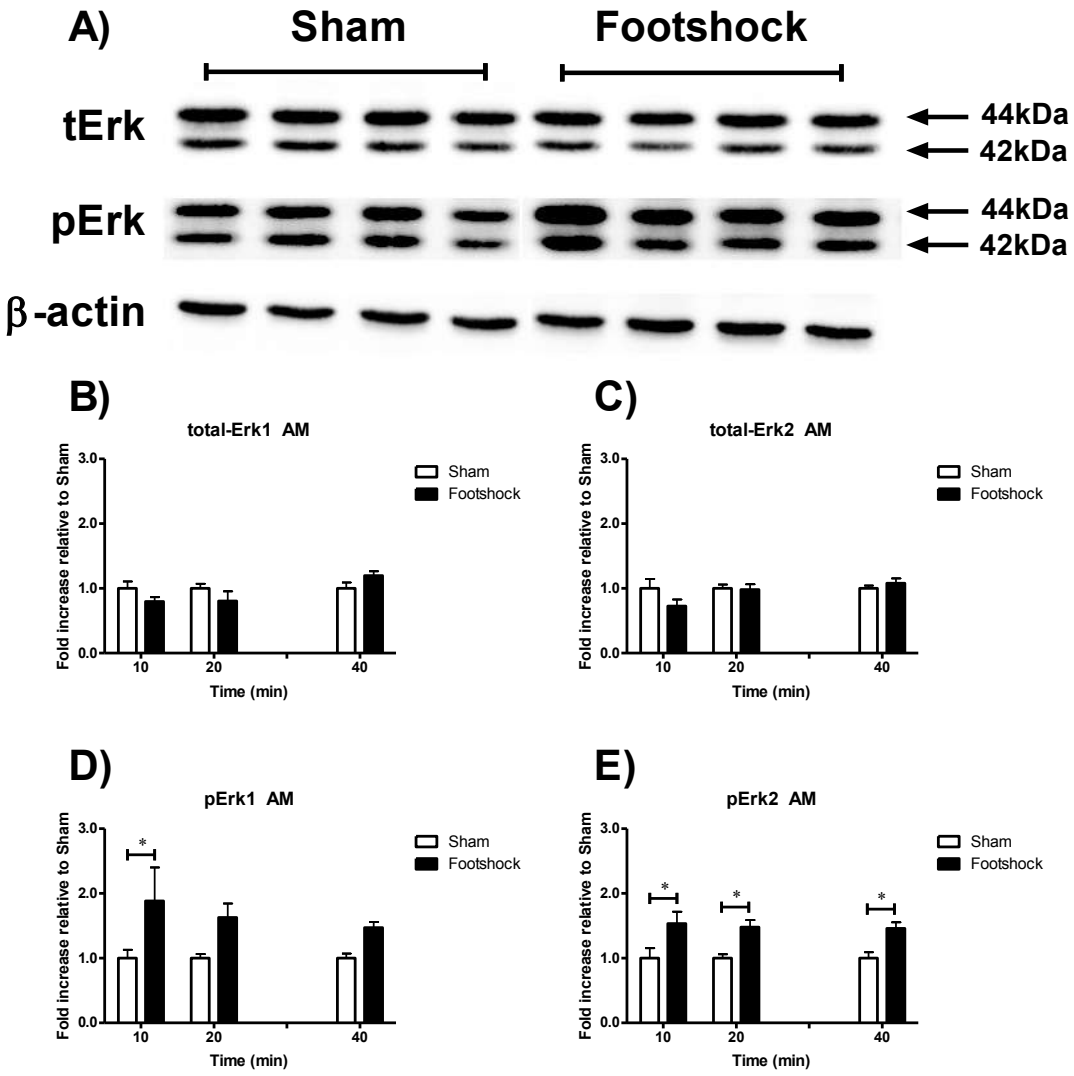
A)
Sham
Footshock
tErk
pErk
β-actin
44kDa
42kDa
44kDa
42kDa
B)
total-Erk1 AM
C)
total-Erk2 AM
D)
pErk1 AM
E)
pErk2 AM
Fold increase relative to Sham
Time (min)
Sham
Footshock
0.0
1.0
2.0
3.0
10
20
40
*

Fig. 2

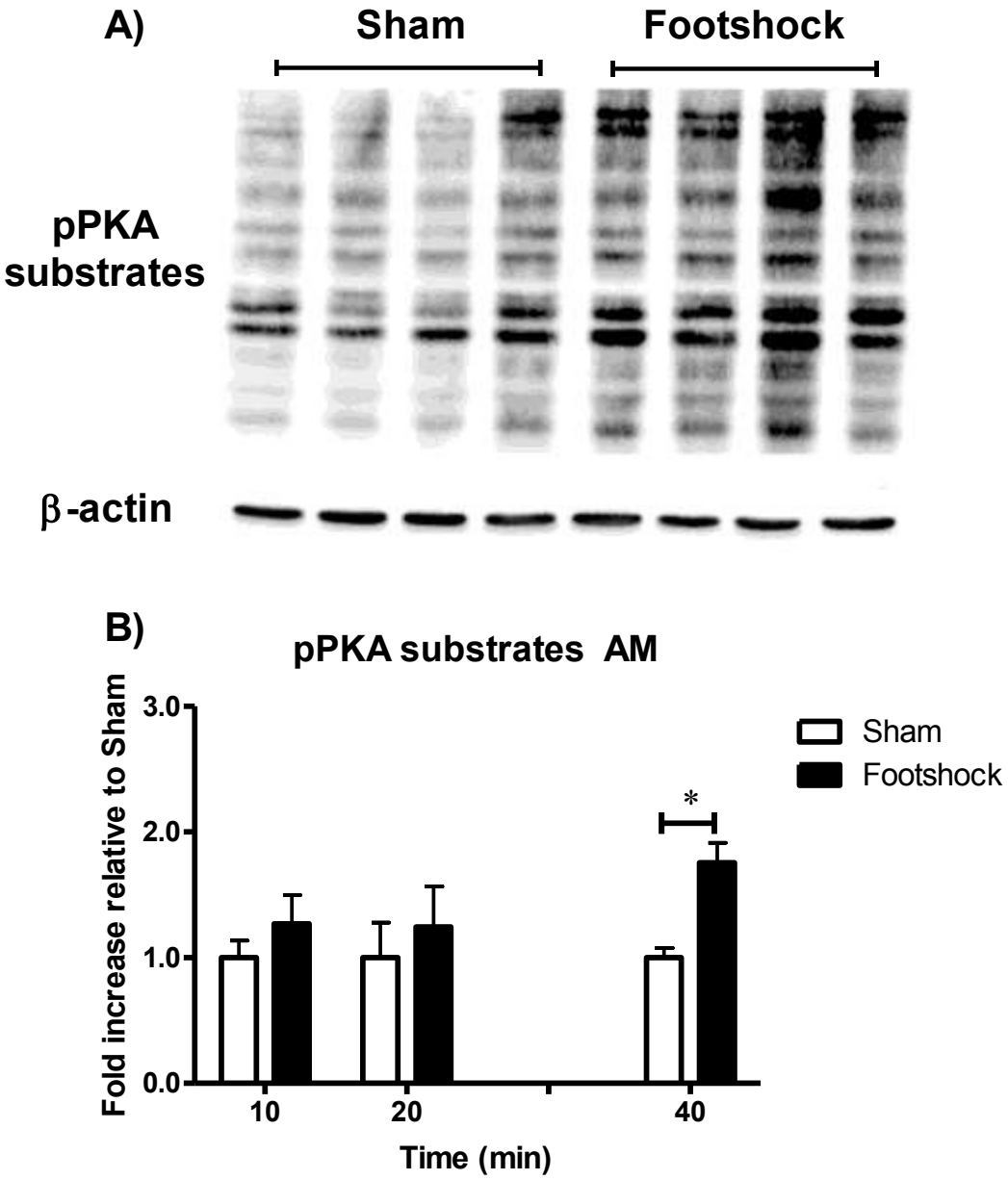
A)
Sham
Footshock
pPKA substrates
β-actin
B)
pPKA substrates AM
Fold increase relative to Sham
3.0
2.0
1.0
0.0
10
20
40
Time (min)
Sham
Footshock
*

Fig. 3

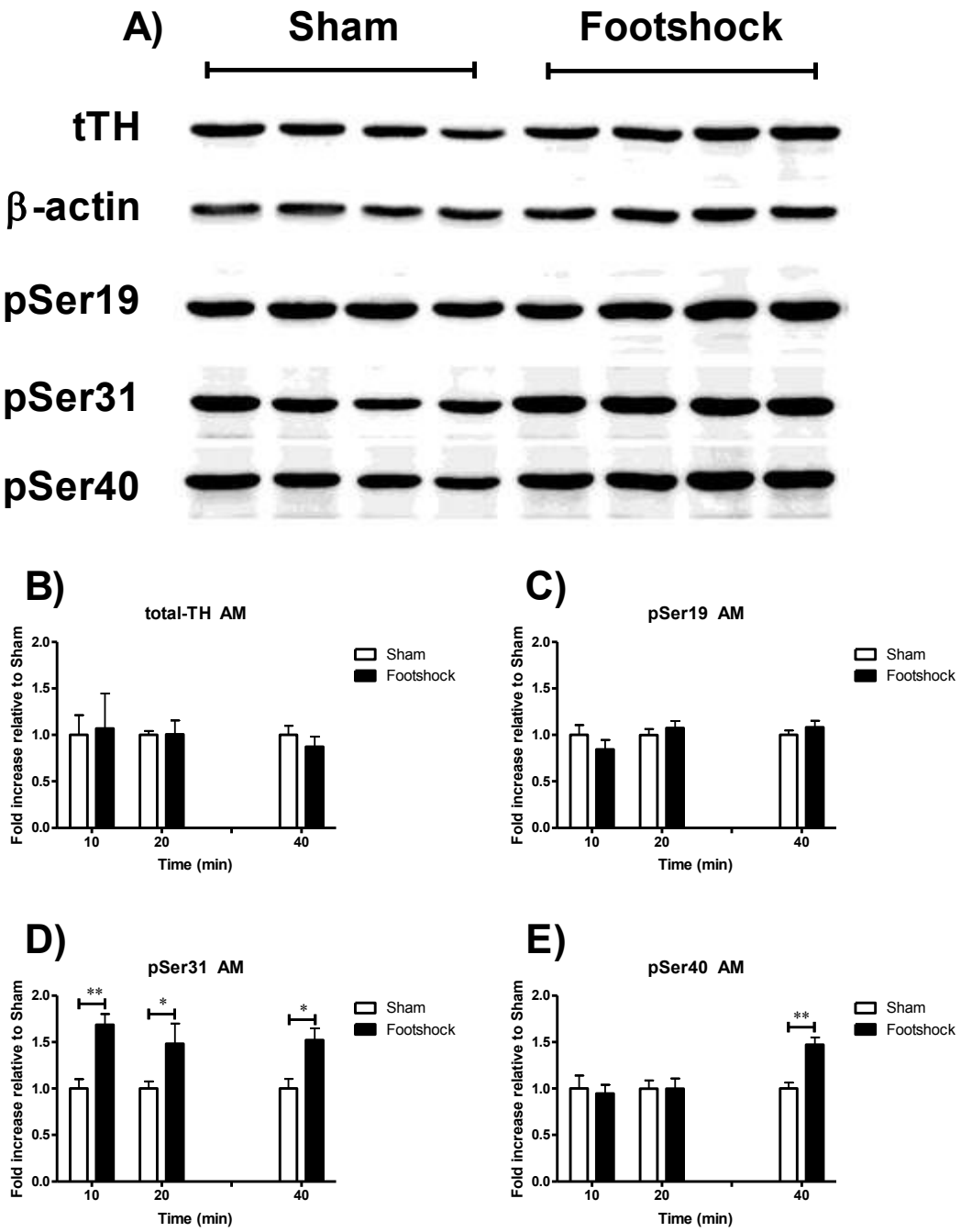
A)
Sham
Footshock
tTH
β-actin
pSer19
pSer31
pSer40
B)
total-TH AM
C)
pSer19 AM
D)
pSer31 AM
E)
pSer40 AM
Fold increase relative to Sham
Time (min)
Sham
Footshock
10
20
40
0.0
0.5
1.0
1.5
2.0
*
**

Fig. 4

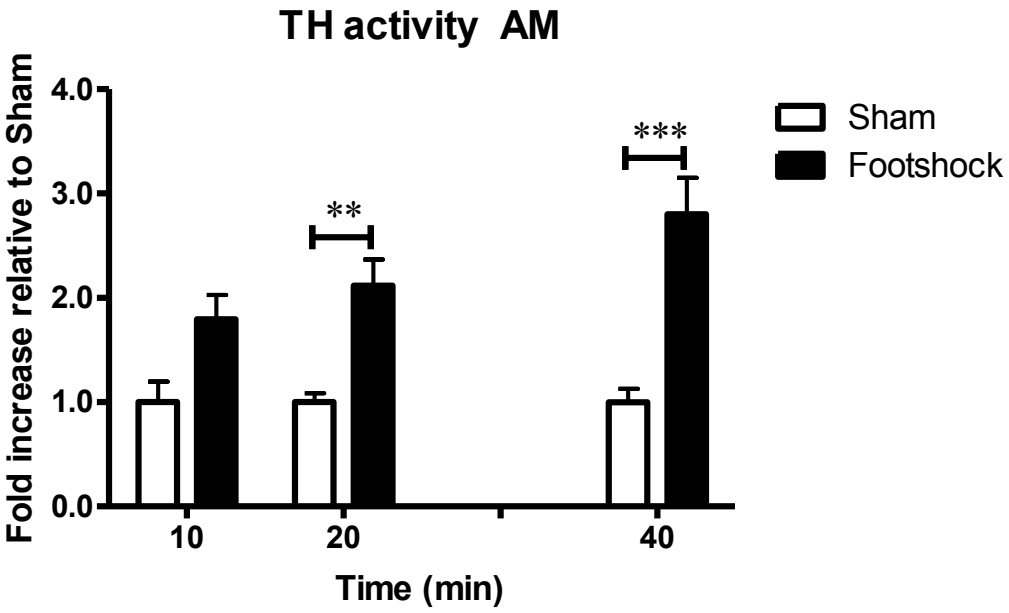
TH activity AM
Fold increase relative to Sham
4.0
3.0
2.0
1.0
0.0
**
***
10
20
40
Time (min)
Sham
Footshock

Fig. 5

Fig. 1: Effect on plasma ACTH, corticosterone, noradrenaline, adrenaline and glucose levels 10, 20 and 40 min after footshock treatment (n=6 per group). *p<0.05, **p<0.01, ***p<0.001

Fig. 2: Effect on total- and phospho-Erk1/2 levels in adrenal medulla 10, 20 and 40 min after footshock treatment (n=6 per group). A) Representative immunoblots show the effect of 10 min footshock treatment on total- and phospho-Erk1/2. The loading controls were performed by analysis of β-actin. *p<0.05

Fig. 3: Effect on phospho-PKA substrates levels in adrenal medulla 10, 20 and 40 min after footshock treatment (n=6 per group). A) Representative immunoblots show the effect of 40 min footshock treatment on phospho-PKA substrates. The loading controls were performed by analysis of β-actin. *p<0.05

Fig. 4: Effect on total- and phospho-TH (pSer19, pSer31 and pSer40) levels in adrenal medulla 10, 20 and 40 min after footshock treatment (n=6 per group). A) Representative immunoblots show the effect of 40 min footshock treatment on total- and phospho-TH. The loading controls were performed by analysis of β-actin. *p<0.05, **p<0.01

Fig. 5: Effect on TH activity levels 10, 20 and 40 min after footshock treatment (n=6 per group). **p<0.01, ***p<0.001

Chapter 4: Signal transduction pathways and tyrosine hydroxylase regulation in the adrenal medulla following glucoprivation: an *in vivo* analysis.

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Acknowledgement of Collaboration

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers.

The work in the following chapter (Chapter 4) was submitted to Neurochemistry International in April 2010. I (Lin Kooi Ong) was the co-author of this manuscript, and the work embodied in this chapter was partly performed by myself:

Figure 3: Analysis of tyrosine hydroxylase phosphorylation and protein levels was performed by myself or Hanafi Damanhuri.

The work embodied by these experiments performed by other authors will NOT be discussed in depth in the final chapter (Chapter 7) of this thesis, however the results from Figure 3 (performed by myself) will be discussed in Chapter 7.

Lin Kooi Ong

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Signal transduction pathways and tyrosine hydroxylase regulation in the adrenal medulla following glucoprivation: An *in vivo* analysis

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ABSTRACT

The regulation of tyrosine hydroxylase (TH, the rate limiting enzyme involved in catecholamine synthesis) is critical for the acute and sustained release of catecholamines from adrenal medullary chromaffin cells, however the mechanisms involved have only ever been investigated under *in vitro/in situ* conditions. Here we explored the effects on, TH phosphorylation and synthesis, and upstream signalling pathways, in the adrenal medulla evoked by the glucoprivic stimulus, 2-deoxy-d-glucose (2DG) administered intraperitoneally to conscious rats. Our results show that 2DG evoked expected increases in plasma adrenaline and glucose at 20 and 60 min. We demonstrated that protein kinase A (PKA) and cyclin dependent kinases (CDK) were activated 20 min following 2DG, whereas mitogen activated protein kinase (MAPK) was activated later and PKC was not significantly activated. We demonstrated that phosphorylation of Ser40TH peaked after 20 min whereas phosphorylation of Ser31TH was still increasing at 60 min. Serine 19 was not phosphorylated in this time frame. TH phosphorylation also occurred on newly synthesized protein 24 h after 2DG. Thus 2DG increases secretion of adrenaline into the plasma and the consequent rise in glucose levels. In the adrenal medulla 2DG activates PKA, CDK and MAPK, and evokes phosphorylation of Ser40 and Ser31 in the short term and induces TH synthesis in the longer term all of which most likely contribute to increased capacity for the synthesis of adrenaline.

Extensive experimentation in *in vitro* systems, using primarily cultured chromaffin cells or PC12 cells, indicates that the regulation of tyrosine hydroxylase (TH, the rate limiting enzyme in catecholamine synthesis) is critical in both the acute and sustained release of catecholamines (Zigmond et al., 1989; Kumer and Vrana, 1996; Dunkley et al., 2004). When adrenomedullary chromaffin cells are activated to secrete catecholamines there is a concomitant increase in the catecholamine synthesis in order to maintain cellular levels of catecholamines constant (Zigmond et al., 1989). In the short term, TH activity is primarily regulated by phosphorylation at Ser40 which relieves feedback inhibition by catecholamines (Kumer and Vrana, 1996; Dunkley et al., 2004). Phosphorylation of Ser31 alone can increase TH activity about 2 fold whereas the phosphorylation of Ser19 and Ser31 contributes to TH activation by increasing the rate of Ser40 phosphorylation (Haycock, 1990; Bevilaqua et al., 2001; Bobrovskaya et al., 2004; Dunkley et al., 2004; Lehmann et al., 2006). In the longer term TH activity is regulated by increased TH protein synthesis (Kumer and Vrana, 1996; Sabban et al., 2004; Tank et al., 2008). Whether such signalling sequences are evoked *in vivo* by physiological stimuli, such as glucoprivation, are as yet unknown. Insulin induced hypoglycemia at least evokes an increase in the affinity of tyrosine hydroxylase (TH) for its cofactors and an increase in TH activity presumably increasing catecholamine synthesis (Fluharty et al., 1983).

The signal transduction pathways activated that lead to increases in TH activity have generally been explored in *in vitro* systems. A large number of neurotransmitter or similar agents including nicotine, muscarine, histamine, PACAP and angiotensin acutely increase TH activation mediated via a range of protein kinase signalling pathways (Dunkley et al., 2004). Which pathways are activated in response to distinct physiological stimuli, over what time frames have yet to be determined.

The aim of this study was to investigate the effects of glucoprivation *in vivo* on these signalling sequences and pathways in the adrenal medulla. Glucoprivation, such as that elicited by 2-deoxy-D-glucose, activates the sympathetic outflow innervating the adrenal medulla releasing adrenaline in order to mobilise

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glucose. Adrenaline release evoked by low effective concentrations of glucose is dependent upon the integrity of the splanchnic nerve (Perlman and Chalfie, 1977). Glucoprivation increases plasma adrenaline, the only source of which is the adrenal medulla. Some elevation of plasma noradrenaline is also seen but this is most likely a result of sympathetic activation relating to other glucoregulatory mechanisms rather than activation of the adrenal medullary noradrenergic chromaffin cells (Medvedev et al., 1990).

Splanchnic nerve stimulation releases acetylcholine to evoke adrenaline release via a calcium dependent mechanism (Douglas and Rubin, 1961), however this response desensitises even though catecholamine release continues under sustained stimulation. PACAP has been shown to influence catecholamine secretion, at least in slices obtained from adrenal gland, and this is evoked via PLC and PKC dependent mechanisms and may be recruited under conditions of sustained stress (Kuri et al., 2009). Little other functional evidence is available as to what neurotransmitters or signalling systems are activated by physiological stimuli.

In order to define the signal transduction pathways and identify the TH signalling systems activated *in vivo* following physiological stimuli we sought to determine the time course and effects of a single episode of glucoprivation. We used 2-deoxy-d-glucose (2DG) to evoke glucoprivation in conscious Sprague–Dawley rats. 2DG is a glucose analogue that does not undergo glycolysis providing an environment of low effective concentrations of glucose. The 2-DG effects on plasma adrenaline and adrenal medullary TH mRNA are similar to those evoked by insulin induced hypoglycaemia (Rusnak et al., 1998; Ishihara et al., 2009).

1. Experimental procedures

1.1. Materials

2-deoxy-d-glucose, ethylene glycol tetraacetic acid (EGTA), ethylene diamine tetraacetic acid (EDTA), Tween-20, reduced glutathione and anti-β-actin antibody were purchased from Sigma Chemical Co. (St Louis, MO, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents were from Bio-Rad Laboratories (Hercules, CA, USA), molecular-weight PAGE standards, nitrocellulose membrane (Hybond ECL), ECL plus kit, anti-rabbit immunoglobulin (horseradish peroxidase-linked whole antibody from donkey), anti-mouse immunoglobulin (horseradish peroxidase-linked whole antibody from sheep) were from GE Health Care (Little Chalfont, UK). Anti-sheep immunoglobulin (horseradish peroxidase-linked whole antibody from rabbit) was from Pierce Biotechnology (Rockford, USA). Total TH, phospho-Ser40TH, phospho-Ser31TH, and phospho-Ser19TH specific antibodies were generated and tested for specificity as described by (Bobrovskaya et al., 2004; Gordon et al., 2009). Phospho-PKA Substrate (RRXS/T) (100G7E) Rabbit mAb antibody (#9624), phospho-(Ser) PKC substrate antibody (#2261), phospho-MAPK/CDK substrates (PXSP or SPXR/K) (34B2) rabbit mAb (#2325) antibody, phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (#9101), p44/42 MAPK (Erk1/2) antibody (#9102) were obtained from Cell Signalling technology (Beverly, MA, USA).

1.2. Animals

All experiments were carried out on male Sprague-Dawley (SD) rats (300–500 g, 10–13 weeks of age; n = 44) with the approval of the Animal Ethics Committees of Macquarie University, Sydney and conducted in accordance with the Australian Code of Practice for the Care and Use of animals for scientific purposes. Animals were purchased from Animal Resource Centre (Perth, Australia). One day prior to experimentation rats were housed singly in a temperature controlled room (21 ± 1 °C) with ad libitum access to food and water. The cages were housed in a single room for a minimum of 12 hours in order to minimize the stress of isolation.

1.3. Animal treatments

Animals were handled routinely in order to reduce stress associated with injection. 2-deoxy-d-glucose (2DG, 400 mg/kg), or saline, was administered intraperitoneally. Following injection of the 2DG or saline, food and water were removed from the cage. Animals were sacrificed at four time points (5 min (n = 4), 20 min (n = 6), 60 min (n = 6) and 24 h (n = 6)) following 2DG or saline injection. The period of stimulation was determined from injection until administration of an anaesthetic overdose, sodium pentobarbital (Lethabarb, 80 mg/kg i.p.). Once surgical levels of anaesthesia were induced (tested by lack of response to painful stimuli such as foot pinch) animals were decapitated by guillotine (< 5 min). Blood and tissue samples were then collected.

1.4. Blood and tissue collection

5 ml of whole trunk blood was collected and placed immediately into tubes containing 4 mM EGTA and 4 mM reduced glutathione (Lambert et al., 1995). Blood samples were centrifuged for 10 min at 1000 rpm (4 °C). Resulting plasma was then spun for 10 min at 2700 rpm (4 °C). Plasma samples were kept at −80 °C until further catecholamine analysis. Blood glucose was measured using Accu-check performa glucometer from Roche (Mannheim, Germany). Whole adrenals were removed rapidly, frozen in dry ice and kept at −80 °C until used. In some experiments the adrenal medulla was dissected to remove adrenal cortex prior to freezing for the analysis of cell signalling substrates.

1.5. Measurement of plasma catecholamines

Samples were prepared using a liquid-liquid extraction procedure (Winter et al., 2008). Measurement of adrenaline (Ad) and noradrenaline (NAd) was performed using a Agilent Model 1200 HPLC system equipped with a ESA Coulochem III detector. Mobile phase consisted of 7.4 g citric acid, 4.6 g disodium phosphate, 300 mg octanesulphonic acid, 500 mg disodium edetate and 40 mL acetonitrile in water adjusted to pH4.1. An internal standard DHBA was used in the assay.

1.6. Measurement of phospho-MAPK1/2, phospho-PKA, phospho-PKC, phospho-MAPK/CDK substrates in the adrenal medulla

Adrenal medullas were weighed and homogenised in 40 volumes of homogenization buffer (2% SDS, 2 mM EDTA, 50 mM Tris (pH 6.8)) by sonication on ice (3 times × 30 sec at 10,000 A), then boiled for 5 min and centrifuged for 20 min. Supernatants were carefully removed. 200 μL of supernatant were mixed with 10 μL of 10% dithiothreitol (DTT) and 70 μL of sample buffer (40% glycerol, 50 mM Tris, minimal bromophenol blue, pH 6.8). 10 μL of sample was run on 10% SDS-PAGE and then transferred to nitrocellulose as described (Bobrovskaya et al., 2007b). Membranes were immunoblotted with primary antibodies for phospho-MAPK1/2 and phospho-PKA, phospho-PKC, phospho-MAPK/CDK substrates according to the manufacturer's instructions. The immunoblots were visualized and quantified on the Fuji LAS 3000 imaging system using ECL-plus detection reagent. The density of the bands was measured and expressed as a fold increase relative to the control samples (animals treated with saline). The loading controls were performed by analysis of the total MAPK1/2 and β-actin protein.

1.7. Measurement of site-specific TH phosphorylation and TH protein in whole adrenals

Whole adrenals were processed and run on SDS-PAGE and transferred to nitrocellulose as described above. Membranes were immunoblotted with total or phospho-specific TH antibodies for 1 h at room temperature or overnight at 4 °C. The levels of pSer19TH, pSer31TH, pSer40TH, total TH (tTH) protein and β-actin protein were determined using specific antibodies that have previously been characterized (Bobrovskaya et al., 2004; Gordon et al., 2009). Secondary antibodies (donkey anti-rabbit or rabbit anti-sheep immunoglobulin) were applied to the membranes for 1 h at room temperature. The immunoblots were visualized and quantified on the Fuji LAS 3000 imaging system (Fuji, Stamford, CT, USA) using ECL-plus detection reagent. The density of the bands was measured and expressed as a fold increase relative to the control samples (animals treated with saline). The loading controls were performed by analysis of the total TH protein and β-actin protein. Site-specific TH phosphorylation was expressed as the ratio of TH phosphorylation at Ser19, Ser31 or Ser40 to total TH protein, to account for variability in total TH protein between samples. Total TH protein levels were expressed as the ratio of TH protein to β-actin as β-actin levels are invariable and commonly used as a housekeeping protein.

1.8. Statistical analyses

Statistical analyses were performed using Student's unpaired t-tests for single comparisons. Differences were considered to be significant at the $p < 0.05$ level.

2. Results

2.1. The effect of 2DG on plasma Ad, NAd and glucose levels

Plasma Ad and NAd levels were increased in response to 2DG 20 min after injection (Fig. 1A,B). Ad concentrations increased from 2.73 ng/ml to 7.2 ng/ml ($p < 0.01$) while NAd concentrations increased from 1.8 ng/ml to 3.14 ng/ml ($p < 0.05$). No significant increases were observed for Ad or NAd 24 h after treatment (not shown). Blood glucose levels were significantly increased by 2DG 20 min after injection (1.8 fold, $p < 0.001$, Fig. 1C) and further elevated at 60 min (2.5 fold, $p < 0.001$, not shown). 2DG evoked an

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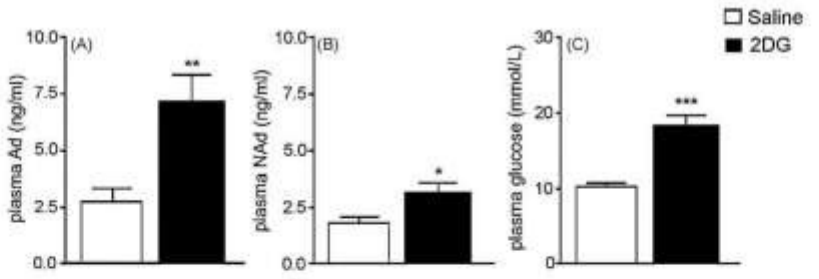

Fig. 1. Effect on plasma levels of adrenaline (Ad), noradrenaline (NAd) and glucose 20 min after the administration (i.p.) of 2-deoxy-D-glucose (2DG; n = 6) or saline (n = 6) in conscious rat. 2DG evoked a large increase in adrenaline (A), a small increase in noradrenaline (B) and an increase in glucose (C) compared with saline. The increase in glucose is associated with the glucoprivic nature of 2DG. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

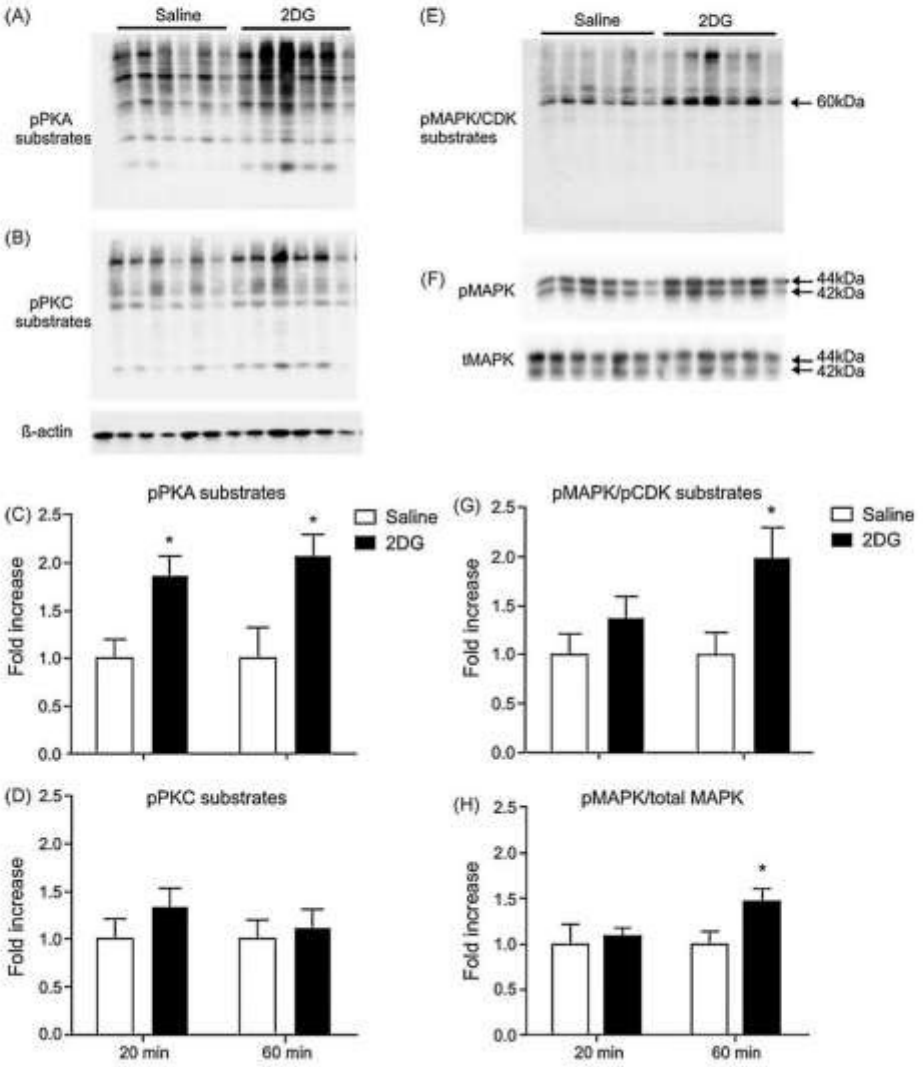

Fig. 2. Effect on substrates in the adrenal medulla phosphorylated by PKA (A,C), PKC (B,D) and MAPK/CDK (E,G) and on phosphorylated or total MAPK (F,H) 20 min and 60 min after administration (i.p.) of 2-deoxy-d-glucose (2DG) (n = 6 for each time point) or saline (n = 6 for each time point), in conscious rat. Representative immunoblots show the effects of 2DG and saline on substrates phosphorylated by PKA (A), PKC (B), MAPK/CDK (E) and on phosphorylated or total MAPK (F). The loading controls for phosphorylated substrates were performed by analysis of the β-actin protein. Analysis of all substrates phosphorylated by each kinase indicates that 2DG activated PKA at 20 min and 60 min (C) but did not change PKC (D). Some individual sites are phosphorylated by PKC but overall no change was seen. Although analysis of all substrates phosphorylated by MAPK/CDK were not significantly elevated until 60 min (G) a prominent band of a substrate of 60kDa was phosphorylated by MAPK/CDK at 20 min. Phosphorylation of MAPK was only elevated significantly after 60 min (H) compared to saline. 2DG evoked a distinct kinase signalling pattern in the adrenal medulla. $^{*}P < 0.05$.

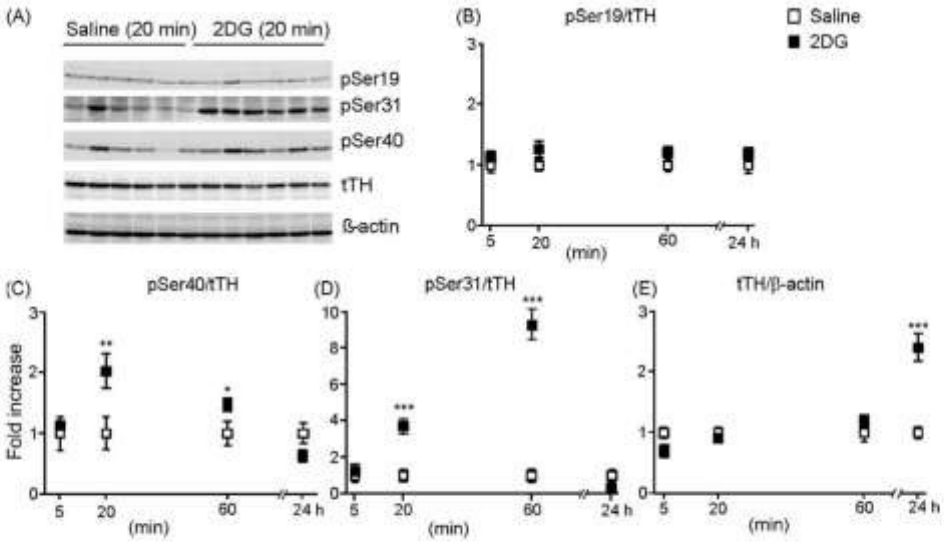

Fig. 3. Effect on the phosphorylation of serine (Ser) residues 19, 31 and 40 of tyrosine hydroxylase (TH) (with respect to total TH) and the level of total TH protein (with respect to β-actin), in the adrenal gland, 5, 20 and 60 min, as well as 24 h, after the administration (i.p.) of 2-deoxy-D-glucose (2DG) ($n = 6$ for each time point) or saline ($n = 6$ for each time point), in conscious rat. Representative immunoblots show the effects of saline and 2DG (A). Compared with saline, 2DG had no effect on the phosphorylation of Ser19 at any time point (B). Phosphorylation of Ser40 occurred at 20 min with a lesser effect evident at 60 min and no effect was seen 24 h after 2DG (C). Phosphorylation of Ser31 was evident at 20 min with a much larger effect seen at 60 min but this had returned to levels seen following saline 24 h after 2DG (D). No change in the amount of TH protein (total TH/β-actin) was evident until 24 h following 2DG (E). A distinct pattern of phosphorylation of existing TH protein was seen following 2DG. A long term effect of a single dose of 2DG was seen on total TH protein 24 hours after administration. Note the different scale used for D. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

increase in plasma Ad and NAd most likely causing the elevation of blood glucose.

2.2. The effects of 2DG on adrenal medullary protein kinase activation

The adrenal medullas were isolated and analysed by immunoblotting with antibodies that recognise specific phosphorylated substrates of protein kinase A (PKA), protein kinase C (PKC) and the proline directed protein kinases MAPK and CDK. There was a significant increase in the total phosphorylation of PKA substrates (quantified as optical density of all bands for each lane) (Fig. 2A,C) 20 min and 60 min after treatment with 2DG. An increase is evident in multiple bands (Fig. 2A). In contrast there were no significant changes in the total phosphorylation of PKC substrates after 20 min or 60 min treatment with 2DG (Fig. 2B,D), although some individual bands did show increased phosphorylation (Fig. 2B). 2DG did not significantly increase the total phosphorylation of MAPK/CDK substrates at 20 min, but did significantly increase the total phosphorylation of MAPK/CDK substrates after 60 min (2 fold, $p < 0.05$, Fig. 2E,G).

There was one prominent MAPK1/2 and/or cyclin-dependent kinase substrate band (~60 kDa) which showed a significant increase at both 20 min (2 fold, $p < 0.05$) and 60 min after treatment (1.9 fold, $p < 0.05$) (not shown). Looking at phospho-MAPK1/2 independently it was not significantly increased at 20 min, but was increased 1.4 fold ($p < 0.05$) 60 min after treatment with 2DG (Fig. 2D).

2.3. The effects of 2DG on phospho-specific TH and TH protein levels in the adrenal gland

The kinases described above are known to phosphorylate the major target protein in the adrenal medulla, tyrosine hydroxylase (62kDa) (Dunkley et al., 2004). We sought to determine the time course and pattern of TH phosphorylation elicited by 2DG *in vivo*. Representative immunoblots for site-specific TH phosphorylation, TH protein and β-actin in the adrenal gland 20 min after treatment with 2DG are shown in Fig. 3A. TH phosphorylation at Ser19 was not significantly changed in response to 2DG at 5, 20, 60 min (Fig. 3A,B). TH phosphorylation at Ser40 was maximally increased after 20 min (2 fold, <0.001), declined to 1.5 fold increase ($p < 0.05$) after 60 min and returned to basal levels 24 h after treatment with 2DG (Fig. 3A,C).TH phosphorylation at Ser31 was significantly increased after 20 min (3.8 fold, $p < 0.001$), reached maximum after 60 min (9 fold, $p < 0.001$) and returned to below basal levels 24 h after treatment with 2DG (Fig. 3A,D). TH protein was significantly increased only at 24 h (2.3 fold, $p < 0.001$) after treatment with 2DG (Fig. 3A, E). Phosphorylation of Ser19 was elevated at 24 hours (not shown) but when calculated with respect to total TH protein the ratio was similar to that seen with saline (Fig. 3B).

3. Discussion

The major findings of this study of the adrenal medulla *in vivo* were that a single episode of glucoprivation led to the activation of a range of protein kinases, predominantly PKA, CDK and, MAPK and a distinct pattern of TH phosphorylation, with Ser40 peaking before Ser31 in the short term. Glucoprivation also evoked release of adrenaline from the adrenal medulla into the plasma which in turn raised plasma glucose. These findings indicate in the short term that an increase TH activity occurs permitting the resynthesis of adrenaline, potentially preventing depletion of glandular adrenaline. A single episode of glucoprivation elevated levels of active TH protein 24 hours after the stimulus increasing capacity of the adrenal medulla possibly buffering against future metabolic insults.

Our data are in keeping with the time course and effect of a single dose of 2DG on plasma Ad, NAd and glucose that are well described (Rusnak et al., 1998; Ritter et al., 2001; Madden et al., 2006). The dose of 2DG used here evokes glucoprivation maximally activating brainstem neurons to mediate the sympathoadrenal

effects seen (Ritter et al., 1998). Following 2DG c-Fos is found in catecholaminergic neurons in the ventral medulla, the sympathetic preganglionic neurons in the spinal cord (SPN) and in the adrenal medulla indicating these cells are activated (Ritter et al., 1998). Furthermore following destruction of spinally projecting catecholamine containing neurons of the ventral medulla (Ritter et al., 2001) or cutting of the splanchnic nerve innervating the adrenal gland (Perlman and Chalfie, 1977) 2DG fails to evoke an increase in plasma catecholamine or glucose. These results indicate that spinally projecting catecholaminergic neurons innervating SPN whose axons make up the splanchnic nerve and innervate the adrenal medulla mediate the release of adrenaline and consequent increase in plasma glucose evoked by 2DG. It should be noted that the hyperphagic responses evoked by glucoprivation are mediated by a different group of catecholaminergic neurons in the ventral medulla that project to feeding centres in the hypothalamus (Ritter et al., 2001). Down regulating catecholamine activity reduces the hyperphagic response (Li et al., 2009). Our study shows that 2DG evokes significant increases in plasma Ad and NAd 20 min after stimulation with a greater effect on Ad when compared to saline administration. The levels of plasma Ad following saline treatment are higher than described in untreated rats. This most likely arises due to the use of decapitation (under anaesthesia) and the collection of trunk blood (Lucot JB 2005) nevertheless it is clear there is a 2DG treatment effect. Evidence suggests that Ad is selectively released from the adrenal medulla whereas the plasma NAd is likely of sympathetic origin (Medvedev et al., 1990). Plasma glucose was already significantly elevated at 20 min indicating catecholamine release is effected rapidly following administration of 2DG. Plasma glucose levels were still rising at 60 min in keeping with other studies showing that the glucoprivic period lasts longer than 2 hours. These results suggest that 2DG mobilises glucose causing an environment of hyperglycaemia, however the effective glucose concentrations are low and plasma insulin levels do not rise significantly (Haito et al., 1984; Ishihara et al., 2009).

Our data demonstrate for the first time that 2DG leads to the activation of several protein kinases in the adrenal medulla. At 20 min and 60 min after treatment there was increased phosphorylation of multiple PKA and MAPK/CDK substrates. These results suggest that overall 2DG has a rapid and long lasting effect on PKA activation, an effect on CDK and a delayed impact on MAPK. Our results suggest that PKC is not activated as overall PKC substrates were not affected significantly by 2DG. However, some substrates detected by the PKC substrate antibody were clearly phosphorylated in response to 2DG. There are two possible interpretations of this result. Firstly, the PKC substrates may have actually been phosphorylated by another protein kinase and secondly, they may have been phosphorylated by a subset of PKC enzymes suggesting that not all of the available PKC enzymes were activated. Many sites on proteins are phosphorylated by more than one protein kinase and if for example PKA and PKC both phosphorylate the same site, both the PKA and the PKC substrate antibodies would show increased phosphorylation even though only PKA was activated. It is necessary to see increases in many substrates, such as we saw with PKA, to be confident that PKC was activated. However, it is also possible that a subset of PKC enzymes was activated, such as one isoform of PKC or only PKC at certain locations within the cell.

Our data also show that 2DG evokes phosphorylation of TH, the major target protein in the adrenal medulla. Ser40 phosphorylation was maximally increased at 20 min and then declined but remained elevated 60 min after treatment with 2DG. Under *in vitro* conditions phosphorylation at Ser40 always correlates with TH activation (Haycock, 1996; Bobrovskaya et al., 2004; Dunkley et al., 2004; Bobrovskaya et al., 2007a) suggesting that TH was activated in response to 2DG within the first 20 min to increase Ad synthesis in order to replenish the Ad stores in the adrenal medulla. As total TH protein was not changed at 60 min the phosphorylation at Ser40 during the first 60 min occurred on pre-existing TH. A range of kinases including PKA and PKC are known to phosphorylate Ser40 *in vitro* (Haycock, 1996; Bobrovskaya et al., 1998; Bobrovskaya et al., 2004; Dunkley et al., 2004; Bobrovskaya et al., 2007b). Although we do not yet know which kinase is responsible for the effects evoked by 2DG, PKA most likely contributes. Together these data indicate that, *in vivo*, glucoprivation evokes an increase in TH activation in the adrenal medulla driven by phosphorylation of Ser40. We can only speculate on which neurotransmitter/s mediate this effect at the sympathoadrenal boundary in the time frames addressed here. Cholinergic agonists could mediate this effect by increases in intracellular calcium or peptidergic agents, including PACAP, could mediate this effect via the commonly described PKA effect (Bobrovskaya et al., 2007a) or by a recently described PKC mechanism (Kuri et al., 2009).

We found that pSer31TH was increased at 20 min and continued to increase 60 min following 2DG injection but declined by 24 hr. The contribution of Ser31 phosphorylation to TH activation under *in vitro* conditions is primarily to enhance the phosphorylation of Ser40 via a hierarchical mechanism (Lehmann et al., 2006) although a two fold increase in TH activity is also described that is independent of Ser40 phosphorylation (Haycock, 1996). Phosphorylation of Ser31TH may have helped maintain the phosphorylation of Ser40 at 60 min although it is clear that pSer31TH levels are still increasing at 60 min so an independent role is also indicated. Under *in vitro* conditions TH is phosphorylated at Ser31 most commonly by MAPK (Haycock et al., 1992; Bobrovskaya et al., 2001; Dunkley et al., 2004), although some evidence also supports a role for CDK (Moy and Tsai, 2004). These kinases were not activated at 20 min, but were at 60 min. There was one prominent CDK substrate protein which showed significant increases in phosphorylation after both 20 and 60 min and this is likely to be TH, although this has not been shown here. In contrast MAPK phosphorylation was not increased at 20 min but was increased at 60 min after 2DG. These findings suggest that CDK but not MAPK mediates phosphorylation of Ser31 early although either or both kinases may mediate Ser31 phosphorylation between 20 and 60 min stimulation following 2DG administration.

Our data show that 2DG increases the phosphorylation of pre-existing TH at Ser40 and Ser31, but not at Ser19. As shown *in vitro*, phosphorylation of Ser19 occurs most rapidly (Dunkley et al., 2004) so it may have occurred within the first 5 min. It is also possible that Ser19 phosphatases were rapidly activated reducing our ability to measure increases in pSer19TH. However, in an isolated perfused adrenal gland, electrical field stimulation (mimicking activation of the splanchnic nerve) at 10 Hz for 30 s evoked a large increase in phosphorylation at Ser19 and Ser40 whereas stimulation at 1 Hz for 5 min evoked significant increases in phosphorylation at all 3 serine residues (Haycock and Wakade, 1992) suggesting that as Ser19 was not phosphorylated at any time point investigated. Kinases that activate Ser19 were not activated by 2DG in the present study. Thus it appears that Ser31 and Ser40 play an important role in response to glucoprivation and activation of these sites may contribute to maintaining catecholamine synthesis/release in the adrenal medulla.

A single dose of 2DG increased the amount of TH protein in the adrenal medulla 24 hours after administration in spite of the fact that plasma adrenaline levels at 24 hours were similar to saline treated animals. This is in keeping with previous studies that demonstrate elevated TH mRNA/protein following a range of stressors (Sabban and Kvetnansky, 2001). It appears however that different mechanisms may be responsible as increases in TH mRNA

evoked by hypoglycemia and cold exposure but not immobilisation were mediated by the splanchnic sympathetic innervation of the adrenal gland (Sabban and Kvetnansky, 2001). We also found at 24 hours following 2DG that the TH phosphorylation at Ser19 was increased in parallel with the increase in TH protein but that the ratio of pSer40 to TH was not changed relative to control animals indicating that the new TH protein was active. These results suggest that 2DG activated TH gene expression that resulted in the synthesis of new TH protein which became phosphorylated at Ser19. It is unlikely that 2DG itself is acting at 24 hours as behavioural alterations such as increased feeding and the hyperglycemia induced by 2DG have returned to normal within 4 hours of administration (Ritter et al., 2006). This suggests that the heightened level of potential TH activity would permit replenishment of adrenal catecholamine release in response to the initial dose of 2DG and may represent a regulatory mechanism which is activated to increase the capacity of the adrenal gland in order to respond to another stimulus when required.

In conclusion our study provides evidence, for the first time, that a single episode of glucoprivation that leads to an elevation in plasma Ad and glucose levels, activated multiple signalling pathways in the adrenal medulla via PKA, CDK and MAPK and caused an increase in activity of pre-existing TH in the short-term via phosphorylation at Ser40 and Ser31 and an increase in the amount of active TH protein at 24 h. Identification of these pathways is of major importance in understanding homeostatic responses to metabolic stressors.

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Chapter 5: The effect of social defeat on tyrosine hydroxylase phosphorylation in the rat brain and adrenal gland.

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Acknowledgement of Collaboration

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers.

The work in the following chapter (Chapter 5) was submitted to Neurochemical Research in July 2010. I (Lin Kooi Ong) was the first author of this manuscript, and the work embodied in this chapter was primarily performed by myself, with the exception of the following:

Resident rats for social defeat animal model were prepared by Frederick R. Walker and his laboratory members.

The work embodied by these experiments is inextricably tied to the overall findings of the manuscript, and therefore this work will be discussed in the final chapter (Chapter 7) of this thesis.

Lin Kooi Ong

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ORIGINAL PAPER

The Effect of Social Defeat on Tyrosine Hydroxylase Phosphorylation in the Rat Brain and Adrenal Gland

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Abstract Tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis, is regulated acutely by protein phosphorylation and chronically by protein synthesis. No studies have systematically investigated the phosphorylation of these sites in vivo in response to stressors. We specifically investigated the phosphorylation of TH occurring within the first 24 h in response to the social defeat stress in the rat adrenal, the locus coeruleus, substantia nigra and ventral tegmental area. Five groups were investigated; home cage control (HCC), two groups that underwent social defeat (SD+) which were sacrificed either 10 min or 24 h after the end of the protocol and two groups that were put into the cage without the resident being present (SD−) which were sacrificed at time points identical to the SD+. We found at 10 min there were significant increases in serine 40 and 31 phosphorylation levels in the locus coeruleus in SD+ compared to HCC and increases in serine 40 phosphorylation levels in the substantia nigra in SD+ compared to SD−. We found at 24 h there were significant increases in serine 19 phosphorylation levels in the ventral tegmental area in SD+ compared to HCC and decreases in serine 40 phosphorylation levels in the adrenal in SD+ compared to SD−. These findings suggest that the regulation of TH phosphorylation in different catecholamine-producing cells varies considerably and is dependent on both the nature of the stressor and the time at which the response is analysed.

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Keywords Stress · Tyrosine hydroxylase · Serine residues · Locus coeruleus · Substantia nigra · Ventral tegmental area

Abbreviations

TH	Tyrosine hydroxylase
Ser	Serine residue
LC	Locus coeruleus
SN	Substantia nigra
VTA	Ventral tegmental area
SDS	Sodium dodecyl sulphate
TBST	Tris-buffered saline with Tween 20
ANOVA	Analysis of variance

Introduction

The catecholamines, including dopamine, noradrenaline and adrenaline, act as neurotransmitters in the nervous system and hormones in the endocrine system [1–3]. Although catecholamines modulate a diverse array of biological functions they are of particular interest in relation to stress, as both physical and psychological stressors potently induce their release within the brain and from the adrenal glands [4]. Moreover, it has been demonstrated that catecholaminergic responses following stress are quite dynamic varying according to the nature, duration and intensity of the stressor [5].

An important feature of the catecholaminergic responses to stress are the molecular responses that enable the level of catecholamines in the brain and the adrenal glands to remain constant during the stress response [6]. The principal enzyme for controlling catecholamine synthesis is

tyrosine hydroxylase (TH; EC 1.14.16.2) which is the rate limiting enzyme for catecholamine biosynthesis [7]. Research over recent years has revealed that in the rat the N-terminal domain of TH includes three specific serine residues 40, 31 and 19 (Ser40, Ser31 and Ser19) that can be phosphorylated [8]. Phosphorylation of these sites is able to increase the activity of TH, either directly or indirectly [9] and this helps to maintain the levels of catecholamines in the tissues and the capacity to respond to stress. Phosphorylation of Ser40, Ser31 and Ser19 has been investigated in perfused adrenal glands [10], but no studies have systematically investigated the phosphorylation of these sites in vivo in response to stressors. Ser40, Ser31 and Ser19 have all been found to be phosphorylated in vivo in the brain under resting conditions [11] and in response to a range of drugs [9, 12]. However, no studies have systematically investigated the phosphorylation of these sites in vivo in response to stressors. We are interested in the changes in TH phosphorylation that occur over the first 24 h in response to exposure of rats to stressors. In cell culture studies we previously defined two distinct phases of TH phosphorylation, the acute phase (which occurs from secs to mins) and the sustained phase (which occurs from hours up to 24 h) [13, 14]. Both of these changes occurred without any changes in TH protein levels. We have found that the protein kinases are responsible for TH phosphorylation in these two phases were different [13, 14]. The sustained phase of TH phosphorylation has not been assessed in vivo.

Although it is recognised that psychological stressors result in catecholaminegic responses [15, 16], how this process is regulated by TH phosphorylation has not been examined. We therefore examined TH phosphorylation in vivo following exposure of rats to either a novel environment or after social defeat, a well characterised psychological stressor [17]. Specifically, we investigated the effects of these stressors on TH phosphorylation at Ser40, Ser31 and Ser19 after either 10 min (the acute phase) or 24 h (the sustained phase). We have analysed tissue from rat adrenal, as well as the locus coeruleus (LC), substantia nigra (SN) and ventral tegmental area (VTA) as these are the major sites of catecholamine synthesis in the rat brain. To our knowledge this is the first study to investigate the phosphorylation of TH in vivo in response to social defeat.

Materials and Methods

Materials

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) reagents were from Bio-Rad Laboratories (Hercules, CA, USA). Molecular weight PAGE standards, nitrocellulose membrane (Hybond ECL), ECL plus kit, anti-rabbit immunoglobulin (horseradish peroxidase-linked whole antibody from donkey) and anti-mouse immunoglobulin (horseradish peroxidase-linked whole antibody from sheep) were obtained from GE Health Care (Little Chalfont, UK). EGTA, EDTA, Tween-20, bovine serum albumin, sodium azide, isopentane and β-actin antibody were from Sigma Chemical Co. (St Louis, MO, USA). Anti-sheep antibody (horseradish peroxidise-linked whole antibody from rabbit) was obtained from Pierce Biotechnology (Rockford, IL, USA). Total-TH antibody and phospho-specific TH antibodies (pSer40, pSer31 and pSer19) were generated and tested for specificity according to previously described methods [18].

Social Defeat Protocol

All experiments were approved by the University of Newcastle Animal Care and Ethics Committee and performed in accordance with the New South Wales Animal Research Act and the "Australian code of practice and use of animals for scientific purposes". Adult male Sprague–Dawley rats (300–400 g) were obtained from Animal Resources Centre (ARC), Perth, Australia. Animals were maintained in group housing under standard laboratory conditions in temperature controlled rooms (21 ± 1°C), reverse 12 h light cycle with darkness from 02:00 to 14:00 h, food and water ad libitum. Animals were habituated and handled for 7 days prior to experiments. Resident rats were at least 6 months old and weighing between 500 and 700 g. Each male resident was co-housed with a female which underwent tubal ligation surgery. Prior to commencement of the study these pairs co-habited for at least 6 weeks and all residents received 2 weeks of attack training sessions. Residents were screened to ensure that they would attack and defeat the intruders and this occurred consistently throughout this study.

A total of five separate groups ($n = 6$/group) were investigated in the current experiment. Specifically, we examined two groups that underwent social defeat (SD+), both underwent identical defeat procedures according to previously reported methods [19], but here rats were exposed to three consecutive 20 min defeat sessions. One group was then sacrificed 10 min after the end of the last defeat session while the second group was sacrificed 24 h after the end of the last defeat session. Briefly, the defeat process involved placement of a naïve male intruder into the home cage of a dominant male resident after which the intruder was attacked and defeated by the resident. Additionally, we investigated two groups of rats that were placed into the home cage of the resident on three consecutive 20 min sessions but without the resident being present (SD−). SD− groups were sacrificed at time points

identical to the SD+ groups (10 min and 24 h). The final group we investigated were animals that were left in their home cage and are referred as our home cage control group (HCC). All animals were sacrificed by stunning and rapid decapitation. The brain and adrenals were dissected and rapidly frozen in −80°C isopentane. The tissues were kept at −80°C until further processing.

Sample Preparation

Brain regions were identified by reference to the Paxinos and Watson stereotaxic brain atlas [20]. Tissue punches were collected from the SN (Bregma −4.80 to −6.30 mm), VTA (Bregma −4.80 to −6.30 mm) and LC (Bregma −9.86 to −10.52 mm) by using a customized freezing microtome (SM2000R, Leica) with an attached micrometer for large distance measurement. Sections were cut at −20°C and transferred rapidly to a refrigerated working bench (4°C) and were punched using a 1 mm tissue punch. Tissues were kept frozen at all times until homogenization. The tissues were weighed (wet weight). The adrenals were homogenised using a sonicator (Soniprep 150, MSE) in homogenisation buffer (2% SDS, 2 mM EDTA, 50 mM Tris, pH 6.8) with ratio 1 mL of buffer per 25 mg tissue. The brain tissue punches were homogenised in homogenisation buffer with ratio 1 mL of buffer per 100 mg tissue. Samples were then centrifuged at 15,000 rpm for 20 min at 4°C. The clear supernatants were collected and 200 µL were mixed with 70 µL of sample buffer (40% glycerol, 50 mM Tris, minimal bromophenol blue, pH 6.8) and 10 µL of 20% dithiotheitol.

SDS–Page and Western Blotting

Samples were subjected to SDS–polyacrylamide gel electrophoresis before being transferred to nitrocellulose according to previously reported methods [21]. Membranes were then stained with Ponceau S (0.5% ponceau in 1% acetic acid) to assess the efficacy of the transfer. Membranes were washed in Tris-buffered saline with Tween (TBST) (150 mM NaCl, 10 mM Tris, 0.075% Tween-20, pH 7.5) and incubated with blocking solution (5% bovine serum albumin, 0.04% sodium azide in TBST) for 2 h at 25°C. Membranes were washed in TBST and incubated with different primary antibodies overnight at 4°C. Membranes were washed in TBST and incubated with horseradish peroxidase-linked anti-IgG secondary specific antibodies for 1 h at 25°C. Membranes were visualized on Fugifilm Las-3000 imaging system (Fuji, Stamford, CT, USA) using ECL plus detection reagents. The density of total-TH and phospho-specific TH bands were measured using a MultiGauge V3.0 (Fuji, Stamford, CT, USA).

Statistical Analysis

The optical densities for total TH and for the phosphorylation of TH at Ser40, Ser31 and Ser19 were determined for each sample. We found no significant differences in the levels of TH protein for any of the groups from any of the tissues. The level of TH phosphorylation was then divided by the level of TH protein in each of the samples to account for differences in tissue recovery. The average values were then determined for each of the treatment groups with the mean of the HCC group being set as equal to 1.0. The data for SD+ and SD− groups were expressed as fold increase of the mean ± SEM of the group relative to the mean of the HCC group. These data were analysed by using PRISM V4.02 (GraphPad Software, Inc., CA, USA). One-way ANOVA followed by Tukey's test for multiple comparisons was used to examine whether there were any significant differences in the SD+ , the SD− and the HCC groups at either 10 min or 24 h after the protocols. All differences were considered to be significant at $P < 0.05$.

Results

In the adrenal samples total-TH protein and phospho-TH (pSer40, pSer31 and pSer19) appeared as single bands corresponding to molecular masses of approximately 60 kDa (Fig. 1). No significant differences were observed in the adrenals between the HCC, SD+ or SD− groups with respect to TH protein at either 10 min or 24 h (Fig. 1a, e). There was a significant decrease ($P < 0.05$) for pSer40 for the SD+ group (1.5 fold) when compared to the SD− group (0.8 fold) at 24 h (Fig. 1f). No significant differences were observed between the HCC, the SD+ and the SD− groups with respect to pSer40 levels at 10 min and with respect to pSer31 or pSer19 levels at either 10 min or 24 h in the adrenals (Fig. 1b, c, d, g and h).

In LC, pSer40 levels were significantly increased relative to HCC in the SD+ group (1.6 fold; $P < 0.01$) at 10 min (Fig. 2a). In addition, pSer31 levels were significantly increased relative to HCC in both the SD+ (2.8 fold; $P < 0.01$) and SD− (2.3 fold; $P < 0.05$) groups at 10 min (Fig. 2b). No significant differences were observed between the HCC, the SD+ and the SD− groups with respect to pSer40 or pSer 31 levels at 24 h or pSer19 levels at either 10 min or 24 h (Fig. 2c, d, e and f).

In SN, pSer40 levels were significantly increased ($P < 0.05$) in the SD+ group (1.1 fold) when compared to SD− group (0.7 fold) at 10 min (Fig. 3a). There were no significant differences observed between the HCC, the SD+ and the SD− groups for pSer40 levels at 10 min or for pSer31 and pSer19 levels at either 10 min or 24 h (Fig. 3b, c, d, e and f).

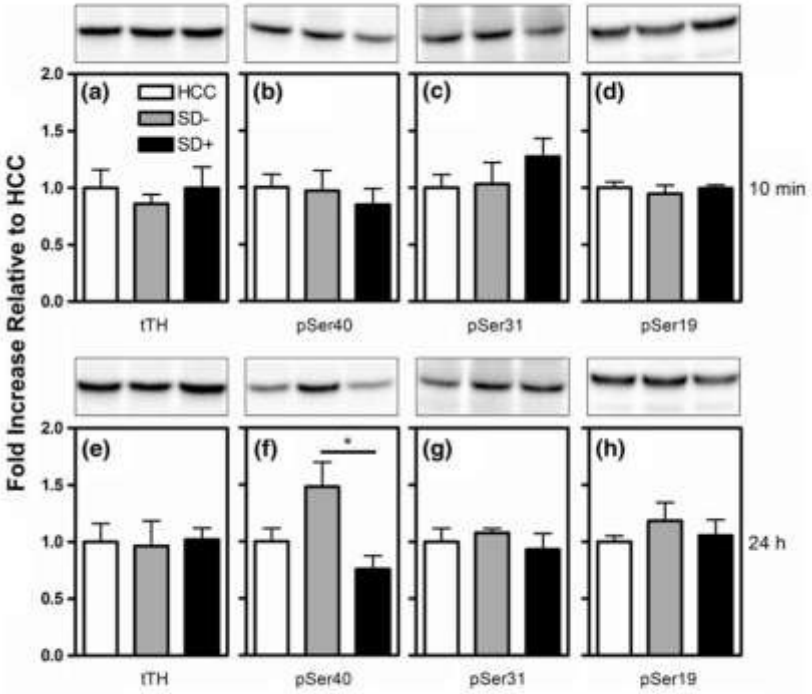

Fig. 1 The regulation of TH protein and TH phosphorylation in rat adrenal at 10 min and 24 h after the end point of social defeat. The SD+ and SD− groups were expressed as a fold increase of the mean ± SEM of the group, relative to the mean of the HCC group which was set as equal to 1.0. Statistical analysis was performed by one-way ANOVA followed by Tukey's test for multiple comparisons. * $P < 0.05$

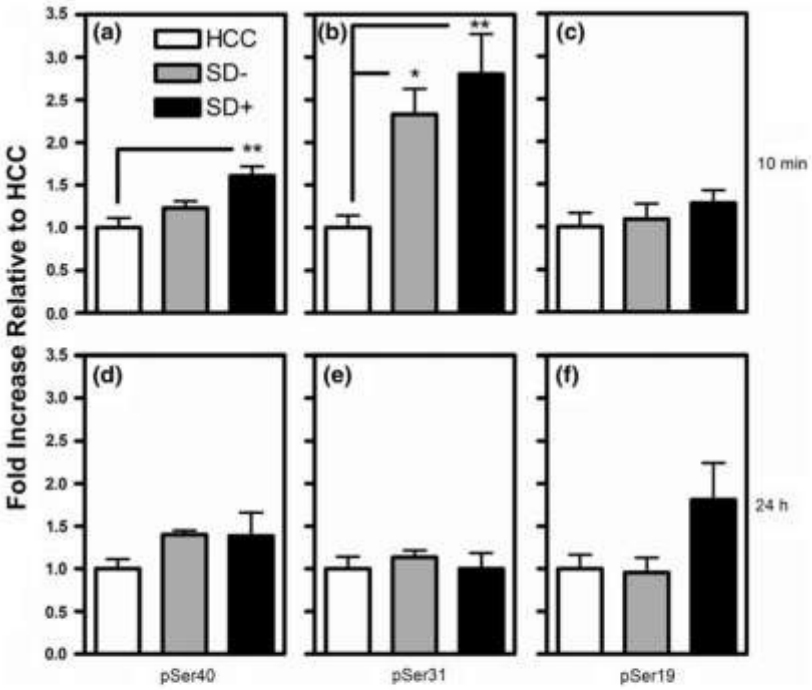

Fig. 2 The regulation of TH phosphorylation in rat locus coeruleus at 10 min and 24 h after the end point of social defeat. The SD+ and SD− groups were expressed as a fold increase of the mean ± SEM of the group, relative to the mean of the HCC group which was set as equal to 1.0. Statistical analysis was performed by one-way ANOVA followed by Tukey's test for multiple comparisons. * $P < 0.05$ ** $P < 0.01$

In VTA, pSer19 levels were significantly increased ($P < 0.01$) relative to HCC in the SD+ group (1.9 fold) at 24 h (Fig. 4f). No significant differences were observed between the HCC, the SD+ and the SD− groups with respect to pSer40 and pSer31 levels at either 10 min or 24 h and the pSer19 levels at 10 min (Fig. 4a, b, c, d and e).

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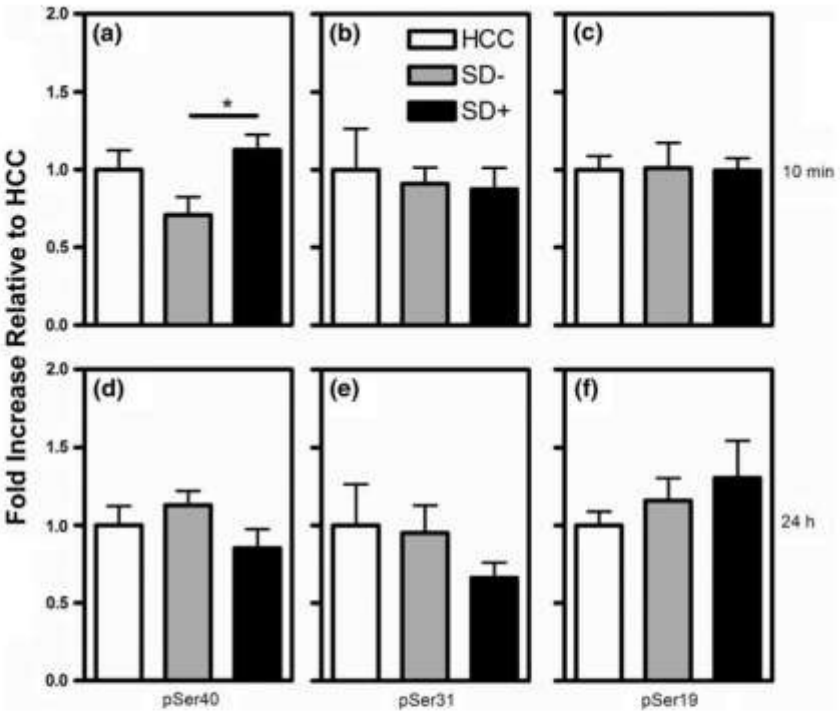

Fig. 3 The regulation of TH phosphorylation in rat substantia nigra at 10 min and 24 h after the end point of social defeat. The SD+ and SD− groups were expressed as a fold increase of the mean ± SEM of the group, relative to the mean of the HCC group which was set as equal to 1.0. Statistical analysis was performed by one-way ANOVA followed by Tukey's test for multiple comparisons. * $P < 0.05$

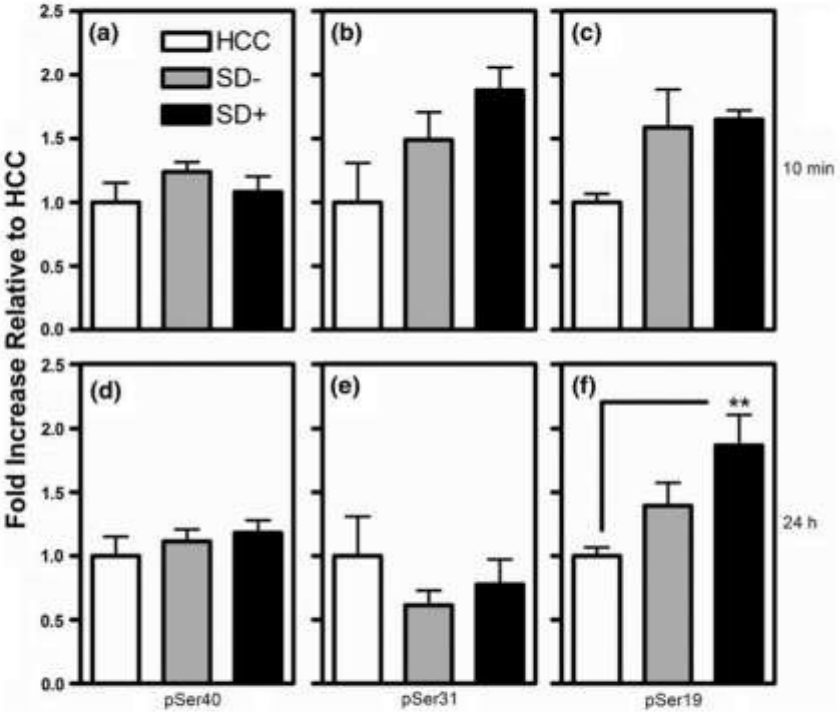

Fig. 4 The regulation of TH phosphorylation in rat ventral tegmental area at 10 min and 24 h after the end point of social defeat. The SD+ and SD− groups were expressed as a fold increase of the mean ± SEM of the group, relative to the mean of the HCC group which was set as equal to 1.0. Statistical analysis was performed by one-way ANOVA followed by Tukey's test for multiple comparisons. ** $P < 0.01$

Discussion

Our aim in this study was to investigate the effects of social defeat on TH phosphorylation at Ser40, Ser31 and Ser19 10 min and 24 h after the exposure to social defeat, in the adrenal gland and in three catecholamine-enriched regions of the brain, including the LC, the SN and the VTA. TH phosphorylation in dynamically controlled by

neuronal activation leading to depolarization of neurons and the opening of voltage-gated-calcium channels [22–24]. An influx of extracellular calcium into the neurons will then activate a number of signal transduction pathways that are able to induce TH phosphorylation at Ser40, Ser31 and Ser19 [9, 25–27]. It has been suggested that this process allow activation of TH and replacement of catecholamines lost by release from the activated cells. Our findings demonstrate that the regulation of TH phosphorylation varies considerably depending upon the cell population examined and further appears to be highly time specific.

The 10 min time point was chosen to determine the acute effects of social defeat on the adrenal and brain catecholaminergic cells as we have previously determined that in vitro stimulation of chromaffin cells led to changes in the levels of TH phosphorylation without any changes in total TH protein in this time domain [9]. Consistent with this work, we found that no significant change in TH protein levels occurred in the adrenal gland, or any of the brain regions, 10 min after the end of the last defeat session. We did observe that social defeat resulted in significant increases in pSer40 levels in the LC and SN and increases in pSer31 in the LC at 10 min after the end of the last defeat session. The increase in the LC was not induced in the SD− group suggesting that neuronal activation required both exposure to a novel environment and social defeat. The increase in the SN was due to social defeat alone. We did not find an increase in pSer40 in the adrenals after 10 min as had been observed in vitro [13, 14]. This could have been due to either a reduced stimulus in vivo, from acetylcholine and/or PACAP, not leading to any changes in TH phosphorylation at Ser40, or an increased stimulus in vivo leading to an increase in pSer40 earlier than the 10 min time point investigated here.

Recent studies suggest that Ser31 phosphorylation is important in TH regulation in the catecholaminergic cells [12]. In the present study, we found that there was a significant increase in pSer31 levels in both the SD+ and the SD− groups 10 min after the end point of social defeat in the LC. The pSer31 response therefore occurred in response to both a novel environment and to social defeat and it was most likely to be a general response to cell activation after stress and not a specific response to social defeat. Phosphorylation of Ser31 has been shown to directly mediate an increase in TH activity in vivo [12]. If this was the case then it may be that the LC uses both Ser40 and Ser31 phosphorylation while the SN uses mainly Ser40 phosphorylation to activate TH. The reason for this difference in response is could be related to the frequency of cell firing in the different regions leads to activation of different signalling pathways and hence different mechanisms of TH activation, both of which will lead to replacement of released catecholamines.

The 24 h time point was chosen to determine the sustained effects of social defeat on adrenal and brain catecholaminergic cells. It had been established in chromaffin cells in vitro that stimulation of these cells with a range of effectors led to a sustained increase in TH phosphorylation at Ser40, but not at Ser31 or Ser19, without any changes in total TH protein [13, 14]. The results of this study demonstrate that no significant change in total TH levels occurred in the adrenal gland 24 h after the end point of social defeat. This result is in agreement with some previous studies that showed no significant change in adrenal TH protein levels 24 h after exposure to a 2 h immobilization stress protocol [28]. However, other studies have shown changes in adrenal TH protein levels 24 h after exposure to 1 h cold stress [29], or to glucoprivation [30]. The nature of the stress exposure therefore determines the sustained effects on TH protein levels, with some stressors having no effects and others having substantial effects.

In the VTA we found an increase in pSer19 levels in SD+ , but not SD− compared to HCC at 24 h after the end point of social defeat. These findings suggest that social defeat increases the activity of Ser19 kinases in vivo but only in the VTA. The effects are most likely to be due to increased kinase activity as the same protein phosphatases that dephosphorylate Ser31 also act on Ser19 [9] and as there was clear dephosphorylation of Ser31 between 10 min and 24 h in the VTA then these phosphatases must have been active.

In the adrenal gland we found a significant decrease in pSer40 levels in SD+ compared to SD− at 24 h after the end point of the social defeat protocol. The results from these studies indicate that sustained phosphorylation of TH at Ser40 does not occur in vivo under the conditions of social defeat used here, but does occur in response to exposure to a novel environment. The reasons for the differences are unknown but it is possible that the increased stress of social defeat also activated protein phosphatases leading to dephosphorylation of TH at Ser40.

In summary, we demonstrated that the regulation of TH phosphorylation in different catecholamine-producing cells varies considerably and is dependent on both the nature of the stressor and the time at which the response is analysed.

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Conflict of interests None.

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Chapter 6: The sustained phase of tyrosine hydroxylase activation *in vivo*.

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Acknowledgement of Collaboration

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers.

The work in the following chapter (Chapter 6) was submitted to Neurochemical Research in January 2012. I (Lin Kooi Ong) was the first author of this manuscript, and the work embodied in this chapter was primarily performed by myself, with the exception of the following:

Rat treatments were performed by Luba Sominsky and her laboratory members.

The work embodied by these experiments is inextricably tied to the overall findings of the manuscript, and therefore this work will be discussed in the final chapter (Chapter 7) of this thesis.

Lin Kooi Ong

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ORIGINAL PAPER

The Sustained Phase of Tyrosine Hydroxylase Activation In vivo

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Abstract Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the biosynthetic pathway for catecholamine synthesis. Stress triggers an increase in TH activity, resulting in increased release of catecholamines from both neurons and the adrenal medulla. In response to stress three phases of TH activation have been identified (acute, sustained and chronic) and each phase has a unique mechanism. The acute and chronic phases have been studied in vivo in a number of animal models, but to date the sustained phase has only been characterised in vitro. We aimed to investigate the effects of dual exposure to lipopolysaccharide (LPS) in neonatal rats on TH protein, TH phosphorylation at serine residues 19, 31 and 40 and TH activity in the adrenal gland over the sustained phase. Wistar rats were administered LPS (0.05 mg/kg, intraperitoneal injection) or an equivolume of non-pyrogenic saline on days 3 and 5 postpartum. Adrenal glands were collected at 4, 24 and 48 h after the drug exposure on day 5. Neonatal LPS treatment resulted in increases in TH phosphorylation of Ser40 at 4 and 24 h, TH phosphorylation of Ser31 at 24 h, TH activity at 4 and 24 h and TH protein at 48 h. We therefore have provided evidence for the first time that TH phosphorylation at Ser31 and Ser40 occurs for up to 24 h in vivo and leads to TH activation independent of TH protein synthesis, suggesting that the sustained phase of TH activation occurs in vivo.

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Abbreviations

2DG	2-deoxy-D-glucose
LPS	Lipopolysaccharide
PND	Postnatal day
Ser	Serine residue
TBST	Tris-buffered saline with Tween
TH	Tyrosine hydroxylase,

Introduction

The catecholamines, including dopamine, noradrenaline and adrenaline, have many functions and influence the activity of almost every tissue. They are especially important in the stress response where stored catecholamines are secreted from the central nervous system and sympathetic neurons as well as the adrenal medulla [1, 2]. However, it has been shown that there is no significant change in catecholamine levels within these cells. This is because in parallel to catecholamine secretion there is a concomitant increase in catecholamine synthesis [3, 4]. Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the biosynthetic pathways for catecholamine synthesis [5]. TH is regulated acutely by TH phosphorylation at serine residues (Ser19, Ser31 and Ser40) and chronically by TH protein synthesis [6]. Both these mechanisms are well established in vivo [7–10]. Recently, we have demonstrated a third mechanism for the control of TH activity in vitro, referred to as the sustained phosphorylation of TH [11, 12]. This mechanism has not been demonstrated to occur in vivo.

Stress triggers an increase in TH activity. In response to stress there are three phases of TH activation and each phase is mediated by distinct mechanisms. The acute phase of TH activation is mediated by TH phosphorylation primarily at Ser40, but also to a lesser extent at Ser31, without any changes in TH protein levels. This phase lasts up to approximately 1 h after which TH phosphorylation and TH activity generally return to baseline levels [6]. The sustained phase of TH activation is mediated by TH phosphorylation primarily at Ser40 which occurs from 1 to 24 h and again there are no changes in TH protein levels [11, 12]. The sustained phase of TH activation is distinguishable from the acute phase of TH activation as it only occurs in response to selected stimuli, the protein kinases mediating the two phases are different and it lasts for 24 h. The chronic phase of TH activation is mediated by an increase in TH mRNA expression between 1 and 24 h and subsequent TH protein synthesis which occurs from 6 to 72 h [6].

TH activation in response to stressors has been studied in vivo in many different animal models. Most of the studies have focused on changes in TH mRNA and TH protein levels [10, 13–15]. We have recently discovered that different stressors lead to different patterns of TH phosphorylation at all three serine residues and TH protein levels [7–9, 16]. However, to date we have not found any stress protocol in vivo that matches the previous findings in vitro using chromaffin cell cultures and which leads to increased TH phosphorylation and TH activity at 24 h, but with no change in TH protein levels.

Here we investigated whether immunological challenge elicited by dual exposure to lipopolysaccharide (LPS) in neonatal rats causes the sustained phase of TH activation in vivo. This protocol of LPS challenge on days 3 and 5 postpartum has been previously employed to study immediate and long-term behavioural and physiological alterations, induced by neonatal immune activation [16–20]. To achieve our aims we investigated the effects of dual exposure to LPS on TH phosphorylation at Ser19, Ser31 and Ser40, TH activity and TH protein synthesis in the adrenal gland at 4, 24 and 48 h after the second LPS treatment.

Materials and Methods

Materials

LPS (*Salmonella enterica*, serotype enteritidis), EGTA, dithiothreitol (DTT), ammonium molybate, sodium pyrophosphate, sodium vanadate, B-glycerolphosphate, microcystin, sodium chloride, Tris, Tween-20, bovine serum albumin, sodium azide, β-actin antibody, catalase, β-mercaptoethanol and activated charcoal were from Sigma Chemical Co. (St Louis, MO, USA). Protease inhibitor cocktail tablets were from Roche Diagnostics Australia (Castle Hill, NSW, Australia). Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) reagents were from Bio-Rad Laboratories (Hercules, CA, USA). PageRuler Prestained Protein Ladder was from Thermo Fisher Scientific (Rockford, IL, USA). Anti-rabbit immunoglobulin (horseradish peroxidase-linked whole antibody from donkey) and anti-mouse immunoglobulin (horseradish peroxidase-linked whole antibody were from sheep) and 3,5-[^{3}H]-L-tyrosine were from GE Health Care (Little Chalfont, UK). Total-TH antibody (tTH) and phospho-specific TH antibodies (pSer19, pSer31 and pSer40) were generated and were tested for specificity as described [21]. Anti-sheep antibody (horseradish peroxidise-linked whole antibody from rabbit) was from Pierce Biotechnology (Rockford, IL, USA). L-tyrosine was from DBH Biochemicals (Poole, UK). Tetrahydrobiopterin was supplied by Dr. Schirck's Laboratory (Jona, Switzerland). Optiphase HiSafe scintillation cocktail was from Perkin-Elmer (Waltham, MA, USA).

Animal Protocols

All animal protocols were approved by the University of Newcastle Animal Care and Ethics Committee and performed in accordance with the New South Wales Animal Research Act and the "Australian code of practice and use of animals for scientific purposes". Wistar rats were mated at the University of Newcastle Psychology vivarium. Neonatal rats (n = 30) were randomly allocated into either saline control or LPS conditions at birth (postnatal day [PDN] 1). On PND 3 and PND 5, neonatal rats were removed from their home cages, weighed and administered intraperitoneally with either 0.05 mg/kg LPS or an equivolume of non-pyrogenic 0.9 % saline as describe previously [16]. Neonatal rats were euthanized at 4, 24 or 48 h (n = 5 per group) following drug exposure on PND 5. Whole adrenal glands were dissected and were kept frozen at −80 °C until further analysis. Adrenal samples were sonicated in 100 µL of homogenizing buffer (2 mM potassium phosphate buffer pH 7.4, 1 mM EGTA, 1× protease inhibitor cocktail tablet, 1 mM DTT, 80 µM ammonium molybate, 1 mM sodium pyrophosphate, 1 mM sodium vanadate, 5 mM β-glycerolphosphate, 2 µM microcystin, final concentration) with a microsonicator (UP50H, Hielscher Ultrasonics GmbH, Teltow, Germany) for 3 × 30 s pulses at 4 °C. Samples were centrifuged at 16,000 rpm for 20 min at 4 °C. The clear supernatants were collected and protein concentrations were determined by Pierce BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Samples were diluted with homogenizing buffer to equalize protein

concentrations (5 mg/mL), were aliquoted and were kept frozen at −80 °C for further analysis.

Western Blotting

Western blotting were performed as previously described with some modifications [7]. Aliquots (one-half volume) of these samples were mixed with sample buffer (1 % SDS, 10 % glycerol, 0.5 % DTT and minimal bromophenol blue, final concentration). 30 µg of each samples were subjected to SDS-PAGE gel electrophoresis and were transferred to nitrocellulose membranes (Hybond ECL, GE Health Care). Nitrocellulose membranes were stained with Ponceau S (0.5 % ponceau in 1 % acetic acid) to assess the efficacy of the transfer. Membranes were washed in Tris-buffered saline with Tween (TBST) (150 mM sodium chloride, 10 mM Tris, 0.075 % Tween-20, pH 7.5) and incubated with blocking solution (5 % bovine serum albumin, 0.04 % sodium azide in TBST) for 2 h at 25 °C. Membranes were washed in TBST and incubated with primary antibodies (total- and phospho-TH, β-actin) for 1 h at 25 °C for adrenal medulla samples. Membranes were washed in TBST and incubated with horse-radish peroxidase-linked anti-IgG secondary specific antibodies for 1 h at 25 °C. Membranes were visualized on Fugifilm Las-3000 imaging system (Fuji, Stamford, CT, USA) using detection reagents (Amersham ECL Plus Western Blotting Detection Reagents, GE Health Care). The density of the bands was measured using a MultiGauge V3.0 (Fuji, Stamford, CT, USA). Total-TH protein levels were normalized to β-actin. Site-specific phospho-TH at pSer19, pSer 31 and pSer40 levels were normalized to total-TH protein levels and were expressed as a fold increase relative to the saline control.

TH Activity Assay

TH activity was measured using a method based on the tritiated water release assay with slight modification [22]. Briefly, aliquots of these samples were mixed in the reaction mixture (50 µg sample, 36 µg catalase, 2 mM potassium phosphate pH 7.4, 0.008 % β-mercaptoethanol, 24 µM L-tyrosine, 1 µCi 3,5-[3H]-L-tyrosine, final volume 50 µL). The 50 µL reactions were initiated with the addition of 100 µM tetrahydrobiopterin in 5 mM HCl. Control representing background reactions were added with 5 mM HCl but did not contain tetrahydrobiopterin. Assays were performed for 20 min at 30 °C and were stopped by addition of 700 µL charcoal slurry (7.5 % activated charcoal in 1 M HCl). Mixtures were vortexed for 1 min and were centrifuged at 16,000 rpm for 10 min at 30 °C. 350 µL supernatants were added to 3 mL scintillation cocktail and were vortexed for 10 s. Mixtures were assayed by scintillation spectrometry (Wallac1410, Pharmacia, Turko, Finland) for 20 min per sample. TH activity assays which were performed under these conditions were linear. The changes in TH activity were normalized to total-TH protein levels and expressed as a fold increase relative to the saline control.

Statistical Analysis

The data for saline and LPS groups were expressed as a fold increase of the mean ± SEM for each group relative to the mean of the saline group. These data were analysed by using Prism 5 for Windows (Version 5.04, GraphPad Software, Inc., CA, USA). Two-Way ANOVA was used to determine whether there were any significant effects of LPS treatment and/or time across the groups. Additional Bonferroni post tests were used to analyse differences between saline and LPS groups at each of the time points (4, 24 and 48 h), where an overall LPS treatment or time effects was found. The significant differences shown on the graphs with asterisks (*) refer to the post hoc tests for LPS treatment effects. All differences were considered to be significant at $p < 0.05$.

Results

The adrenal glands were analysed by Western Blotting with antibodies that recognise total-TH (tTH) and site-specific phospho-TH. Total- and phospho-TH appeared as single bands corresponding to molecular masses of approximately 60 kDa (Fig. 1a). The results for total-TH levels were calculated relative to β-actin levels (Fig. 1b). The results for phospho-TH (pSer19, pSer31 and pSer40) levels were calculated relative to total-TH levels because the ratios more accurately represent phosphorylation states and account for variability in total-TH among samples (Fig. 1c, d and e). The adrenal glands were also analysed for TH activity levels using a tritiated water release assay. The results for TH activity levels were calculated relative to total-TH levels.

As illustrated in Fig. 1b, there was a significant effect of LPS treatment on total-TH levels ($F_{(1,24)} = 4.7$, $p < 0.05$). Post hoc analysis further indicated that LPS caused a significant increase in total-TH levels relative to saline controls at 48 h (1.2 fold, $p < 0.05$) (Fig. 1b). There was no effect of LPS treatment or time on pSer19 levels (Fig. 1c). However, a significant effect of LPS treatment ($F_{(1,24)} = 5.9$, $p < 0.05$) and time ($F_{(2,24)} = 12.7$, $p < 0.001$) was found on pSer31 levels (Fig. 1d). Post hoc analysis indicated that LPS caused a significant increase in pSer31 levels relative to saline controls at 24 h (1.5 fold, $p < 0.001$) (Fig. 1d). There was a significant effect of LPS treatment ($F_{(1,24)} = 50.8$, $p < 0.001$) and time ($F_{(2,24)} =$

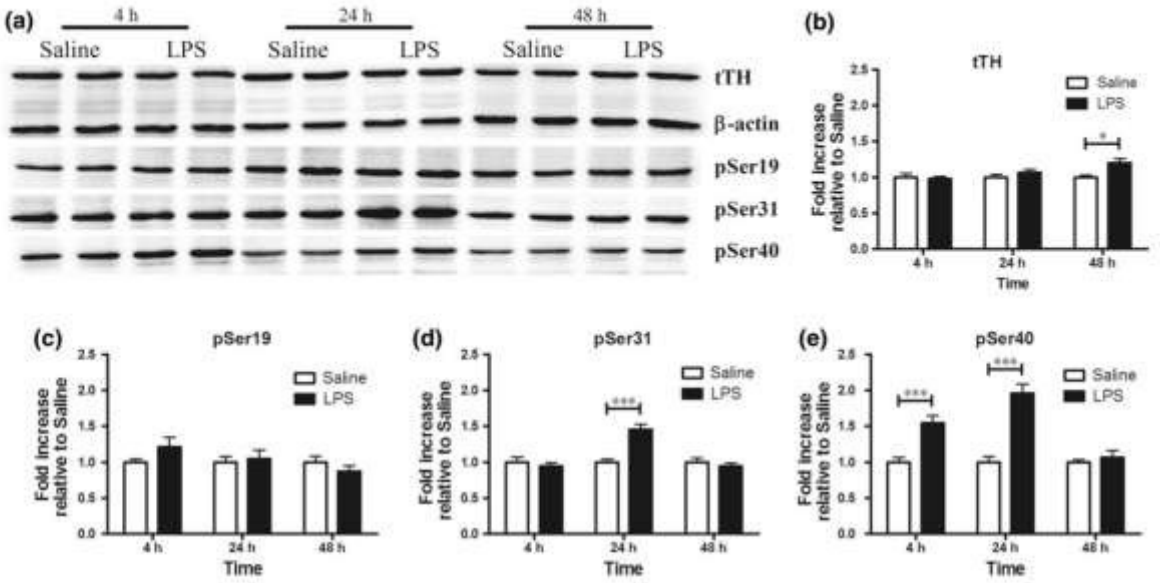

Fig. 1 Effect on total- and phospho-TH (pSer19, pSer31 and pSer40) levels in adrenal glands 4, 24 and 48 h after LPS treatment (n = 5 per group). **a** Representative immunoblots show the effect of LPS treatment on total- and phospho-TH. The loading controls were performed by analysis of β-actin. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

12.3, $p < 0.001$) on pSer40 levels (Fig. 1e). Post hoc analysis indicated that LPS caused a significant increase in pSer40 levels relative to saline controls at 4 h (1.5 fold, $p < 0.001$) and 24 h (2.0 fold, $p < 0.001$) (Fig. 1e). A significant effect of LPS treatment ($F_{(1,24)} = 22.1$, $p < 0.001$) and time ($F_{(2,24)} = 9.3$, $p < 0.01$) was also found in regards to TH activity levels (Fig. 2). Post hoc analysis indicated that LPS caused a significant increase in TH activity levels relative to saline controls at 4 h (2.0 fold, $p < 0.01$) and 24 h (2.2 fold, $p < 0.001$) (Fig. 2).

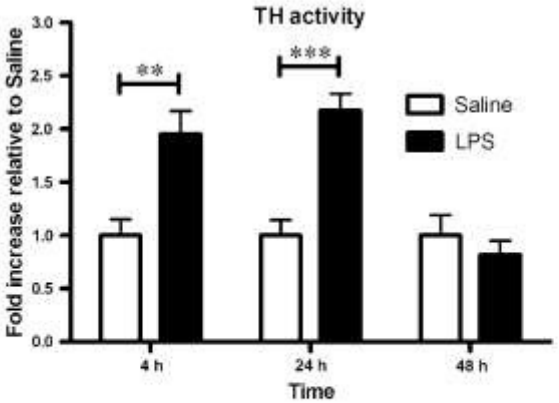

Fig. 2 Effect on TH activity levels 4, 24 and 48 h after LPS treatment (n = 5 per group). $^{**}p < 0.01$, $^{***}p < 0.001$

Discussion

The major findings of this study of the adrenal gland in vivo were that dual exposure to LPS during neonatal life led to an increase in TH phosphorylation of Ser40 at 4 and 24 h, TH phosphorylation of Ser31 at 24 h, TH activity levels at 4 and 24 h and TH protein levels at 48 h. This is the first study to our knowledge to demonstrate in vivo sustained phase of TH activation, as indicated here by increased TH phosphorylation and TH activity at 24 h, with no change in TH protein levels at this time point,

TH is regulated acutely by phosphorylation of its serine residues and chronically by protein synthesis [6]. In bovine adrenal chromaffin cell cultures, acute incubation (<1 h) with nicotine leads to TH phosphorylation at Ser19, Ser31 and Ser40 [23, 24] and chronic incubation (48 h) with nicotine leads to TH protein synthesis [25]. Previous studies in vivo showed that TH phosphorylation changes are detectable as early as 10 min in response to a range of stressors in the acute phase of TH activation [7–9]. Whereas, TH protein changes are only detectable many hours after exposure to stressors in the chronic phase of TH activation [8, 26–28] with the earliest detectable change being 6 h after a 2 h immobilization stress [29]. In this study, we particularly investigated the effects of dual exposure to LPS on TH phosphorylation at three serine residues, TH activity and TH protein synthesis in adrenal gland at 4, 24 and 48 h after the second exposure to LPS. Our data showed that dual exposure to LPS evokes an

increase in TH protein in the adrenal gland only after 48 h. These findings suggested that dual exposure to LPS has a delayed effect on TH protein synthesis. However, this increase in TH protein did not lead to an increase in TH activity. Previous studies showed that chronic cold stress evokes a significant increase in TH protein synthesis without inducing an increase in TH activity [30, 31]. Therefore increases in TH protein do not always induce increases in TH activity. This may be because the newly synthesised TH protein binds to catecholamines and becomes inactivated before it is able to be phosphorylated at Ser40 which is required to keep it active. The increase in TH protein without an increase in activity may represent a regulatory mechanism to increase the capacity of the adrenal gland to response to subsequent stressors.

Recently, we found sustained phosphorylation of TH at Ser40 to be a novel mechanism for maintenance of catecholamine synthesis in vitro [11, 12]. In bovine adrenal chromaffin cell cultures, sustained incubation (1–24 h) with nicotine leads to TH phosphorylation at Ser19, Ser31 and Ser40. Phosphorylation of TH at Ser19 and Ser31 was substantially increased at 10 min to maximum levels and then there was a marked decreased in phosphorylation of TH at these sites between 10 min to 1 h. The level of phosphorylation of TH at Ser31 and Ser19 remained very low up to 24, but was still significantly above the unstimulated control levels. However, phosphorylation of TH at Ser40 was initially increased at 10 min, decreased somewhat from 1 to 8 h and then increased again from 8 to 24 h. Ser40 levels were very significantly above basal levels at all times and no significant differences were observed at 24 h from that observed at 10 min [11]. This increase in Ser40 phosphorylation occurred in response to nicotine, angiotensin, histamine and PACAP but did not occur in response to bradykinin and muscarine [11, 12]. We found that the phosphorylation of TH at Ser40 in response to nicotine and PACAP occurred via unique mechanisms and both led to an increase in TH activity. Phosphorylation of TH at Ser19 does not directly influence TH activity, Ser31 increases TH activity modestly and Ser40 plays a major role, by abolishing the feedback inhibition of TH caused by catecholamine binding, and increases TH activity both in vitro and in vivo [24, 32, 33].

Previous studies in vivo have investigated TH phosphorylation at 24 h but no evidence for the sustained phase of TH activation were found as TH phosphorylation at Ser40 was decreased and TH protein was not altered 24 h after exposure to a 1 h social defeat protocol [9] and TH phosphorylation at Ser40 was not altered and TH protein was increased 24 h after exposure to glucoprivation stress elicited by 2DG [8]. Here we found that TH phosphorylation occurs at Ser40 at 4 h leading to an increase in TH activity without any changes in phosphorylation of TH at Ser19 or Ser31. This suggests that there is a sustained phase of TH activation in vivo that extends beyond the acute changes that are generally over by 1 h after exposure to a stressor. In addition, we showed that dual exposure to LPS also evokes an increase in phosphorylation of TH at Ser31 and Ser40 at 24 h and these changes led to an increase in TH activity, without an increase in TH protein. The appearance of Ser31 phosphorylation at 24 h but not at 4 h suggests activation of different signalling pathways occurs later in the stress response and implies that there is a unique pattern of protein kinase activation at 24 h. Overall, we have provided evidence for the first time that TH phosphorylation occurs for up to 24 h and leads to TH activation independent of TH protein synthesis, suggesting that the sustained phase of TH activation occurs in vivo.

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Conflict of interest The authors declare no conflicts of interest.

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Chapter 7: Conclusions and Future Directions

7.1 Overview

Stress is a major burden in our society. One of the major cell types involved in the stress response are the catecholaminergic cells (Kvetnansky et al., 2009). Stress triggers the activation of the catecholaminergic cells and the release of catecholamines. When the catecholamines are released, it has been shown that there is no significant change in their levels within the catecholaminergic cells. This is because in parallel to catecholamine secretion there is also a concomitant increase in the rate of catecholamine biosynthesis (Wakade et al., 1988; Zigmond et al., 1989). TH is the rate-limiting enzyme in catecholamine biosynthesis and is subjected to a range of regulatory mechanisms (such as feedback inhibition by catecholamines, phosphorylation of the three serine residues, mRNA expression and protein synthesis) (Kumer and Vrana, 1996). There are three phases of TH activation (acute, sustained and chronic) when these regulatory mechanisms offer several levels of control over enzyme activity. These mechanisms come into play to maintain homeostasis in response to stress, but dysregulation of these regulatory mechanisms plays a central role in the development of diseases such as hypertension, stroke, obesity, autoimmune and inflammatory disorder in vulnerable individuals.

This thesis comprises 5 individual research chapters which aimed at investigating the different phases of TH activation, particularly in the acute and sustained phases by measuring TH phosphorylation and TH protein in the adrenal medulla and the brain at different time points in response to a range of short-term stressors *in vivo* (Figure 7.1).

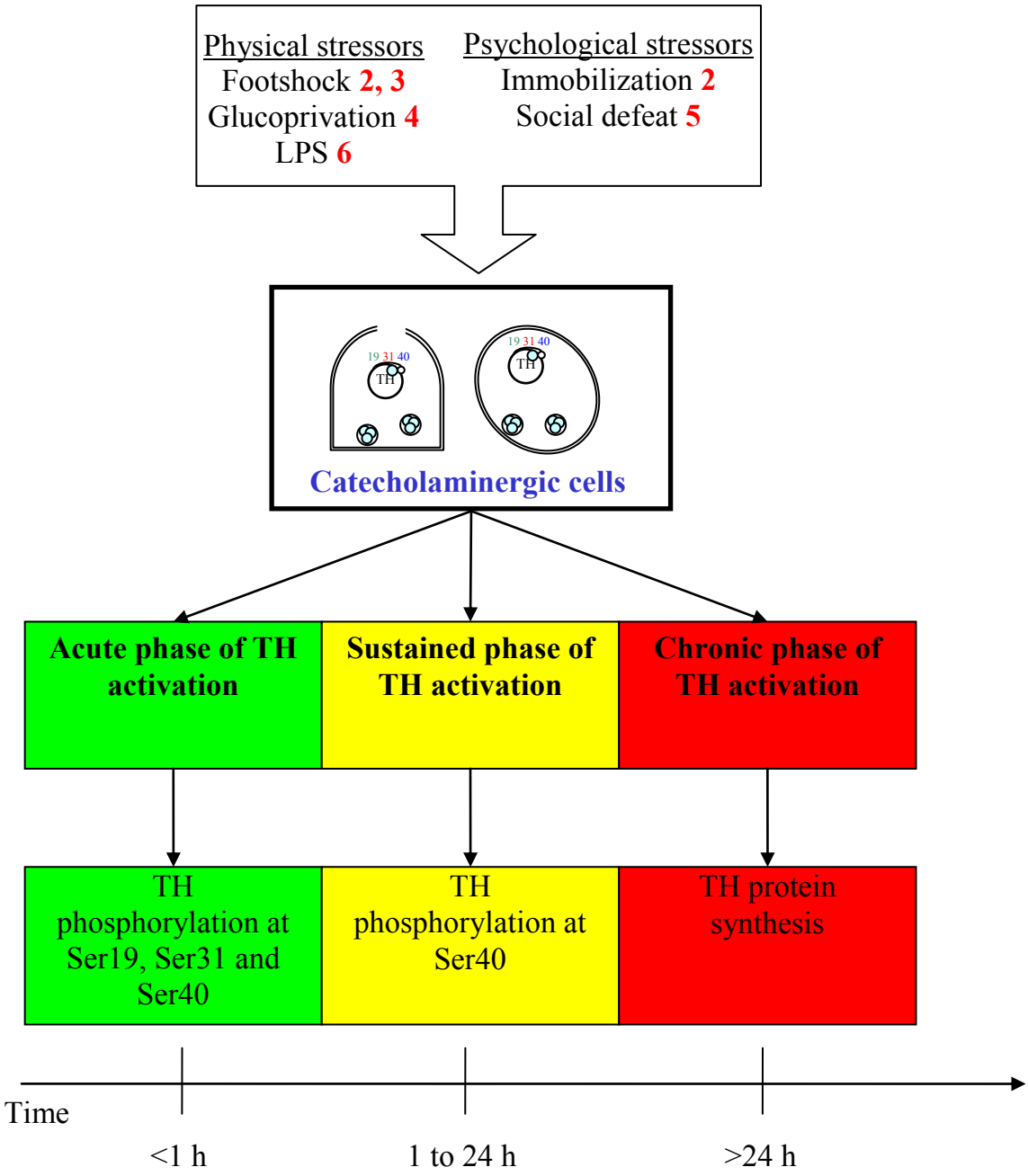

Figure 7.1: The three phases of TH activation. The acute phase of TH activation is characterised by TH phosphorylation at Ser31 and Ser40. It was examined in Chapters 2 – 5. The sustained phase of TH activation is characterised by TH phosphorylation only at Ser40. It was examined in Chapters 4 – 6. The chronic phase of TH activation is characterised by TH protein synthesis. It was examined in Chapters 4 – 6.

We have proposed two hypotheses in Chapter 1. Here we summarize our findings in relation to these hypotheses.

Hypothesis 1. We confirmed that different stressors will induce different patterns of TH phosphorylation without any change in TH protein levels in the adrenal medulla and the LC over a 1 hr period.

Hypothesis 2. We confirmed that one stressor (LPS) will induce TH phosphorylation at Ser40 without any change in TH protein in the adrenal medulla at 24 hr

On the basis of these findings we found that different stressors induce different responses in different catecholaminergic cells in terms of their temporal profile of TH phosphorylation at Ser19, Ser31 and Ser40 and TH protein synthesis, with responses varying according to the nature of the stressors and the time at which the responses are analysed.

7.2 The acute phase of TH activation

Initial studies in this thesis were aimed at examining the acute phase of TH activation *in vivo*. This phase is mediated by TH phosphorylation at Ser19, Ser31 and Ser40, without any change in TH protein levels. This phase lasts up to approximately 1 h after which TH phosphorylation and TH activity return to basal levels (Kumer and Vrana, 1996).

In adrenal chromaffin cell cultures *in vitro*, acute incubation (<1 h) with nicotine leads to TH phosphorylation which follows a pattern: Ser19 phosphorylation is rapidly increased to maximal levels within 1 min and then dephosphorylation occurs; Ser40 phosphorylation begins more slowly that Ser19 reaching a plateau by 4 min, without any subsequent dephosphorylation; Ser31 phosphorylation is delayed until 4 min but rapidly increased up to 10 min. Ser19, Ser31 and Ser40 phosphorylation is then returned toward basal levels by 1 h (Haycock, 1993). To date, there have been limited studies that have investigated the acute phase of TH activation *in vivo*. We have compared the profile of TH phosphorylation at Ser19, Ser31 and Ser40 and TH protein elicited by two stressors tentatively classified as physical (footshock or glucoprivation stress) and two stressors tentatively classified as psychological (immobilization or social defeat stress) in the adrenal medulla and the LC over a 1 h period (see Chapters 2 – 5).

<u>Adrenal medulla</u>

Collectively, the results of the thesis show that the acute phase of TH activation is mediated by different patterns in TH phosphorylation at Ser19, Ser31 and Ser40,

without any change in TH protein levels in the adrenal medulla over 1 h period in response to a range of short-term stressors (Table 7.1). In Chapter 2, immobilization stress did not alter TH phosphorylation at Ser19, increased TH phosphorylation at Ser31 only at 20 min and did not alter TH phosphorylation at Ser40. In contrast, footshock stress decreased TH phosphorylation at Ser19 at 10 and 20 min, increased TH phosphorylation at Ser31 at 10, 20 and 40 min and did not alter TH phosphorylation at Ser40. In Chapter 3, footshock stress did not alter TH phosphorylation at Ser19, increased TH phosphorylation at Ser31 at 10, 20 and 40 min and increased TH phosphorylation at Ser40 at 40 min. The reason for these differences in the effects of footshock stress (Chapters 2 & 3) must be related to whether the rats were habituated or not to the footshock chamber prior to the footshock stress protocols. The fact that in Chapter 2 the rats were not habituated to the footshock chamber prior to the footshock protocols suggests that the stress of exposure to a novel environment contributed to the changes in TH phosphorylation at Ser19. Whereas, in Chapter 3 prior habituation to the footshock chamber contributed to the changes in TH phosphorylation at Ser40. In Chapter 4, glucoprivation stress did not alter TH phosphorylation at Ser19, increased TH phosphorylation at Ser31 at 20 min and 1 h and increased TH phosphorylation at Ser40 at 20 min and 1 h. In Chapter 5 social defeat did not alter TH phosphorylation at Ser19, Ser31 and Ser40 at 10 min. Overall, the physical stressors (footshock and glucoprivation stress) activate the adrenal medulla to a greater extent compared to the psychological stressors (immobilization and social defeat stress). The fact that TH phosphorylation at Ser31 and Ser40 was increased in the physical stressors would suggest that TH activity is likely to be increased as Ser31 and Ser40 phosphorylation increases TH activity directly.

Stressors	Phospho -TH	5 min	10 min	20 min	40 min	60 min
Immobilization 2	Ser19		No	No	No	
	Ser31		No	↑↑	No	
	Ser40		No	No	No	
Footshock 2	Ser19		↓↓↓	↓↓	No	
	Ser31		↑	↑	↑↑	
	Ser40		No	No	No	
Footshock 3	Ser19		No	No	No	
	Ser31		↑↑	↑	↑	
	Ser40		No	No	↑	
Glucoprivation 4	Ser19	No		No		No
	Ser31	No		↑↑↑		↑↑↑
	Ser40	No		↑↑		↑
Social defeat 5	Ser19		No			
	Ser31		No			
	Ser40		No			

Table 7.1: TH phosphorylation in the adrenal medulla *in vivo*. Numbers represent research chapters. Arrows indicate increases or decreases. "No" indicates no changes.

Locus coeruleus

Collectively, the results of the thesis also show that the acute phase of TH activation is mediated by different patterns in TH phosphorylation at Ser19, Ser31 and Ser40, without any change in TH protein levels in the LC over 1 h period in response to a range of short-term stressors (Table 7.2). In Chapter 2, immobilization stress did not alter TH phosphorylation at Ser19, increased TH phosphorylation at Ser31 only at 10 min and did not alter TH phosphorylation at Ser40. In contrast, footshock stress

decreased TH phosphorylation at Ser19 at 40 min, increased TH phosphorylation at Ser31 at 20 and 40 min and did not alter TH phosphorylation at Ser40. In Chapter 5 social defeat did not alter TH phosphorylation at Ser19, increased TH phosphorylation at Ser31 and Ser40 at 10 min. Overall, the psychological stressors (immobilization and social defeat stress) have a rapid and short lasting effect on TH phosphorylation in the LC, whereas the physical stressor (footshock stress) has a delayed effect on TH phosphorylation in the LC.

Stressors	Phospho -TH	10 min	20 min	40 min
Immobilization 2	Ser19	No	No	No
	Ser31	↑↑	No	No
	Ser40	No	No	No
Footshock 2	Ser19	No	No	↓
	Ser31	No	↑↑↑	↑↑↑
	Ser40	No	No	No
Social defeat 5	Ser19	No		
	Ser31	↑↑		
	Ser40	↑↑		

Table 7.2: TH phosphorylation in the LC *in vivo*. Numbers represent research chapters. Arrows indicate increases or decreases. "No" indicates no changes.

Results (presented in Chapters 2 – 5) have shown here that the pattern of TH phosphorylation at Ser19, Ser31 and Ser40 varies considerably in the adrenal medulla and the LC, and is dependent on both the type of stressor and the time when the response is analysed.

7.3 The sustained phase of TH activation

Subsequent studies in this thesis were aimed at examining the sustained phase of TH activation *in vivo*. This phase is mediated by TH phosphorylation at primarily at Ser40, also without any change in TH protein levels. This phase occurs from 1 to 24 h (Bobrovskaya et al., 2007a; Bobrovskaya et al., 2007b).

In adrenal chromaffin cell cultures *in vitro*, sustained incubation (1 – 24 h) with nicotine leads to TH phosphorylation which follows a pattern: Ser19 and Ser31 phosphorylation are very low but significantly above the unstimulated control levels at 24 h; Ser40 phosphorylation is decreased below acute levels from 1 to 8 h (but still significantly above basal levels) and then substantially increased from 8 to 24 h (Bobrovskaya et al., 2007a; Bobrovskaya et al., 2007b). The hallmarks of this phase are 1) TH phosphorylation of Ser40 is increased, 2) TH protein is not altered and 3) the signal transduction pathways activation is different from the acute phase. To date, there have been no studies that have investigated the sustained phase of TH activation *in vivo*. We have compared the profile of TH phosphorylation at Ser19, Ser31 and Ser40 and TH protein elicited by two stressors tentatively classified as physical (glucoprivation or LPS stress) and one stressor tentatively classified as psychological (social defeat stress) in the adrenal medulla at 24 h (in Chapters 4 – 6).

Collectively, the results of the thesis show that the sustained phase of TH activation only occurs in the adrenal medulla at 24 h in one of the rodent stress models used, namely LPS stress (Table 7.3). In Chapter 4, glucoprivation stress did not alter TH phosphorylation at Ser40 but increased TH protein at 24 h. In Chapter 5, social defeat

stress decreased TH phosphorylation at Ser40 but did not alter TH protein at 24 h. In Chapter 6, LPS stress increased TH phosphorylation at Ser40 and TH activity, but did not alter TH protein in neonatal adrenal medulla at 24 h. The differences are due to the different rodent stress models. Although glucoprivation and LPS stress are classified as physical stressors, glucoprivation stress causes disturbance in metabolic status and LPS stress causes disturbance in immunity status.

Stressors		**24 h**	**The sustained phase of TH activation**
Glucoprivation 4	Ser40 TH protein	No ↑↑↑	**NO**
Social defeat 5	Ser40 TH protein	↓ No	**NO**
LPS 6	Ser40 TH protein	↑↑↑ No	**YES**

Table 7.3: The effects of different stressors on TH phosphorylation at Ser40 and TH protein in the adrenal medulla *in vivo*. Numbers represent research chapters. Arrows indicate increases or decreases. "No" indicates no changes.

Results (presented in Chapters 4 – 6) have shown here that TH phosphorylation at Ser40 occurs for up to 24 h and leads to TH activation independent of TH protein synthesis, suggesting that the sustained phase of TH activation occurs *in vivo*. This phenomenon that was defined *in vitro* has also been shown for the first time to occur *in vivo* (although only in one rodent stress model).

7.4 The chronic phase of TH activation

The chronic phase of TH activation is mediated primarily by the activation of transcription factors, which over time increases TH mRNA expression and TH protein synthesis leading to increased TH activity (Kumer and Vrana, 1996).

In adrenal chromaffin cell cultures *in vitro*, chronic incubation (48 h) with nicotine leads to increased TH protein synthesis and TH activity (Craviso et al., 1992). To date, there have been extensive studies that have investigated the chronic phase of TH activation *in vivo*. Therefore, this phase was not a major focus of this thesis.

Nevertheless, the results of this thesis show that the chronic phase of TH activation occurs in the adrenal medulla in the rodent stress models used. In Chapter 4, glucoprivation stress increased TH protein in the adrenal medulla at 24 h. In Chapter 6, LPS stress increased TH protein in adrenal medulla at 48 h, but did not increase TH activity. This may be because the newly synthesised TH protein binds to catecholamines and becomes inactivated before it is able to be phosphorylated at Ser40 which is required to keep it active. The increase in TH protein without an increase in activity may represent a regulatory mechanism to increase the capacity of the adrenal gland to response to subsequent stressors. Results (presented in Chapters 4 & 6) have shown that the chronic phase of TH activation is detectable earlier in glucoprivation stress compared to LPS stress.

7.5 The advantages of measuring TH phosphorylation

Overall, we provided evidence that different catecholaminergic cells respond differently in terms of the temporal profiles of TH phosphorylation at Ser19, Ser31 and Ser40 presumably due to differences in the frequency of cell firing and/or the nature of the neurotransmitters released onto the cells. In addition, we demonstrated that the activation of the enzyme is associated with TH phosphorylation at Ser19, Ser31 and Ser40 *in vivo*, an effect that had previously been demonstrated in cultured cells. We also demonstrated that TH phosphorylation at either Ser31 and/or Ser40 can contribute to increases in TH activity *in vivo*.

The results of the thesis show that different stressors induce the acute phase of TH activation but provide different patterns in TH phosphorylation at Ser19, Ser31 and Ser40, without TH protein synthesis in the adrenal medulla and the LC over 1 h period. Different patterns in TH phosphorylation at Ser19, Ser31 and Ser40 suggest that cell activation varies in different catecholaminergic cells (the adrenal medulla chromaffin cells vs. the LC neurons), that there is a unique pattern of protein kinases activated and that there is a difference in the probability of catecholamine biosynthesis being activated.

In response to stressors, both the adrenal medulla chromaffin cells and catecholaminergic neurons are activated by depolarization of the cells, leading to an influx of extracellular calcium via voltage-sensitive calcium channels (de Diego et al., 2008). The influx of extracellular calcium causes the exocytosis of catecholamines from the cells (as neurotransmitters from neurons and as hormones from the adrenal medulla chromaffin cells) (de Diego et al., 2008) and the activation of various signal

transduction pathways in the cells (Sabban and Kvetnansky, 2001; Salvatore et al., 2001; Wong and Tank, 2007). When catecholamines are released from cells there is a concomitant increase in TH activity in order to replenish the released catecholamines (Wakade et al., 1988; Zigmond et al., 1989). TH is primarily regulated by a feedback inhibition mechanism by the catecholamines (Spector et al., 1967) and phosphorylation at three key serine residues *in vivo* (Dunkley et al., 2004).

Depolarizing stimuli and increases in intracellular Ca^{2+} have been shown to activate CaMPKII and TH phosphorylation at Ser19 (Padmanabhan and Prasad, 2009). However, Ser19 phosphorylation alone does not increase TH activity *in vitro* and *in situ* (Haycock et al., 1998; Salvatore et al., 2001). The functional roles of TH phosphorylation at Ser19 remain unclear *in vivo*. Depolarizing stimuli have been shown to activate ERK1/2 and TH phosphorylation at Ser31. There is strong evidence that the activation of ERK1/2 mediates TH phosphorylation at Ser31 and leads to TH activation and an increase in the biosynthesis of catecholamines *in vivo* (Nunez et al., 2007; Almela et al., 2008; Nunez et al., 2008). Depolarizing stimuli have been shown to activate PKA and TH phosphorylation at Ser40. There is strong evidence that the activation of PKA mediates TH phosphorylation at Ser40 and leads to TH activation and an increase in the biosynthesis of catecholamines *in vivo* (Dunkley et al., 2004; Almela et al., 2008; Raghuraman et al., 2009). On the basis of our understanding of these phenomena, this thesis presents evidence to support the advantages of measuring TH phosphorylation at different time points *in vivo*. In adrenal medulla chromaffin cells (Figure 7.2) and catecholaminergic neurons (Figure 7.3), changes in TH phosphorylation over time provide information on:

1) **The extent of cell activation.** TH phosphorylation was found to be a sensitive and reliable marker for cell activation at times between 10 min to 1 h after the stimulus. We were able to detect subtle differences between cell types not seen with other cell activation markers such as c-Fos which is optimal after 2 h, TH mRNA which is optimal from 6-18 h and TH protein which is optimal from 24-48 h.
2) **The nature of the signal transduction pathways activated.** TH phosphorylation at Ser31 occurs most likely via ERK1/2 activation and TH phosphorylation at Ser40 occurs most likely via PKA activation in the adrenal chromaffin cells.
3) **The extent of TH activation.** TH phosphorylation at either Ser31 or Ser40 can contribute to increases in TH activity in the adrenal chromaffin cells.

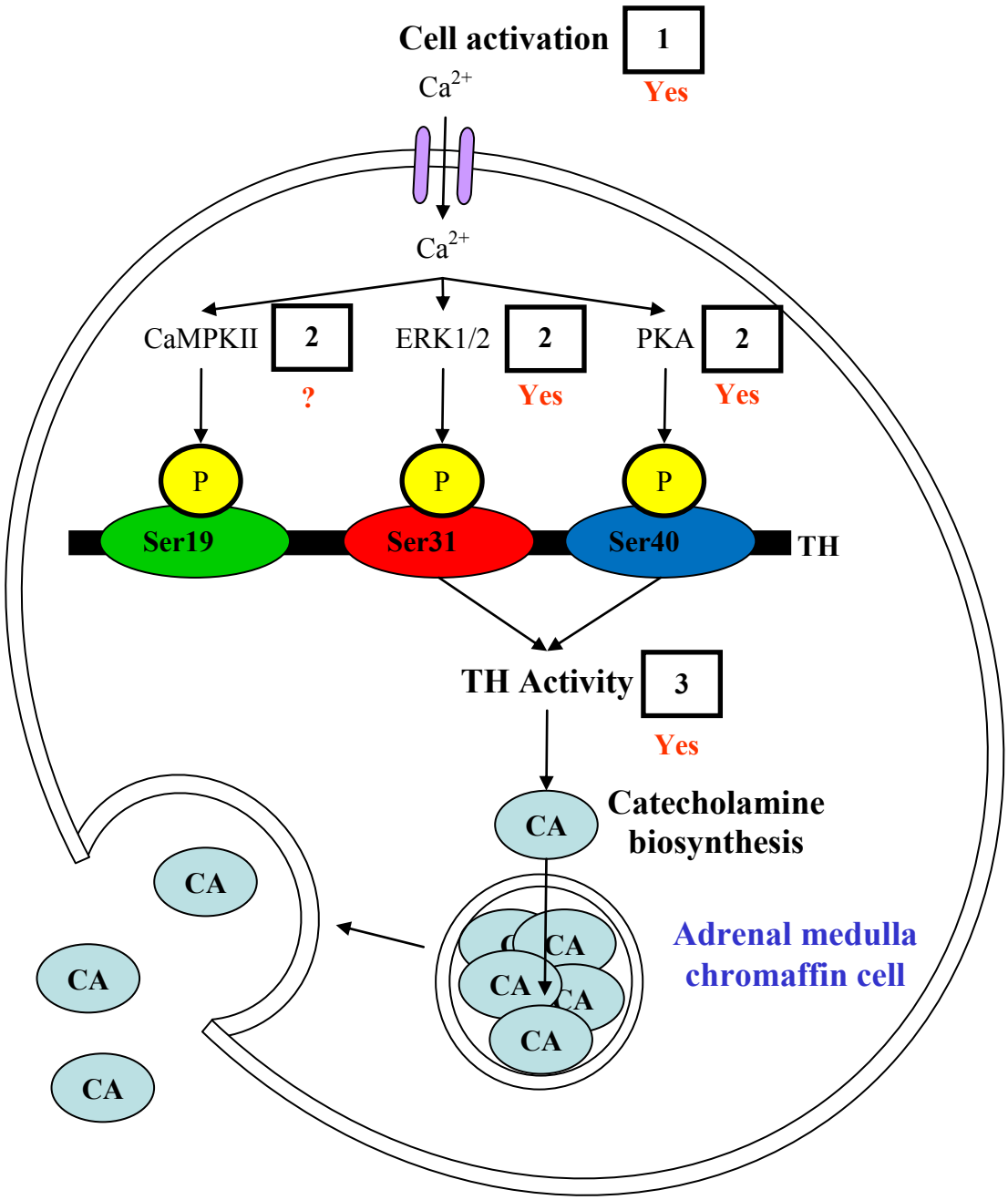

Figure 7.2: TH phosphorylation at Ser19, Ser31 and Ser40 in adrenal medulla chromaffin cells *in vivo*. Numbers in squares represent information provided by changes in TH phosphorylation. "Yes" indicates that this was supported by the data provided. "?" indicates that this still requires further investigation. 1 was evaluated in Chapters 2 – 6, 2 in Chapters 3 & 4 and 3 in Chapters 3 & 6.

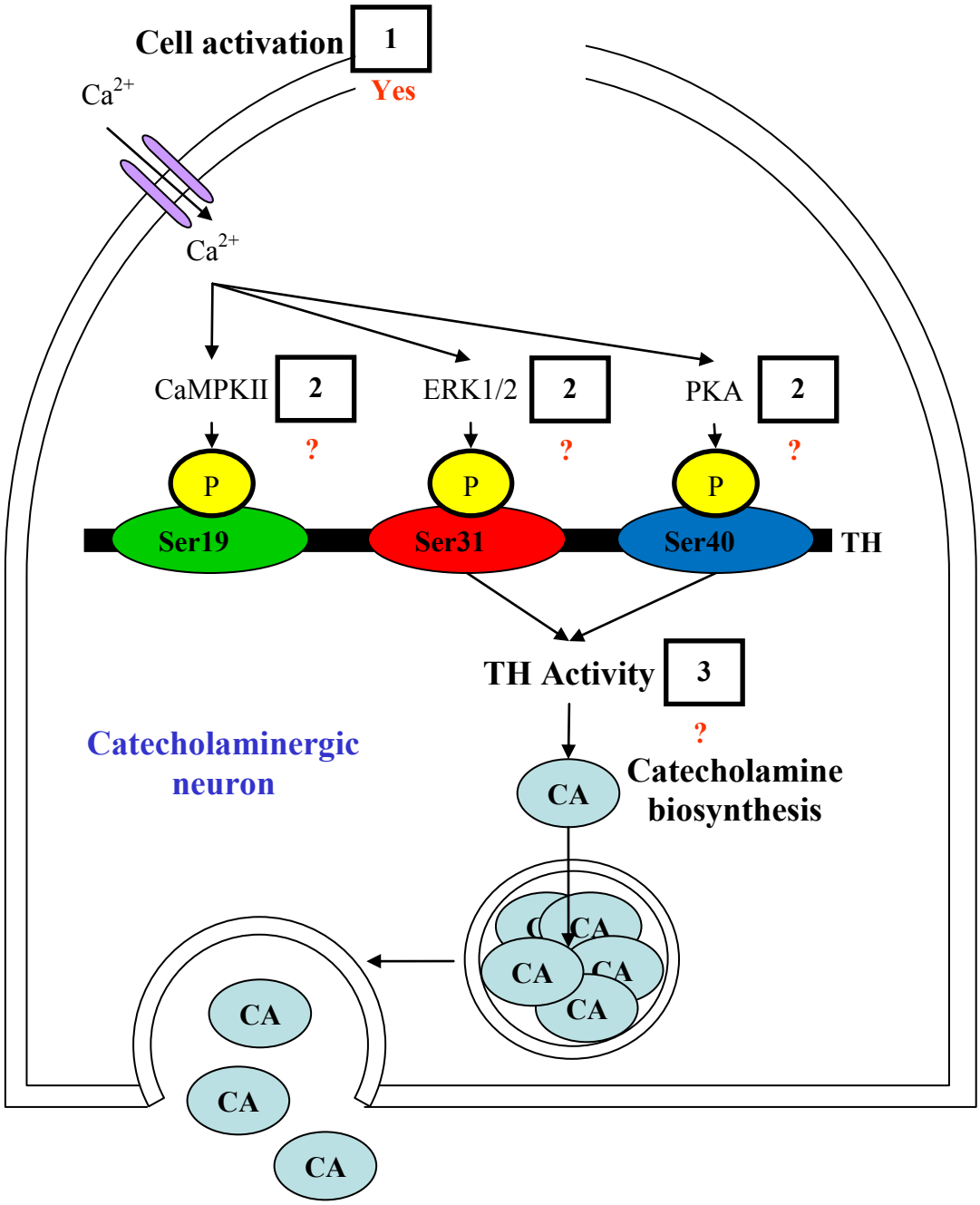

Figure 7.3: TH phosphorylation at Ser19, Ser31 and Ser40 in catecholaminergic neuron *in vivo*. Numbers in squares represent information provided by changes in TH phosphorylation. "Yes" indicates that this was supported by the data provided. "?" indicates that this still requires further investigation. Hypothesis 1 was tested in Chapters 2 & 5.

7.5.1 TH phosphorylation indicates the extent of cell activation

TH phosphorylation is a sensitive and reliable marker for the extent of cell activation in adrenal chromaffin cells and brain catecholaminergic neurons *in vivo*. Cell activation is defined as changes or modifications of the cell "basal" conditions. These changes or modifications include the frequency of cell firing, the nature of the neurotransmitters released onto these cells. Different neurotransmitters lead to activation of different receptors, which then lead to activation of different second messengers and changes in TH phosphorylation. Collectively, the results in this thesis (Chapters 2 - 5) demonstrate that a range of stressors induce very different patterns in TH phosphorylation at Ser19, Ser31 and Ser40 in the adrenal medulla (Table 7.1) and the LC (Table 7.2) over a 1 h period immediately following exposure to a stressor. These results facilitate an understanding of two issues:

1) Whether cell activation occurs or not?
2) If cell activation occurs, is the cell activation different in terms of either the quantitative aspects (large or small changes) or the time point (when the differences occur)?

In the adrenal medulla, footshock stress (Chapters 2 & 3) caused cell activation at 10, 20 and 40 min, immobilization stress (Chapter 2) caused transient cell activation only at 20 min, glucoprivation (Chapter 4) caused cell activation at 20 and 60 min and social defeat (Chapter 5) did not cause any acute cell activation. In LC, footshock stress caused cell activation at 20 and 40 min, while immobilization stress and social defeat caused transient cell activation (only at 10 min). Results in Chapter 2 demonstrate that

footshock and immobilization stress lead to different extents of cell activation in the adrenal medulla and LC as the quantitative extent of the TH phosphorylation changes differed at 10, 20 and 40 min. Results in Chapter 4 demonstrate that social defeat stress leads to different extents of cell activation in the adrenal medulla, LC, SN and VTA at 10 min. The reason for these differences in response must be related to the nature of the neurotransmitters released onto these cells and the extent of calcium entry, or the different frequency of cell firing that occurs with these different stressors. In summary, cell activation varies in different catecholaminergic cells and is very dependent on both the type of stressors and the time when the response is analysed.

7.5.2 TH phosphorylation indicates the nature of the signal transduction pathways activated

TH phosphorylation indicates the nature of the signal transduction pathways activated in the adrenal chromaffin cells *in vivo*. Collectively, the results of the thesis (Chapters 3 & 4) demonstrate that TH phosphorylation at Ser31 is most likely through a mechanism of ERK1/2 activation and TH phosphorylation at Ser40 is most likely through a mechanism involving PKA activation in the adrenal medulla (Table 7.4). Results in Chapter 3 demonstrate that footshock stress caused ERK1/2 activation and increased TH phosphorylation at Ser31 in the adrenal medulla at 10, 20 and 40 min. Footshock stress also caused PKA activation and increased TH phosphorylation at Ser40 in the adrenal medulla at 40 min. Results in Chapter 4 demonstrate that glucoprivation stress caused ERK1/2 activation at only 60 min but increased TH phosphorylation at Ser31 in the adrenal medulla at both 20 and 60 min. Detailed discussion on why TH phosphorylation at Ser31 is increased but not ERK1/2 activation

at 20 min is available in Chapter 4. Glucoprivation stress also caused PKA activation and increased TH phosphorylation at Ser40 in the adrenal medulla at 20 and 60 min. However, these results simply reflect the possible signal transduction pathways that are likely to mediate TH phosphorylation in the adrenal medulla. A comprehensive investigation of all of the protein kinases that are known to mediate TH phosphorylation is required to confirm the identity of the protein kinases involved.

Stressors		10 min	20 min	40 min	60 min
Footshock 3	*Signalling pathways*				
	ERK1/2	↑↑	↑	↑	
	PKA	No	No	↑	
	Phospho-TH				
	Ser31	↑↑	↑	↑	
	Ser40	No	No	↑	
Glucoprivation 4	*Signalling pathways*				
	ERK1/2		No		↑
	PKA		↑		↑
	Phospho-TH				
	Ser31		↑↑↑		↑↑↑
	Ser40		↑↑		↑

Table 7.4: The effects of footshock and glucoprivation stress on signalling pathways activation and TH phosphorylation at Ser31 and Ser40 in the adrenal medulla *in vivo*. Numbers represent research chapters. Arrows indicate increases or decreases. "No" indicates no changes.

It should be mentioned that we did not investigate the relationship between CaMPKII activation and TH phosphorylation at Ser19 in the adrenal medulla. CaMPKII is activated more rapidly by depolarizing stimuli in a Ca^{2+}-dependent manner. Ser19 is

more rapidly phosphorylated and dephosphorylated (within 1 min) and therefore, it is less of a focus in experiments that were done here (>5 min). It should also be mentioned that we did not investigate the signal transduction pathways activated in the brain catecholaminergic neurons. The obstacle is the difficulty in isolating specific brain catecholaminergic neurons by using the "slice and punch" method used in this thesis and the presence of all of the protein kinases in most cells of the brain including both neuronal and glial cells. Therefore, other methods such as laser capture microdissection which can isolate specific TH containing neurons are required in future investigations, but a large number of cells would have to be pooled to be able to see any changes that might have taken place *in vivo*.

7.5.3 TH phosphorylation indicates the extent of TH activation

TH phosphorylation suggests TH activation in the adrenal chromaffin cells *in vivo*. Collectively, the results of the thesis (Chapters 3 & 6) demonstrate that TH phosphorylation at Ser31 and Ser40 contribute to the increases in TH activity seen in the adrenal medulla (Table 7.5). Results in Chapter 3 demonstrate that footshock stress caused TH phosphorylation at Ser31 at 20 min (1.5 fold) and 40 min (1.5 fold) and TH phosphorylation at Ser40 at 40 min (1.5 fold). Therefore, Ser31 phosphorylation increased TH activity at 20 min (2.1 fold) when Ser40 was not increased, while Ser40 further increased TH activity at 40 min (2.8 fold) when there is no further increase in Ser31 phosphorylation. Results in Chapter 6 demonstrate that LPS stress caused TH phosphorylation at Ser31 at 24 h (1.5 fold) and TH phosphorylation at Ser40 at 4 h (1.5 fold) and 24 h (2.0 fold). Therefore, Ser40 phosphorylation increased TH activity at 4 h

(2.0 fold) while Ser40 (and Ser31) phosphorylation further increased TH activity at 24 h (2.2 fold).

Stressors		10 min	20 min	40 min
Footshock 3	*Phospho-TH*			
	Ser31	↑↑	↑	↑
	Ser40	No	No	↑
	TH activity	No	↑↑	↑↑↑
		4 h	**24 h**	**48 h**
LPS 6	*Phospho-TH*			
	Ser31	No	↑↑↑	No
	Ser40	↑↑↑	↑↑↑	No
	TH activity	↑↑	↑↑↑	No

Table 7.5: The effects of footshock and LPS stress on TH phosphorylation at Ser31 and Ser40 and TH activity in the adrenal medulla *in vivo*. Numbers represent research chapters. Arrows indicate increases or decreases. "No" indicates no changes.

As mentioned earlier, *in vitro,* TH phosphorylation at Ser19 does not increase TH activity directly, TH phosphorylation at Ser31 increases TH activity about 2 fold and TH phosphorylation at Ser40 increases TH activity up to 40 fold, by abolishing the feedback inhibition by catecholamines (Dunkley et al., 2004). However, results in Chapters 3 & 6 demonstrate that the same extent of increase in TH phosphorylation at Ser31 and Ser40 leads to similar increases in TH activity. The lack of correlation of the results *in vivo* with those *in vitro* maybe due to two reasons: 1) the sensitivity of the phospho-specific TH antibodies and 2) the stoichiometry of TH phosphorylation *in vivo*. Under basal conditions, TH is phosphorylated at Ser31 to variable stoichiometery levels (about 30 %) whereas TH is phosphorylated at Ser40 to quite low stoichiometery levels

compared to Ser31 (about 5 %) (Salvatore et al., 2000; Saraf et al., 2007). Therefore, 1.5 fold increases in Ser31 phosphorylation (about 45 %) is much more than 1.5 fold increases in Ser40 phosphorylation (about 7.5 %). It appears that a 1.5 fold increase in TH phosphorylation at Ser31 (about 45 %), increases TH activity in the adrenal chromaffin cells *in vivo*. It also appears that a 1.5 fold increase, even though TH phosphorylation at Ser40 is about 7.5 % is sufficient to increase TH activity in the adrenal chromaffin cells *in vivo*. Thus, TH phosphorylation at Ser31 and Ser40 contribute to the increases in TH activity and most likely contribute to catecholamine biosynthesis in the adrenal medulla. The investigation of TH phosphorylation and TH activity in the brain catecholaminergic system is not presented in this thesis as our laboratory and associates are currently investigating the effects of stressors (footshock and glucoprivation stress) on the central catecholaminergic system by measuring TH phosphorylation and TH activity in the brain catecholaminergic neurons. These results will be presented in another thesis and are therefore not discussed here.

7.6 Perspective

Overall, the results of the thesis show that different stressors induce the acute phase of TH activation but provide different patterns in TH phosphorylation at Ser19, Ser31 and Ser40, without TH protein synthesis in the adrenal medulla and the LC over 1 h period. LPS stress induces the sustained phase of TH activation by inducing a sustained TH phosphorylation without TH protein synthesis being increased in the adrenal medulla at 24 h period, which bridges the gap between the acute and chronic phases of TH activation. Glucoprivation led to chronic TH activation, by increased TH protein synthesis in the adrenal medulla. The results of these studies not only provide original data, but they allow us to correlate this data with previous findings *in vitro* using adrenal chromaffin cell cultures.

In addition, the results of the thesis show that the physical stressors (footshock, glucoprivation or LPS stress) activate the adrenal medulla to a greater extent compared to the psychological stressors (immobilization or social defeat stress). The basal rate of cell firing and/or input of neurotransmitters are increased by the stressors. The numbers and types of neurotransmitters release onto these cells lead to activations of different receptors, which in turn lead to activation of different second messengers and TH phosphorylation. The fact that TH phosphorylation at Ser31 and Ser40 was increased by the physical stressors would suggest that TH activity is likely to be increased as Ser31 and Ser40 phosphorylation increases TH activity directly. TH protein was increased in physical stressors (glucoprivation or LPS stress) and was not increased in psychological stressors (social defeat stress). Therefore, physical stressors could be considered

stronger stressors as they required substantial TH activation and the biosynthesis of catecholamines.

We also found that measurement of TH phosphorylation at Ser19, Ser31 and Ser40 will provide an indication on 1) the extent of cell activation, 2) the nature of the signal transduction pathways activated and 3) the extent of TH activation. These findings have substantially improved our understanding of the effects of different stressors on the central catecholaminergic and/or sympathetic-adrenomedullary systems *in vivo* and a number of clear cut conclusions have been made. These findings open up opportunities for the use of the methodologies that were developed in future studies.

7.7 Future directions

Work presented here has demonstrated for the first time that the sustained phase of TH activation occurs *in vivo*. A number of studies that could be conducted to further elucidate the sustained phase of TH activation. The signal transduction pathways activated and catecholamine synthesis/release must be determined. While the study in Chapter 6 was undertaken using neonatal rats, further studies need to be done to investigate whether the sustained phase of TH activation also occurs in adult rats.

A systemic investigation of the catecholamine neuronal circuits in response to different stressors has not yet been undertaken. Most of the neuronal circuit mapping has concentrated on c-fos expression as an indication of cell activation, in spite of uncertainties the exact functional roles of c-fos, as a transcription factor. Our laboratory and associates are currently investigating the effects of different stressors (footshock and glucoprivation stress) in different brain regions such as dopaminergic and noradrenergic neurons by measuring TH phosphorylation at its serine residues and TH protein.

As mentioned earlier the signal transduction pathway activation and the catecholamine synthesis/release in the brain catecholaminergic neurons has not yet been undertaken. To investigate the signal transduction pathways activated, specific brain catecholaminergic neurons can be isolated by using methods such as laser capture microdissection. To investigate the catecholamine release, specific brain catecholaminergic neurons can be measured by using methods such as *in vivo* dialysis.

All preclinical models of psychological disorders such as anxiety and depression are based around exposure of animals to stressors. A central finding of this thesis is that exposure to stress triggers changes in the catecholaminergic cells in term of acute, sustained and chronic TH activation. It still remains unclear whether these changes are entirely beneficial (protective) adaptive responses mediating resilience to stress or whether they may be in part detrimental (harmful) responses leading to vulnerability to the many stress-related disorders. In order to further elucidate the role of these changes, the neurobiological and behavioural consequences of longer term and repeated stress must be determined. This will include investigation of the HPA axis, downstream metabolic pathways and behavioural testing.

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Appendix

Increased microglial activation in the rat brain following neonatal exposure to a bacterial mimetic

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Short communication

Increased microglial activation in the rat brain following neonatal exposure to a bacterial mimetic

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ABSTRACT

Neonatal lipopolysaccharide (LPS) exposure increases anxiety-like behaviour in adulthood. Our current aim was to examine whether neonatal LPS exposure is associated with changes in microglial activation, and whether these alterations correspond with alterations in behaviour. In adulthood, LPS-treated animals exhibited significantly increased anxiety-like behaviour and hippocampal microglial activation. The efficacy of the LPS challenge was confirmed by increased neonatal plasma corticosterone and tyrosine hydroxylase (TH) phosphorylation in the adrenal medulla. These findings suggest a neuroimmune pathway which may underpin the long-term behavioural and neuroendocrine changes following neonatal infection.

Research over the last decade has shown that perinatal immune activation with lipopolysaccharide (LPS) can induce long term functional alterations in the metabolic [1], immune [2–5], behavioural [6–10], and neuroendocrine [3,11] systems. Our laboratory has been particularly interested in the increased levels of anxiety-like behaviours observed in adult rats exposed to LPS on postnatal days 3 and 5. We have consistently reported that this exposure paradigm increases anxiety-like behaviour in adulthood across a range of behavioural tests [8–10]. Recently, several studies have shown that glia, and in particular microglia, respond vigorously to LPS, and appear to be involved in modulating the expression of certain behaviours. Interestingly, Bilbo and colleagues, using a similar form of postnatal challenge, have shown that exposure to *Escherichia coli* on postnatal day 4 induces changes in microglia that persist until adolescence (28 days later). The same group later observed that adult rats challenged postnatally with *E. coli* and then with LPS produced higher levels of CD11b, a putative marker of microglial activation. Together, these findings suggest that exposure to Gram-negative bacteria, the source from which LPS is derived, causes persistent changes in microglial responsiveness. As yet, however, no studies have examined whether alterations in microglial activation may occur in rats challenged with LPS on postnatal days three and five, the most commonly used model of early life bacterial driven immune activation. The association between such increases in microglial activation and anxiety-like behaviour also remains to be determined. Here we tested postnatally challenged animals using the Elevated Plus Maze (EPM) and Holeboard tests. Immediately following testing, we collected the brains of these animals and assessed them for changes in microglial activation status using immunohistochemical labelling of the ionized calcium-binding adaptor molecule (Iba-1) protein. Iba-1 is recognised to be an effective method for identifying changes in microglial activation status [12–15]. Finally, we confirmed the efficacy of the LPS challenge in activating neonatal stress pathways

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by assessing plasma corticosterone concentrations and tyrosine hydroxylase (TH) phosphorylation (a rate-limiting enzyme crucial for catecholamine synthesis) in the adrenal medulla.

68 Wistar males deriving from 17 litters were used in this study. Animals were mated in the University of Newcastle Psychology vivarium. Litters were randomly allocated into either LPS (8 litters) or saline control conditions (9 litters) at birth (postnatal day [PND] 1). On PND 3 and PND 5, animals were briefly removed from their home cages, weighed, and administered intraperitoneally with either 0.05 mg/kg LPS (*Salmonella enterica*, serotype enteritidis; Sigma–Aldrich Chemical Co., USA) or an equivolume of non-pyrogenic 0.9% saline (Livingstone International, Australia) as described previously [10]. A subgroup of rats ($n = 56$ males, derived from 13 litters; 6 LPS-treated, 7 saline-treated) were euthanized 4 or 24 h following drug exposure on PND 5 to determine whether neonatal LPS administration was effective in activating the neonatal neuroendocrine and sympathetic nervous system stress responses. 4 or 24 h following drug exposure on PND 5 the immediate effects of LPS on plasma corticosterone, as well as TH protein and phosphorylation levels in adrenals were assessed. The remaining 4 litters were left with their dams until weaning (PND 22), at which time they were segregated into same-sex paired housing (41.5 cm × 28.0 cm × 22.0 cm cages; Mascot Wire Works, Sydney, Australia). Rats were left undisturbed from weaning until behavioural testing in adulthood (PND 85) apart from weights collected weekly. Housing conditions were identical to those previously reported [10]. All experimentation occurred in accordance with the 2004 NH&MRC Australian Code Of Practice For The Care And Use Of Animals For Scientific Practice.

All behavioural testing was conducted in adulthood (PND 85) in complete darkness under infrared lighting. Detailed protocols and conditions for the EPM and Holeboard have been previously described [9,10]. Anxiety-related variables assessed in the EPM included the percentage of time spent in the open arms and the number of closed and open arm entries. Distance and activity measures were recorded for indications of locomotor activity and freezing. Exploratory head dips, time spent in the central square, distance travelled, and activity were recorded to assess anxiety-like behaviours in the Holeboard. The order of the behavioural tests was counterbalanced across tasks and subjects.

Animals allocated for assessment of HPA axis activation following neonatal drug administration were rapidly decapitated 4 h and 24 h following drug administration on PND 5 and trunk blood was collected into EDTA-coated tubes (Livingstone International, Australia). Plasma corticosterone concentrations were assessed using a rat corticosterone 125I radioimmunoassay kit following manufacturer's instructions (MP Biomedicals, USA)

Tyrosine hydroxylase protein and phosphorylation levels were analysed as previously described with some modifications [16]. Briefly, the adrenals were homogenised using a sonicator (Soniprep 150, MSE) in 200 μl homogenisation buffer (50 mM Tris–HCl, pH 7.5; 1 mM EGTA; 1 protease tablet; 1 mM sodium vanadate; 1 mM sodium pyrophosphate; 80 μM ammonium molybdate; 5 mM β-glycerophosphate; 2 μM microcystin). Samples were then centrifuged at 16,000 rpm for 20 min at 4 °C. The clear supernatants were collected and protein concentration was determined by a BCA assay according to the manufacturer's general protocol for protein analysis. Samples were diluted with homogenisation buffer to same concentration and mixed with sample buffer (1% SDS, 10% glycerol, 0.5% DTT and minimal bromophenolblue). 30 μg of each samples were subjected then to SDS–polyacrylamide gel electrophoresis before being transferred to nitrocellulose [17]. Membranes were then stained with Ponceau S (0.5% ponceau in 1% acetic acid) to assess the efficacy of the transfer. Membranes then were washed in Tris-buffered saline with Tween (TBST) (150 mM NaCl, 10 mM Tris, 0.075% Tween-20, pH 7.5) and incubated with blocking solution (5% bovine serum albumin, 0.04% sodium azide in TBST) for 2 h at 25 °C. Membranes were washed in TBST and incubated with total or phosphor-specific TH antibodies for 1 h at 25 °C. The levels of total TH (tTH) protein, pSer40 and β-actin protein have previously been characterized [18]. Membranes were washed in TBST and incubated with horse-radish peroxidase-linked anti-IgG secondary specific antibodies for 1 h at 25 °C. Membranes were visualized on Fugifilm Las-3000 imaging system (Fuji, Stamford, CT, USA) using ECL plus detection reagents. The density of total TH, phospho-specific TH and β-actin bands were measured using a MultiGauge V3.0 (Fuji, Stamford, CT, USA). Total TH protein levels were expressed as the ratio of TH protein to β-actin as β-actin levels are used as house-keeping proteins. Site-specific TH phosphorylation at Ser40 was expressed as the ratio to total TH protein to account for variability in total TH between samples.

Two hours following the conclusion of behavioural testing, animals were deeply anaesthetised with sodium pentobarbitone and perfused transcardially with heparinised phosphate buffered saline (PBS) followed by 4% formaldehyde (pH 9.5) in 0.1 M phosphate buffer (PB). Brains were then extracted and postfixed in a 15% sucrose solution containing the same fixative solution. After fixation, brains were transferred to a 15% sucrose solution in 0.1 M PBS for cryoprotection. Serial coronal sections (30 μm) were cut using a freezing (−25° C) microtome (Leica SM 2000R) and were divided into a one-in-six series, which was stored in an anti-freeze solution (4° C) until required for immunoperoxidase labelling. Sections were then processed using immunohistochemistry to assess microglia using ionized calcium-binding adaptor molecule (Iba-1) protein. The antibody is not expressed in neurons, astrocytes, or oligodendroglia [19–21], and is constitutively expressed and upregulated in activated microglia [21]. Previous research indicated that Iba-1 and Mac-1 (CD11b) antibodies overlap in their ability to label microglia [22]. However, Iba-1 and CD11b differ in the subcellular locations that they label. While CD11b is detected predominantly in the cytoplasm, with some labelling of the processes, Iba-1 labels the cytoplasm, nucleus and processes strongly. Accordingly, Iba-1 provides superior labelling, in particular for quantitative analysis.

For immunoperoxidase labelling, a series of sections from all animals in both treatment conditions was processed simultaneously. Sections were rinsed with 0.1 M PB and then endogenous peroxidases were destroyed in 0.1 M PB containing 3% hydrogen peroxide. Non-specific binding was blocked with 3% normal horse serum. The sections were then incubated with the primary antibody (anti-rabbit Iba-1, Wako, 1:10,000) in 0.1 M PB containing 1% horse serum, 0.1% bovine serum albumin (BSA) and 0.3% Triton-X for 48 h at 4 °C. Sections were then rinsed, incubated in the corresponding secondary antibody (Amersham donkey anti-rabbit, 1:300) in phosphate buffered horse serum for 2 h, rinsed, incubated in 0.1% extravidin peroxidise for 1 h, and then rinsed again. The reaction was detected under a microscope after applying 2% nickel sulphate in 0.1 M PB containing 0.05% 3,30-diaminobenzidine, and was stopped and rinsed with 0.1 M PB once optimal staining with minimal background labelling had been achieved. Sections were then mounted onto chrome alum coated slides, dehydrated using a series of graded alcohols (70%, 95%, 100%, absolute), cleared in xylene and coverslipped with ultramount (Fronine Laboratory Supplies, Australia).

Iba-1 immunolabelling data was analysed by an experimenter blind to the experimental conditions. Images from hippocampal and amygdala regions were taken using an Olympus BX51 microscope fitted with an Olympus DP71 camera and an Olympus UPlan-Fi objective (10×/0.30). The images were processed using DP Manager software (Version 3.1.1.208; Olympus Corporation) and stored at a resolution of 4086 × 3072 pixels (1 pixel = 0.429 μm^2

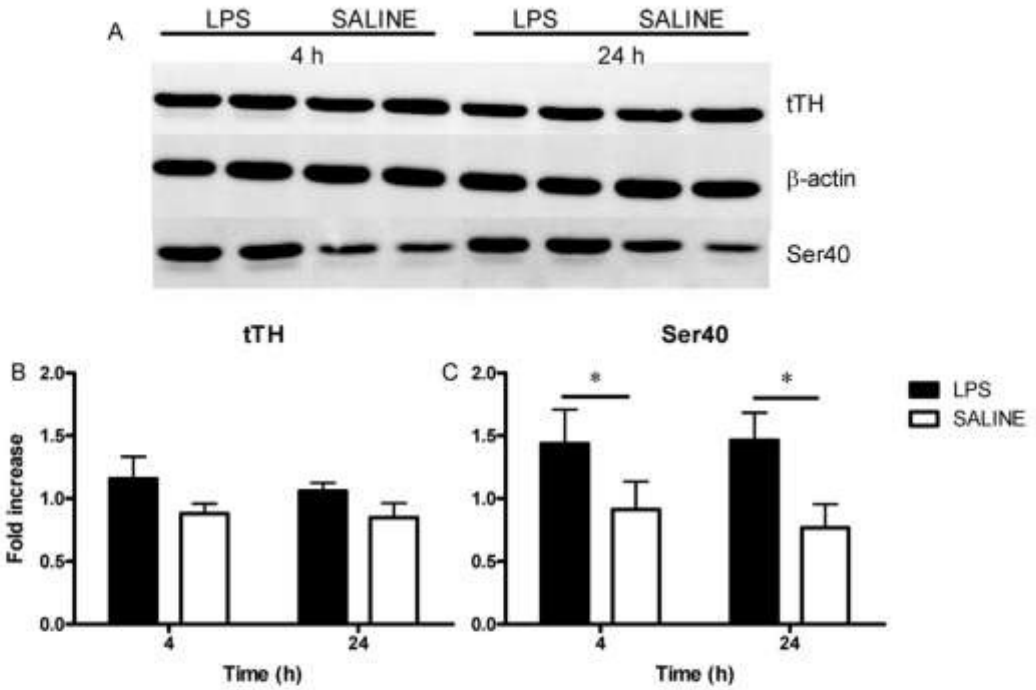

Fig. 1. Effect of neonatal LPS exposure on total tyrosine hydroxylase (TH) protein (±SEM) with respect to β-actin and the phosphorylation of serine (Ser) residue 40 of TH with respect to total TH in the adrenal gland 4 and 24 h after the administration of LPS or saline. (A) Representative immunoblots show the effect of LPS and saline. (B) Compared with saline, LPS had no effect on the TH protein at anytime point. (C) Phosphorylation of Ser40 occurred at 4 h and was sustained at 24 h. Filled bars represent neonatally challenged LPS males ($n = 6$ at each time point) and hollow bars represent neonatally challenged saline males ($n = 6$ at each time point). $^*p < .05$.

at 100× magnification). A rat brain atlas [23] was used to identify the anatomical location for each of the 3 regions of interest for Iba-1 immunolabelling, specifically, the CA1 region and the dentate gyrus of the hippocampus, and basolateral amygdala (CA1, DG and BLA, three sections, bregma −2.12 to −2.56 mm). In each of the regions, left and right hemispheres were recorded independently to assess inter-hemispheric asymmetries. The total density of immunoreactive material in the aforementioned regions for Iba-1 and Iba-1 density restricted to the soma region were determined using Metamorph software (Version 7.1.3.0; Molecular Devices). This program has been successfully employed to determine levels of Iba-1 immunolabelling previously [15].

Statistical analyses were conducted using the Statistical Package for the Social Sciences for Windows, Volume 18 (SPSS Inc.). All data were analysed using analyses of covariance (ANCOVA) controlling for litter effects such as litter size and male-to-female

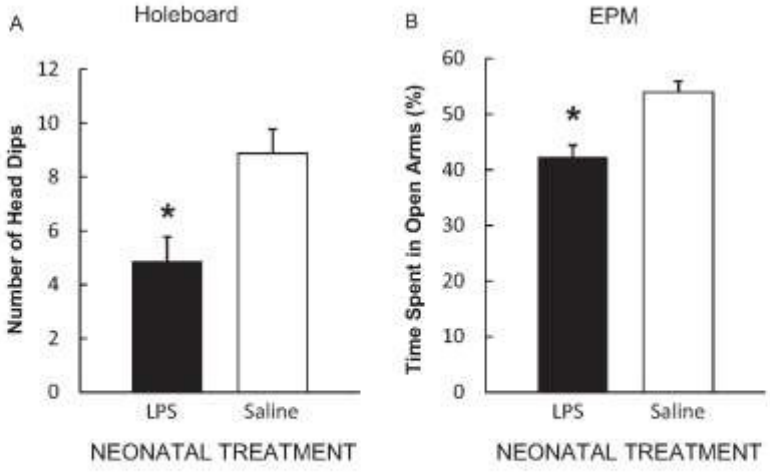

Fig. 2. Effect of neonatal LPS exposure on anxiety-related behaviour in adulthood. (A) LPS-treated males spent significantly less time (%; ±SEM) on the open arms of the EPM compared to saline-treated controls. The filled bar represents neonatally challenged LPS males ($n = 6$) and the hollow bar represents neonatally challenged saline males ($n = 5$). $^*p < .05$. (B) LPS-treated males exhibited significantly fewer exploratory head dips compared to saline-treated controls. The filled bar represents neonatally challenged LPS males ($n = 6$) and the hollow bar represents neonatally challenged saline males ($n = 6$). $^*p < .05$.

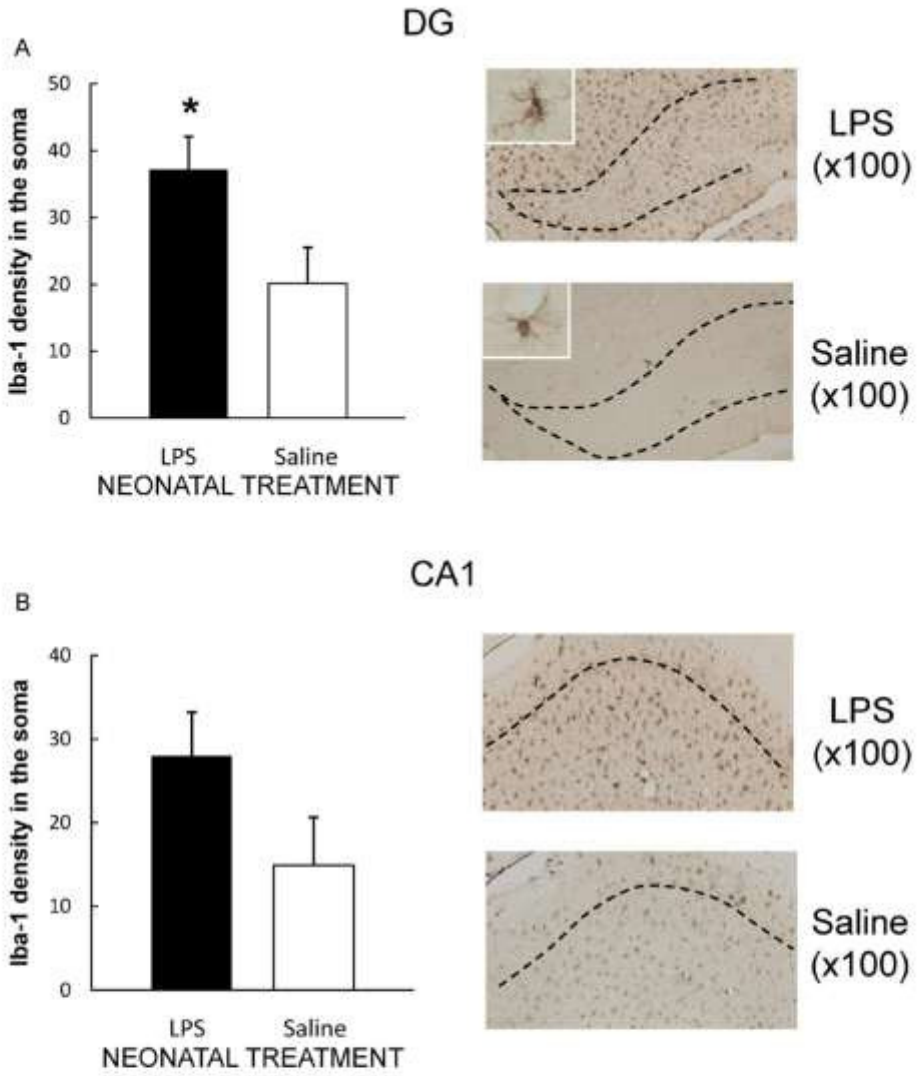

Fig. 3. Effect of neonatal LPS exposure on microglial activation in the hippocampus. (A and B) LPS-treated males ($n = 6$) exhibited higher Iba-1 immunolabelling in the dentate gyrus and CA1 compared to saline-treated ($n = 5$) controls, $^*p < .05$. Filled bars represent neonatally challenged LPS males and hollow bars represents neonatally challenged saline males. Representative photomicrographs exemplify greater Iba-1 immunolabelling in the dentate gyrus and CA1 of LPS-treated males compared to saline-treated controls.

ratio. The ANCOVA revealed that litter effects did not have an impact on any of the behavioural or physiological measures assessed.

LPS-treated males gained significantly more weight between PND 3 and PND 5 (M = 2.89 g, SEM = 0.08) compared to saline-treated controls (M = 2.55 g, SEM = 0.09), $F(14,70) = 9.17$, $p < .001$. No differences in weight gain were observed following weaning.

LPS-treated males exhibited significantly greater corticosterone concentrations 4 h following neonatal treatment on PND 5 (M = 19.84 ng/ml, SEM = 0.91) compared to saline-treated controls (M = 16.82 ng/ml, SEM = 1.12), $F(1,43) = 4.43$, $p < .05$.

While no differences were observed in regards to total TH protein levels, a significant and sustained increase in TH phosphorylation at Ser40 was evident in LPS-treated males 4 h and 24 h following injection on PND 5 compared to saline-treated controls, $F(1,21) = 6.19$, $p < .05$. See Fig. 1.

LPS-treated males spent a significantly lower proportion of time in the open arms of the EPM compared to saline-treated controls, $F(1,7) = 15.92$, $p < .05$. A main effect of neonatal treatment was observed in regards to exploratory head dips in the holeboard apparatus, $F(3,8) = 5.58$, $p < .05$. LPS-treated males exhibited significantly fewer head dips compared to saline-treated controls. See Fig. 2A and B.

A significant effect of neonatal treatment was observed for Iba-1 immunolabelling in the dentate gyrus, $F(1,9) = 5.28$, $p < .05$, whereby LPS-treated males exhibited significantly greater density of Iba-1 within the cell body of the microglia compared to saline-treated controls (Fig. 3A). Trends for Iba-1 immunolabelling of CA1 reflected that of the dentate gyrus, whereby LPS-treated males exhibited increased density of Iba-1 immunolabelling restricted to the soma region compared to saline-treated controls, however this failed to reach significance ($p = .1$; Fig. 3B). No difference in

total density of Iba-1 immunolabelling across the entire image was observed for either the dentate gyrus or CA1. No significant differences were observed in regards to Iba-1 immunolabelling within the soma region nor across the entire image in the basolateral amygdala.

Early life is exquisitely sensitive to environmental inputs. In particular, interaction with the early postnatal microbial environment is essential for setting the tone of both the endocrine and immune systems. Dual exposure to LPS during neonatal life is the most well characterized model of postnatal bacterial exposure, and has been extensively used to examine immune-neuroendocrine communication. We and others have previously shown that this dynamic interplay in early life determines the trajectory of a range of physiological and behavioural responses. Here, we have demonstrated that dual postnatal LPS exposure protocol produces an increase in anxiety-like behaviour that is co-incident with an increase in levels of microglial activation.

In the present study, we demonstrated that LPS challenge in the neonate robustly increased corticosterone release and TH phosphorylation in the adrenal medulla, the former measure indexing engagement of the hypothalamic-pituitary-adrenal (HPA) axis and the later the sympathetic nervous system. In adulthood, LPS challenged neonates exhibited increased anxiety-like behaviour on the EPM and Holeboard apparatus, evidenced by reduced time spent in the open arms of the EPM and a reduction in exploratory head dips in the Holeboard apparatus. Both the EPM and Holeboard are widely used to assess anxiety-like behaviour in rodents, and we have previously found that this dual LPS exposure protocol increases anxiety-like behaviour as demonstrated by these standard behavioural measures [8–10]. The particularly novel finding reported in the current study is the co-incident increase in microglial activation, as indicated by greater Iba-1 immunolabelling. Increase in microglial density following LPS challenge has been shown to be associated with prolonged activation and proliferation of microglia [24]. While our findings do not unequivocally implicate activated microglia in playing a central role in the enhanced anxiety observed, it is worthwhile to note that microglial activation can potently influence neuronal signalling [25].

Interestingly, changes in microglial activation were observed within the dentate gyrus but not as we had expected in the basolateral amygdala. Despite the absence of obvious changes in microglial activity within the BLA, functional alterations within the dentate gyrus have been linked with enhanced levels of anxiety [26–28]. While it is difficult to fully account for the regional differences in microglial activation observed, one possibility relates to the timing of the LPS challenge in relation to CNS development. In the current study, exposure to LPS occurred during the critical stress hyporesponsive period of development for the HPA axis, of which the hippocampus plays a fundamental role [29,30]. The increases in microglial activation in the hippocampus may reflect a pronounced susceptibility of primary HPA axis-related regions, of which the amygdala is involved but less influential. However, examination of microglial activation should be extended in the future to additional amygdaloid anxiety-related regions, such as the bed nucleus of the stria terminalis, in order to fully account for the discrepancies found in this study. Finally, it should be noted that while Iba-1 is a commonly used marker for microglia, it also stains for macrophages. However, it is generally accepted that microglia predominantly exist within the parenchyma, whereas macrophages are mostly considered to be a hematogenous cell type [31]. Thus, one can be relatively confident that the current findings in the hippocampus and BLA pertain almost solely to microglia.

The current study has provided insight into the complex interaction between the brain and immune system during early life of which microglia clearly play an interesting and intriguing role. Of importance are the seemingly divergent changes in microglial activation in regions of the hippocampus. These findings may represent potential mechanisms for the well-documented increases in anxiety-like behaviour in animals neonatally exposed to LPS. Certainly the evidence demonstrating that increased microglial activation in the hippocampus corresponds with induced anxiety and stress responses [32,33] lends credence to this assertion. However, it is clear that while both increased microglia and increased behavioural regulation in adulthood occur following neonatal LPS challenge, their contributions have not been directly linked to one another. Previous research investigating mechanisms in the generation of anxiety-like behaviour and microglial activation revealed potential pathways, by means of pharmacological intervention. Inhibition of IL-1β synthesis, in neonatally infected rats, prevented cognitive impairments induced by subsequent LPS challenge in adulthood, implicating the important role of IL-1β in the context of neonatal programming by immunological challenge [34]. Another study showed that increased anxiety-like behaviour, microglial activation and central levels of IL-1β induced by social defeat can be prevented by administration of propranolol – a β-adrenergic receptor antagonist. Moreover, IL-1 receptor type-1 deficient mice did not exhibit anxiety-like behaviour or microglial activation in the same context [35]. These latest findings indicate the changes induced by social defeat were dependent on activation of β-adrenergic and IL-1 receptors. Investigation of these and other pathways (such as GABA) in our model of postnatal LPS exposure can shed light on the mechanisms mediating behavioural changes in adulthood.

Conflict of interest

The authors declare there are no conflicts of interest.

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Neonatal respiratory infection and adult re-infection: Effect on glucocorticoid and mineralocorticoid receptors in the hippocampus in BALB/c mice

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Neonatal respiratory infection and adult re-infection: Effect on glucocorticoid and mineralocorticoid receptors in the hippocampus in BALB/c mice

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ABSTRACT

Stressful events during the perinatal period in both humans and animals have long-term consequences for the development and function of physiological systems and susceptibility to disease in adulthood. One form of stress commonly experienced in the neonatal period is exposure to bacterial and viral infections. The current study investigated the effects of live *Chlamydia muridarum* bacterial infection at birth followed by re-infection in adulthood on hippocampal glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) and stress response outcomes. Within 24 h of birth, neonatal mice were infected intranasally with *C. muridarum* (400 inclusion-forming units [ifu]) or vehicle. At 42 days, mice were re-infected (100 ifu) and euthanized 10 days later. In males, infection in adulthood alone had the most impact on the parameters measured with significant increases in GR protein compared to adult infection alone; and significant increases MR protein and circulating corticosterone compared to other treatment groups. Neonatal infection alone induced the largest alterations in the females with results showing reciprocal patterns for GR protein and TH protein. Perinatal infection resulted in a blunted response following adult infection for both males and females across all parameters. The present study demonstrates for the first time that males and females respond differently to infection based on the timing of the initial insult and that there is considerable sex differences in the hippocampal phenotypes that emerge in adulthood after neonatal infection.

1. Introduction

The impact of early-life insults on adult physiology is well established (McMillen and Robinson, 2005; Strachan, 1989). Events such as infection, dietary restriction or psychological stress have the capacity to detrimentally affect adult immune and endocrine systems as well as behavior (Buitelaar et al., 2003; McGowan et al., 2008; Strachan, 2000). Epidemiological studies have demonstrated that early-life stress leads to unfavorable outcomes for adult pathology (Barker, 1995; Barker and Bagby, 2005; McMillen and Robinson, 2005). An increasing body of evidence indicates that perinatal events involving the immune system may contribute to the development of adult disease (Hall and Peckham, 1997). Furthermore, the immune system in the neonatal period is not fully developed (Garvy, 2003) and consequently the neonate is more at risk of infection than an adult, with newborns particularly susceptible to bacterial and fungal infections (Garvy, 2003). Nevertheless, the long term impact of early-life infection and its effects on adult health are poorly understood. Therefore, it is important to examine the impact of pre- and post-natal infection on adult physiology and to determine the potential mechanisms that may lead to subsequent disease.

The hippocampus plays a central role in the modulation of the hypothalamic–pituitary–adrenal (HPA) axis by tonic inhibition. A decrease in glucocorticoid receptors (GRs) in the hippocampus attenuates the effects of circulating glucocorticoids leading to the HPA response being terminated (Herman et al., 1996; Jacobson and Sapolsky, 1991; Sapolsky et al., 1984). It has been shown that dysregulation of the HPA axis can result from changes to hippocampal GR and mineralocorticoid receptor (MR) abundance (Liu et al., 2001). Perinatal stress has been shown to alter morphology and function in the hippocampus and modify the adult stress response in a number of animal models that have used mice (Macri et al., 2009; Macrì S et al., 2009), rats (Szuran et al., 2000; Zhu et al., 2004), guinea pig (Matthews, 1998; Owen and Matthews, 2007; Setiawan et al., 2007), sheep (Sloboda et al., 2008) and monkey (Tauber et al., 2006). Typically, early-life stress is linked to adult offspring that exhibit stress hyper-responsiveness

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due to altered ontogeny of the HPA axis (Kapoor et al., 2006). Perinatal stress results in altered GR and MR expression and function in the brain as well as altered responsivity to stress (Avishai-Eliner et al., 2001; Kapoor et al., 2008; Levitt et al., 1996; Sutanto et al., 1996; Tuchscherer et al., 2004; Vallee et al., 1999). One form of stress commonly experienced in the neonatal period is exposure to bacterial and viral infections. Exposure to infection during the perinatal period in both humans and animals has long-term consequences for the development and function of physiological systems and susceptibility to disease in adulthood (Brown, 2006; Nelson and Willoughby, 2000; Ozawa et al., 2006; Shi et al., 2009). For example, early-life infection with Chlamydia, a common respiratory pathogen, increases the severity of subsequent allergic airways disease (Horvat et al., 2007, 2010). Other animal studies demonstrate that neonatal exposure to the bacterial lipopolysaccharide (LPS) affects reactivity to stress, immune regulation and susceptibility to disease in adulthood (Hodgson et al., 2001; Shanks et al., 2000). These effects are not limited to LPS as polyriboinosinic–polyribocytidilic acid (Ellis et al., 2006) and live *Escherichia coli* (Bilbo et al., 2005) elicit similar consequences. It is also becoming increasingly apparent that the outcomes of pre- and post-natal events are dependent on the sex of the individual (Avitsur et al., 2006; Clifton, 2005; Kikusui et al., 2005; Zhu et al., 2004).

To date no studies have investigated the effects of neonatal infection-induced stress on GRs and MRs in the adult hippocampus. Given the integral role of the hippocampus in regulating the stress response (Kretz et al., 2001), investigating the potential effects of infection in early-life on programming of hippocampal GR and MR responses in different sexes will facilitate the elucidation of the mechanisms that underpin the detrimental programming effects of the early-life environment. The current study aimed to assess the outcomes of early-life and subsequent adult lung infection with *Chlamydia muridarum* on hippocampal GRs and MRs in the adult hippocampus. *C. muridarum* is an intracellular bacterium, a natural mouse pathogen and an appropriate strain for the investigation of natural host-pathogen interactions. The temporal, histological and immunological progression of disease mirrors reactions observed in human *Chlamydia pneumoniae* (Adkins et al., 1993; Hansbro et al., 2004; Horvat et al., 2007). Like any infection, bacterial exposure in the mouse causes HPA axis activation resulting in increased systemic glucocorticoid concentrations (Ruzek et al., 1999). Thus, neonatal exposure to *C. muridarum* in the mouse represents a valid model of both infection and physiological stress exposure. We hypothesized that animals exposed to postnatal infection would have altered GR and MR mRNA and protein. Given the causal effect of GR and MR expression on circulating corticosterone and adrenocorticotropic hormone (ACTH), we expect infection to impact on adult levels of those hormones. Infection is a stressor which also affects sympathetic nervous system (SNS) parameters including catecholamine levels. Adrenal tyrosine hydroxylase (TH) is the rate-limiting enzyme in catecholamine biosynthesis (Nagatsu et al., 1964) and gives a stable indication of catecholamine activity; as such we expect increases in TH after infection.

2. Methods

2.1. Animals

BALB/c mice were housed in an accredited animal care facility under specific pathogen free conditions and were obtained from the animal services unit and used with approval from the animal ethics committee, University of Newcastle (Newcastle, Australia). Dams were left undisturbed until birth of the young. Within 24 h of birth, offspring were infected with Chlamydia or sham infected and then returned to their home cage until weaning. Offspring are weaned and sexed at 21 days of age, whereupon male and female mice are separated and housed in groups no larger than six. Mice are monitored on a daily basis and are immediately separated following any evidence of aggressive interaction, regardless of sex. Animals were given access to food ("Rat and Mouse Pellets", Specialty Feeds, Glen Forrest, Western Australia) and tap water *ad libitum*, and housed either per litter (mothers with young) or in groups no larger than six (adult and weaned young) under controlled environmental conditions with a 12:12 h light:dark cycle, with lights on at 6 am. Samples were taken from an experimental pool in which whole litters were assigned to a treatment condition. Litter sizes were between two and 10 mice. Mice were weaned and sex separated, with animals of the same sex combined from different litters in groups no bigger than six. Individual litters were not kept separate. The experimental pool consisted of a mix of mice from 38 litters of different sizes with 8–14 mice in each treatment and sex condition. Approximately nine litters were used for each condition excluding the infection at birth alone group which was made from 11 litters. Non-infected mice were not litter mates of infected mice.

2.2. Infection

C. muridarum strain ATCC VR-123 (formerly known as the mouse pneumonitis biovar of *Chlamydia trachomatis*) was obtained from the American Type Culture Collection (ATCC, Virginia, USA). Stocks were cultured and stored at −80 °C in sucrose-phosphate-glutamate buffer (SPG) (Horvat et al., 2007, 2010). Within 24 h of birth, neonatal mice were infected intranasally with *C. muridarum* without anesthesia. Mice were held in the upright position and the inoculum (400 inclusion-forming units [ifu]) in 5 μl of SPG was gently pipetted onto the nares until the whole inoculum was inhaled (Horvat et al., 2007, 2010). The neonates were separated from the dam for between 2 and 3 min during the infection protocol. At 42 days of age mice were infected intranasally with *C. muridarum* (100 ifu in 30 μl of SPG). Control groups for infection were sham-infected with SPG. At 52 days old mice were euthanized by sodium pentobarbital overdose (Abbott Australasia, Kurnell, Australia). No animals died due to the infection protocol. We have previously shown that infection peaks between 10 and 15 days after inoculation, bacterial clearance occurs by 21 days and pulmonary inflammation is largely resolved by 45 days (Horvat et al., 2007). The current protocol is one of chronic infection, rather than the standard acute infection modeled when using endotoxin. The brain was removed and placed in RNA later solution (Applied Biosystems/Ambion, Austin, TX, USA) and stored at −80 °C. Blood was collected by cardiac puncture, stored on ice for no more than 1 h, and then serum was collected by centrifugation (18,000g, 10 min) and stored at −20 °C until assayed.

2.3. RNA/DNA/protein extraction from hippocampus

The whole hippocampus was dissected from the rest of the brain then RNA and DNA extracted using a RNA/DNA kit according to the manufacturer's instructions (Qiagen, Doncaster, VIC, Australia), with the following modifications to the protocol to allow for the parallel extraction of protein (Tolosa et al., 2007). From the first elution step all flow-through was collected. Once extraction was complete, 2 ml acetone was added and allowed to precipitate overnight at −20 °C. The solution was then centrifuged (10,000g, 30 min, 4 °C), the supernatant removed and the pellet re-suspended in 300 μl 2D buffer (30 mM Tris; 7 M urea; 2 M thiourea; 4% CHAPS). Again acetone (1200 μl) was added and the solution was left to precipitate for 2 h at −20 °C before centrifugation. The protein pellet was then re-suspended in 60 μl 2D buffer. RNA concentration was assessed by spectrophotometer (NanoDrop 3300,

Thermo Scientific, Wilmington, DE, USA). Protein was determined using a 2D quantitation kit following the manufacturer's instructions (GE Healthcare, Piscataway, NJ, USA).

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

Analysis of GR and MR mRNA was carried out using quantitative real-time polymerase chain reaction (qRT-PCR). RNA was purified and DNase treated using RNeasy mini kits and RNase-free DNase sets (Qiagen) then reverse transcribed using Superscript III first-strand kits following the manufacturer's instruction (Invitrogen, Mount Waverly, VIC, Australia). qRT-PCR was performed using Sybrgreen PCR master mix (Applied Biosystems, Foster City, CA, USA) and an ABI prism 7700 sequence detector (Applied Biosystems). Primer sequences were as previously published, with β actin as the reference gene (Berry et al., 2004; Muller et al., 2007). Products from pooled sample were sequenced at an external facility (Australian Genome Research Facility, St Lucia, Australia) to confirm specificity.

2.5. Hippocampal protein analysis

Hippocampal protein was assayed using western blot. Proteins (20 μg per well) were separated on 7% tris–acetate gels (Invitrogen), then transferred onto Hybond C Extra nitrocellulose membrane (GE Healthcare). Membranes were left to dry overnight and stored at 4 °C. Membranes were blocked in 5% bovine serum albumin for one hour, washed with Tris-buffered saline with Tween (TBS-T, 150 mM NaCl; 10 mM; Tris 0.75%; Tween-20; pH 7.5) and incubated overnight with primary antibody for GR (ab2768, Abcam, Cambridge, MA, USA), MR (H-300, Santa Cruz, Santa Cruz, CA, USA) and loading control actin (ab8227, Abcam). Membranes were then washed 5 × 5 min in TBS-T and incubated with secondary antibody (Goat anti-rabbit, Millipore, Billerica, MA, USA; Rabbit anti-mouse, Abcam) for one hour followed by 5 × 5 min washes in TBS-T. Proteins were visualized using electrochemiluminescence (ECL) western blotting detection reagents (GE Healthcare) on Fujifilm Las-3000 imaging system (Fuji, Stamford, CT, USA) and quantified using densitometry and MultiGauge V3.0 software (Fuji).

2.6. Extraction and Western blot of protein from adrenal glands

The adrenals were homogenized using a sonicator (Soniprep 150, MSE, London, UK) in homogenization buffer (2% SDS; 2 mM EDTA; 50 mM Tris; pH 6.8) with ratio 1 mL of buffer per 25 mg tissue. Samples were then centrifuged (10,000g 20 min, 4 °C). The clear supernatants were collected and 200 μl was mixed with 70 μl of sample buffer (40% glycerol; 50 mM Tris; minimal bromophenol blue; pH 6.8) and 10 μl of 20% dithiothreitol. Samples were subjected to SDS–polyacrylamide gel electrophoresis before being transferred to nitrocellulose (Jarvie and Dunkley, 1995). Membranes were then stained with Ponceau S (0.5% ponceau in 1% acetic acid) to assess the efficacy of the transfer, before being washed in TBS-T and incubated with blocking solution (5% bovine serum albumin, 0.04% sodium azide in TBS-T for 2 h at 25 °C). Membranes were washed in TBS-T and incubated with total-TH antibody for 1 h at 25 °C. Membranes were washed in TBS-T and incubated with anti-rabbit immunoglobulin (horseradish peroxidase-linked whole antibody from donkey) for 1 h at 25 °C. Membranes were visualized on Fujifilm Las-3000 imaging system (Fuji) using ECL plus detection reagents (GE Healthcare). The process was repeated with β-actin and secondary antibody anti-mouse immunoglobulin (horseradish peroxidase-linked whole antibody from sheep). The densities of total-TH and β-actin bands were measured using a MultiGauge V3.0 (Fuji).

2.7. Radioimmunoassay

Plasma corticosterone levels were determined by radioimmunoassay using corticosterone ^{125}I radioimmunoassay kits according to the manufacturer's instructions (MP biomedicals, Seven Hills, NSW, Australia). The reported recovery of exogenous corticosterone is 100% and the intra- and inter-assay coefficients of variation were lower than 8% and 10%, respectively. Plasma ACTH concentrations were assessed using a double antibody ACTH ^{125}I radioimmunoassay kit (MP Biomedicals). The recovery of exogenous ACTH is 100%, with an inter- and intra-assay variability of 3.9% and 6.8%, respectively.

2.8. Data analysis

All data were analyzed using a between subjects three way ANOVA using SPSS software (V18, SPSS Inc., Chicago, IL, USA), with the factors being Treatment at birth (control and infection), Treatment in adulthood (control and infection) and Sex (male and female). Data for GR mRNA, GR protein, TH protein and corticosterone were log transformed as residual plots revealed the data were not normally distributed. For all comparisons α was set at $p < 0.05$. Planned comparisons were conducted using pairwise comparisons of means when significant interactions were observed. Figures show means ± standard error of the means on the original scale of the data. For the logged variables the means and standard error bars were obtained by back transformation from the log scale by exponentiation.

3. Results

3.1. GR and MR gene expression

Hippocampal GR mRNA abundance for adult animals neonatally infected with *C. muridarum* (CMU) or sham infected (SPG) and subsequently challenged with CMU (SPG/CMU and CMU/CMU) or SPG (SPG/SPG and CMU/SPG) in adulthood was assessed. Analysis was conducted using a three way ANOVA and revealed no significant main effects or interaction effects due to Treatment at birth, Treatment in adulthood or Sex on GR mRNA abundance (data not shown).

A significant interaction of treatment at birth and treatment in adulthood was observed for hippocampal MR mRNA, F (1, 55) = 6.295, p = .015. Pairwise comparisons showed that within the SPG at birth group, CMU infection in adulthood significantly increased MR mRNA expression compared to SPG, p = .008 whereas for the CMU infected at birth group, adult infection with CMU resulted in no change compared to SPG, p = .38 (see Fig. 1).

3.2. GR and MR protein expression

Fig. 2 shows the expression of GR protein relative to actin as determined by Western blot. A significant Sex by Treatment at birth interaction was observed for GR protein, F (1, 67) = 7.271, p = .009. Planned comparisons revealed that the SPG at birth treated females had significantly less GR protein in adulthood than males of the same condition, p = .02. While for CMU treatment at birth group resulted in no difference between the sexes, p = .15.

The between subjects ANOVA conducted on hippocampal MR protein revealed a main effect of Treatment in adulthood, F (1, 60) = 8.578, p = .005; significant interactions between Sex and Treatment at birth, F (1, 60) = 8.462, p = .005 and Sex and Treatment in adulthood, F (1, 60) = 6.135, p = .016. There was also a significant three way interaction, F (1, 60) = 4.773, p = .033. Follow up tests to elucidate the three way interaction was focused on sex

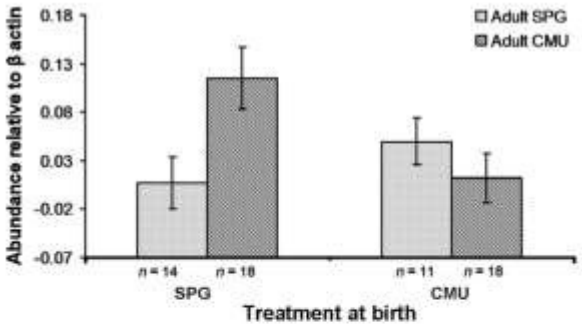

Fig. 1. Hippocampal MR mRNA abundance for mice neonatally infected with *C. muridarum* (CMU) or sham infected (SPG) and subsequently challenged with CMU (SPG/CMU and CMU/CMU) or SPG (SPG/SPG and CMU/SPG) in adulthood. A two way ANOVA using Treatment at birth and Treatment in adulthood as the factors showed only the interaction to be significant. Within the SPG at birth group, CMU infection in adulthood significantly increased MR mRNA expression compared to SPG, whereas for the CMU infected at birth group, adult infection with CMU resulted in no change compared to SPG.

differences (see Fig. 3). Males treated with SPG at birth and CMU in adulthood had significantly more MR protein than the females in the same condition, $p < .001$. For the remaining three combinations only males treated with CMU at birth and SPG in adulthood had significantly less MR protein compared to females in the same condition, $p = .046$.

3.3. *Adrenal tyrosine hydroxylase*

Adrenal TH protein in animals neonatally infected with *C. muridarum* (CMU) or sham infected (SPG) and subsequently challenged with CMU (SPG/CMU and CMU/CMU) or SPG (SPG/SPG and CMU/SPG) in adulthood was assessed (Fig. 4). The between subjects three way ANOVA revealed a significant main effect of Treatment at birth, $F(1, 40) = 5.56$, $p = .023$, and Treatment in adulthood, $F(1, 40) = 9.248$, $p = .004$; with a significant interaction of Sex and Treatment in adulthood, $F(1, 40) = 4.578$, $p = .039$. The three way interaction of Sex, Treatment at birth and Treatment in adulthood was also significant, $F(3, 40) = 3.76$, $p = .018$. When focusing on the sex comparisons, females with infection at birth alone (CMU/SPG)

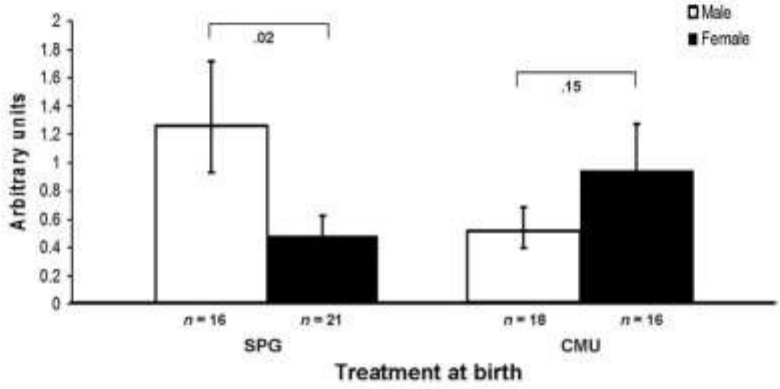

Fig. 2. Hippocampal GR protein relative to actin for mice neonatally infected with *C. muridarum* (CMU) or sham infected (SPG) and subsequently challenged with CMU (SPG/CMU and CMU/CMU) or SPG (SPG/SPG and CMU/SPG) in adulthood. The between subjects three way ANOVA revealed a significant effect of the interaction between Sex and Treatment at birth. The pattern of GR expression is sexually differentiated, with males treated with SPG at birth having significantly more GR protein than same treatment females. While not significant, the pattern of GR protein expression is reversed in animals infected with CMU at birth.

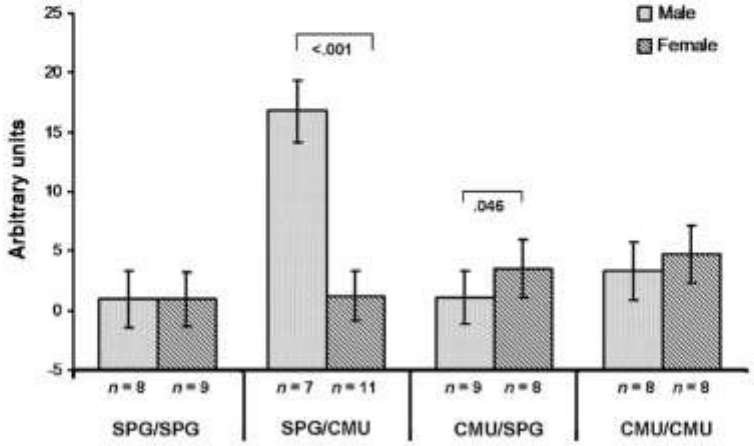

Fig. 3. Hippocampal MR protein relative to actin for mice neonatally infected with *C. muridarum* (CMU) or sham infected (SPG) and subsequently challenged with CMU (SPG/CMU and CMU/CMU) or SPG (SPG/SPG and CMU/SPG) in adulthood. Males have more MR protein in the adult infection alone group (SPG/CMU) compared to all other treatment groups. Females show no alteration of MR protein due to treatment.

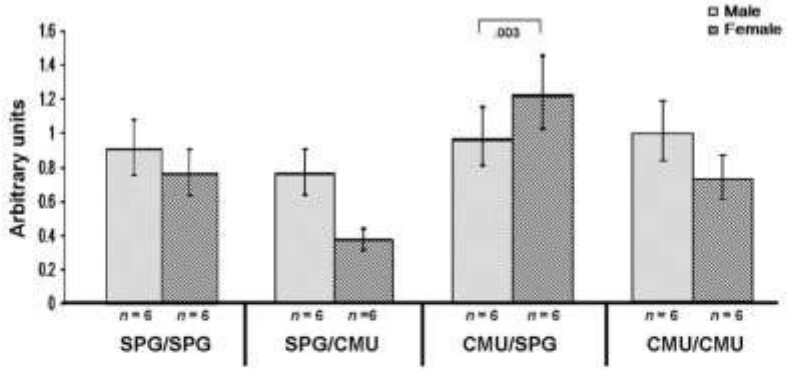

Fig. 4. Adrenal TH protein relative to β-actin for mice neonatally infected with *C. muridarum* (CMU) or sham infected (SPG) and subsequently challenged with CMU (SPG/CMU and CMU/CMU) or SPG (SPG/SPG and CMU/SPG) in adulthood. When focusing on the sex comparisons, females with infection at birth alone (CMU/SPG) had significantly more TH protein than males in the same condition. While there were no significant differences for the other combinations, it is worth noting that for all groups except CMU/SPG, there is a pattern that the males had higher levels of TH protein than the females.

had significantly more TH protein than males in the same condition, $p = .003$. There were no significant differences for the other combinations. It is worth noting that for all groups except CMU/SPG, there is a pattern that males had higher levels of TH protein than females.

3.4. Circulating corticosterone

Circulating corticosterone for animals neonatally infected with *C. muridarum* (CMU) or sham infected (SPG) and subsequently challenged with CMU (SPG/CMU and CMU/CMU) or SPG (SPG/SPG and CMU/SPG) in adulthood was assessed and the three way ANOVA identified a significant main effect of Treatment at birth and a significant interaction of Treatment at birth and Treatment in adulthood. The model was reduced to a two way ANOVA revealing the main effect of Treatment at birth, $F(1, 66) = 7.041$, $p = .01$, and significant interaction between Treatment at birth and Treatment in adulthood, $F(1, 66) = 4.875$, $p = .031$. Pairwise comparisons showed that within the SPG at birth group, adult CMU infection resulted in significantly more circulating corticosterone than adult treatment with SPG, $p = .007$ (Fig. 5).

3.5. Adrenal weight

Analysis revealed no significant difference in adrenal weight due to Sex and/or Treatment at birth or Treatment in adulthood (data not shown).

3.6. Circulating ACTH

Plasma ACTH was not significantly altered in any of the groups due to Sex or Treatment at birth or Treatment in adulthood (data not shown).

4. Discussion

This is the first study to examine the effect of neonatal bacterial infection and adult re-infection on hippocampal corticosterone receptor expression. The results demonstrate that infection can

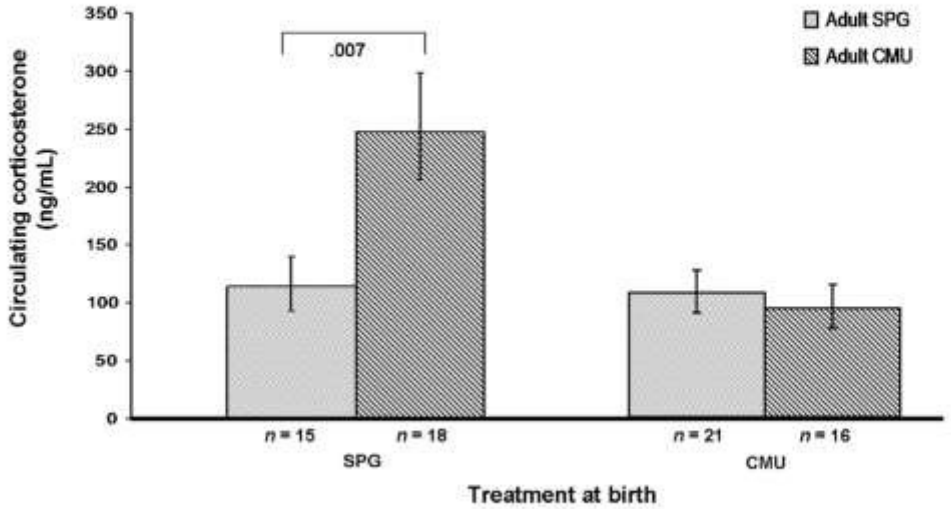

Fig. 5. Circulating corticosterone for mice neonatally infected with *C. muridarum* (CMU) or sham infected (SPG) and subsequently challenged with CMU (SPG/CMU and CMU/CMU) or SPG (SPG/SPG and CMU/SPG) in adulthood. A two way ANOVA revealed the main effect of Treatment at birth and significant interaction between Treatment at birth and Treatment in adulthood. Within the SPG at birth group, adult CMU infection resulted in significantly more circulating corticosterone than adult treatment with SPG. Within the CMU at birth group, the CMU in adulthood treated animals had significantly less circulating corticosterone than SPG at birth group.

alter hippocampal corticosterone receptors, adrenal TH and circulating corticosterone. Endocrine protein production has been shown to be sensitive to the cumulative effects of repeated infection as observed for corticosterone receptor and TH protein. We see no difference due to sex for MR mRNA and circulating corticosterone, suggesting that these factors may not be specifically sex regulated. Perinatal infection resulted in a blunted response following adult infection for both males and females across all parameters. The rate of infection is high in newborns due to their relatively immature, naive immune system and our results suggest that the neonatal neuroendocrine system is exquisitely sensitive to infection and can produce long term changes in its function.

Early-life events are known to modify the hippocampal-HPA axis. Neonatal stress has been shown to alter morphology and function of the hippocampus and adult stress responses in a number of animals, including the mouse (Macri et al., 2009; Macrì S et al., 2009), rat (Szuran et al., 2000; Zhu et al., 2004), guinea pig (Matthews, 1998; Owen and Matthews, 2007; Setiawan et al., 2007), sheep (Sloboda et al., 2008) and monkey (Tauber et al., 2006). While no other studies have used live infection, our results support previous work showing that neonatal stress results in significant and enduring alteration of hippocampal GR and MR protein. Generally, in the adult after exposure to perinatal stress, such as endotoxin or synthetic glucocorticoids, there are decreases in GR and MR with increases in corticosterone and ACTH (Hodgson et al., 2001; Kapoor et al., 2006, 2008; Kapoor and Matthews, 2005; Shanks et al., 1995; Ward et al., 2000). Thus, the perinatal period is a critical determinant of hippocampal and HPA function across the life span, with alterations potentially accounting for increased susceptibility to a number of adverse health conditions.

In the present study, the males in all groups exhibited altered patterns for GR and MR protein expression suggesting that infection at any time may affect the corticosterone receptors. Previous studies using perinatal immune stressors have found decreases in corticosterone receptors and increases in corticosterone in the adult (del Rey et al., 1996; Meaney et al., 1996; Reul et al., 1994; Shanks et al., 2000). However, the current results show infection at adulthood alone induced significant increases in circulating corticosterone in both males and females with significant increases in hippocampal GR and MR protein in the males alone. Corticosterone and GR are negatively correlated, and the occupation of GR by corticosterone in the hippocampus results in the cessation of the HPA axis-driven stress response. The results of the current study suggest that males display a HPA axis response with a reduced capacity for termination following adult infection. The inconsistency in findings compared to previous research may be due to the current study using live bacteria as opposed to endotoxin, in addition to the differences in timing of neonatal infection. Studies using endotoxin generally use a protocol of repeated exposure at three and five days of age (i.e. Shanks et al., 2000; Walker et al., 2009a,b), while the present study utilized one persistent infection initiated within 24 h of birth. The advantage of the current protocol is that we have modeled the real-world conditions of a chronic live infection, rather than the acute infection that is modeled with endotoxin. Future experiments will aim to investigate this issue further.

Neonatal infection alone resulted in the biggest response in the females with reciprocal patterns for GR protein and TH protein observed in the CMU/SPG group. Few studies have examined hippocampal corticosterone receptors or TH in adult females after neonatal immune stimulus. One study using neonatal endotoxin exposure found increases to GR protein in the hypothalamus but no change to GR protein in the adult hippocampus (Nilsson et al., 2002). Another study found a decrease in hippocampal GR binding in the adult females following neonatal exposure to endotoxin (Shanks et al., 1995). Furthermore, to date no studies have examined adult adrenal TH in female animals after neonatal immune stress. Such a parameter should be investigated as TH is a reliable indicator of the activity of the animals' sympathetic–adrenomedullary system (SAS) (Haemisch and Gartner, 1996), the partner system to the HPA in generating the stress response. For the females, high basal levels of TH could be indicative of anxiety-like behavior (Ely and Henry, 1978), also hyper-activation of the SAS is related to the etiology of cardiovascular disease (Henry, 1982, 1992). Overall, the data indicate that further investigation of the parameters involved in the stress response of females is warranted, such as the potential immune modulating effects of estrogens.

Our sexually dimorphic findings have importantly demonstrated that it is insufficient to examine the stress biology of only one sex and attempt to extrapolate the results to the other. These findings are consistent with sexually differentiated observations in stress-behavior and indicate a gonadal hormone influence (Bale and Vale, 2003; Marcondes et al., 2001). Such sex differences extend to the perinatal stress paradigm. For example, one study using neonatal exposure to endotoxin showed evidence of behavioral sexual dimorphism in response to an adult immune challenge. Neonatally treated males demonstrated significantly less open field activity after adult treatment with endotoxin, while females showed no changes to behavior due to treatment or estrus cycle (Tenk et al., 2008). Adult re-exposure to immune stimulus also results in a sexually differentiated response, for example, female adult offspring having significantly lower basal leukocyte number after re-exposure to endotoxin compared to males (Hodyl et al., 2007). In general, females known to have higher glucocorticoid levels in response to HPA axis activation (Handa et al., 1994) regardless of the oestrous cycle (Spinedi et al., 1992). Primary targets for the cause of such differences are the sex hormones (Kudielka and Kirschbaum, 2005). Estrogen enhances immune responses (Cutolo, 1998; Cutolo and Wilder, 2000), whereas progesterone and androgens, such as testosterone and dehydroepiandrosterone (DHEA), suppress the immune response (Cutolo et al., 2003; Cutolo and Wilder, 2000).

The estrus cycle of the females was not assessed in the present study and may have been a factor in the results as the female response to stress has been shown to be affected by estrogen levels. For example, in response to the Trier Social Stress Test, men have a higher ACTH response to the stressor compared with women, but the plasma cortisol response is the same for both sexes (Kirschbaum et al., 1999; Young and Korszun, 2010). However, the female cortisol response is dependent on menstrual cycle phase (Young and Korszun, 2010; Young et al., 1991). This outcome appears to be due to more than just the influence of estrogens. The differences are also related to levels of progesterone, with decreases in progesterone resulting in cortisol suppression (Young et al., 1991). Estradiol does influence HPA functioning as it has been shown to decrease stress responsiveness in rodents (Young et al., 2001), sheep (Komesaroff et al., 1998) and humans (Komesaroff et al., 1999). Human studies suggest that estradiol inhibits the HPA axis response and progesterone impairs glucocorticoid negative feedback (Young and Korszun, 2010). As such, it would be necessary in the future to closely monitor the estrus cycle of the female animals.

In the current study, GR mRNA does not correspond to GR protein. This inconsistency may be attributable to a number of factors: (1) GR mRNA has not been shown to be directly indicative of GR protein due to factors such as translational modifications, preferential transcription of GR or mRNA stability. (2) GRs have many isoforms and its expression and transcription is tissue specific and still being investigated in the research (Duma et al., 2006). (3) In humans, GR heterogeneous nuclear RNA (hnRNA) has been shown to be directly related to cortisol, but modifications to mRNA and protein results in that relationship being lost (Hodyl et al.,

2010). Experiments to investigate this would include measuring hnRNA in addition to assessing various post-translational modifications such as phosphorylation. One interesting aspect of the results is that different corticosterone levels were detected in the SPG/CMU group for both males and females but no changes were detected for females after adulthood alone for any of the other parameters tested. Possible mechanisms underlying this discrepancy could be the interaction of sex hormones with immune and HPA axis functioning. Experiments to investigate the potential effects of estrogens would include, assessing levels of estrogens in addition to identifying stage in estrus cycle. Also, castration experiments with animals being castrated and then given sex hormones such as progesterone, estrogens and testosterone to both males and females to investigate a possible dose-dependent response. Another interesting result is that corticosterone increases in the SPG/CMU treatment group is associated with higher MR and GR expression in males. Generally, decreases in GR and MR in the brain result in an increase in circulating corticosterone due to the negative feedback mechanisms of the HPA axis (Dallman, 2000). It is possible that GR and MR affinity was reduced. Statistical tests showed that the incongruent expression of corticosterone receptors and corticosterone was not due to any ratio changes of GR and MR (data not shown). It is also possible that modifications to the GR protein impacted on glucocorticoid sensitivity, as we did not assess factors such as phosphorylation in the current study. GR phosphorylation has been shown to result in reduced GR binding affinity (Irusen et al., 2002). As such future studies will examine the potential effect of GR and MR post-translational modifications on binding affinity.

The current study shows that males and females respond differently to infection based on the timing of the initial insult. Males were relatively unaffected by a neonatal infection compared to a primary adult infection; while females were more affected by neonatal infection alone compared to other treatments. Different stressors can have diverse effects on the developing animal depending on the timing, duration and dose of the stress (Seckl, 2001). Animal studies have shown that prenatal programming can be altered by the immediate postnatal environment (Maccari et al., 1995; Seckl, 2001). In the rodent model, manipulations such as handling, which has negative effects for the offspring if the dam is handled during pregnancy, can be beneficial for the stress response if the handling takes place in the first few weeks of life (Meaney et al., 1989). Cross-fostering also reverses the effects of prenatal exposure to excess corticosterone (Maccari et al., 1995) and 'environmental enrichment' has been shown to normalize HPA activity and increase play behavior (Laviola et al., 2004; Morley-Fletcher et al., 2003). Furthermore, maternal behavior has been demonstrated to determine offspring outcomes following perinatal stress (Walker et al., 2004). During the current study, maternal behavior was not assessed, as such we can not rule out maternal care as a contributing factor. Therefore, future studies should assess maternal behavior in parallel with the offspring's functional and physiological parameters. Given the results of the current study, further work is needed on the paradigm, with sampling at successive time points after early-life infection in order to identify potential critical windows for intervention.

The early-life environment is a critical determinant of health outcomes in later life. The present study demonstrates for the first time that neonatal bacterial infection leads to alterations in the hippocampus in the adult and that there are sex differences in the hippocampal phenotypes that emerge in adulthood after perinatal stress. Furthermore, given the role of the hippocampus in the regulation of HPA function, the observations of the current study have implications for immune and endocrine function as well as stress responsivity.

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