Expression of Substance P and the Tachykinin NK1 Receptor in the Medullary Serotonergic Network of the Human Infant During Development; Implications for Sudden Infant Death Syndrome (SIDS)

Fiona Maree Bright

Ph.D. in Medicine

December 2016

A thesis submitted to the University of Adelaide in fulfillment of the requirements for the degree of Doctor of Philosophy
**Table Of Contents**

Thesis Declaration............................................................................................................7
Preface..............................................................................................................................8
Thesis Dedication................................................................................................................9
Acknowledgements...........................................................................................................10
Conference presentations related to thesis.........................................................................12
Glossary of terminology.....................................................................................................13-14
List of figures and tables...................................................................................................15-17
Introduction .....................................................................................................................18
Thesis research objectives and methods overview............................................................20-22

**Chapter 1: Literature review**

1.1 Significance and Epidemiology of SIDS....................................................................24-25
1.2 SIDS Definition and diagnosis...................................................................................26-27
1.3 Pathophysiology of SIDS overview...........................................................................28
1.4 Underlying vulnerability and the brainstem hypothesis...............................................29
1.5 Brainstem respiratory network overview.....................................................................30-31
1.6 Respiratory defence mechanisms and arousal failure in SIDS.......................................31-32
1.7 Putative role of multi-transmitter homeostatic network dysfunction in SIDS..............33-34
1.8 Neurotransmitters and the critical developmental period in SIDS...............................35-36
1.9 Medullary serotonergic (5-HT) network in SIDS........................................................37-39
1.10 Neuropeptide SP and SIDS......................................................................................40-45
1.11 Serotonin and substance p in SIDS..........................................................................46-49
1.12 Synopsis....................................................................................................................50

**Chapter 2. Normative distribution investigation**

*Normative distribution of substance p and its neurokinin-1 receptor in the medullary serotonergic network of the human infant during development*

2.1 Introduction.................................................................................................................54-55

2.2 Materials and methods

2.2.1 Clinicopathological database: Fresh frozen and fixed tissue ..................................55
2.2.2 Anatomical identification of nuclei within the medullary 5-HT network................56
2.2.3 Determination of SP specific binding density to NK1R in normal human infant medullae with $^{125}$I Bolton Hunter SP autoradiography.................................................................58
2.2.4 Quantitative analysis of brainstem autoradiograms………………………………………………58
2.2.5 Photomicrograph production………………………………………………………………………..58-59
2.2.6 Normative distribution of SP/NK1R relative to medullary 5-HT network in human infant using fixed tissue immunohistochemistry and immunofluorescence………………………………….59
2.2.7 Single label immunohistochemistry in formalin fixed paraffin embedded tissue………………59
2.2.8 Double label immunofluorescence in formalin fixed paraffin embedded tissue………………59-60
2.2.9 Image capture and processing……………………………………………………………………62

2.3 Results
2.3.1 $[^{125}]$I Bolton Hunter SP binding to NK1R in normal human infant medulla…………………62-63
2.3.2 Normative distribution of NK1R and SP immunoreactivity in human infant medulla using single label immunohistochemistry ………………………………………………………………..69
2.3.3 Normative distribution of NK1R and SP relative to 5-HT in medullary serotonergic network of human infant using double label immunofluorescence…………………………………..73

2.4 Discussion
2.4.1 Normative distribution of SP and NK1R immunoreactivity within human infant medulla………………………………………………………………………………………………….78
2.4.2 Normative developmental distribution of NK1R binding in the human infant medullary 5-HT network………………………………………………………………………………………..78-79
2.4.3 Distribution of SP and NK1R immunoreactivity relative to 5-HT in the medullary serotonergic network of human infant during development …………………………………………79-82

2.5 Conclusions……………………………………………………………………………………………..82

Chapter 3. SIDS vs. Controls investigation: Medullary SP/NK1R study

Developmental abnormalities in substance p neurokinin-1 receptor binding in brainstem nuclei related to prematurity and sex

3.1 Introduction…………………………………………………………………………………………………..86

3.2 Methods summary………………………………………………………………………………………..86-87

3.3 Results
3.3.1 $[^{125}]$I labelled SP binding to NK1R in human infant medulla in SIDS vs. controls………..88
3.3.2 Analysis of $[^{125}]$I labelled SP binding to NK1R by age and prematurity status……………….88-89
3.3.3 Analysis of $[^{125}]$I labelled SP binding to NK1R by sex………………………………………….96
3.4 Discussion

3.4.1 Absolute reductions in NK1R binding in NTS and IO medullary nuclei in SIDS.............99-100
3.4.2 Significant differential developmental profile of NK1R binding in SIDS cases related to prematurity..................................................100-101
3.4.3 Significant sexual dimorphism in NK1R binding in medullary nuclei and implications for SIDS.................................................................101
3.5 Conclusions........................................................................................................102

Chapter 4. SIDS vs. Controls investigation: Medullary 5-HT study

Serotonin neuron abnormalities in the serotonergic network in a South Australian SIDS cohort

4.1 Introduction........................................................................................................105

4.2 Methods and materials

4.2.1 Clinical database..........................................................................................106
4.2.2 Determination of number, morphology and density of 5-HT neurons in the caudal, mid and rostral medulla........................................................................106-107
4.2.3 Statistical analysis.........................................................................................107

4.3 Results

4.3.1 Clinicopathological data..............................................................................107-108
4.3.2 Risk factors identified in SIDS cohort.........................................................108
4.3.3 All medullary levels combined....................................................................113
4.3.4 Caudal medullary level...............................................................................115
4.3.5 Mid-rostral medullary level..........................................................................116
4.3.6 Rostral medullary level.................................................................................117

4.4 Discussion.........................................................................................................120-122

4.5 Conclusions.......................................................................................................123
Chapter 5. General Discussion

5.1 Summary of major thesis outcomes .................................................................126
5.2 General discussion and future research directions .........................................127-129
5.3 Concluding remarks ......................................................................................129

Bibliography .........................................................................................................132-145
Thesis Declaration

I certify that this work contains no material which has been accepted for the award of any other degree of diploma in my name, 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. In addition, I certify that no part of this work in the future will be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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.

I also give permission for the digital version of my thesis to be made available on the web, via the University’s digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Fiona Maree Bright
Preface

An Australia Postgraduate Award (APA) 2013 to 2016 at the University of Adelaide, School of Medicine, Department of Anatomy and Pathology funded Ms Fiona Bright’s Ph. D. candidature. The entirety of the scientific research was funded by River’s Gift Australia. Professor Roger Byard and Professor Robert Vink provided primary supervision to the candidate at the University of Adelaide, with co-supervision by Dr Anna Leonard. Dr. Jhodie Duncan, University of Melbourne, Florey Institute of Neuroscience, provided external co-supervision. In 2014 Ms Fiona Bright received the River’s Gift International Fellowship and undertook an 18-month fellowship in Boston, MA, USA collaborating with the Kinney Laboratory at Harvard Medical School and Boston Children’s Hospital, under the supervision of Dr David Paterson, with academic support and guidance given by Professor Hannah Kinney. The format of this thesis is a combination of conventional and publication format with each core study written in manuscript style.

Author and Supervisor Affiliations

**Fiona M. Bright:** ¹Discipline of Anatomy and Pathology, Adelaide Medical School, University of Adelaide, Adelaide, SA, Australia ²Department of Pathology, Boston Children’s Hospital and Harvard Medical School, Boston, MA, USA

**Professor Roger W. Byard:** ¹Discipline of Anatomy and Pathology, Adelaide Medical School, University of Adelaide, Adelaide, SA, Australia ²Forensic Science South Australia.

**Professor Robert Vink:** Sansom Institute for Health Research, University of South Australia, Adelaide, SA, Australia

**Dr David S. Paterson:** Department of Pathology, Boston Children’s Hospital and Harvard Medical School, Boston, MA, USA

**Dr Jhodie R. Duncan:** Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville, VIC, Australia

**Dr. Anna V Leonard:** Discipline of Anatomy and Pathology, Adelaide Medical School, University of Adelaide, Adelaide, SA, Australia
Thesis Dedication

In memory of

~ River Jak Adam Waddell ~

02.07.2011 - 07.11.2011

And each and every infant lost to unexpected infant death, accidental death and infants tragically lost to circumstances beyond their control.

This Ph.D. thesis is dedicated sincerely to Alexandra and Karl Waddell, the extended River’s Gift family and to all of the families and communities who have tragically lost infants and children suddenly and unexpectedly in Australia and around the world.

~There is no footprint too small that cannot leave an imprint in this world~
Acknowledgements

First and foremost I would like to express my sincere gratitude to my parents Josephine and Kevin, my brother Ryan and extended family for their unyielding support and utmost belief in my ability to achieve what I set out to accomplish. To my close friends who have been a source of endless support and encouragement to me throughout the course of this doctorate, I thank you sincerely. To Alexandra and Karl Hamilton Waddell and River’s Gift, who in the face of hardship and grief have worked tirelessly to establish a foundation for collaborative SIDS research your dedication and determination is inspirational, thank you for making this research possible.

I would like to sincerely thank my supervisors, mentors and colleagues acknowledged below, for their various contributions, scientific teaching, guidance and support throughout my Ph.D. candidature. I have been especially fortunate to have been surrounded by some of the best scientific minds in the fields of pathology, neuroscience and general research practice and have learnt more than I could ever have hoped from each and every one of you.

Professor Roger Byard

Professor Robert Vink

Dr. David Paterson

Professor Hannah Kinney

Dr Jhodie Duncan

Dr Anna Leonard

Dr Kevin Broadbelt

Dr Robin Haynes

Mrs Felicia Tratchenberg

Mr Ryan Harris

Dr Lynn Sleeper

Dr Minmin Liu

Ms Kimberley Mander

Ms Amber Meservey

Ms Katie Kritikos

Ms Kelley Journey

Dr Hoa Tran

Mrs Melissa Walker

Mr Jim Manavis

Ms Kelly McAteer

Ms Marie Anastasi
Conference Presentations Related To Thesis (2013-2016)


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARC</td>
<td>Arcuate nucleus</td>
</tr>
<tr>
<td>BH-SP</td>
<td>Bolton Hunter Substance P</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COCH</td>
<td>Cochlear nuclei</td>
</tr>
<tr>
<td>DAO</td>
<td>Dorsal accessory olivary nuclei</td>
</tr>
<tr>
<td>DMX</td>
<td>Dorsal motor nucleus of vagus nerve</td>
</tr>
<tr>
<td>DRN</td>
<td>Dorsal Raphe nucleus</td>
</tr>
<tr>
<td>GA</td>
<td>Gestational age</td>
</tr>
<tr>
<td>GC</td>
<td>Gigantocellularis lateralis nuclei</td>
</tr>
<tr>
<td>HG</td>
<td>Hypoglossal nuclei</td>
</tr>
<tr>
<td>IO</td>
<td>Inferior olivary nuclei</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IRZ</td>
<td>Intermediate reticular zone</td>
</tr>
<tr>
<td>MAO</td>
<td>Medial accessory olivary nuclei</td>
</tr>
<tr>
<td>NK</td>
<td>Neurokinin</td>
</tr>
<tr>
<td>NK1R</td>
<td>Tachykinin NK-1 receptor</td>
</tr>
<tr>
<td>NTS</td>
<td>Nucleus tractus solitarii</td>
</tr>
<tr>
<td>PBC</td>
<td>Prebotzinger complex</td>
</tr>
<tr>
<td>PCA</td>
<td>Post-conceptional age</td>
</tr>
<tr>
<td>PGCL</td>
<td>Paragigantocellularis lateralis nuclei</td>
</tr>
<tr>
<td>PIO</td>
<td>Principal inferior olivary nuclei</td>
</tr>
<tr>
<td>PMI</td>
<td>Post mortem interval</td>
</tr>
<tr>
<td>PNA</td>
<td>Postnatal age</td>
</tr>
<tr>
<td>RS</td>
<td>Rett's syndrome</td>
</tr>
<tr>
<td>SIDS</td>
<td>Sudden infant death syndrome</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>SUB</td>
<td>Subtrigeminal nucleus</td>
</tr>
<tr>
<td>SUDC</td>
<td>Sudden unexpected death in childhood</td>
</tr>
<tr>
<td>SUID</td>
<td>Sudden unexpected death in infancy</td>
</tr>
</tbody>
</table>
List of figures and tables

**Figure 2.1** Autoradiographic grey scale images of 125I Bolton Hunter SP binding to NK1R in transverse sections of the caudal and rostral human infant medulla..............................57

**Figure 2.2** Autoradiograms displaying mean 125I Bolton Hunter SP binding to NK1R in transverse tissue sections of caudal and rostral human infant medulla .........................65

**Figure 2.3** Linear regression displaying NK1R binding density (fmol/mg) with PCA........66

**Figure 2.4** NK1R binding density in premature vs. term infants across multiple medullary nuclei.............................................................................................................67

**Figure 2.5** NK1R binding in male vs. female infants.........................................................68

**Figure 2.6** Distribution of NK1R immunoreactivity in nuclei of medullary 5-HT network using single label IHC ..........................................................70

**Figure 2.7** Distribution of SP immunoreactivity in nuclei of medullary 5-HT network using single label IHC ..................................................................................71

**Figure 2.8** Single label IHC images of NK1R, SP and 5-HT immunoreactivity in transverse sections of rostral midline raphe nuclei.........................................................72

**Figure 2.9** Double label IF images of NK1R and 5-HT immunoreactivity in transverse sections of rostral midline raphe nuclei..........................................................74

**Figure 2.10** Double label IF images of NK1R and 5-HT immunoreactive neurons in transverse sections of rostral midline raphe (RMID) and extra raphe nuclei (PGCL)........75

**Figure 2.11** Double label IF images showing localization of SP and 5-HT immunoreactive neurons in transverse sections of rostral midline raphe nuclei (RMID)..................76

**Figure 2.12** Double label IF images showing localization of SP and 5-HT immunoreactive neurons in transverse sections of rostral midline raphe (RMID) and rostral extra raphe nuclei (PGCL)........................................................................................................77

**Figure 3.1** Mean total NK1R binding density (fmol/mg) across medullary nuclei analyzed, presented as highest to lowest binding density.................................................90
Figure 3.2 Autoradiograms displaying NK1R binding (fmol/mg) in medullary nuclei in SIDS vs. control. ..................................................................................................................................................................................91

Figure 3.3 Autoradiograms displaying NK1R binding (fmol/mg) in select nuclei in a SIDS vs. control case..........................................................................................................................................................................................................................................................92

Figure 3.4 NK1R binding by PCA across diagnoses in multiple medullary nuclei.................94

Figure 3.5 NK1R binding by prematurity status..............................................................................................................................95

Figure 3.6 NK1R binding density in male SIDS compared to male combined controls........97

Figure 4.1 The four morphological subtypes of 5-HT expressing neurons within human infant medullary 5-HT network (Kinney et al., 2007) stained for TPH2 (PH8 antibody).........................109

Figure 4.2 Mean 5-HT neuron count and density adjusted for sex, PMI and PCA across all medullary levels (caudal to rostral) in SIDS vs. controls ............................................................................................................114

Figure 4.3 Distribution of Caudal, Mid-rostral and rostral medullary serotonergic neurons in an infant dying from SIDS and an infant with acute death from a cause other than SIDS control.118

Figure 4.4 Transverse tissue sections of caudal extra raphe in SIDS vs. control infant medulla..............................................................................................................................................................................................................................................119

Figure 4.5 Transverse tissue sections of mid-rostral midline raphe nuclei in SIDS vs. control infant medulla. .............................................................................................................................................................................................................................................................119

Table 1.1 Previously published research investigating substance P in post mortem human infant brain tissue in SIDS.................................................................45

Table 2.1 Selected antibodies and sources ..............................................................................................................................61

Table 2.2 Normative distribution of SP and NK1R in the medullary 5-HT network in human infant medulla during postnatal development......................................................64

Table 2.3 NK1R binding by prematurity status ..........................................................................................................................67

Table 2.4 NK1R binding by sex.........................................................................................................................................................68

Table 3.1 Clinicopathological data..................................................................................................................................................87

Table 3.2 Effect of prematurity on NK1R binding. ..........................................................................................................................93
Table 3.3 NK1R binding by sex…………………………………………………………98

Table 4.1 Epidemiological and Clinicopathological data for SIDS and control cases ..........110-112

Table 4.2 5-HT neuron number, density and morphology in SIDS vs. combined controls across all medullary levels and sub nuclei…………………………………………………………113

Table 4.3 5-HT neuron number, density and morphology in SIDS vs. controls in caudal medulla…………………………………………………………………………………………115

Table 4.4 5-HT neuron number, density and morphology in SIDS vs., controls in mid-rostral medulla ………………………………………………………………………………………116

Table 4.5 5-HT neuron number, density and morphology in SIDS vs. controls in rostral medulla……………………………………………………………………………………117
Introduction

Sudden infant death syndrome (SIDS) is a devastating and unexpected event in which a seemingly healthy infant dies in the first year of life during a sleep period, with no warning or prior indication of any adverse pathology to cause alarm (Kinney and Thach, 2009a). It is one of the most significant causes of post neonatal mortality in developed countries, profoundly affecting families and their communities. SIDS is complex, heterogenous and a diagnosis based solely on exclusion where the exact cause of death remains largely unexplained following complete post mortem examination and investigation of the circumstances of death (Krous 2004). By attempting to identify those children who may be at risk, medical professionals and scientific researchers endeavour to uncover and understand the pathogenesis of SIDS not only to prevent its occurrence, but also to provide some form of closure for families who are left to make sense of not only the death of their child but the heartache and stigma that comes with the “nonentity” of SIDS (Thach, 2008, Wender, 2012).

Multiple definitions, theories, animal and human studies have been established in an attempt to decipher the pathogenesis of SIDS. Unfortunately there are no available biomarkers for SIDS; no single universally accepted definition or theory and the direct cause remains relatively unknown. However, multiple neuropathologic studies have provided evidence that a certain subset of SIDS infants are not entirely ‘normal’ prior to death (Filiano and Kinney, 1994, Takashima and Becker, 1985, Sridhar et al., 2003, Paterson et al., 2006b). Instead these infants possess some form of underlying vulnerability exposing them to an increased risk for sudden death (Kinney, 2009a, Kinney and Thach, 2009a, Paterson et al., 2006b). It is thought that SIDS or a certain subset of SIDS is caused by some form of underlying neural or systematic abnormality in medullary homeostatic control that impairs critical responses to life-threatening challenges such as hypoxia during a sleep period (Kinney and Thach, 2009a). This failure is thought to result from abnormalities in a multi-neurotransmitter network of neural pathways in the medulla oblongata that control respiration, chemosensitivity, autonomic function and arousal. Indeed abnormalities in various brainstem neurochemicals including catecholaminergic, nicotinic, muscarinic, cholinergic, glutamatergic and neuropeptide systems have been reported (Kinney, 2009b, Kinney et al., 2009b). Abnormalities in the medullary serotonergic (5-HT) system have been the most significantly and consistently observed in the brainstem of SIDS infants, however it remains unclear whether these abnormalities are the primary event in SIDS or an epiphenomenon, with the underlying pathogenesis of these specific abnormalities still undetermined.

The neuropeptide substance P (SP) functions within key medullary nuclei to regulate cardiorespiratory and autonomic function in conjunction with 5-HT and other neurochemicals.
Actions of SP are primarily mediated by tachykinin NK1 receptors (NK1R) in the CNS and SP is recognized as a primary excitatory neurotransmitter and central mediator of cardiovascular reflexes such as baroreceptor sensitivity and chemoreceptor reflex modulation in response to hypoxia. Abnormalities in SP neurotransmission may play, therefore, a role in homeostatic dysfunction in SIDS. Previous studies analyzing SP and NK1R in the brainstem in SIDS have, however, been inconsistent and inconclusive. Furthermore a potential functional relationship between the 5-HT and SP neurotransmitter systems may be of critical importance to the pathogenesis of SIDS, where deficiencies in 5-HT which is already well established in the literature, may stimulate a compensatory response by SP. Previous animal studies and post-mortem human infant tissue research have investigated both 5-HT and SP individually in relation to homeostatic control and failure underlying the pathogenesis of SIDS, however the role of SP in association with the medullary 5-HT network in SIDS has not been fully examined.

In a collaborative effort combining two independent cohorts of human infant brainstem tissue and associated digital autopsy databases from Australia and the USA, the overall objective of this research was to investigate the expression of SP and its NK1R in the medullary 5-HT network during neurodevelopment, with specific investigation of the potential role of both neurotransmitter systems in contributing to a multi-transmitter medullary homeostatic network dysfunction in a subset of SIDS cases. The thesis is comprised of three core studies, each of which are closely interrelated. Collectively these studies resulted in significant outcomes that contribute immensely to the continued investigation of the underlying pathogenesis of SIDS and have provided a foundation for promising future research directions.
Thesis Research Aims and Methods overview

Chapter 2: Normative distribution investigation

Normative distribution of substance P and its tachykinin NK-1 receptor in the medullary serotonergic network of the human infant during development

Specific Aim:

Characterization of the normative distribution of SP and the NK1R in the medullary 5-HT network of the human infant medulla during development in control cases (non-SIDS).

Hypothesis:

The NK1R and SP are extensively co-distributed but do not co-localize with 5-HT neurons in the nuclei of the medullary 5-HT network.

Methods:

1. Descriptive analysis using formalin-fixed paraffin-embedded human infant medullae specimens (non-SIDS controls) (N=10) obtained from Forensic Science South Australia (FSSA). Single labelled immunohistochemistry (IHC) was performed for TPH2 (5-HT neurons), SP and NK1R and double label immunofluorescence (IF) performed for SP relative to 5-HT and NK1R relative to 5-HT respectively. Descriptive immunohistochemical distribution of SP and NK1R was assessed within the medullary 5-HT system.

2. Quantitative analysis using fresh frozen human infant medullae specimens (non-SIDS controls) (N=15) accrued from autopsy services at the Department of Pathology, Boston Children’s Hospital and the office of chief medical examiner San Diego, CA. SP specific binding density was performed using 0.15nM [^{125}I]- Bolton Hunter labelled SP autoradiography and quantitative densitometry analysis of total and non-specific binding density performed using MCID core computer based software, to determine the normative distribution of SP binding to NK1R in medullary 5-HT network nuclei.
Chapter 3. SIDS vs. Controls study: Medullary SP/NK1R study

*Developmental abnormalities in SP NK-1 receptor binding in brainstem nuclei in sudden infant death syndrome related to sex and prematurity*

**Specific Aim:**

Determination of NK1R binding density in the medullary 5-HT network in the human infant medulla in SIDS vs. non-SIDS control cases.

**Hypothesis:**

NK1R binding density, and thus putatively SP neurotransmission, is significantly altered in key autonomic and respiratory control nuclei in the medulla oblongata of SIDS cases compared to controls.

**Method:**

Quantitative analysis using fresh frozen infant brainstem specimens (n=76) accrued from the Office of the Chief Medical Examiner in San Diego, from three separate tissue datasets over the period 2004-2015. Tissue section autoradiography with 0.15nM [125I] Bolton Hunter labelled SP and quantitative densitometry with MCID core software, was used to determine NK1R binding density (fmol/mg) in 14 medullary nuclei in 55 SIDS and 21 non-SIDS control infant brainstems. Binding results were adjusted for age, sex, prematurity and post-mortem interval.
Chapter 4. SIDS vs. Controls study: Medullary 5-HT study

Serotonin neuron abnormalities in the medullary serotonergic network in a South Australia SIDS cohort

Specific Aim:
To determine if the number and density of 5-HT neurons in the medullary 5-HT network is altered in an independent cohort of SIDS cases from South Australia replicating the same methodology as previously published by Paterson et al., 2006.

Hypothesis:
A significant medullary 5-HT abnormality exits in South Australian SIDS cases characterized by a significantly higher number and density of 5-HT neurons and altered neuron morphology in SIDS cases compared to controls.

Method:
Formalin-fixed paraffin-embedded medullae from infants who died from SIDS (41) and control cases (39) who died from definitive causes of death other than SIDS, were obtained from FSSA. Sections of medulla (4μm) were cut and immunostained for tryptophan hydroxylase (TPH2) using PH8 antibody to label 5-HT neurons. Quantitative 5-HT neuron cell count analysis was then performed using the Neurolucida (MBF bioscience) computer based software. Number, density and different morphological types of 5-HT neuron were compared in SIDS cases to controls. Results were adjusted for age, sex, prematurity and post-mortem interval.
Chapter 1. Literature Review
1.1 Significance and epidemiology of SIDS

In Australia every year over 3,500 families experience the sudden and unexpected death of a foetus, infant or child, either through stillbirth or during the first year of life from sudden unexpected death in infancy (SIDS or fatal sleeping accidents), sudden unexpected death in childhood (SUDC) or accidental death (ABS, 2015). Between 1989 and 2010 there were 3,934 SIDS deaths in Australia (ABS, 2003) and most recent data show that in 2013 alone, there were 54 SIDS deaths in Australia with an overall incidence of 0.2 per 1000 live births (ABS, 2015). SIDS remains the third leading cause of death in infancy and the leading cause of death between 1 month and 1 year of age in developed countries with 2,300 infants succumbing to SIDS alone in the United States each year (Moon and Fu, 2012).

A reduction in SIDS rates has been observed in a number of developed western countries including Australia (Hauck and Tanabe, 2008, Blair et al., 2006, Mitchell et al., 2016, Trachtenberg et al., 2012), with SIDS rates decreasing by approximately 80% between 1981 and 2011 (ABS, 2015). Decline in SIDS rates and a consequent reduction in total post neonatal infant mortality has been attributed to the identification and greater understanding of risk factors for SIDS, thus resulting in numerous risk reduction campaigns and public health initiatives implemented around the world in the late 1980’s and early 1990’s (Moon and Fu, 2012, Goldstein et al., 2016). It is estimated that approximately 7,000 infant lives have been saved since risk reduction campaigns began in Australia alone (ABS, 2003). The ‘back to sleep’ campaign is the most recognised of these global initiatives, emphasising the dangers of prone sleep position, bed sharing or co-sleeping with infants and unsafe sleeping environments as being key factors in the pathogenesis of SIDS (Bourne et al., 1994, Byard, 1994, Byard, 2015, Stanley and Byard, 1991). For most westernised countries, risk reduction campaigns had a major impact in decreasing the number of infants put to sleep in the prone position and hence the reduction in SIDS rates of around 50-90% (Wennergren et al., 1997, Ponsonby et al., 2002, Moon and Fu, 2012, Mitchell et al., 2016). While the implementation of vigorous public health campaigns around the world are acknowledged to have had a substantial role in decreasing SIDS rates, it has also been argued that the decline is due to a genuine decrease, with a greater understanding of SIDS risk factors aiding in the decline of residual SIDS rates (Goldstein et al., 2016). However differences in SIDS rates and trends across countries are understood to be in part influenced by diagnostic shifts that have occurred (Hauck and Tanabe, 2008). Improvement in diagnostic approaches due to adjustments in the use of terminology such as ‘positional asphyxia’, ‘accidental suffocation’ or ‘undetermined’ on death certificates opposed to the diagnosis of ‘SIDS’ is supportive of a diagnostic shift influence on SIDS rates (Byard, 2013). Instrumental to the diagnostic shift, with changes in deaths away from being classified as ‘SIDS’ towards other causes
of infant death, is the improvement of death scene investigations and autopsies that has led to increased awareness of potential risk factors associated with SIDS (Mitchell et al., 2000, Byard, 2013) and a greater appreciation of the complex interplay between medical science, forensic practices and epidemiology (Goldstein et al., 2016).

Despite the significant decline in SIDS rates in Australia, the USA and other developed countries, recent investigations report a plateau in SIDS rates suggesting that the burden of SIDS is still unacceptably high and that the syndrome remains a major public health concern (Hauck and Tanabe, 2008, Moon and Fu, 2012, Goldstein et al., 2016). Further to these concerns, are the discrepancies in SIDS rates between male and female infants and varying racial, cultural and ethnic populations. Male sex is recognized as a risk factor for SIDS, with mortality rates showing male predominance (3:2) (Blair et al., 2006, Kinney and Thach, 2009b). SIDS rates are also substantially higher in African Americans, American Indians, Northern Plains Indians, Maori, Indigenous Australians and mixed ancestry populations in Cape Town (Moon and Fu, 2012, Kinney et al., 2003, Thornhill-Scott et al., 2016, Panaretto et al., 2002, Tuohy et al., 1998). In Australia during the period of 2000-2004, indigenous Australian infants died from SIDS at a rate twelve times higher than non-indigenous infants and between 2008-2012, the mortality rate of indigenous infants was 1.7 times the non-indigenous rate (ABS and AIHW, 2005, ABS, 2015). Furthermore, under-reporting of indigenous SIDS and infant death has been suggested, implying that observed differences between indigenous and non-indigenous mortality rates are likely underestimates of true differences (ABS and AIHW, 2005). Ethnic and lower socioeconomic subgroups have been associated with a lower awareness of SIDS prevention (Ponsonby et al., 2002). In order to reduce such disparity, families from different races, ethnic and cultural groups must be constantly counselled regarding the recommendations for reducing the risk of SIDS (Hauck and Tanabe, 2008, Hauck et al., 2003). Racial and ethnic disparity in SIDS is of major concern and as such race and other related epidemiologic parameters must be taken into consideration when pursuing scientific investigation using human infant databases.

Interpretation of SIDS epidemiology is challenging, as not all jurisdictions adhere to standardised diagnostic practices and often there is a failure to frequently report changes in infant mortality rates (Hauck and Tanabe, 2008). Variability in the interpretation of infant mortality rates across jurisdictions must be dealt with by constant monitoring of trends and exercising vigilance in defining infant death diagnosis and classification (Byard, 2013). The provision of annual statistics should be actively undertaken by jurisdictions around the world, with regards to the number and rate of SIDS deaths, postneonatal deaths, rates for the leading causes of death within these categories, age ranges for SIDS diagnosis and diagnostic practices or standards, if any, that are used (Hauck and Tanabe, 2008).
1.2 SIDS definition and diagnosis

SIDS research has become a literature minefield having been extensively investigated and reported. Multiple and variable definitions of SIDS have been established and despite a multitude of research, to date no single theory or definition of SIDS has been or is universally accepted. Diagnosis of SIDS is therefore one based on exclusion and unfortunately the syndrome remains a ‘diagnosis in search of a disease’ (Byard, 1995). Consequently the use of widely divergent definitions and classification systems impedes the ability to make comparisons of data between SIDS studies and continues to obstruct research activity (Byard and Lee, 2012).

The term ‘SIDS’ has been erroneously applied and consistently misused in research practice (Krous et al., 2004, Willinger et al., 1991). Byard et al performed two audits of the use of definitions in SIDS peer-reviewed literature in 2005 and 2010-2011 (Byard and Marshall, 2007, Byard and Lee, 2012). In the 2005 audit, over half of papers reviewed failed to specify a definition of SIDS in their research or did not cite a standard definition at all. In the latter re-audit, the authors reported a significant improvement in the use of definitions in SIDS research however, 1 in 3 authors continued to omit a standard definition from published works. In addition it was reported that there was an apparent tendency for pathologists to use the terms ‘undetermined’ or ‘unascertained’ as opposed to diagnosing cases as SIDS, in the absence of diagnostic features at autopsy, contributing to further difficulty (Krous et al., 2004, Byard, 2013, Blair et al., 2012). The aberrant use of such terms indicated the inability of pathologists to make a specific diagnosis, significantly impacting victim’s families, who are left without answers or closure and with an unfortunate stigma that is associated with an ‘undetermined’ death (Blair et al., 2012). It is possible that inconsistencies among authors and institutions in the use of SIDS definitions and their application to research, may contribute to considerable contradictions and difficulties in interpreting SIDS research and the use or misuse of variable definitions may impact results. There may be considerable potential for bias if uniformity is not achieved in research activity in the future (Krous, 2013, Byard and Lee, 2012).

The definition of SIDS has undergone numerous modifications, including mandating death scene investigations, circumstances of death and the association of SIDS with sleep in order to establish confidence in the diagnosis of infant deaths as SIDS (Krous et al., 2004, Willinger et al., 1991). In 1969 the first standardised definition of SIDS, the Seattle definition, was established (Beckwith, 1970) mandating an autopsy for infants whose deaths are diagnosed as SIDS and established a set of criteria for a cohort of infants with similar characteristics for whom vital statistics, research, and family counselling were essential (Kinney and Thach, 2009b). In 1989 the NICHD definition was established, to include age; ‘sudden death of an infant under 1 year of age’, ‘examination of death scene’ and ‘review of clinical history, which were absent from previous definitions (Willinger et al., 1991). In the following years a vast amount of research and additional information emerged,
justifying the need for adjustment of previous definitions to incorporate epidemiologic and pathologic features, risk factors and ancillary test findings (Krous et al., 2004).

In 2004 an expert panel of paediatric and forensic pathologists and clinicians recognised the need for standardisation of diagnosis and classification of infant deaths and considered the necessity for these adjustments to improve administrative and vital statistical practices. The San Diego definition resulted, providing a more defined approach to monitoring the ever-changing epidemiologic patterns in SIDS deaths and a means for greater validity in international comparisons of SIDS data. Furthermore it established distinct definitions and criteria for subsets of SIDS deaths, including ‘sudden unexpected death in infancy’ (SUDI) and specification of requirements for diagnosis of sudden death in infant (Blair et al., 2012). SUDI not only standardised diagnosis and classification of infant deaths, but also to a certain extent assisted in avoiding the stigma or suspicion associated with the death of an infant, with pathologists able to use sub classifications based on possible mechanisms of death. This provided parents and families with a term and associated definition that may be easier for them to accept, however still enabling pathologists to discuss critical aspects of the case without compromise (Krous, 2010, Byard, 2009).

SIDS research receives extensive funding and support from parents, families and their communities who are desperate for answers, and research outcomes have a significant impact on victims’ families and the wider community. Furthermore a lack of consistency with regards to assigning causes of sudden death in infants greatly affects paediatricians, health care professionals, scientists, medical examiners, coroners and law enforcement officers (Hunt et al., 2015). Therefore it is crucial that accuracy in the use of SIDS and other diagnoses of infant death is maintained when selecting or defining SIDS cases and non-SIDS controls in research (Krous et al., 2004). Unfortunately this has not always been the case and consequently SIDS research has often been criticised due to the array of contradictory results from multiple investigations that impede the determination of aetiological pathways (Byard and Lee, 2012). The San Diego definition was intended to benefit researchers with all cases of infant sudden death able to be reported and registered providing an invaluable tool in facilitating research activity (Blair et al., 2012, Krous et al., 2004). While it made immense improvements to date, future progress in SIDS research requires constant vigilance in the use of terminology and its application to scientific investigation (Goldstein et al., 2016) in addition to standardisation of diagnostic criteria across jurisdictions (Byard, 2009, Krous, 2010). Going forward, an interdisciplinary international approach has been suggested, in order to review current methods and determine consensus strategies to improve consistency in assigning cause of death. Strategies must consider intrinsic risk factors, infant vulnerability and the known environmental risk factors to include the progressively expanding knowledge base associated with SIDS (Hunt et al., 2015).
1.3 Pathophysiology of SIDS overview

SIDS is identified as having a complex and heterogeneous pathogenesis with multiple abnormalities in a number of physiological functions and systems including neurological, cardiovascular, respiratory, gastrointestinal, nutritional, endocrine, metabolic, infectious, immunological, and environmental and genetics (Thach, 2008, Weese-Mayer et al., 2007, Kinney, 2009b, Thach, 2005, Veereman-Wauters et al., 1991, Opdal and Rognum 2011). Typically without warning, an apparently healthy infant is found deceased sometime after being placed to sleep (Kinney and Thach, 2009b). There has been a vast array of theories and animal and human studies attempting to understand the pathophysiology of SIDS. Unfortunately to date there are no biomarkers available to aid in the prevention or diagnosis of exclusion in SIDS. The aim of scientific research is therefore to determine the mechanism of failure in SIDS infants that is undetectable prior to death and that remains just as ambiguous following death, with non-pathognomonic features. While the precise cause of death in SIDS has not been identified, there is formidable evidence that the syndrome results from a combination of circumstances of a cardiorespiratory challenge that occurs in a neurologically compromised infant at a specific period of postnatal development (Kinney, 2009b, Harper and Kinney, 2010, Garcia et al., 2013b). The following review will focus on the cardiorespiratory and autonomic control failure associated with the neuropathology of SIDS.

An important step in understanding the complex pathophysiology of SIDS was the establishment of the triple risk model, which successfully conceptualised the epidemiological, physiological and neuropathological data associated with SIDS. The triple risk model proposes three coinciding factors in the pathogenesis of SIDS, these factors being: (1) underlying vulnerability of the infant; (2) a critical developmental period in homeostatic control the infant is transitioning through and (3) the application of an exogenous stressor/s such as an asphyxiating environment (Filiano and Kinney, 1994). The model states that an infant may die of SIDS if all three factors are simultaneously present, that is to say that the infant's vulnerability remains underlying until they enter the critical period in development in which they are exposed to an exogenous stressor/s resulting in sudden death (Filiano and Kinney, 1994, Kinney and Thach, 2009b). All three factors are identified as contributing to the risk of an adverse event that occurs suddenly in an otherwise ‘healthy’ infant. Therefore, consideration of the triple risk model is of key importance to SIDS research with the model providing a well-established foundation for researchers to use as a basis to build upon in the generation of research activity. This research thesis has utilized all aspects of the TRM, and wherever possible risk factors, both intrinsic and extrinsic were considered for a thorough and comprehensive investigation.
1.4 Underlying vulnerability and the brainstem hypothesis

Multiple neuropathologic studies of SIDS victims have supported the notion that SIDS infants are not entirely 'normal' prior to death; instead these infants possess some form of underlying vulnerability exposing them to an increased risk for sudden death (Kinney and Thach, 2009b, Kinney, 2009b). Interest in investigation of the brainstem in SIDS began with the findings of Naeye (1976) who reported astrogliosis in the brainstem in 50% of SIDS cases and controls, with hypoxia thought to be the underlying cause. Further research by Kinney et al. (1983), showed reactive gliosis in one fifth of SIDS cases. Building upon these observations, research was then directed at the network of neurotransmitters in brainstem respiratory-related pathways in SIDS infants.

To date, there is well-established evidence that SIDS or a certain subset of SIDS is caused by some form of underlying neural or systematic dysfunction in medullary homeostatic control that impairs critical responses to life threatening challenges such as hypoxia, hypercarbia and asphyxia during a sleep period (Kinney, 2009b, Kinney, 2009a, Kinney and Thach, 2009b). This failure is thought to result from abnormalities in a multi-neurotransmitter network of neural pathways in the medulla oblongata that control respiration, chemosensitivity, autonomic function and arousal (Kinney, 2009a, Harper and Kinney, 2010, Garcia et al., 2013b), hence the term ‘brainstem hypothesis’. This concept is based foremost on established evidence that the brainstem has a crucial role in respiratory control, blood pressure, central chemosensitivity, thermoregulation and upper airway reflexes particularly during sleep. Additionally investigations of defects in medullary control, consistent with brainstem dysfunction in infants who subsequently died of SIDS including impaired auto resuscitation (gasing), abnormal respiratory patterning, episodic obstructive apnoea during sleep, autonomic dysfunction (episodic tachycardia/bradycardia, abnormal heart rate variability), and arousal deficits (Kinney, 2009b, Sridhar et al., 2003, Sawaguchi et al., 2003, Poets, 2004, Kato et al., 2003, Poets et al., 1999, Schechtman et al., 1996, Franco et al., 1998). Therefore continued research into the neuropathology and physiology associated with SIDS is critically important to provide insight into mechanisms that underlie the brainstem hypothesis.
1.5 Brainstem respiratory network overview

Respiration is both a spontaneous and autonomic physiological function crucial for survival. Respiratory drive plays a critical role in homeostatic control by regulating blood oxygen, carbon dioxide and pH levels (Feldman et al., 2003, Mellen and Thoby-Brisson, 2012) and is controlled by rhythmic respiratory signals generated by extensive neural networks located in the medulla oblongata (Bianchi et al., 1995a, Gray et al., 1999a). Excitatory amino acids are proposed to be the primary source of neurochemical signals in the generation of respiratory rhythm and inspiratory drive to spinal and cranial motoneurons (Greer et al., 1991, Al-Zubaidy et al., 1996) and basic respiratory rhythm pattern is modulated by multiple amine and peptide neurotransmitter and neuromodulator systems (Al-Zubaidy et al., 1996). Breathing must be constantly adapted to metabolic demands and is therefore a highly integrative process. Breathing behaviours are exerted via the integration of multiple respiratory neurons concentrated in the ventral respiratory column including the prebotzinger, botzinger, retrotrapezoid nucleus, parafacial respiratory group, kolliker fuse and some cortical and cerebellar networks (Ramirez et al 2012). Respiratory rhythm and inspiratory and expiratory motor patterns emerge from the dynamic interactions between these structural and functional components (Smith et al 2009).

The core of breathing rhythm generation is the prebotzinger complex (PBC). Identified physiologically as an essential part of the medullary respiratory and rhythm-generating network in mammals by Smith et al in 1991, the PBC is well established as a critical region for the generation and coordination of respiratory rhythm and breathing cessation (Gray et al., 2001, Doi and Ramirez, 2008, Ramirez, 2011). Three types of respiratory rhythmic control are identified to originate in the PBC, eupnea, sighs and gasping (Lieske et al 2000, Garcia et al 2013), and the region is particularly sensitive to hypoxia (Lieske et al 2000, Telgkamp et al 2002, Hill et al 2011). Lesioning of the PBC results in cessation of breathing in experimental animals (Wenninger et al., 2004, McKay et al., 2005) and pacemaker neurons within the PBC are postulated to have a role in the control of breathing as a contingency system that may be activated when normal respiratory rhythmogenesis fails (Nattie, 1999, Fewell, 2005). Therefore the structure and function of the PBC is of considerable focus with regards to brainstem respiratory control and failure in SIDS. Although well described anatomically in experimental animals, the precise location of the PBC in the human brainstem has remained ambiguous. However distinct cytoarchitectural characteristics of neighbouring nuclei and fibre tracts in addition to markers for interneurons of the PBC may be utilised to identify the region. Interneurons of the PBC have been shown to express high levels of the tachykinin NK1 receptor (NK1R) (Gray et al 1999) and elevated somatostatin (Stornetta et al 2003), and Schwarzacher et al (2011) utilised these characteristics to identify a circumscribed region of the ventrolateral medulla containing a high number of NK1R and somatostatin.
immunoreactive neurons indicative of the PBC region in experimental animals and determined this region to be the presumptive human homologue of the PBC (Schwarzacher et al., 2011).

1.6 Respiratory defence mechanisms and arousal failure in SIDS

During infancy, exposures to respiratory challenges occur frequently, however infants are usually able to overcome these challenges due to the highly evolved respiratory network consisting of protective respiratory defence mechanisms (Fewell, 2005). These mechanisms consist of complex processes; they exist at several neuroanatomic levels and are controlled by different underlying neural pathways and neurochemical actions to produce an integrated response (Harper and Kinney, 2010).

Under normal conditions increased blood carbon dioxide levels (hypercapnia) or decreased oxygen levels (hypoxia) stimulate an infant to produce respiratory and motor defence mechanisms including sighs, thrashing, eye opening, head lift or tilt and cries to trigger arousal (Lijowska et al., 1997, McNamara et al., 1998). Arousal from sleep then successfully overcomes the respiratory challenge and restores the respiratory network to the normal ‘eupneic’ breathing state (McNamara et al. 1998, Garcia et al 2013). This arousal response to harmful stimuli is a key feature of breathing control development in newborns (Gallego & Matrot 2010) protecting the infant from prolonged respiratory distress (Thach, 2002, Fewell, 2005) and any interruption or depression of arousal will have significant implications on the normal response to respiratory challenges (McNamara et al., 1998). In the event of arousal failure, the normal breathing state shifts to gasping which is a strong indicator of hypoxia. If oxygen becomes available during gasping, recovery from respiratory challenge is still possible by ‘auto resuscitation’ where complete and rapid return of function in all organs is achieved (Fewell, 2005, Guntheroth and Kawabori, 1975, Adolph, 1969). Gasping and auto resuscitation are however the final defence in overcoming respiratory challenge and failure of both results in a failure to restore blood oxygen levels and drive heart rate is lost (Harper and Kinney, 2010, Lijowska et al., 1997, McNamara et al., 1998, Fewell, 2005, Garcia et al 2013). The infant will therefore experience further respiratory distress, failing to overcome respiratory challenge and will succumb to death rapidly (Garcia et al 2013).

Indeed SIDS infants have a markedly reduced ability to turn their faces or lift their head away from a dangerous microenvironment in conjunction with an inability to produce adequate respiratory musculature activity (Harper 2000, Paluszynska et al 2004) indicating an underlying neuropathology in the control of such mechanisms at the neural and subcellular levels. Studies of infants on a monitor, who eventually succumbed to SIDS, have provided indirect evidence for a sleep-related impairment or a delayed maturation of these defence mechanisms (Kato et al., 2003, (Poets, 2004)). Future SIDS victims from these studies exhibited decreased spontaneous and
induced arousals during sleep (Sawaguchi et al., 2005, Kato et al., 2003), altered sleep patterns (Schechtman et al., 1992) and had significantly more obstructive and mixed apnoea’s that were associated with an altered autonomic response to these challenges (Guilleminault et al., 1984, Franco et al., 1999, Kahn et al., 1992, Kato et al., 2003, Kato et al., 2000, Sawaguchi et al., 2005). Gasping has also been identified to be a common feature of recordings of future SIDS infants, with reports of unusual repeated double and triple gasps that had were ineffective and had minimal effects on increasing heart rate (Sridhar 2003, Poets et al 1999). Other studies have indicated that SIDS may not always be sudden but rather death may be preceded by episodic cycles of tachycardia, bradycardia or apnoea in the hours to days before the lethal event (Kinney & Thach 2009). This is further evident from markers of chronic tissue hypoxia (Vege et al 1994, Cutz et al 2007, Jones et al 2003) including brainstem gliosis and apoptosis observed in the brains of SIDS victims (Kinney et al 1983, Naeye 1980, Machalaani and Waters 2008).
1.7 Putative role of the multi-neurotransmitter homeostatic network dysfunction in SIDS

Neurochemicals are the real mediators of sensory, motor, integrative and modulatory processing in the respiratory network, with these neurochemicals including multiple inhibitory and excitatory neurotransmitters and neuromodulators (Telgkamp et al., 2002b, Wong-Riley and Liu, 2005). Specifically, in regions of the brainstem involved in the control of respiration, notably the PBC, raphe magnus and raphe obscurus of the medulla, are found neurotransmitter amino acids including glutamate, GABA, taurine and glycine as well as the neurotransmitters serotonin, dopamine and SP and the neuromodulator adenosine (Burton and Kazemi, 2000). The respiratory system is defined by the balance and specific action of these neurotransmitter and neuromodulator systems which have diverse roles in regulating the amplitude and frequency of central rhythm generation and respiratory output (Doi and Ramirez, 2008, Bonham, 1995, Telgkamp et al., 2002b) by functioning at motoneurons, sensory neurons and neurons of the central nervous system (CNS).

Neurotransmitters are expressed in a state-dependent manner and are centrally involved in reconfiguring the respiratory network under normal conditions, as well as the homeostatic response to changes in oxygen and carbon dioxide levels during various states of breathing (Doi and Ramirez, 2008, Burton and Kazemi, 2000), by modifying membrane and synaptic properties of rhythm generating neurons (Telgkamp et al., 2002) and altering their activity during different states particularly during hypoxia (Burton and Kazemi, 2000). Actions of neurochemicals are determined by the concurrent modulation and interaction with one another (Doi and Ramirez, 2010) and any deficiencies in one will be immediately compensated by the action of others (Doi and Ramirez, 2010, 2008). Following the deprivation of a specific modulatory input over a prolonged period, rhythmic activity is restored by the respiratory network functioning in an independent neuromodulator manner (Haji et al 2000, Golowasch et al., 1999, Thoby-Brisson and Simmers, 1998). Therefore varying networks likely adapt to changes in neurotransmitter and neuromodulator expression by altering the concentration of other endogenously released neurochemicals that are still expressed (Telgkamp et al., 2002).

As mentioned, the underlying vulnerability of SIDS infants is thought to be characterised by abnormalities in a multi-neurotransmitter network of neural pathways in the medulla oblongata that control critical homeostatic mechanisms. Indeed abnormalities in various brainstem neurochemicals including catecholamines, neuropeptides, acetylcholinergic, indole amines (predominantly serotonin and its receptors), amino acids (predominantly glutamate), brain derived neurotropic growth factor (BDNF), and some cytokine systems have been reported in infants who died of SIDS (Duncan et al 2008, Kinney et al 1995, Kopp et al 1993, Machaalani and Waters 2003, Mallard et al 1999, Nachmanoff et al 1998, Obonai et al 1998, Panigrahy et al 1997, Yamanouchi et al 1993, Paterson et al 2006). Observations of abnormal neurochemicals across the medullary network may be the
primary defect in SIDS responsible for failure of protective mechanisms to counteract homeostatic imbalances that impinge upon an infant during a sleep state. While the multi-transmitter hypothesis for SIDS acknowledges that neurochemical abnormalities are not limited to one neurochemical system, it has yet to be established how abnormalities in individual systems may influence one another, or the impact of a particular neurotransmitter dysfunction on other systems within the same or closely associated medullary nuclei.

Typically, more than one transmitter marker has not been studied in the same medullary region in the same case and not all of the abnormalities currently established have been confirmed by independent investigators. Therefore independent replication to validate neurochemical abnormalities already established within key medullary nuclei in SIDS, in conjunction with investigation of other neurotransmitters in concert with these defective systems is crucial. Hence the focus of this thesis was pathways of the medulla involving the neuropeptide neuromodulator SP, its tachykinin NK1R and their relationship with the medullary 5-HT system in SIDS.
Development of respiratory control is complex and begins early in gestation with the respiratory network continually undergoing extensive refinement and adjustment after birth to reach adult levels of maturity (Carroll, 2003). Humans experience a long gestation and prolonged period of postnatal maturation, therefore infants experience a heightened vulnerability and increased risk of the interaction of a number of environmental factors both prenatally and postnatally, exposing the infant to harmful stimuli such as hypoxia, hyperoxia and potential toxins (Carroll, 2003b, Bavis et al., 2002). In addition during development there is an enhanced C0₂ sensitivity which is thought to be, in part, mediated by the transition from a fetal to neonatal pattern of breathing (Wickstrom et al, 1999). Postnatal developmental changes in networks generating respiratory rhythm are likely occurring concurrently across several brainstem nuclei and therefore must be seamless and well synchronized to prevent any interruption of breathing (Feldman et al., 2003, Herlenius and Lagercrantz 2004). Adverse events during this ‘critical period of development’ may result in long-term alterations to the structure and function of the respiratory network, including dysfunction of the ventilatory response to an hypoxic challenge (Liu and Wong-Riley, 2002, Carroll, 2003, Liu and Wong-Riley, 2005). Alterations during this period are likely to have a greater effect on respiratory control and maturity than insults outside this period (Carroll, 2003, Moss, 2002, Wong-Riley and Liu, 2008, Liu and Wong-Riley, 2002).

At birth a cascade of neurotransmitters and transcriptional factors are activated and there is increasing evidence that neurotransmitters, neuromodulators and their receptors are used as developmental signals, important for the maturation of synapses and formation of neuronal networks, by modulating plasticity of brain circuits (Herlenius & Lagercrantz 2004, Gaspar et al, 2003, Zhang and Poo 2001). Shortly after birth, the respiratory system operates under ‘alert’ conditions, defined by increased excitability in central respiratory networks (Shvarev and Lagercrantz, 2006) and a switch in dominance from inhibitory to excitatory neurotransmission (Lagercrantz and Slotkin, 1986, Lagercrantz, 1987, Moss and Inman, 1989 Wong-Riley and Liu, 2005). The various neurochemicals expressed within the respiratory network have been identified to either increase their expression with age (i.e. glutamate, serotonin, norepinephrine, thyrotropin releasing hormone) or decrease in expression with age (i.e. GABA, 5-HT1A receptor, SP, NK1R, somatostatin) (Wong-Riley and Liu, 2005).

Animal studies have provided insight into what may constitute a critical period in the development of the respiratory network. Wong-Riley and Liu (2005) reported that the end of the second postnatal week was the most dynamic in the development of brainstem respiratory control in rat pups. However at postnatal day 12, a dramatic shift occurred, where a transient dominance of inhibitory over excitatory neurotransmission was observed in addition to multiple neurochemical and
physiological adjustments and switches being simultaneously orchestrated. During this period rat pups had a reduced ability to respond to hypoxia and experienced multi-faceted growth and adjustment of the respiratory system in order for the transition from neonate to adult form of ventilatory control (Wong-Riley and Liu, 2008).

Extrapolating the critical developmental period identified in animal studies to that of the developing human infant respiratory network has its challenges, however these studies contribute immensely in understanding and potentially explaining why 90% of SIDS deaths occur in the first 6 months of life (Kinney 2009a). The peak incidence in SIDS at 2-4 months of age (Kinney and Thach, 2009, Kinney et al., 2009) may constitute a period of major brainstem respiratory network development in which an infant’s abilities to respond and overcome respiratory insults are diminished. Indeed abnormalities in neurochemicals and their systems, such as that of the medullary 5-HT network in SIDS, are thought to originate during prenatal development (Harper and Kinney, 2010). However the effects of these abnormalities are thought to be uncovered after birth during the early postnatal period (Kinney et al., 2009, Haddad and Mellins, 1984). It is not known exactly when changes in the expression and activity of the neurotransmitters and neuromodulators occurs in the developing human brain, or the extent to which these changes may impact on normal respiration and contribute to increased vulnerability of a SIDS infant. It is unlikely that a critical developmental period ends abruptly, but rather that it tapers off gradually. Therefore identifying when neurochemical switches occur in the human infant brainstem in particular is necessary to fully understand the critical developmental period and potential abnormalities during this time frame associated with the pathogenesis SIDS.
1.9 Medullary Serotonergic (5-HT) system in SIDS

Monoaminergic pathways represent a key component of the reticular activating system within the mammalian brain and are involved in multiple physiological functions (Haxhiu et al., 2001). 5-HT is one of several biologic monoamines located in axon terminals that are widely distributed throughout the CNS (Molliver, 1987). The 5-HT system is spread throughout the brainstem however is primarily situated in the medulla oblongata where it is referred to as the ‘medullary 5-HT system’ (Azmitia, 1999, Kinney, 2009a, Kinney et al., 2011). The system is comprised of two core domains, caudal and rostral, which are distinct in their anatomic location, development, functions and connectivity. The caudal domain projects to the cerebellum and spinal cord and is critical for respiratory and autonomic output. The rostral domain projects to the cerebral cortex, thalamus, hypothalamus, basal ganglia, hippocampus and amygdala and mediates arousal, cognition, mood, motor activity and cerebral blood flow (Kinney et al 2009, Tork and Hornung 1990). This system is recognised as a key regulator of the brain’s homeostatic control systems including upper airway control, ventilation and gasping, autonomic control, thermoregulation, chemosensitivity, arousal and hypoxia-induced plasticity (Azmitia, 1999, Kinney and Thach, 2009, Kinney et al., 2009).

5-HT plays a fundamental role in the control and modulation of breathing (Bianchi et al., 1995, Bonham, 1995) exhibiting both inhibitory and excitatory effects (Doi and Ramirez, 2008) and acting via a large array of receptors that function to facilitate diverse respiratory effects (Barnes and Sharp, 1999, Richter et al 2003). The synaptic projections of 5-HT neurons are present across all major respiratory nuclei including PBC, and arise from the midline raphe pallidus and raphe obscurus (Manaker and Tischler 1993). Several neurotransmitters and neuropeptides are released by 5-HT neurons and directly enhance the excitability of multiple neuron subsets within the respiratory network (Kinney et al 2009). 5-HT terminals also contain SP and thyrotropin releasing hormone (TRH) and receptors for 5-HT, SP and TRH are localized on neurons across the major respiratory nuclei (Richerson et al 2004). Activation of these receptors in vitro provokes modulatory effects on respiratory neurons to enhance their excitability and activity of the respiratory network. There is also considerable evidence recognizing 5-HT neurons as putative central respiratory chemoreceptors that assist in the detection of CO₂ and the implementation of ventilatory responses in order to maintain blood homeostasis (Corcoran et al 2009).

It is well established that the exogenous release of 5-HT exerts complex modulatory effects on respiratory drive, as observed in in-vivo preparations (Bianchi et al., 1995, Morin et al., 1990). Pena and Ramirez (2002) demonstrated that bursting respiratory neurons rely on endogenously released 5-HT acting on 5-HT2A receptors and that blockage of these receptors abolishes the critical bursting property of neurons in order to generate normal breathing. Doi and Ramirez (2008) found
that 5-HT increased and subsequently decreased bursting frequency in pre-inspiratory and inspiratory neurons, highlighting its important modulatory effects. Furthermore, inhibition of 5-HT medullary raphe and extra raphe neurons has been reported to decrease ventilatory sensitivity to CO₂ and also results in alterations to cardiovascular variables and sleep cycling (Messier et al 2004). These observations reinforce the importance of neuromodulators such as 5-HT, in adjusting ionic conductances crucial for regulating pacemaker and network properties of the rhythm-generating network.

There is substantial evidence for multiple neural mechanisms contributing to the fatal event in SIDS. However, the most compelling and reproducible research to date is focused on the hypothesis that SIDS is due to a developmental disorder of medullary serotonergic and related neurotransmitter systems that occurs prenatally, however exerts its effects in the postnatal period (Kinney et al., 2012, Harper and Kinney, 2010, Paterson et al., 2009, Duncan et al., 2010). 5-HT abnormalities have been reported across multiple SIDS data-sets from varying ethnic, social and cultural backgrounds (Panigrahy et al., 2000a)(Kinney et al., 2003, Paterson et al., 2006, Duncan et al., 2010) and involve raphe, extra-raphe, and ventral (Arcuate) populations of the brainstem containing 5-HT neurons and their projection sites such as the dorsal motor nucleus of the vagus and the nucleus of the solitary tract (Kinney et al., 2011). Abnormalities identified include alterations in 5-HT receptor binding patterns (5-HT1A and 5-HT2A receptors) (Panigrahy et al., 2000, Ozawa and Okado, 2002, Machaalani et al., 2009, Paterson et al., 2006, Kinney et al., 2005), reduced brainstem levels of 5-HT and tryptophan hydroxylase (TPH2; the rate limiting enzyme regulating 5-HT synthesis) (Duncan et al., 2010), decreased binding to 5-HT transporter relative to 5-HT cell density (Kinney, 2009a, Paterson et al., 2006), increased 5-HT cell number and density of 5-HT neurons, morphological immaturity of 5-HT neurons (Paterson et al., 2006, Kinney, 2009a) and reductions in the level of the 14-3-3 signal transduction family of proteins in regions of the medulla oblongata critically involved in the regulation of homeostatic function (Broadbelt et al., 2012). Given the complex role of 5-HT within the medulla, associated abnormalities are likely responsible for impaired reflexes and responses of critical autonomic respiratory defence mechanisms to exogenous stressors such as hypoxia (Harper and Kinney, 2010, Panigrahy et al., 2000, Kinney, 2009b).

While medullary 5-HT abnormalities are the most prominent findings in SIDS research to date, the precise pathogenesis remains unknown, with uncertainty as to whether these abnormalities are the primary event in SIDS or an epiphenomenon. However it has been postulated that 5-HT abnormalities are multifactorial involving a combination of environmental and genetic risk factors (Kinney et al., 2012), with 87% of SIDS cases having medullary 5-HT abnormalities and having been exposed to an exogenous stressor at the time of death (Paterson et al 2006). Furthermore it is important to determine how substantial evidence for a serotonergic component of the
underlying vulnerability in SIDS fits within the multi-transmitter hypothesis. Given 5-HT responds to numerous neuropeptides, neurochemicals and steroids, in turn influencing both the production and release of these substances (Azmitia, 1999, Jacobs and Azmitia, 1992), investigation of other brainstem nuclei neurochemical networks in conjunction with 5-HT abnormalities is therefore of fundamental importance.
1.10 Neuropeptide Substance P and SIDS

SP is an undecapeptide belonging to the tachykinin family of neuropeptides, the largest peptidergic family described in animal organisms. SP is widely distributed throughout the central, peripheral and enteric systems (Hökfelt et al., 2001, Ribeiro-da-Silva and Hökfelt, 2000), however nervous tissue represents the most important location of tachykinins. SP acts as a neurotransmitter and neuromodulator and its biological actions on target sites are mediated via three types of tachykinin, G-protein coupled receptors NK1, NK2 and NK3 (Helke et al., 1990). SP preferentially binds to the NK1 receptor, which mediates the biological effects of SP within the CNS (Mantyh, 2002). Animal and post-mortem human brain studies have shown the extensive distribution of SP and NK1R throughout the CNS (Ljungdahl et al., 1978, Quirion and Dam, 1986, Nakaya et al., 1994, Ribeiro-da-Silva and Hökfelt, 2000, Rikard-Bell et al., 1990, Coveñas et al., 2003) and SP has been demonstrated to contribute to the widespread regulation of autonomic and physiological function, with roles in nociception, pain transmission, smooth muscle contraction, respiration, thermoregulation and cardiovascular control (Von Euler and Pernow, 1956, Harrison and Geppetti, 2001, Mistrova et al., 2016, Ebner and Singewald, 2006, Snijdelaar et al., 2000). The following overview will primarily discuss the role of SP in brainstem homeostatic control associated with the underlying pathogenesis of SIDS including respiration, chemosensitivity, cardiovascular reflexes and sleep/wake physiology.

SP and the respiratory network

SP has been shown to play an integral role in the modulation of respiratory network activity in experimental models both in-vivo (Gray et al., 2001) and in-vitro (Telgkamp et al., 2002a, Lagercrantz et al., 1991, Wickström et al., 2004). The application of SP to the medulla causes an increase in respiratory drive, reflecting its excitatory properties (Hedner et al., 1981, Yamamoto and Lagercrantz, 1985, Gray et al., 1999b, Ptak et al., 1999, Shvarev et al., 2002). The NK1R mediates these respiratory responses to SP (Mazzone and Geraghty, 2000, Quartara and Maggi, 1998) and is present on many respiratory neurons (Ellenberger et al., 1992, Guyenet and Wang, 2001). The importance of NK1R in respiratory control is emphasized by experimental models in which SP induced increases in respiratory frequency or inspiratory motor output in wild-type mice, but not in NK1R knockout mice (Ptak et al., 2000).

SP is found in neurons within multiple medullary nuclei including the nucleus tractus solitaries (NTS), nucleus accumbens, the dorsal motor nucleus of vagus and hypoglossal nucleus (Ribeiro-da-Silva and Hökfelt, 2000) each of which either receive input or project to PBC (Bianchi et al., 1995b). As mentioned the PBC has been suggested to play a role in the pathogenesis of SIDS given that it is the site for the generation of respiratory defence mechanisms (sighs and gasps) in response
to harmful stimuli (Garcia et al., 2013b). Furthermore, normal breathing in mammals requires an intact PBC, which is especially sensitive to SP modulation of respiratory activity (Gray et al., 2001, Gray et al., 1999b). SP exerts its excitatory effects by acting on NK1R present in the PBC. NK1Rs expressed on respiratory neurons within the PBC are highly characteristic; hence they are used anatomically to identify the PBC in experimental models (Schwarzacher et al., 2011, Gray et al., 1999b, Gray et al., 2001).

Experimental animal studies have been pivotal in investigating not only the importance of the PBC in respiratory rhythm generation, but also the critical role of SP within the PBC in the control of normal breathing rhythmogenesis. Rodent studies have shown that although NK1R-deficient mice have a normal PBC maturation and functionality at birth (Ptak et al., 2002), reduction of SP expression within the PBC offsets ventilatory rhythm generation in neonates (Morgado-Valle and Feldman, 2004, Lavezzi and Matturri, 2008). Destruction of NK1Rs has been shown to impair or eliminate breathing (McKay et al., 2005) and block somatosympathetic reflexes (Makeham et al., 2005), while lesions of NK1R-expressing neurons within the PBC result in profoundly abnormal respiratory patterns and altered blood gases and pathological responses to hypoxia (Gray et al., 2001, Wenninger et al., 2004a, Wenninger et al., 2004b, Telgkamp et al., 2002a). Furthermore Peña and Ramirez (2004) observed that the degree of pacemaker activity within the PBC and respiratory network was not fixed but rather dynamically regulated by SP neuromodulation and these pacemaker neurons are not only sensitive to SP (Gray et al 1999) but also to hypoxia (Thoby-Brisson and Ramirez, 2001).

Although the endogenous release of SP is important for maintenance and regulation of respiratory activity, respiratory rhythm dependency on SP release and the tonic activation of NK1R is partly determined by the interaction of both with other excitatory neuromodulators such as serotonin and norepinephrine (Doi and Ramirez, 2010, Peña and Ramirez, 2004). Therefore further investigation is required to determine the relationship between SP and other neurochemicals during varying states within the PBC and greater brainstem respiratory network, in order to fully understand the implications of abnormalities in these systems during development that may contribute to the underlying pathogenesis of SIDS.

**Substance P and cardiovascular and chemoreceptor reflex**

Aside from its role in the regulation of normal breathing, SP is known to exert its actions when the CNS is challenged or when respiratory network demands are heightened in response to stressful stimuli. SP is recognized as a primary excitatory neurotransmitter and central mediator of cardiovascular reflexes such as baroreceptor sensitivity and chemoreceptor modulation in response to hypoxia within the mammalian brainstem (Hökfelt et al., 2001, Harrison and Geppetti, 2001, ...
The magnitude of SP release is proportional to the intensity and frequency of the stimulation (Mantyh, 2002). Increases or decreases in SP release and neuronal sensitivity to its modulation have been suggested to assist neural systems in adapting to trauma, enhancing survival and promoting recovery from harmful stimuli (Hokfelt and Kuteeva, 2006). In transverse slice preparations, short term block of SP decreases the frequency and regulation of rhythmic activity, whereas long term SP deficiency initiates compensatory mechanisms that are capable of adapting central respiratory activity during normoxia, however these significantly disturb respiratory network response during anoxia (Telgkamp et al., 2002a, Mazzone and Geraghty, 2000). Furthermore depletion of SP in rodents by chronic exposure to capsaicin has been shown to significantly reduce the magnitude of hypoxic ventilatory response by diminishing respiratory frequency (De Sanctis et al., 1991).

Hypoxia has been shown to facilitate SP release within the carotid body and SP enhances the hypoxic response of the carotid body in vivo (Kumar and Prabhakar, 2003, Kumar and Prabhakar, 2012). Furthermore SP release has been shown to mediate hypoxic drive via peripheral chemoreceptors by acting directly on the respiratory rhythm generating network (Srinivasan et al., 1991, Lindefors et al., 1986, Yamamoto et al., 1992). The stimulatory effect of hypoxia on SP is significantly diminished by SP and NK1R antagonists indicating an important effect of SP in mediating peripheral chemoreceptor input to the brainstem (Yamamoto and Lagercrantz, 1985). SP may mediate the hypoxic sensory activation particularly in the carotid body by activation of NK1R (Kumar and Prabhakar, 2003). Certainly NK1R are thought to have a key role not only in respiratory generation but also in the maintenance of respiratory timing during sustained hypoxia (Gray et al., 2001), especially given that all of the putative central chemoreceptor sites contain cells expressing NK1Rs (Wickström et al., 2004, Mazzone and Geraghty, 2000). Given SIDS infants are associated with an inability to adequately respond to harmful stimuli such as hypoxia, the mechanisms by which neurochemicals participate in the hypoxic and ventilatory responses to hypoxia in the human infant, such as SP, require further investigation.

### Development of the substance P, NK1 receptor system

The development of SP-containing neuronal pathways begins early in development and is already largely established well before adult transmitter contents are reached. The SP-ergic system is considered to be more active in respiratory responses during early postnatal development in neonatal and newborn rats than in adults (Shvarev and Lagercrantz, 2006), and during this period SP activation of NK1R is essential when an organism is challenged by hypoxia (Berner et al., 2007). Yamamoto and Lagercrantz, (1985) identified juvenile rodents as being inherently more sensitive to SP than mature animals, supporting a more important role of SP regulation on respiratory activity during the perinatal period. Indeed Ptak et al. (1999) reported that SP does not
control ventilatory rhythm generation in fetal rats and does not modulate respiratory rhythm prior to birth, but rather exerts its effects on phrenic motoneurones in the postnatal period. Furthermore, Shvarev and Lagercrantz (2006) reported that with advancing postnatal age in rat pups, respiratory output was considerably modified and SP had an age-dependent excitatory effect on respiratory activity. The density of NK1R binding sites in both animal and human studies has been observed to peak at or prior to birth, before decreasing thereafter over the course of postnatal development to reach adult levels (Quirion and Dam, 1986, Rodier et al., 2001, Bergström et al., 1984, Ljungdahl et al., 1978). In post-mortem human infant tissue samples, Bergström et al. (1984) reported a spike increase in SP expression at birth, which levels off during the first 6 months of development, a period consistent with peak incidence of SIDS (Kinney and Thach, 2009a). A transiently higher level of expression of NK1R transcripts early in the postnatal period overlapping with a critical period of development of the mammalian CNS (Srinivasan et al., 1991, Berner et al., 2007) suggested a physiological role of SP and NK1R in synaptic plasticity.

The PBC also undergoes extensive maturation during the postnatal period (Ellenberger, 1999, Thoby-Brisson and Greer, 2008, Greer et al., 2006) and SP within the PBC is thought to play a potentially important role in this maturation (Pagliardini et al., 2003, Ptak et al., 1999, Quirion and Dam, 1986). Altered SP neurotransmission or NK1R binding during the postnatal period could contribute to abnormal development or maturation of the respiratory network.

**Substance P and sleep/wake physiology**

As previously discussed, SIDS infants have fewer arousals from sleep and abnormal sleeping patterns. SP levels are reduced during sleep states, as is understood for 5-HT, given that SP possesses excitatory properties (Doi and Ramirez, 2010). SP and NK1R have been implicated in sleep/wake behaviour (Nattie and Li, 2002, Andersen et al., 2006) and SP administration at various regions of the brain has been reported in experimental models to exhibit diverse effects on sleep state (Andersen et al., 2006, Zhang et al., 2004, Strittmatter et al., 1996b) likely mediated by NK1R (Ursavas, 2008, Andersen et al., 2006). NK1 receptors have been detected in a number of brain structures related to regulation of sleep and waking, including the DRN (Commons and Valentino, 2002, Lacoste et al., 2006). A reported side effect of NK1R antagonists is excessive sleepiness, suggesting an inverse relationship between the diminished concentration of SP and augmentation of sleep (Kramer et al., 2004). As discussed previously, destruction of NK1R in animal studies results in profound adverse effects on respiratory rhythm, including cessation of respiration when these animals fell asleep (McKay et al., 2005, McKay and Feldman, 2008). This supports the concept that sleep may be a catalyst in unmasking neurotransmitter deficits in SIDS, which may explain why SIDS infants appear normal during awake states. Hence SP/NK1R dysfunction within critical nuclei
in the control of sleep/wake behaviour may contribute to the inability of a vulnerable infant to arouse from sleep in response to harmful stimuli.

**Implications for the pathogenesis of SIDS**

Given the extensive role of SP across multiple homeostatic systems, a number of human pathologies have been associated with an altered SP/NK1R system within the CNS (Muñoz and Coveñas, 2014). Of particular interest is the role of SP in Rett’s syndrome (RS), which underscores the investigation of SP in the pathogenesis of SIDS. Children with RS have significantly decreased levels of SP in the cerebrospinal fluid (Matsuishi et al., 1997) and in key nuclei within the brainstem (Deguchi et al., 2000, Saito et al., 2001). Breathing behaviour in RS is highly unstable and results in increased episodes of irregular respiratory frequency, apnoea’s and hyperventilation (Ogier and Katz, 2008, Katz et al., 2009).

The role of SP in the pathogenesis of SIDS has been previously explored in a number of studies, which have been summarized in Table 1.1. The results of SP expression in SIDS have lacked consensus across studies, even with respect to the normative distribution of SP and its receptor in the human infant brainstem. Among these studies are reports of increased SP immunoreactivity (Biondo et al., 2004, Bergström et al., 1984, Obonai et al., 1996, Yamanouchi et al., 1993, Takashima et al., 1994), lowered expression of SP fibres and tracts (Lavezzi et al., 2011) and reports of no change in SP binding density (Jordan et al., 1997, Sawaguchi et al., 2003) within various brainstem nuclei in SIDS cases compared to controls. The definition of SIDS is a significant factor in the investigation of SIDS pathogenesis using human infant tissue, however many of these studies did not cite a definition or provide explanations as to how cases were classified. SIDS cases that had extended post mortem interval were also included in analyses in some of the studies. These factors may explain the discrepancies observed between published results and unfortunately contribute to an inability to compare across pathological studies. To date a potential role for an altered SP/NK1R system within the brainstem in conjunction with abnormalities in other neurochemical networks in SIDS remains ambiguous.

The concentration of neuromodulators such as SP depends upon the state of the respiratory network and in part the dependence of this network on the simultaneous modulation and interaction with one another (Doi and Ramirez, 2010). While previous studies focused on the expression of SP and NK1R solely in SIDS, in line with the multi-neurotransmitter hypothesis we recognized the need for analysis of SP in conjunction with neurotransmitter networks abnormalities already implicated in SIDS. Hence our investigation has focused on the expression of the SP/NK1R system within the medullary 5-HT network, in nuclei intimately related to cardiorespiratory and autonomic control.
<table>
<thead>
<tr>
<th>Author/Year</th>
<th>Brain region/nuclei analyzed</th>
<th>Objective &amp; method used</th>
<th>Key findings</th>
<th>Definition classification of SIDS cited</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bergstrom et al 1984</td>
<td>Cortex, medulla oblongata, pons, hypothalamus</td>
<td>Investigate expression of Met-enkephalin and SP. Radioimmunolabeled assay</td>
<td>Significantly increased SP in medulla oblongata of SIDS cases</td>
<td>12 SIDS, no definition cited</td>
</tr>
<tr>
<td>Takashima et al 1994</td>
<td>Brainstem, Ventrolateral medulla</td>
<td>Developmental brainstem pathology in SIDS. Golgi and IHC</td>
<td>Increased SP nerve fibres in pons of SIDS cases</td>
<td>No definition cited</td>
</tr>
<tr>
<td>Yamanouchi et al 1993</td>
<td>Reticular formation and pontine nuclei</td>
<td>IHC</td>
<td>Increased SP in trigeminal fibres in SIDS cases</td>
<td>20 SIDS cases, no definition cited</td>
</tr>
<tr>
<td>Obonai et al 1996</td>
<td>Medulla oblongata, dorsal vagal, reticular formation, NTS</td>
<td>IHC, cell count analysis</td>
<td>Increased SP in trigeminal nucleus and NTS</td>
<td>15 SIDS, no definition cited</td>
</tr>
<tr>
<td>Jordan et al 1997</td>
<td>Raphe magnus obscurus, hypoglossal, locus coeruleus, nucleus cuneiformis, NTS, inferior olive, nucleus parabrachialis</td>
<td>Distribution of SP binding density in medulla oblongata. Autoradiography</td>
<td>No significant change in SP binding site density in SIDS cases</td>
<td>9 SIDS, Classified according to criteria from Taylor et al 1990: Minor diseases not normally fata or essentially unexplained</td>
</tr>
<tr>
<td>Sawaguchi et al 2003</td>
<td>Nuclei spinal, mesencephalic and principal sensory nervi trigemini, nucleus parabrachialis</td>
<td>Correlation of sleep apnoea data and SP expression. Polysomnography sleep data, IHC, cell density</td>
<td>No significant correlation between sleep apnoea and density of SP in SIDS cases</td>
<td>26 SIDS, no definition cited</td>
</tr>
<tr>
<td>Biondo et al 2004</td>
<td>NTS</td>
<td>Investigation of functional and morphological alterations in neurons and glia with SP expression</td>
<td>Significant increase in expression of SP in medulla in SIDS</td>
<td>23 SIDS, no definition cited</td>
</tr>
<tr>
<td>Lavezzi et al 2011</td>
<td>Medulla oblongata, spinal trigeminal nucleus</td>
<td>SP expression in medulla oblongata</td>
<td>Negative/low SP expression in fibres and tracts of SIDS in sp. trigeminal</td>
<td>32 SIDS cases, not defined</td>
</tr>
</tbody>
</table>
1.11 Serotonin and Substance P in SIDS

Experimental animal studies have provided the basis for a potential functional relationship between 5-HT and SP neurotransmission across brainstem-mediated homeostatic control, a relationship that may be of critical importance to the underlying pathogenesis of SIDS. As discussed previously, the actions of neurotransmitters are determined by the concurrent modulation and interaction with one another and deficiencies in one will likely be immediately compensated by the action of others (Doi and Ramirez, 2010a). Therefore varying networks likely adapt to changes in neurotransmitter expression by altering the concentration of other endogenously released neurochemicals that are still expressed (Telgkamp et al., 2002). With respect to the pathogenesis of SIDS, medullary 5-HT dysfunction already established within critical brainstem regions such as the raphe nuclei in SIDS, may stimulate a compensatory response by SP or have adverse affects on SP neurotransmission within the same medullary nuclei. Withdrawal or alteration of the combination of 5-HT and SP mediated homeostatic control within the developing infant brainstem could therefore contribute to the multi-transmitter homeostatic network dysfunction in a subset of SIDS cases. The following overview discusses previous published research, primarily animal studies, supporting a dual role of 5-HT and SP in homeostatic control within the brainstem.

Co-distribution/localization of 5-HT and SP

Interactions between 5-HT and SP are known to occur within the CNS and the distribution of monoamine synthesizing and SP immunoreactive neurons has been shown to significantly overlap, particularly within ventral medullary neurons in the brainstem (Léger et al., 2002, Otsuka and Yoshioka, 1993, Ptak et al., 2009, Hökfelt et al., 2000). Indeed, 5-HT synthesizing cells are identified as the main source of SP in rodent models (Ljungdahl et al., 1978). SP is located in the same neurons as 5-HT in the raphe nuclei, nucleus tractus solitarii, nucleus accumbens, dorsal motor nucleus of vagus nerve and hypoglossal nuclei (Hokfelt 2001). Given that 5-HT and SP coexist within crucial respiratory centres of the brainstem, there is a likely interplay between the two systems in the control of respiratory drive, in which they may exhibit a synergistic relationship (Jacquin et al., 1989, Ptak et al., 2009, Doi and Ramirez, 2010b, Hadjjeri and Blier, 2001, Berner et al., 2007). Indeed receptors for 5-HT, SP and thyrotropin releasing hormone (TRH) are located in neurons within each of the major respiratory nuclei and their actions in vitro induces modulatory effects on respiratory neurons, enhancing their excitability and that of respiratory network activity (Holtman et al., 1984). Furthermore the endogenous release of both 5-HT and SP has been demonstrated to result in similar effects on respiratory frequency as both systems partly converge on the same second messengers (Doi and Ramirez, 2010b).
Respiratory rhythm generation

Experimental animal studies have shown that spontaneous raphe neuron activity results in the endogenous release of both 5-HT and SP and their combined effects are critical for the generation of inspiratory rhythm and motor output in both neonatal and juvenile rodent systems (Ptak et al., 2009). 5-HT and SP containing neurons within the raphe project to and excite neurons in the PBC (Ptak et al., 2009) and 5-HT and SP co-localize in presynaptic terminals within the PBC (Hodges and Richerson, 2008). The neuromodulatory effects of both 5-HT and SP on respiratory rhythm generation, non-5-HT chemosensitive neurons and respiratory motor neurons likely combine to enhance ventilation at the level of the PBC (Richerson, 2004, Hodges and Richerson, 2008). Therefore deficiencies in 5-HT and SP expression within the PBC may contribute to dysfunctional respiratory drive and failure of the generation of respiratory defence mechanisms.

Chemosensitivity

In addition to a combined role in the modulation of respiratory drive, both the 5-HT and SP systems are also involved in respiratory chemosensitivity and in the neural response to hypoxia (Cuello and McQueen, 1980, Bach and Mitchell, 1996). Like SP, 5-HT is also released during hypoxia and the dual release of both has been suggested to be a coordinated response to counteract hypoxic states (Ptak et al., 2000). The elimination of central 5-HT neurons which co-express SP has been shown to result in severe apnoea, hypoventilation and increased mortality in transgenic neonatal mice (Hodges et al., 2009). Furthermore, 5-HT/SP synthesizing cells in the raphe nuclei of the brainstem have been identified as candidates for central chemoreceptors stimulated by CO₂ and pH (Iceman et al., 2013). Iceman and Harris (2014) identified a previously unrecognizable pool of chemosensitive medullary raphe neurons in the juvenile rat that were not 5-HT, however did express NK1R and were adjacent to 5-HT expressing cells in the medullary raphe. In this study CO₂ stimulation of both SP/5-HT and non-SP/5-HT cells, constituted distinct groups that had different firing frequencies and hypercapnic sensitivities. The authors concluded that at least two distinct groups of raphe cells are activated by hypercapnia, those that co-express 5-HT/SP and those that are non SP/5-HT expressing, with the raphe providing both 5-HT and non 5-HT innervation to the diaphragm.

NK1R expressing neurons are intermingled among 5-HT neurons and are distributed widely in the brainstem of rodents (Nakaya et al., 1994). Nattie et al. (2004) showed that both 5-HT and NK1R expressing neurons are required for the normal response to systemic CO₂ in juvenile rodents and that the specific killing of medullary 5-HT neurons or of adjacent NK1R expressing neurons reduces the ventilatory response to systemic CO₂ in wakefulness in non rapid eye movement sleep (NREM). The investigators suggested that NK1R-expressing neurons may provide a necessary excitatory modulation
that enables the full breathing response of 5-HT chemosensitive neurons to CO\textsubscript{2}, or that NK1R expressing neurons may respond to SP that is released by CO\textsubscript{2} sensitive 5-HT neurons, in which case 5-HT would likely excite NK1R expressing neurons to stimulate breathing.

SP/NK1R modulation of 5-HT system

Neuropeptides, particularly SP, may induce 5-HT mediated behaviours by enhancing the release of endogenous 5-HT (Liu et al., 2002). Consistent with this are reports that SP plays a key role in the modulation of 5-HT neurotransmission in the brain, with NK1R antagonists combined with a selective 5-HT reuptake inhibitor augmenting 5-HT release by modulating SP/5-HT interactions in the dorsal raphe nucleus (DRN) (Guiard et al., 2004), a region where 5-HT neurons are extensively located with a substantial population of SP synthesizing cells (Baker et al., 1991). The facilitated 5-HT neurotransmission by NK1R antagonists purportedly associated with the NK1R’s ability to limit increased endogenous release of 5-HT at the level of the DRN (Commons and Valentino, 2002). A functional relationship may also exist between NK1R and 5-HT receptors in the NTS nuclei in cardiobaroreflex modulation, especially in response to stressful conditions such as a hypoxic microenvironment (Raul, 2003).

Two types of neurotransmitters have been suggested to act in opposition, one as an accelerator and one as a brake to modulate respiratory responses to fluctuations in blood oxygen levels (Richerson et al., 2001). Given their co-localization in key respiratory regions in the brainstem and that both exhibit important roles in chemosensitivity, it is reasonable to suggest that 5-HT and SP may not only interplay, but may act in opposition to produce responses to respiratory challenges that alter the homeostasis of the respiratory system. As integration of multiple systems is required to trigger respiratory defence mechanisms such as gasping, any response modulated by 5-HT is likely in balance with other neuromodulators in the brainstem’s rostral system, SP being a feasible candidate. Therefore 5-HT dysfunction within key medullary sites in SIDS may have significant implications for SP neurotransmission.

Sleep/wake physiology

In addition to the potential modulation of 5-HT by SP in cardiorespiratory control, sleep apnoea and narcolepsy studies have provided insight into SP’s involvement in not only sleep modulation and arousal, but also its potential modulation of 5-HT neurotransmission and receptor sensitivity during sleep states with SP acting as a co-transmitter in sleep/wake cycling (Strittmatter et al., 1996, Adori et al., 2016). Strittmatter et al (1996) revealed significant correlations between neuropeptides, specifically SP, and monoaminergic transmitter metabolites such as 5-HT. The investigators suggested that SP might affect 5-HT levels in narcolepsy, as lower levels of SP decrease the activity of the 5-HT
system therefore affecting the sleep cycle and arousal. This potential interplay of the two systems in sleep modulation is further supported by findings that within the DRN, a region known to be significant in the regulation of sleep and waking, SP has been found to be co-expressed with 5-HT in approximately 40-50% of ascending serotonergic neurons in both human and animal studies (Baker et al., 1991).

**Neurodevelopment**

5-HT is considered essential for the maturation of the neuronal network having a role in respiratory plasticity, and serving as a trophic factor with direct effects on neuronal proliferation, migration and differentiation (Herlenius and Lagercrantz, 2004, Sodhi and Sanders-Bush, 2004, Di Pasquale et al., 1994). Similarly, SP and NK1R have a role in the development and maturation of medullary raphe neurons (Horie et al., 2000). Therefore interactions between 5-HT, SP and their receptors may have a significant role in the formation of raphe nuclei and neurogenesis within the medulla. Indeed there is increasing evidence that neurotransmitters are developmental signals, crucial for the development of synapses and formation of neuronal networks, by modulating the construction and plasticity of brain circuits (Herlenius and Lagercrantz, 2001, Whitaker-Azmitia, 1991, Gaspar et al., 2003). Moreover, the development of different neurotransmitter systems may be directly related to one another (Whitaker-Azmitia, 1991). Various neurotransmitters may therefore play a role within the immature brain that is entirely different to their role in the mature brain (Horie et al., 2000). As already mentioned, any dysfunction in SP or 5-HT transmission during early life could cause long lasting structural and functional alterations (Hornung, 2003). Further investigation is required to determine the potential dual role of these neurotransmitters in development and to identify when possible time dependent neurochemical switches of these systems occur, the alteration of which may coincide with a heightened vulnerability to environmental challenges. Such vulnerability is thought to underlie SIDS pathology, particularly during the first six months of life (Moon et al., 2007, Kinney et al., 2009a).
1.12 Synopsis

This thesis is organized as a combination of conventional and publication formats and is comprised of three core study chapters each of which are closely interrelated and written in manuscript style. The thesis will examine the potential role that the neuropeptide SP may play in the pathophysiology of SIDS in brainstem nuclei previously shown to have abnormal expression of neurotransmitters such as 5-HT in a subset of SIDS infants. The thesis will test the overall hypothesis that abnormalities in the SP and 5-HT neurotransmitter systems in critical medullary nuclei during development may contribute to a multi-transmitter medullary homeostatic network dysfunction in a subset of SIDS cases. A brief introduction will precede each experimental investigation, along with details of the methodological protocol. Although each individual chapter will report results specific to a particular study, many of the results will have implications not only for that study chapter but also for other aspects raised in the thesis. This will result in some overlap across the chapters in interpretation and discussion.

In Chapter 2 emphasis will focus on characterizing the normative distribution and expression of SP within the human infant brainstem in key nuclei intimately associated with cardiorespiratory and autonomic control, which have been previously implicated in pathogenesis of SIDS. This study will test the hypothesis that SP and the NK1R are extensively co-distributed but do not co-localize with 5-HT in the medullary serotonergic network of the normal human infant during development. Thus chapter 2 provides a foundation for further specific investigation of SIDS pathology in Chapter 3.

Chapters 3 and 4 present a SIDS vs. controls (non-SIDS) analysis of SP/NK1R and 5-HT neurotransmitter expression within these key medullary nuclei. Firstly in Chapter 3, an investigation of the expression of SP binding to the tachykinin NK1R in the medulla of SIDS vs. controls is presented testing the hypothesis that NK1R binding density and thus putatively SP neurotransmission is significantly altered in key autonomic and respiratory control nuclei in the medulla oblongata of SIDS vs. control infants. Thereafter in Chapter 4 the thesis will investigate the distribution and expression of 5-HT neurons in the medullary serotonergic network in an independent cohort of SIDS cases from South Australia replicating the methodology published by Paterson et al., 2006. The study will test the hypothesis that a significant medullary 5-HT abnormality exists in South Australian SIDS cases characterized by a significant increase in the number and density of 5-HT neurons and altered neuron morphology in SIDS vs. control infants. Finally, a concluding general discussion will integrate and summarize the major findings drawn from each study chapter before addressing the general implications and future research directions emerging from this thesis.
Chapter 2. Normative distribution investigation
Normative distribution of substance P and its NK-1 receptor in the medullary serotonergic network of the human infant during development

Fiona M Bright¹,², Roger W Byard¹, Anna V Leonard¹, Robert Vink³, David S Paterson²

1. Discipline of Anatomy and Pathology, Adelaide Medical School, University of Adelaide SA, Australia
2. Department of Pathology, Boston Children's Hospital and Harvard Medical School, Boston MA, USA
3. Sansom Institute for Health Research, University of South Australia, Adelaide SA, Australia

ABSTRACT

Substance P (SP) and its tachykinin NK1 receptor (NK1R) function within key medullary nuclei to regulate cardiorespiratory and autonomic control. In the present study, 0.15 nM [¹²⁵I] labelled Bolton Hunter SP tissue receptor autoradiography (n=15), single label immunohistochemistry and double label immunofluorescence (n=10) were used to characterize the normative distribution profile of SP and NK1R in the medullary serotonergic (5-HT) network of the human infant during postnatal development, a period coinciding with extensive adjustment and refinement of respiratory and autonomic control, in part modulated by neurotransmitters and their receptors. Autoradiography revealed high-density NK1R binding in the midline raphe nuclei at the caudal and rostral level and in all subdivisions of the inferior olivary complex: the principal inferior olive, medial accessory olive and dorsal accessory olive. Intermediate to low binding was present in the hypoglossal nucleus, gigantocellularis, intermediate reticular zone, paragigantocellularis lateralis, nucleus tractus solitarii, dorsal motor nucleus of vagus and subventricular nuclei. A trend for binding to decrease with age was observed across the majority of nuclei analysed, with a significant age-related reduction in NK1R binding in the raphe obscurus (p=0.009). Prematurity status significantly influenced age-related reductions in binding observed, with premature infants exhibiting significantly higher binding than term infants across multiple nuclei. Furthermore a significant sex effect was observed with higher binding in male compared with female infants in multiple nuclei. The pattern of NK1R immunoreactivity using single label IHC paralleled NK1R binding density and revealed widespread distribution of both SP and NK1R immunoreactivity throughout the nuclei analyzed. Double label IF revealed extensive interactions of SP and NK1R with the medullary 5-HT network, specifically in the midline raphe and extra raphe nuclei. These observations provide support for the involvement of the SP/NK1R network in cardiorespiratory function and autonomic control, and further evidence for the interaction with 5-HT in key medullary nuclei during postnatal development. This investigation provides a baseline for analysis of the SP/NK1R system and its interaction with 5-HT within paediatric brainstem disorders in early life.
Keywords: Substance P, neurokinin-1 receptor, serotonin, medulla oblongata, human infant, neurotransmitters, neurodevelopment

2.1 INTRODUCTION

During the first year of life, extensive adjustment and refinement of respiratory and autonomic control are known to occur, in part modulated by the extensive array of neurotransmitters and their receptors (Doi and Ramirez, 2008). In neonates specifically, the neurochemical control of respiratory homeostasis is critical, with neurodevelopment being associated with an increased vulnerability in which any adverse event could potentially result in long-term alterations to the structure and function of the respiratory network (Liu and Wong-Riley, 2003, Carroll, 2003a, Liu and Wong-Riley, 2005). Given that a number of neurochemical systems have been identified to be abnormally expressed during development in paediatric disorders associated with brainstem respiratory and autonomic control, there is increased interest in the human infant brainstem expression of neurotransmitters and neuromodulators during postnatal development. The medullary serotonergic (5-HT) network in particular has been a focus of investigation with abnormalities in 5-HT being consistently implicated in paediatric brainstem disorders (Paterson et al., 2006a, Richter et al., 2003). However alterations in other neurotransmitters including SP have also been identified (Jordan et al., 1997, Deguchi et al., 2000).

SP and its NK1R, function within key medullary nuclei to regulate cardiorespiratory and autonomic function, and with 5-HT, are recognized as a primary excitatory neurotransmitter and central mediator of cardiovascular reflexes in response to hypoxia (Hökfelt et al., 2001, Harrison and Geppetti, 2001). The medullary 5-HT network in the human medulla, previously defined by Kinney et al. (2007), is vital for homeostatic control and is known to play an important role in the regulation of respiratory and autonomic function and neurodevelopment (Azmitia, 1999, Kinney and Thach, 2009b, Kinney et al., 2009a). Interactions between SP and 5-HT are known to occur throughout the CNS given their co-localization in ventral medullary neurons within the brainstem (Otsuka and Yoshioka, 1993, Ptak et al., 1999, Johansson et al., 1981) and their co-expression in multiple medullary nuclei in both animal and human studies (Baker et al., 1991, Halliday et al., 1988, Hökfelt et al., 2000, Ptak et al., 2009, Hodges and Richerson, 2008). 5-HT synthesizing cells are thought to be the main source of SP (Johansson et al., 1981) and of particular interest to the present study, the largest proportion of 5-HT neurons occupying the raphe region of the lower medulla that are concerned with respiration and considered putative sites for central chemosensitivity, have been shown to express SP (Halliday et al., 1988, Wong-Riley and Liu, 2005, Del Fiacco et al., 1984, Rikard-Bell et al., 1990, Richerson, 2004).
While multiple studies have investigated the anatomical localization of SP and NK1R within animal and human brainstems, no study has specifically investigated the normal developmental distribution of SP and NK1R specifically within the medullary 5-HT network. Therefore in a collaborative effort combining two independent cohorts of fresh frozen and formalin-fixed human infant brainstem tissue, this study has quantitatively analyzed the normative developmental distribution of NK1R binding and provided a descriptive normative distribution profile of SP and NK1R immunoreactivity within the medullary 5-HT network during postnatal development. Establishing a normal distribution profile of SP/NK1R and its interaction with 5-HT in the infant medulla will provide a baseline for future analysis of this system and its potential interaction with 5-HT in paediatric brainstem disorders in early life.

2. 2 MATERIALS AND METHODS

2.2.1 Clinicopathological database: fresh frozen and formalin fixed tissue

Fresh frozen human infant medullae specimens (N=15) were accrued from autopsy services at the Department of Pathology Boston Children’s Hospital and the office of chief medical examiner San Diego, CA. All autopsy information and specimens were obtained under the auspices of the San-Diego Medical Examiner system in accordance with California law. The committee on clinical investigation at Boston Children’s Hospital approved this study. Eight cases were male and seven female. Post conceptional age (PCA) ranged from 26-68.6 weeks and gestational age (GA) ranged from 25.9-41 weeks, with 2 premature cases. Post mortem interval (PMI) ranged from 0.5-27.7 hours with a median PMI of 17.4 hours. All 15 cases were classified as acute deaths, defined as a healthy infant who had died suddenly and in whom a complete autopsy established a known cause of death. None of the medullae demonstrated pathology by standard histopathological criteria and none of the cases were classified as undetermined or SIDS deaths.

Formalin fixed paraffin embedded human infant medullae specimens (N=10) were obtained from Forensic Science South Australia (FSSA) with permission for use granted by FSSA and the University of Adelaide Human ethics committees granted human tissue research approval. Cases dated from 1999 to 2006 with all cases having had autopsies performed in South Australia by Forensic Pathologists at FSSA. Seven cases were male and three female. Cases ranged from 40-57.7 PCA weeks, GA ranged from 29-39 weeks, with 2 cases being premature. PMI ranged from 16-47 hours with a median PMI of 27.6 hours. All 10 cases were classified as acute deaths, defined as a healthy infant who had died suddenly and in whom a complete autopsy established a known cause of death. None of the medullae demonstrated pathology by standard histopathological criteria and none of the cases were classified as undetermined or SIDS deaths.
2.2.2 Anatomical identification of nuclei within the medullary 5-HT network

The normative distribution of the medullary 5-HT network defined previously by Kinney et al. (2007) consists of neurons with cell bodies located in the raphe nuclei (raphe obscurus and raphe magnus), extra raphe nuclei (paragiganotcellularis lateralis, gigantocellularis, intermediate reticular zone, and sub trigeminal nucleus) and ventral surface (arcuate nuclei). SP and NK1R distribution throughout the medulla oblongata and medullary 5HT network was analyzed in 14 nuclei in each specimen (fresh frozen and formalin fixed paraffin embedded tissue respectively) at defined levels of the brainstem, including the caudal-mid medulla level 5 which constituted the level of the nucleus of Roller (Plate X), and the rostral medulla level 7 (Plate XII) according to the atlas of Olszewski (1954). The raphe’ nuclei were classified according to Tork (1990) as previously described (Duncan et al., 2008, Paterson and Darnall, 2009). Nuclei included the caudal-mid raphe obscurus (ROb), rostral midline Raphe (RMid), nucleus of the solitary tract (NTS), dorsal motor nucleus of the vagus (DMX), hypoglossal nucleus (HG), intermediate reticular zone (IRZ), gigantocellularis nucleus (GC), paragigantocellularis lateralis nucleus (PGCL), dorsal accessory olive (DAO), principle inferior olive (PIO), medial accessory olive (MAO), sub trigeminal nucleus (SUB), arcuate nucleus (Arc) and cochlear nucleus (COCH) (Fig 2.1). All 14 nuclei were not available for analysis in some cases.
Fig 2.1 Autoradiographic grey scale images of $^{125}$I Bolton Hunter SP binding to NK1R receptors in transverse sections of the caudal and rostral human infant medulla. 14 nuclei in total were analyzed including caudal-mid raphe obscurus (ROb), rostral midline Raphe (RMid), nucleus of the solitary tract (NTS), dorsal motor nucleus of the vagus (DMX), hypoglossal nucleus (HG), intermediate reticular zone (IRZ), gigantocellularis nucleus (GC), paragigantocellularis lateralis nucleus (PGCL), dorsal accessory olive (DAO), principle inferior olive (PIO), medial accessory olive (MAO), subtrigeminal nucleus (SUB), arcuate nucleus (Arc) and cochlear nucleus (COCH, not shown).
2.2.3 Determination of SP receptor specific binding density to NK1R in the normal human infant medullae with $[^{125}\text{I}]$ Bolton Hunter SP autoradiography

Unfixed brainstems were stored frozen at -80°C and subsequently sectioned at 20μm on a Leitz cryostat and thaw mounted onto super frost plus, glass microscope slides (Thermo Fisher Scientific). Receptor binding density expressed as the specific activity of tissue-bound ligand was analyzed. SP specific binding density was performed using 0.15nM $[^{125}\text{I}]$- Bolton Hunter labelled Lys3 Substance P autoradiography. Sections were pre-incubated in 50nM TrisHCl (pH 7.4), 0.02% bovine serum albumin for 15 minutes at room temperature, then incubated in the same buffer containing 0.1nM $^{125}$I BH-SP (Perkin-Elmer Inc., Wellesley Mass), 3nM MnCl$_2$, Chymostatin 2ug/ml, Leupeptin 4ug/ml and Bacitracin 40ug/ml for 60 minutes at room temperature. Nonspecific binding was determined in the presence of 5um SP added to the incubation solution. Sections were then washed 1x 2 minutes at room temperature in 50nM Tris-HCl (pH 7.4), 6x1 minute in ice cold 50nM Tris-HCl (pH 7.4) and finally 1x 2 minutes at room temperature in distilled H$_2$O + 0.02% BSA. Sections were then dried in warm air before being placed in cassettes and exposed to $^{125}$I-sensitive film (Kodak BMR) for 10 days along with a set of $^{125}$I standards (Amersham) for conversion of optical density of silver grains to fmol/mg tissue.

2.2.4 Quantitative analysis of brainstem autoradiograms

Film autoradiograms were generated according to standard laboratory procedure for development of light-sensitive film. Digital autoradiogram images of SP specific receptor binding in target nuclei of the human infant medulla were generated as TIFF files from the autoradiography film using MCID Imaging system (Imaging Research, Ontario, Canada). Autoradiograms were generated in grey scale prior to using MCID software to calibrate the images to $^{125}$I radioactive standards for normalization. Quantitative densitometry analysis of total and non-specific binding density was then measured in fmol/mg in the specific nuclei of interest. Total receptor binding was determined in 2 sections and non-specific receptor binding in 1 section for each nucleus analyzed. Specific receptor binding density was determined by subtracting nonspecific binding from total binding.

2.2.5 Photomicrograph production

Images of NK1R binding in the human infant medulla were generated as TIFF files from the autoradiography film by the MCID Imaging system (Imaging Research, Ontario, Canada). All images were then imported into Photoshop 6.0 (Adobe Systems, San Jose, CA) where they were scaled
relative to each other and appropriate labels were added to form composite images for subsequent assessment.

2.2.6 Normative distribution of SP/NK1R relative to medullary 5-HT network in human infant using fixed tissue immunohistochemistry and immunofluorescence

Single labelled immunohistochemistry (IHC) was performed for tryptophan hydroxylase 2 (TPH2, 5-HT neurons), SP and NK1R. Double label immunofluorescence (IF) was performed for 5-HT relative to SP and 5-HT relative to NK1R, respectively. The antibodies selected for analysis are summarized in Table 1. The distribution of each marker was determined in two adjacent sections of medulla selected from medullary levels 5-7 (caudal level of postrema to rostral level of paragigantocellularis nuclei), based on defined discrete nuclear landmarks previously described (Kinney et al., 2001). In all assays additional tissue sections were assigned as controls and incubated in 4% NGS overnight in the absence of primary antibody to determine that the IHC/IF observed was specific for target protein.

2.2.7 Single label immunohistochemistry in formalin fixed paraffin embedded tissue

Five micron, formalin-fixed paraffin embedded sections of human infant medulla were de-paraffinized before antigen retrieval with 1x citrate buffer solution (pH 6.0) in a temperature controlled antigen retrieval microwave for 10 minutes at 95°C. Sections were cooled for 10 minutes at room temperature before being washed in running water for 5 minutes. Sections were then washed in 1x phosphate buffered saline with 0.2% tween for 30 minutes. Sections were rinsed and then blocked in appropriate serum (horse or goat) and PBS plus 0.05% tween for 60 min before being incubated in primary antibody at optimal dilution, overnight at 4°C. On day two, sections were then washed 3x20min in PBS with 0.2% tween before being incubated in a biotinylated secondary antibody (Table 2.1) for 60min, rinsed briefly in PBS + tween and incubated in avidin horseradish peroxidase solution for 1 hour. Staining was then developed by DAB vector kit and sections washed in running water before counterstained in Mayer’s haematoxylin, and then dehydrated in a series of alcohols and cleared in histolene before finally cover slipped with paramount for assessment and analysis.

2.2.8 Double label immunofluorescence in formalin fixed paraffin embedded tissue

Double label immunofluorescence was performed to determine the distribution and cellular localization of SP relative to 5-HT and NK1R relative to 5-HT in 5µm paraffin embedded fixed tissue medullae sections using the same panel of primary antibodies used for single label (Table 2.1). Tissue sections were de-paraffinized using a series of incubations in xylene 5 minutes each (or 2x 5 minutes histolene) followed by quick dips in 100%, 95%, 80% and 70% alcohol. Sections were then rinsed
under running tap water for 2-3 minutes prior to antigen retrieval in 1x citrate buffer (pH 6.0). Sections were heated to 95°C in temperature controlled microwave for 10 minutes, then left to cool at room temp for 5-10 minutes prior to being rinsed under running tap water for 2-3 minutes. Blocking of non-specific binding was then performed by incubating sections in PBS + 4% serum (goat or horse) +0.05% triton for 1 hour. Block solution was removed and sections were then incubated simultaneously with both primary antibodies diluted in PBS and 4% serum and 0.05% triton overnight at 4°C. On day two, sections were rinsed in PBS and 2% tween prior to application of secondary immunofluorescent antibodies (Table 2.1) diluted in PBS and 4% serum and incubated for 30 mins at room temp in a dark box. Sections were then rinsed in PBS prior to being mounted with vectashield mounting medium for fluorescence with DAPI, cover slipped and stored at 4°C in a dark box for subsequent assessment and analysis.
Table 2.1. Selected antibodies and sources

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration</th>
<th>Species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Single label immunohistochemistry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK1R ab466</td>
<td>1:100</td>
<td>Rabbit polyclonal</td>
<td>ABCAM</td>
</tr>
<tr>
<td>SP ab14184</td>
<td>1:1000</td>
<td>Mouse monoclonal</td>
<td>ABCAM</td>
</tr>
<tr>
<td>PH8 (TPH2, 5-HT neurons) MAB5278</td>
<td>1:2000</td>
<td>Mouse monoclonal</td>
<td>Millipore</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG BA-1000</td>
<td>1:200</td>
<td>Goat Biotinylated</td>
<td>Vector</td>
</tr>
<tr>
<td>Horse anti-mouse IgG BA-2000</td>
<td>1:200</td>
<td>Horse Biotinylated</td>
<td>Vector</td>
</tr>
<tr>
<td><strong>Double label immunofluorescence</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK1R ab466</td>
<td>1:100</td>
<td>Rabbit polyclonal</td>
<td>ABCAM</td>
</tr>
<tr>
<td>SP ab10353</td>
<td>1:150</td>
<td>Guinea pig polyclonal</td>
<td>ABCAM</td>
</tr>
<tr>
<td>PH8 (TPH2) MAB5278</td>
<td>1:1000</td>
<td>Mouse monoclonal</td>
<td>Millipore</td>
</tr>
<tr>
<td>Alexa fluor IgG 488 A-11034</td>
<td>1:250</td>
<td>Goat anti-rabbit polyclonal</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Alexa fluor IgG 568 A-11031</td>
<td>1:250</td>
<td>Goat anti-mouse polyclonal</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Alexa fluor IgG 488 A-11073</td>
<td>1:250</td>
<td>Goat anti-guinea pig</td>
<td>Thermo Scientific</td>
</tr>
</tbody>
</table>
2.2.9 Image capture and processing

Single label IHC sections were scanned using a NanoZoomer-XR C12000 digital slide scanner (Hamamatsu Photonics) to produce NDPI files that could then be exported to NDP.view2 viewing software U12388-01 (Hamamatsu photonics), where adjustment of contrast and clarity, the addition of appropriate labels and scaling of images was performed. TIFF and JPEG files of tissue sections were then generated for presentation. Double-labelled immunofluorescent sections were scanned using a Virtual Slide Microscope and slide scanner VS120 (Olympus) to produce VSI files that were then visualized with an Olympus BX51 microscope using FITC and TRITC filters. VSI files were then exported into OlyVia ver2.9 software and Olympus Cell-cens software to produce TIFF and JPEG files for assessment. Both OlyVia and Cell-cens software were used to adjust the contrast and clarity of images, apply appropriate labels and scale images to one another for presentation.

2.3. RESULTS

2.3.1 $^{[35]}$I-BH-SP binding to NK1 receptors in the normal human infant medulla

The overall distribution of SP within the medulla oblongata in the current study was generally consistent with previous studies in both human infant and adult brainstem (Del Fiacco et al., 1984, Halliday et al., 1988, Rikard-Bell et al., 1990) and is summarized in Table 2.2. We observed highest NK1R binding density (fmol/mg) in the RMID and PIO nuclei. High binding was also present in the MAO, ROB and DAO. Intermediate to low binding was present in HG, GC, IRZ, PGCL, NTS, DMX and SUB and very low to negligible binding was present in the ARC and COCH (Table 2.2, Fig 2.2) likely due to a small sample of cases with these nuclei available for analysis. Therefore the ARC and COCH were subsequently omitted from further analysis given the limited number of cases in which these nuclei were observable. Post mortem interval had no significant effect on binding across nuclei analyzed. Analysis of binding by PCA revealed a significant reduction in binding in the ROB nuclei (p=0.009) and a trend for binding to decrease with age was observed across multiple nuclei analyzed (Fig 2.3). To determine if prematurity influenced binding, the density of NK1R sites in premature infants (defined as infants with gestational age <36 weeks) was compared to term infants (gestational age ≥ 36 weeks). Although only a small number of premature infants were present in this control cohort (n=2), premature infants were observed to have significantly higher binding (more than 50%) than term infants in the HG (p=0.005), ROB (p=0.02), GC (p=0.008), IRZ (p=0.02), PGCL (p=0.01), RMID (p=0.03) and PIO (p=0.02), with borderline significance observed in the DAO (p=0.06)(Table 2.3, Fig 2.4). Despite the limited number of premature cases in the cohort these observations suggest that reduced NK1R binding with PCA may be driven at least in part by higher binding in premature
infants compared to term infants. To determine differences between the sexes, further analysis by sex revealed significantly higher NK1R binding in male infants compared to females in the ROb (p=0.02) and DAO (p=0.02) with borderline significance observed in the GC (p=0.07) and PGCL nuclei (0.09) (Table 2.4, Fig 2.5). Significantly higher binding in males was also observed in the ARC (p=0.006), however the small sample size available for analysis of this nucleus would suggest that validation in a larger cohort is required.
Table 2.2 Normative distribution of SP, NK1R in the medullary 5-HT network in the human infant medulla.

<table>
<thead>
<tr>
<th>Nuclei</th>
<th>$^{125}$I BH-SP NK1R (fmol/mg)</th>
<th>Single label immunohistochemistry</th>
<th>Double label immunofluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SP</td>
<td>NK1R</td>
<td>5-HT</td>
</tr>
<tr>
<td>HG</td>
<td>++</td>
<td>+</td>
<td>X</td>
</tr>
<tr>
<td>NTS</td>
<td>++</td>
<td>+++</td>
<td>X</td>
</tr>
<tr>
<td>DMX</td>
<td>++</td>
<td>+++</td>
<td>X</td>
</tr>
<tr>
<td>RMID</td>
<td>++</td>
<td>+++</td>
<td>X</td>
</tr>
<tr>
<td>Rob</td>
<td>++</td>
<td>+++</td>
<td>X</td>
</tr>
<tr>
<td>GC</td>
<td>++</td>
<td>+++</td>
<td>X</td>
</tr>
<tr>
<td>IRZ</td>
<td>++</td>
<td>+++</td>
<td>X</td>
</tr>
<tr>
<td>PGCL</td>
<td>++</td>
<td>+++</td>
<td>X</td>
</tr>
<tr>
<td>SUB</td>
<td>++</td>
<td>+++</td>
<td>X</td>
</tr>
<tr>
<td>PIO</td>
<td>++</td>
<td>+++</td>
<td>X</td>
</tr>
<tr>
<td>MAO</td>
<td>++</td>
<td>+++</td>
<td>X</td>
</tr>
<tr>
<td>DAO</td>
<td>++</td>
<td>+++</td>
<td>X</td>
</tr>
<tr>
<td>ARC</td>
<td>++</td>
<td>+++</td>
<td>X</td>
</tr>
</tbody>
</table>

Binding and immunoreactivity intensity: + low, ++ intermediate, +++ moderately high, ++++ high, X not present
Fig 2.2 Autoradiograms displaying mean $^{125}$I Bolton Hunter SP binding to NK1R in transverse tissue sections of caudal and rostral human infant medulla. Highest density binding (fmol/mg) was observed in Midline Raphe (rostral) and PIO nuclei. High binding was also present in MAO, ROb and DAO. Intermediate to low binding was present in HG, GC, IRZ, PGCL, NTS, DMX and SUB. Very low to negligible binding was present in the ARC and, COCH (not shown here) likely due to a small sample of cases with these nuclei available for analysis.
Fig 2.3. Linear regression displaying NK1R binding density (fmol/mg) with PCA. Binding significantly decreased with PCA in ROb (p=0.009) and there was a trend for binding to decrease with PCA across multiple nuclei analyzed.
**Table 2.3.** NK1R binding by prematurity status. Premature infants had significantly higher NK1R binding density across multiple nuclei.

<table>
<thead>
<tr>
<th>Effect of prematurity</th>
<th>Premature N=2</th>
<th>Term N=13</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HG</td>
<td>2.74 (0.41)</td>
<td>1.16 (0.16)</td>
<td>0.005</td>
</tr>
<tr>
<td>DMX</td>
<td>0.97 (0.25)</td>
<td>0.89 (0.14)</td>
<td>0.78</td>
</tr>
<tr>
<td>NTS</td>
<td>0.05 (1.13)</td>
<td>0.8 (0.34)</td>
<td>0.53</td>
</tr>
<tr>
<td>SUB</td>
<td>0.93 (0.25)</td>
<td>0.6 (0.14)</td>
<td>0.3</td>
</tr>
<tr>
<td>ROb</td>
<td>4.55 (0.66)</td>
<td>2.62 (0.24)</td>
<td>0.02</td>
</tr>
<tr>
<td>GC</td>
<td>2 (0.33)</td>
<td>0.91 (0.12)</td>
<td>0.008</td>
</tr>
<tr>
<td>IRZ</td>
<td>1.79 (0.33)</td>
<td>0.84 (0.12)</td>
<td>0.02</td>
</tr>
<tr>
<td>PGCL</td>
<td>1.66 (0.32)</td>
<td>0.72 (0.11)</td>
<td>0.01</td>
</tr>
<tr>
<td>RMID</td>
<td>7.89 (1.11)</td>
<td>3.93 (0.61)</td>
<td>0.03</td>
</tr>
<tr>
<td>DAO</td>
<td>5.29 (1.36)</td>
<td>2.26 (0.48)</td>
<td>0.06</td>
</tr>
<tr>
<td>PIO</td>
<td>8.3 (1.47)</td>
<td>4 (0.53)</td>
<td>0.02</td>
</tr>
<tr>
<td>MAO</td>
<td>4.49 (1.46)</td>
<td>3.67 (0.59)</td>
<td>0.59</td>
</tr>
<tr>
<td>ARC</td>
<td>0.1 (0.77)</td>
<td>0.29 (0.37)</td>
<td>0.84</td>
</tr>
</tbody>
</table>

**Fig 2.4.** NK1R binding density in premature vs. term infants across multiple nuclei. Binding was significantly higher in premature infants in the HG, ROb, GC, IRZ, PGCL, RMID, and PIO with borderline significance in the DAO. **p=<0.01, * p=<0.05**
Table 2.4. NK1R binding by sex. Male infants had significantly higher NK1R binding in ROb, DAO and ARC with a trend for binding to be higher in males across nuclei analyzed.

<table>
<thead>
<tr>
<th>Effect of sex controlling for PCA</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>Male N=8</td>
<td>Female N=7</td>
</tr>
<tr>
<td>HG</td>
<td>1.54 (0.29)</td>
<td>0.91 (0.37)</td>
</tr>
<tr>
<td>DMX</td>
<td>0.69 (0.18)</td>
<td>1.04 (0.13)</td>
</tr>
<tr>
<td>NTS</td>
<td>0.53 (0.44)</td>
<td>1.13 (0.55)</td>
</tr>
<tr>
<td>SUB</td>
<td>0.63 (0.22)</td>
<td>0.71 (0.17)</td>
</tr>
<tr>
<td>ROb</td>
<td>3.36 (0.29)</td>
<td>2.06 (0.34)</td>
</tr>
<tr>
<td>GC</td>
<td>1.22 (0.18)</td>
<td>0.65 (0.22)</td>
</tr>
<tr>
<td>IRZ</td>
<td>1.1 (0.18)</td>
<td>0.63 (0.21)</td>
</tr>
<tr>
<td>PGCL</td>
<td>0.99 (0.17)</td>
<td>0.5 (0.2)</td>
</tr>
<tr>
<td>RMID</td>
<td>5.73 (1.1)</td>
<td>4.12 (1.1)</td>
</tr>
<tr>
<td>DAO</td>
<td>3.55 (0.57)</td>
<td>1.12 (0.66)</td>
</tr>
<tr>
<td>PIO</td>
<td>4.98 (0.83)</td>
<td>3.36 (1)</td>
</tr>
<tr>
<td>MAO</td>
<td>4.11 (0.66)</td>
<td>2.91 (1)</td>
</tr>
<tr>
<td>ARC</td>
<td>-0.45 (0.01)</td>
<td>0.46 (0)</td>
</tr>
</tbody>
</table>

Fig 2.5. NK1R binding density in male vs. female infants. Binding was significantly higher in male infants in the ROb, DAO and ARC (not shown, small sample size) with a trend for binding to be higher in males across nuclei analyzed. * p<0.05
2.3.2 Normative distribution of NK1R and SP immunoreactivity in the medulla using single label immunohistochemistry

NK1R distribution observed with single label IHC in formalin-fixed tissue confirmed the NK1R distribution profile observed in frozen tissue section autoradiography. The distribution of NK1R was comparable to that of SP however there were differences in their expression across nuclei. In the caudal medullary nuclei (HG, NTS, DMX) NK1R was observed to be extensively localized to large neurons in the HG that were of granular, fusiform and pyramidal morphology, consistent with α-motor neurons. In the NTS and DMX NK1R localized to small to medium granular and pyramidal neurons (Fig 2.6). In contrast, SP localized to very few if any neurons in the HG and NTS and was diffusely expressed, with dense punctate immunoreactivity observed in terminals and tracts. In the DMX SP did localize extensively to small granular neurons, with dense punctate terminal staining also observed (Fig 2.7). In the midline raphe nuclei at both the caudal and rostral levels (ROb and RMID respectively), the distribution of NK1R was distinctly different to that of SP. NK1R was extensively expressed in raphe terminals, with a large proportion of NK1R expressing neurons concentrated in uniform clusters extending vertically along the most central aspect of the raphe nuclei, inside the paramedian tracts. NK1R positive neurons were also observed outside this central cluster and were characteristically medium to large and granular in morphology (Fig 2.8). This distinct distribution of NK1R confirmed the high density NK1R binding in the midline raphe nuclei observed in autoradiography. In contrast, SP was densely expressed in a punctate form, primarily in terminals and tracts of the raphe nuclei with very few SP immunoreactive neurons observed, which were small to medium and granular, fusiform or pyramidal in morphology. These-SP positive neurons were distributed along the vertical paramedian tracts, comparable to the distribution of 5-HT expressing neurons also observed with single label IHC (Fig 2.8). In the extra raphe nuclei NK1R immunoreactive neurons were extensively distributed throughout the ventrolateral medulla from the pontomedullary junction rostrally, to the lateral reticular nucleus caudally, including PGCL (rostral medulla) and SUB (mid-rostral medulla). In the GC, IRZ, PGCL and SUB nuclei NK1R expression was also dense and punctate in terminals, processes and tracts (Fig 2.6). Similar to the distribution in the raphe nuclei, extensive, dense, punctate SP immunoreactivity was observed in fibres and terminals throughout the extra raphe region. In the PGCL and SUB nuclei SP localized to few neurons that were of granular or fusiform morphology (Fig. 2.7). In the IO nuclei, NK1R was extensively localized to small to medium granular and fusiform neurons within the PIO, DAO and MAO. Low to moderate NK1R punctate immunoreactivity was also observed in terminals and tracts of the IO nuclei. In contrast, SP was densely expressed primarily in terminals of the PIO, DAO and MAO. SP localized to few neurons in the PIO and MAO, however did not localize to neurons within the DAO. At the ventral surface (ARC) NK1R was localized to clusters of small granular and fusiform neurons, whereas SP was expressed moderately in fibres and terminals of the ARC (Fig. 2.6-2.7).
Fig 2.6. Distribution of NK1R immunoreactivity in nuclei of medullary 5-HT network, using single label IHC. NK1R immunoreactivity was extensive across all nuclei analyzed, primarily localizing at small to medium neurons. 40x mag. Scale bar = 100 µm.
Fig 2.7. Distribution of SP immunoreactivity in nuclei of medullary 5-HT network, using single label IHC. SP was diffusely expressed across all nuclei analyzed, primarily punctate in appearance in fibres and terminals, with some localization to small granular and fusiform neurons. 40x mag. Scale bar= 100µm
Fig 2.8. Single label IHC images of NK1R, SP and 5-HT immunoreactivity in the rostral midline raphe nuclei. The pattern of distribution of NK1R in the raphe nuclei was distinctively different from that of SP. The pattern of distribution of SP was similar to that of 5-HT and the pattern of distribution observed using single label IHC was consistent with the distribution observed in $^{125}$I BH-SP autoradiography and using DL-IF for all neurochemicals. Scale=100 $\mu$ m
2.3.3 Normative distribution of NK1R and SP relative to 5-HT in the medullary serotonergic network using double label immunofluorescence

Double label immunofluorescence showed extensive co-distribution and co-localization of SP and NK1R with the 5-HT network in key medullary nuclei (Table 2.2). As discussed previously, the pattern of distribution observed in the midline raphe nuclei of NK1R relative to 5-HT was distinctly different to that of SP relative to 5-HT. As observed in single label IHC, uniform clusters of neurons that exclusively expressed NK1R were distributed in the most central part of the upper portion of the midline raphe and did not co-localize with 5-HT at this portion of the raphe (Fig. 2.9). However at the mid level of the raphe, NK1R expressing neurons closely co-localized with 5-HT positive neurons that were expressed in their characteristic vertical and bilateral paramedian tracts or plates, many of which also co-expressed NK1R within the cytoplasm (Fig. 2.9-2.10). In contrast, SP expression within the raphe nuclei was comparable to that of 5-HT. SP co-localized within the cytoplasm in some but not all 5-HT-positive neurons situated along the vertical paramedian tracts. Of particular note was the observation that SP immunoreactive neurons appeared to always co-express 5HT i.e. there were few if any neurons that exclusively expressed SP (Fig.2.11-2.12). Co-localization of SP and 5-HT was observed primarily in the rostral raphe. In the extra raphe nuclei NK1R expressing neurons closely co-distributed with adjacent 5-HT positive neurons in the GC, IRZ, PGCL and SUB. In the GC, PGCL and SUB, some 5-HT expressing neurons also co-expressed NK1R in the cytoplasm, however this was observed more prominently in the rostral medulla at the level of the PGCL. Similarly SP co-localization with adjacent 5-HT expressing neurons was observed in the GC, IRZ, PGCL and SUB. Some adjacent 5-HT positive neurons in the PGCL and SUB co-expressed SP in the cytoplasm, albeit very few, however this was more prominent rostrally at the level of the PGCL. As observed in the raphe nuclei, SP positive neurons almost always co-expressed with 5-HT. 5-HT was not expressed in any of the IO nuclei, therefore no co-localization with NK1R or SP respectively, was observed. At the ventral surface within the ARC nuclei, some NK1R expressing neurons closely co-distributed with adjacent 5-HT positive neurons. Co-distribution of SP terminals with 5-HT expressing neurons was also observed in ARC and some but not all 5-HT expressing neurons also co-expressed SP in the cytoplasm.
**Fig 2.9.** Double label IF images of NK1R and 5-HT immunoreactivity within rostral midline raphe nuclei. Multiple 5-HT neurons co-expressed NK1R within the soma (arrows) merged images, however unlike SP, neurons that exclusively expressed NK1R were in abundance and not all 5-HT neurons co-expressed NK1R. 10x mag, Scale bar=100μm.
Fig 2.10. Double label IF images of NK1R and 5-HT immunoreactive neurons within the rostral midline raphe nuclei and rostral extra raphe nuclei at level of PGCL. Multiple 5-HT neurons also co-expressed NK1R within the cytoplasm (arrows) merged images. Neurons that exclusively expressed NK1R were in abundance (*) and not all 5-HT neurons co-expressed NK1R (*). 40x mag, Scale bar= 100 μ m.
**Fig 2.11.** Double label immunofluorescent images showing localization of SP and 5-HT neurons in rostral midline raphe (RMID). SP immunofluorescent staining, TPH2 immunofluorescent staining, merged images. SP co-localizes to soma and dendrites of a subset of 5-HT immunoreactive neurons. SP expressing neurons were always co-expressed with 5-HT (arrows). 40x mag, Scale bar=100 μ m.
Fig 2.12. Double label IF images showing localization of SP and 5-HT immunoreactive neurons in the rostral midline raphe (RMID) and the rostral extra raphe nuclei (level of PGCL). SP immunoreactive neurons were found to always co-localize to soma and dendrites of a subset of 5-HT immunoreactive neurons in both the raphe and extra raphe nuclei (arrows). 40x mag. Scale bar= 100μm.
2.4. DISCUSSION

2.4.1 Normative distribution of SP and NK1R within the human infant medulla

Multiple human post-mortem tissue studies have investigated the anatomical distribution of SP and its NK1R receptor in the adult and in the infant brainstem during development (Halliday et al., 1988, Rikard-Bell et al., 1990, Coveñas et al., 2003, Del Fiacco et al., 1984, Jordan et al., 1997, Chigr et al., 1991). In general the pattern of distribution observed in the present study was consistent these previous reports, principally that both SP and NK1R are ubiquitously expressed in multiple nuclei across the caudal and rostral medulla. However the present study differed in that the objective was to determine the interaction of SP and NK1R specifically within the medullary 5-HT network. In the present study the pattern of distribution of SP was comparable to that of NK1R, however there were differences in their expression, with SP primarily expressed in fibres and terminals and few cell bodies, whereas NK1R was extensively localized to cell bodies. Indeed studies have described a topographical mismatch between NK1R binding site densities and location of SP immunoreactive fibres and terminals (Halliday et al., 1988, Liu et al., 1994) particularly in the brainstem of the newborn (Jordan et al., 1995, Chigr et al., 1991) and NK1R are found independently of synapses that contain SP (Liu et al., 1994). Although this has been associated with differences in technique, the mismatch between sites of SP release and NK1R has been suggested to indicate that SP like other neuropeptides is capable of long range diffusion to target its receptors (Snijdelaar et al., 2000, Liu et al., 1994). Nevertheless the widespread distribution of the SP/NK1R system in the present study across medullary nuclei analyzed provides further support for the role of this system in the modulation and control of cardiorespiratory, cardiovascular and autonomic function (Gray et al., 2001, Mazzone et al., 1997, Helke and Seagard, 2004).

2.4.2 Normative developmental distribution of NK1R binding within the human infant medullary 5-HT network during postnatal development

The development of SP-containing neuronal pathways begins early in development and the SP network is largely established well before adult transmitter contents are reached, similar to that of the 5-HT system (Herlenius and Lagercrantz, 2001). The significant decrease of NK1R binding with PCA observed in the ROb and a general trend for NK1R binding to decrease with age across all nuclei analyzed, is generally consistent with previous reports that medullary SP/NK1R expression is at peak some point during gestation and normally declines during development after birth to reach adult levels in both animal studies and post-mortem human tissue analysis (Quirion and Dam, 1986, Rodier et al., 2001, Bergström et al., 1984). However some studies have reported contradictory observations of no significant changes in NK1R binding with age (Jordan et al 1995), whereas others observed increased density of SP immunoreactivity with age (Chigr et al 1991). Nevertheless, a relatively greater importance of SP in early life is acknowledged. Indeed animal studies have shown that juveniles are more sensitive to SP, and its modulation of respiratory activity is more important during the early postnatal period particularly when the juvenile is challenged by hypoxia.
(Shvarev and Lagercrantz, 2006, Yamamoto and Lagercrantz, 1985). The observation of significantly higher NK1R binding in premature compared to term infants further suggests that key medullary nuclei are particularly sensitive to SP neurotransmission during early life. Given that SP has been suggested to be involved in the resetting and adaption of an organism to extrauterine life (Herlenias and Lagercrantz), higher NK1R binding observed in premature infants may be a compensatory response to ensure that SP expression is adequate for this role. Alterations in the early postnatal expression of SP and NK1R could therefore have significant implications for pathologies associated with abnormal development of neurotransmitter networks within key cardiorespiratory and autonomic medullary nuclei, as has been shown for the medullary 5-HT system in SIDS. Indeed destruction of NK1Rs in animal studies impairs or eliminates breathing (McKay et al., 2005) and blocks somatosympathetic reflexes (Makeham et al., 2005) and children with Rett’s syndrome who have significantly decreased levels of SP in the cerebrospinal fluid (Matsuishi et al., 1997) and in key nuclei within the brainstem (Deguchi et al., 2000, Saito et al., 2001) exhibit highly unstable breathing patterns, increased apnoeas and alternating episodes of hypoventilation and irregular hyperventilation (Katz et al., 2009, Weese-Mayer et al., 2006, Lugaresi et al., 1985, Ramirez et al., 2013).

In addition to the effects of prematurity on binding, we also observed a significant sexual dimorphism in NK1R binding, with males exhibiting significantly higher binding compared to females in the ROB, DAO and ARC, although a small sample size available for the ARC precluded this nuclei from further analysis. Sexual dimorphisms have been reported in multiple neurotransmitter systems including 5-HT (Cahill, 2006, Nishizawa et al., 1997, Madeira and Lieberman, 1995) and a particular brain region may vary between the sexes in its neurotransmitter function and response to different experiences (Cahill, 2006). Higher NK1R binding in males in key medullary nuclei may provide further evidence for a greater role of SP/NK1R innervation during development in male compared to female infants. These sex differences in brainstem neurotransmitter expression have direct implications for disorders in which the rate of male deaths are significantly higher than that of females (Trachtenberg et al., 2012, Kinney and Thach, 2009b), particularly deaths associated with respiratory conditions (Mage and Donner, 2004). Therefore sex must be considered across all aspects of investigation of paediatric neuropathology.

2.4.3 Distribution of SP and NK1R relative to 5-HT in the human infant medullary serotonergic network during postnatal development

In the present study, SP and NK1R extensively co distributed and co-localized with 5-HT expressing neurons within the medullary 5-HT network. While co distribution of SP/NK1R with 5-HT was observed across both the caudal and rostral medulla, co-localization of both SP and NK1R to 5-HT positive neurons was predominantly observed in the rostral medulla, consistent with previous reports (Halliday et al., 1988). The extensive nature of distribution of both systems in nuclei intimately related to cardiorespiratory and autonomic control, supports the idea that SP and 5-HT exhibit a synergistic relationship within the brainstem

**Medullary raphe nuclei**

Perhaps the most striking feature of SP/NK1R distribution observed within this study was the high-density expression of NK1R binding in the midline raphe that was distinctly different to that of SP. Although we did not use markers for glia or growth factors in the present study, we questioned whether the distinct expression of NK1R vertically along the most central aspect of the raphe nuclei observed with both tissue receptor autoradiography and IHC, could be potentially localizing at midline radial glial cells within the raphe nuclei. Radial glial cells present in the midline brainstem are thought to have role in the maintenance of brain structure during the early developmental period (Horie et al., 2000, Paolicelli et al., 2011, MacFarlane et al., 2016, Wake et al., 2011). Indeed a reported feature of the SP receptor in the fetal brain is its localization on immature glial cells in the midline with a body of evidence suggesting the presence of NK1R in cells of the glial lineage in vitro (Horie et al., 2000, Torrens et al., 1989, Mantyh et al., 1989). In support of this Horie et al. (2000) reported that SP receptor expressing immature glial cells at the medullary midline raphe were involved in the development of SP immunoreactive neurons both in the formation of medullary raphe nuclei and in axon guidance and growth. These observations strongly suggested that during the mid-gestational to early postnatal period, SP and NK1R have a mutual role in the development of raphe neurons. Therefore the significant decline in NK1R binding in the ROb observed in the present study may be indicative of the end role of the SP/NK1R system in the raphe nuclei during the postnatal period.

Further to the striking pattern of NK1R expression was the relative distribution of SP and 5-HT that was observed to be distinctly different to the relative distribution of NK1R and 5-HT in the midline raphe nuclei. SP immunoreactivity mirrored that of 5-HT, with SP-positive neurons expressed along the same bilateral paramedian tracts where 5-HT is characteristically expressed. SP positive neurons also appeared to always co-express 5-HT i.e. there were few if any neurons that exclusively expressed SP. NK1R immunoreactive neurons were expressed in distinct linear uniform clusters vertically along the most central portion of the midline raphe, with neurons that exclusively expressed NK1R being in abundance. In addition to a potential relationship of NK1R with the glial cell lineage in the raphe nuclei, midline glial processes have been shown to guide the migration of 5-HT neurons from the ventricular zone to form the raphe nuclei in rodents (Wallace and Lauder, 1983). Additionally, as already suggested for SP, 5-HT has been identified as necessary during the maturation of the neuronal network and is considered to have a critical role in respiratory plasticity, serving as a trophic factor with direct effects on neuronal proliferation, migration and differentiation (Herlenius and Lagercrantz, 2004, Sodhi and Sanders-Bush, 2004, Di Pasquale et al., 1994).

Interactions between 5-HT, SP and their receptors may therefore have a significant role in the formation of raphe nuclei and neurogenesis within the medulla. Indeed there is increasing evidence that neurotransmitters
and their receptors are used as developmental signals, crucial for the development of synapses and formation of neuronal networks, by modulating the construction and plasticity of brain circuits (Herlenius and Lagercrantz, 2004, Whitaker-Azmitia, 1991, Gaspar et al., 2003), with the development of different neurotransmitter systems being directly related to one another (Whitaker-Azmitia, 1991). Various neurotransmitters may therefore play a role within the immature brain that is entirely different to that of their role in the mature brain (Horie et al., 2000). As already mentioned, any dysfunction in SP or 5-HT neurotransmission during early life could cause long lasting structural and functional alterations (Hornung, 2003). Further investigation of the extent of a dual role of these neurotransmitters in development and identifying possible time-dependent neurochemical switches would assist with determining the critical period of developmental in which alterations in these systems coincide with a heightened vulnerability to environmental challenges. This is of particular importance for disorders such as SIDS where the majority of infants die during the first six months of life (Moon et al., 2007, Kinney et al., 2009a).

In addition to a potential dual role of SP and 5-HT in medullary raphe development, previous experimental studies have reported a large proportion of cell bodies in the raphe region that contain SP (Hökfelt et al., 2000, Ljungdahl et al., 1978) and these 5-HT/SP-synthesizing cells within the raphe are proposed as candidates for central chemoreceptors stimulated by CO₂ and pH (Iceman and Harris, 2014). Experimental studies have shown that spontaneous raphe neuron activity results in the endogenous release of both 5-HT and SP, and their combined efforts are critical for the generation of inspiratory rhythm and motor output in both neonatal and juvenile rodent systems, providing direct evidence for connections that mediate the excitation of respiratory output by a raphe 5-HT/SP system (Ptak et al., 2009). In a study by Iceman and Harris (2014) a previously unrecognizable pool of chemosensitive raphe neurons in the juvenile rat that were not 5-HT, however did express NK1R, were closely apposed to 5-HT expressing cells in the medullary raphe. The non-5HT cells were identified to have faster firing frequencies and larger hypercapnic responses than raphe 5-HT neurons in situ and the authors concluded that at least two distinct groups of raphe cells are activated by hypercapnia, those that are 5-HT/SP co-expressed and those that are non 5-HT expressing but do express NK1R, and are closely apposed to surrounding 5-HT cells. They concluded that the raphe provides both 5HT and non 5-HT innervation to the diaphragm. Furthermore Nattie et al. (2004) showed that both 5-HT and NK1R expressing neurons are required for the normal response to systemic CO₂ in juvenile rodents. Therefore the distribution of NK1R closely with 5-HT in the raphe nuclei of the human infant observed in the present study supports the idea that a population of adjacent non-5-HT, NK1R expressing neurons may interact within the medullary raphe nuclei to affect chemoreception in conjunction with 5-HT, the extent to which is yet to be fully determined. Further investigation of the relationship between SP/NK1R and 5-HT particularly within the raphe nuclei is important given dysfunction of neurotransmission in the raphe nuclei has been associated with paediatric brainstem pathology (Paterson et al., 2006a, Richter et al., 2003).
Medullary extra raphe nuclei

The distribution of the SP/NK1R system throughout the extra raphe nuclei of the ventrolateral medulla (GC, IRZ, PGCL and SUB) was generally consistent with previous studies in both rat and human (Nakaya et al., 1994, Huang and Paxinos, 1995, Benarroch et al., 2003). Clusters of NK1R expressing neurons observed specifically in the PGCL were of particular interest, given that the ventral aspect of the ventrolateral medulla has been reported as representative of the putative human homologue of the prebotzinger complex (PBC) (Schwarzacher et al., 2011, Ramirez, 2011). Although yet to be defined in the human infant medulla to date, the PBC is well characterized in animal studies and is identified as a critical component of the medullary respiratory and rhythm-generating network in mammals (Smith et al., 1991), in addition to being a critical region for the generation of eupnea, sighs and gasps (Gray et al., 2001, Doi and Ramirez, 2008). The extensive distribution of SP terminals and NK1R expressing neurons in the extra raphe nuclei provides further evidence for the importance of the SP/NK1R system in the ventrolateral medulla for respiration and cardiovascular control at the level of the PBC (Benarroch et al., 2003, Wang et al., 2002). Altered SP expression in the PBC is identified to offset ventilatory rhythm generation in neonates (Morgado-Valle and Feldman, 2004a, Lavezzi and Matturri, 2008) and lesioning of the area results in depletion of NK1R and cessation of breathing in experimental animal studies (Wenninger et al., 2004, McKay et al., 2005, Gray et al., 2001). Indeed the presence of NK1R on PBC neurons in animal studies is highly characteristic and used as a marker for localizing the PBC (Gray et al., 2001, Schwarzacher et al., 2011). Furthermore the co-distribution and co-localization of SP and NK1R with 5HT in some neurons within the PGCL, is further evidence for interplay of both neurotransmitter networks within the ventrolateral medulla, consistent with reports that both 5-HT and SP have important roles in modulating PBC activity in experimental animal studies (Morgado-Valle and Feldman, 2004a, Lavezzi and Matturri, 2008).

2.5 CONCLUSIONS

In the present study we have observed an extensive distribution of the SP/NK1R system in the medullary 5-HT network of the normal human infant during development, with a trend for NK1R binding to decrease with PCA. Significantly higher binding was observed in premature and male infants. Our observations therefore provide further evidence suggesting a role for SP in early postnatal period and for the involvement of SP and its receptor in the modulation of medullary cardiorespiratory and autonomic control in conjunction with medullary 5-HT mediated pathways. Furthermore, we provide evidence for sexual dimorphism in the SP/NK1R system within the medulla, which could have implications for paediatric disorders in which males are at greater risk. Taken together our observations provide a baseline to facilitate future investigation of the SP/NK1R system and its interaction with 5-HT within paediatric brainstem disorders in early life.
Author contributions

Principal author (candidate) FMB conducted the experiments, analyzed and interpreted the data and drafted the manuscript. DSP assisted with interpretation of data and manuscript preparation. DSP, RWB and RV conceived the study. RWB, RV and AVL contributed to manuscript preparation.
Chapter 3. SIDS vs. Controls: Medullary SP/NK1R study
Developmental abnormalities in substance P, NK1 receptor binding in brainstem nuclei in sudden infant death syndrome related to sex and prematurity

Fiona M. Bright1,2, Roger W. Byard1, Jhodie R. Duncan3, Henry Krous4, Robert Vink5, David S. Paterson2

1Discipline of Anatomy and Pathology, Adelaide Medical School, University of Adelaide, Adelaide, SA, Australia
2Department of Pathology, Boston Children’s Hospital and Harvard Medical School, Boston, MA, USA
3Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville, VIC, Australia
4Department of Pathology, Children’s Hospital-San Diego, San Diego, CA, USA
5Sansom Institute for Health Research, University of South Australia, Adelaide, SA, Australia

ABSTRACT

Sudden infant death syndrome (SIDS) involves failure of arousal to potentially life-threatening events, including hypoxia during sleep. While neuronal dysfunction and abnormalities in neurotransmitter systems have been implicated, the specific pathways associated with cardiorespiratory failure are unknown. The neuropeptide substance P (SP) functions within key medullary nuclei to regulate cardiorespiratory and autonomic function and is recognized as a primary excitatory neurotransmitter and central mediator of cardiovascular reflexes such as baroreceptor sensitivity and chemoreceptor reflex modulation in response to hypoxia. Abnormalities in SP neurotransmission may therefore result in autonomic dysfunction during sleep and contribute to SIDS deaths. In the present study, we used [125I] labelled Bolton Hunter SP autoradiography to map the distribution and density of the SP tachykinin NK1 receptor (NK1R) to 14 specific nuclei intimately related to cardiorespiratory function and autonomic control, in the human infant medulla of 55 SIDS and 21 control (non-SIDS) infants. Compared to controls, SIDS cases exhibited a differential, abnormal development of the SP/NK1R system in the medulla. NK1R binding was significantly reduced in the nucleus tractus solitarii (NTS) and all three subdivisions of the inferior portion of the olivocerebellar complex; the principal inferior olivary complex (PIO), medial accessory olive (MAO) and dorsal accessory olive (DAO). Altered NK1R binding was influenced by prematurity and male sex, which may explain the increased risk of SIDS in premature and male infants. Together our observations support the concept that abnormal development of a multi-neurotransmitter network in the medullary homeostatic network may underlie the pathogenesis of an important subset of SIDS cases and that SIDS is a complex underlying developmental disorder with a prenatal etiology.

Subject terms: SIDS, substance P, tachykinin NK1 receptor, medulla, cardiorespiratory, somatomotor, pathogenesis
3.1 INTRODUCTION

There is substantial evidence for multiple neural mechanisms contributing to the fatal event in SIDS. However, the most compelling and reproducible research to date is focused on the hypothesis that SIDS is due to a developmental disorder of a multi-neurotransmitter network of neural pathways in the medulla oblongata that control critical homeostatic mechanisms (Kinney, 2005, Paterson et al., 2006a, Kinney, 2009a). Indeed abnormalities in various brainstem neurochemicals including catecholamines, neuropeptides, acetylcholinergic, indole amines (predominantly serotonin and its receptors), amino acids (predominantly glutamate), brain derived neurotropic growth factor (BDNF), and some cytokine systems have been reported in infants who died of SIDS (Duncan et al 2008, Kinney et al 1995, Kopp et al 1993, Machaalani and Waters 2003, Mallard et al 1999, Nachmanoff et al 1998, Obonai et al 1998, Panigrahy et al 1997, Yamanouchi et al 1993, Paterson et al 2006). Previously analysis of the expression of neuropeptide SP within the medulla in SIDS cases compared to controls has been inconsistent and inconclusive, with reports of increases, decreases and no change in SP expression (Bergström et al., 1984, Jordan et al., 1997). Therefore, following on from chapter 2, the present study utilized [125I] labelled BH-SP autoradiography to quantitatively examine the distribution and expression of SP binding to NK1R in 14 specific medullary nuclei intimately related to cardiorespiratory function and autonomic control, which have been previously implicated in the pathogenesis of SIDS, in the human infant medullae of SIDS compared to non-SIDS control cases. In contrast to previous studies, the present investigation used consistent standardized cause of death definitions for SIDS and non-SIDS control cases and incorporated a thorough analysis of key parameters such as age, prematurity status and sex and post mortem interval. Hence this study provides a comprehensive investigation of abnormal SP/NK1R expression within the medulla in SIDS cases contributing to a further understanding of the role of abnormalities in a multi-neurotransmitter network that may underlie the pathogenesis of a subset of SIDS infants.

3.2 METHODS SUMMARY

The committee on clinical investigation at the Children’s Hospital Boston approved the current study protocol. The medullae of 76 infants from 3 separate datasets extending from 2004 to 2015 were sourced from the Office of the Chief Medical examiner in San Diego. The cohort comprised of 41 male and 35 female infants. Deaths were classified as SIDS (n=55), acutely ill controls (n=15), chronically ill controls (n=2) and hypoxic controls (n=4). SIDS cases were classified according to the San Diego definition (Krous et al., 2004). Acute control cases were defined as infants who died acutely and in whom a definitive cause of death was established, chronic controls were defined as an infant under 1 year of age with a history of chronic or repetitive hypoxaemia associated with underlying cardiac, pulmonary, or neurological disorder and hypoxic controls were diagnosed according to definitive pathological findings at autopsy. Brainstems
did not demonstrate pathologic changes by standard histologic criteria. There were 19 premature cases in the cohort ranging from 26 to 69.29 PCA weeks with a median GA of 29.51 weeks. Non-premature cases ranged from 36.3 PCA weeks (term birth) to 76 PCA weeks. Median PMI for the entire cohort was 19.15 hours, with a range of 0.5-30 hours. Clinicopathological data is outlined in Table 3.1.

Fourteen nuclei of the human infant medulla in each specimen were targeted for analysis, although not all 14 nuclei were available in each case. Nuclei included the raphe obscurus (ROb), midline raphe (RMid), nucleus of the solitary tract (NTS), dorsal motor nucleus of the vagus (DMX), hypoglossal nucleus (HG), intermediate reticular zone (IRZ), gigantocellularis nucleus (GC), paragigantocellularis lateralis nucleus (PGCL), dorsal accessory olive (DAO), principle inferior olive (PIO), medial accessory olive (MAO), subtrigeminal nucleus (SUB), arcuate nucleus (Arc) and cochlear nucleus (COCH) at a defined level of the brainstem according to the atlas of Olszewski and Baxter (1954). The raphe nuclei were classified according to Tork and Hornung (1990). Determination of SP receptor specific binding density was performed using 0.15nM $[^{125}\text{I}]$- Bolton Hunter labelled SP autoradiography and expressed as the specific activity of tissue-bound ligand based on a previously reported protocol (Rodier et al., 2001). Quantitative densitometry analysis of total and non-specific binding density was measured in fmol/mg in the specific nuclei of interest in SIDS and in non-SIDS control infants. Total receptor binding was determined in two tissue sections and non-specific receptor binding in one tissue section for each nucleus analyzed. Specific receptor binding density was determined by subtracting non-specific binding from total binding. Statistical analysis of covariance (ANCOVA) adjusting for parameters including postnatal age (PNA), postconceptional age (PCA), sex, prematurity status and post mortem interval were performed across analyses, with dataset of origin also adjusted for in all statistical modelling.

<table>
<thead>
<tr>
<th>Table 3.1. Clinicopathological data</th>
<th>SIDS (n=55)</th>
<th>Acute (n=15)</th>
<th>Combined controls (n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age mean (±SD) weeks</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postconceptional</td>
<td>52.11 (7.88)</td>
<td>42.45 (10.27)</td>
<td>44.98 (11.73)</td>
</tr>
<tr>
<td>Gestational</td>
<td>37.17 (4.82)</td>
<td>35.4 (5.3)</td>
<td>36 (4.72)</td>
</tr>
<tr>
<td>Postnatal</td>
<td>21.86 (34.28)</td>
<td>10.48 (11.78)</td>
<td>11.43 (12.2)</td>
</tr>
<tr>
<td><strong>Birth status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premature</td>
<td>12</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Term</td>
<td>43</td>
<td>13</td>
<td>18</td>
</tr>
<tr>
<td><strong>PMI mean (SD) hours</strong></td>
<td>19 (5.73)</td>
<td>16.82 (7.4)</td>
<td>17.62 (6.64)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>33</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Female</td>
<td>22</td>
<td>7</td>
<td>9</td>
</tr>
</tbody>
</table>
3.3 RESULTS

3.3.1 \(^{[125]I}\) labelled SP binding to NK1R in human infant medulla in SIDS vs. controls

The highest density of \(^{[125]I}\) BH-SP labelled binding (>2 fmol/mg) to the NK1R in the medulla of the normal human infant (non-SIDS controls) was observed in the RMid nuclei and PIO nuclei. High binding (>1 fmol/mg) was also present in the ROb, MAO and DAO, while intermediate to low binding (<1 fmol/mg) was present in the HG, DMX, GC, IRZ, SUB, PGCL and NTS. Very low to negligible binding (<0.05 fmol/mg) was present in the ARC and COCH (Fig. 3.1), which were subsequently excluded from further analysis given the limited number of cases in which these nuclei were observable. Post mortem interval had no significant effect on NK1R binding across nuclei analyzed and analysis controlling for age, sex and PMI showed no significant differences in binding values between acute, chronic and hypoxic control groups. We therefore combined the three groups into a combined control cohort for subsequent analysis. Comparison of absolute binding levels (i.e., corrected for age but not prematurity or sex) revealed a significant reduction in NK1R binding sites in the NTS (p=0.04), DAO (p=0.01) and MAO (p=0.03) in SIDS cases compared to acute controls alone and in the ROB (p=0.048), PIO (p=0.002), DAO (p=0.01) and MAO (p=0.03) with borderline significance in the PIO (p=0.09) when compared to combined controls (Figs. 3.2-3.3).

3.3.2 Analysis of \(^{[125]I}\) labelled SP binding to NK1R by age and prematurity status

Analysis of binding by PCA revealed a trend for binding to decrease with age across all nuclei analyzed although a statistically significant reduction in binding was observed only in the ROB in both acute (p=0.009) and combined controls (0.04). To determine if prematurity status influenced binding, the density of NK1R sites in premature infants (defined as infants with gestational age <36 weeks) was compared to term infants (gestational age \(\geq\) 36 weeks). Although only a small number of premature infants were present in the control cohort (n=2 acute controls, n=1 hypoxic) a striking trend for higher binding was observed in these cases compared to term infants with an increase of more than 50% observed in each nuclei. Statistically significant increases in NK1R binding were observed in the HG (p=0.02), ROb (p=0.006), GC (p=0.008), IRZ (p=0.01) and PGCL (p=0.007), with borderline significance in RMID (p=0.07), DAO (p=0.06) and PIO (p=0.09) in premature combined controls compared to term infants (Table 3.2). Despite the small number of premature cases in the cohort these observations indicate that a trend for NK1R binding to decrease with PCA is driven at least in part by higher binding in premature infants compared to term infants.

Analysis of binding by age revealed a differential developmental pattern of NK1R expression in the medulla of SIDS cases. In contrast to controls, where a trend for NK1R binding to decrease with age was observed across all nuclei, there was a trend for NK1R binding to increase with age in SIDS cases in several nuclei including the DMX, HG, NTS and SUB and a significant PCA vs. diagnosis interaction was evident in the HG (p=0.03) and DMX (p<0.001) in SIDS cases (Fig 3.4). Binding within the DMX in particular indicates...
that a clear differential pattern of NK1R expression is present, given binding decreased with age in controls and increased with age in SIDS cases. In contrast, significant age-related reductions in NK1R binding were observed in the DAO (p=<0.001) and MAO (p=0.02) and a borderline significant reduction observed in the GC (p=0.07). This inconsistent developmental pattern of NK1R binding may be explained by the observation that in contrast to controls, NK1R binding in SIDS premature cases was not observed to be higher than term SIDS infants. Indeed, no significant differences in NK1R binding were observed between premature and term SIDS infants in any of the nuclei analyzed (Table 3.2, Fig 3.5). Moreover NK1R binding in premature SIDS infants was significantly lower in multiple nuclei including the HG (p=0.02), ROb (p=0.04), GC (p=0.001), IRZ (p=<0.001), PGCL (p=<0.001), RMID (p=0.04) and DAO (p=0.003) with borderline significance in the PIO (p=0.08) when compared to premature acute controls and in the ROb (p=0.04), GC (p=0.001), IRZ (p=<0.001), PGCL (p=<0.001), RMid (p=0.04) and DAO (p=0.003) with borderline significance in PIO (p=0.08) when compared to premature combined controls (Table 3.2). The difference in binding between term SIDS cases and term controls was much less marked. Compared to term acute controls, NK1R binding in term SIDS cases was significantly lower only in the NTS (p=0.04), with borderline significance observed in the MAO (p=0.07) and significantly lower in the MAO (p=0.05), with borderline significance observed in the NTS (p=0.08) when compared to combined controls. These observations suggest that the pathological process resulting in reduced NK1R expression in SIDS originates during gestation and is largely affected prior to birth.
**Fig 3.1.** Mean total NK1R binding density (fmol/mg) across medullary nuclei analyzed, presented as highest to lowest binding density.
**Fig 3.2.** Autoradiograms displaying NK1R binding (fmol/mg) in medullary nuclei in SIDS vs. control. NK1R binding density was quantitatively analyzed and shown to be decreased across multiple nuclei analyzed in SIDS vs. controls. Red= highest density, Orange/yellow= intermediate to high density, Green/light blue= intermediate to low density, Dark blue= low density, Black= lowest to negligible density.
Fig 3.3. Autoradiograms displaying NK1R binding (fmol/mg) in select nuclei. NK1R binding was significantly decreased in the NTS (0.04), DAO (p=0.01) and MAO (p=0.03) in SIDS compared with acute controls and in DAO (p=0.01), MAO (0.03) and borderline significance in PIO (p=0.09) compared with combined controls.
Table 3.2. Effect of prematurity status on NK1R binding. Significant effects of prematurity were observed in controls in multiple nuclei, with a trend for increased binding in premature compared with term cases across nuclei in acute and combined controls. No significant effects of prematurity were observed in the SIDS cohort.

<table>
<thead>
<tr>
<th></th>
<th>SIDS</th>
<th>Acute Controls</th>
<th>Combined Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Premature mean (±SE)</td>
<td>Term mean (±SE)</td>
<td>P value</td>
</tr>
<tr>
<td></td>
<td>N=12</td>
<td>N=43</td>
<td>N=2</td>
</tr>
<tr>
<td>HG</td>
<td>0.98 (0.17)</td>
<td>0.7 (0.1)</td>
<td>0.17</td>
</tr>
<tr>
<td>DMX</td>
<td>1.08 (0.26)</td>
<td>0.86 (0.09)</td>
<td>0.43</td>
</tr>
<tr>
<td>NTS</td>
<td>0.52 (0.09)</td>
<td>0.39 (0.05)</td>
<td>0.26</td>
</tr>
<tr>
<td>SUB</td>
<td>0.68 (0.72)</td>
<td>0.89 (0.24)</td>
<td>0.79</td>
</tr>
<tr>
<td>Rob</td>
<td>2.28 (0.36)</td>
<td>1.64 (0.23)</td>
<td>0.14</td>
</tr>
<tr>
<td>GC</td>
<td>0.83 (0.13)</td>
<td>0.64 (0.08)</td>
<td>0.24</td>
</tr>
<tr>
<td>IRZ</td>
<td>0.62 (0.13)</td>
<td>0.64 (0.08)</td>
<td>0.89</td>
</tr>
<tr>
<td>PGCL</td>
<td>0.61 (0.12)</td>
<td>0.55 (0.07)</td>
<td>0.69</td>
</tr>
<tr>
<td>RMDM</td>
<td>3.13 (1.23)</td>
<td>3.73 (0.4)</td>
<td>0.65</td>
</tr>
<tr>
<td>DAO</td>
<td>1.33 (0.21)</td>
<td>1.13 (0.17)</td>
<td>0.43</td>
</tr>
<tr>
<td>PIO</td>
<td>2.81 (0.63)</td>
<td>2.08 (0.39)</td>
<td>0.31</td>
</tr>
<tr>
<td>MAO</td>
<td>1.62 (0.36)</td>
<td>1.2 (0.24)</td>
<td>0.34</td>
</tr>
<tr>
<td>ARC</td>
<td>0.42 (0.21)</td>
<td>0.44 (0.12)</td>
<td>0.94</td>
</tr>
<tr>
<td>COCH</td>
<td>0.25 (0.06)</td>
<td>0.19 (0.03)</td>
<td>0.51</td>
</tr>
</tbody>
</table>
Fig. 3.4. NK1R binding by PCA across diagnoses in multiple medullary nuclei. In controls a trend for binding to decrease with age across nuclei was observed, however a differential developmental pattern was observed in SIDS cases with an inconsistent trend for binding to increase with age in select nuclei.
Fig 3.5. NK1R binding by prematurity status. Compared to combined controls binding was significantly decreased in premature SIDS cases in the ROb (0.04), GC (p=0.001), IRZ (p=<0.001), PGCL (p=<0.001), RMID (p=0.04) and DAO (p=0.003). Borderline significance was observed in PIO (p=0.08).
Analysis of binding by sex revealed an overall trend for higher binding in male compared to female infants. Significantly higher binding was observed in the ROb (p=0.02) and DAO (p=0.02) with borderline significance in PGCL (p=0.09) and GC (p=0.07) in acute male compared to acute female controls. A similar trend for higher binding in males was observed in combined controls with statistically significant increases observed in the DAO (p=0.02), with borderline significance in ROb (p=0.06) and GC (p=0.09) (Table 3.3).

In contrast, no significant differences in NK1R binding were observed between male and female SIDS cases in any of the nuclei analyzed, with the exception of a trend for binding to be higher in the PGCL in male SIDS cases (p=0.07 (Table 3.3). However, NK1R binding in male SIDS cases was significantly lower in the HG (p=0.04) and DAO (p=<0.001) with borderline significance in the PIO (p=0.09) and MAO (p=0.06) compared to male acute controls and significantly lower binding in DAO (p=<0.001), PIO (p=0.04) and MAO (p=0.03) compared to male combined controls (Fig 3.6). There were no significant differences in binding observed between female SIDS and female acute or female SIDS and female combined controls.

Analysis to determine the potential interaction of prematurity and sex on binding in SIDS cases revealed a trend for higher binding in several nuclei in premature male SIDS cases compared to term male SIDS cases. However a statistically significant increase was only observed in the HG (p=0.005) with borderline significance in the ROb (p=0.09). In contrast, no trend for increased binding and no statistically significant differences in binding were observed in premature female SIDS infants compared to term female SIDS in any of the nuclei analyzed.
Fig 3.6. NK1R binding density in male SIDS compared to male combined controls. NK1R binding was significantly lower in the DAO (p=<0.001), PIO (p=0.04) and MAO (p=0.03) in SIDS males, with a trend for binding to be decreased in SIDS males across nuclei analyzed. ***p=<0.001, ** = p < 0.01; * p < 0.05.
Table 3.3. NK1R binding by sex. Binding was significantly higher in multiple nuclei in control males vs. control females, with a trend for binding to be higher in males across nuclei analyzed. In SIDS however, there were no significant differences in binding between male and females, with the exception of trend for binding to be higher in PGCL.

<table>
<thead>
<tr>
<th></th>
<th>SIDS</th>
<th>Acute Controls</th>
<th>Combined Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male mean (±SE)</td>
<td>Female mean (±SE)</td>
<td>P value</td>
</tr>
<tr>
<td></td>
<td>N=33</td>
<td>N=22</td>
<td></td>
</tr>
<tr>
<td>HG</td>
<td>0.69 (0.12)</td>
<td>0.92 (0.13)</td>
<td>0.17</td>
</tr>
<tr>
<td>DMX</td>
<td>0.9 (0.15)</td>
<td>0.86 (0.12)</td>
<td>0.85</td>
</tr>
<tr>
<td>NTS</td>
<td>0.43 (0.06)</td>
<td>0.43 (0.07)</td>
<td>0.99</td>
</tr>
<tr>
<td>SUB</td>
<td>0.54 (0.36)</td>
<td>1.13 (0.32)</td>
<td>0.27</td>
</tr>
<tr>
<td>Rob</td>
<td>1.82 (0.25)</td>
<td>1.85 (0.28)</td>
<td>0.93</td>
</tr>
<tr>
<td>GC</td>
<td>0.77 (0.08)</td>
<td>0.57 (0.1)</td>
<td>0.1</td>
</tr>
<tr>
<td>IRZ</td>
<td>0.7 (0.08)</td>
<td>0.53 (0.1)</td>
<td>0.17</td>
</tr>
<tr>
<td>PGCL</td>
<td>0.64 (0.07)</td>
<td>0.44 (0.09)</td>
<td><strong>0.07</strong></td>
</tr>
<tr>
<td>RMID</td>
<td>3.84 (0.51)</td>
<td>3.41 (0.66)</td>
<td>0.62</td>
</tr>
<tr>
<td>DAO</td>
<td>1.24 (0.14)</td>
<td>1.07 (0.18)</td>
<td>0.37</td>
</tr>
<tr>
<td>PIO</td>
<td>2.35 (0.4)</td>
<td>2.29 (0.5)</td>
<td>0.92</td>
</tr>
<tr>
<td>MAO</td>
<td>1.45 (0.23)</td>
<td>1.13 (0.28)</td>
<td>0.36</td>
</tr>
<tr>
<td>ARC</td>
<td>0.56 (0.09)</td>
<td>0.23 (0.13)</td>
<td><strong>0.08</strong></td>
</tr>
</tbody>
</table>
3.4 DISCUSSION

In this study we observed abnormalities in NK1R binding in multiple nuclei intimately related to the control of cardiorespiratory and autonomic function in the medulla oblongata of SIDS cases. SP functions within key medullary nuclei to regulate cardiorespiratory and autonomic function and is recognized as a primary excitatory neurotransmitter and central mediator of cardiovascular reflexes in response to hypoxia (Gray et al., 2001, Mazzone et al., 1997, Helke and Seagard, 2004). Normal breathing in mammals requires an intact prebotzinger complex (PBC), which is especially sensitive to SP modulation (Gray et al., 2001, Gray et al., 1999). Altered SP neurotransmission within the PBC offsets ventilatory rhythm generation in neonates (Morgado-Valle and Feldman, 2004b) and lesioning of NK1R expressing neurons within the PBC results in profoundly abnormal respiratory patterns (Gray et al., 2001, Wenninger et al., 2004). Therefore disruption of the SP/NK1R system within the medulla may contribute to dysfunction of homoeostatic control, supporting the idea that abnormalities in a multi-neurotransmitter network underlies the pathogenesis of an important subset of SIDS cases.

3.4.1 Absolute reductions in NK1R binding in the NTS and IO medullary nuclei in SIDS

A striking feature of the abnormalities observed is that many were related to either prematurity or male sex. However, absolute reductions in NK1R binding that were not related to either prematurity or sex, were observed in the NTS and all sub-divisions of IO (PIO, MAO, DAO). Indeed, the NTS was the only nuclei in which NK1R binding was significantly lower in term SIDS cases compared to term controls, indicating a critical role of SP neurotransmission within this nuclei. The NTS houses the primary relay station for brainstem transmission of important respiratory and cardiovascular reflexes and is enriched with a high density of SP containing axon terminals (Gillis et al., 1980, Massari et al., 1998). Evidence from animal studies indicates a functional role for SP in the NTS as a central integrator of cardiovascular control (Morilak et al., 1988), modulator of baroreceptor reflex sensitivity (Seagard et al., 2000) and a primary excitatory mediator of the chemoreceptor reflex in response to hypoxia (Lessard et al., 2010, Nichols et al., 2014). In rodent models, activation of NK1R by SP in the NTS stimulates respiration, while loss of NK1R reduces the respiratory response, severely impairing the chemoreceptor reflex (Abdala et al., 2006, Mazzone and Geraghty, 1999, Nattie and Li, 2002). Furthermore, selective lesioning of NK1R expressing neurons in the NTS and other chemoreceptor sites blunts cardiovascular reflexes and decreases ventilation and chemosensitivity. These observations support the concept that the abnormal expression of NK1R in the NTS, as observed in this study, may result in dysfunction of critical cardiorespiratory reflexes in response to harmful stimuli such as hypoxia and may contribute to an inability of an infant to appropriately respond to life threatening challenges during sleep.

In contrast to the NTS, the IO is not generally considered to play role in the regulation and coordination of homeostatic function. Rather the IO is a pre-cerebellar relay network containing climbing fibres to Purkinje cells in the cerebellar cortex, and is considered a central site for the integration of motor and sensory
information (Ausim Azizi, 2007, De Zeeuw et al., 1998). Notably, previous studies of SIDS have described neurotransmitter deficits (Broadbelt et al., 2011, Panigrahy et al., 2000a), significantly altered neuron density (Kinney et al., 2002) and substantial reactive gliosis (Kinney et al., 1983) within the IO with a subset of SIDS deaths. The PIO, DAO and MAO project to multiple regions of the cerebellum (Coffman et al., 2011), which has been directly implicated in SIDS given its influences on upper airway and respiratory muscle regulation and blood pressure control in response to hypotensive challenge (Harper, 2000, Kinney et al., 1992). Afferent input to the cerebellum via the IO or climbing Purkinje fibres from the olivary nuclei have been implicated in the failure of cerebellar mechanisms to produce adequate somatomotor response (i.e. head lift/tilt, respiratory muscle activity) to overcome cardiorespiratory challenges during sleep (Harper et al., 2000). Given that the density and distribution of neuropeptides including SP within the IO has led to the proposal of a modulatory role in olivary neuron output and activity (Gregg and Bishop, 1997), abnormal reductions in NK1R binding in the IO observed in the present study, could have significant implications for cerebellar circuitry and ultimately motor control in SIDS infants.

3.4.2 Significant differential developmental profile of NK1R binding in SIDS cases related to prematurity

As described above one of the striking features of this study was the differential developmental profile of NK1R expression in SIDS cases compared to controls. In animal models, SP expression is reported to peak at birth and decrease over the course of development to reach adult levels of expression (Quirion and Dam, 1986, Rodier et al., 2001). Generally consistent with this, in our control cohort we observed a trend for binding to decrease with PCA across all nuclei analyzed with highest binding observed in the first weeks after birth, thereafter declining to a level at six months of age that remained constant up to one year. The significantly higher density of binding observed in premature infants is likely to have strongly influenced this observation and the small number of premature control infants (n=3) likely explains why statistical significance was not reached in any of the nuclei. In contrast to controls, an inconsistent pattern of age-related changes was observed in SIDS cases. Indeed a paradoxical trend for binding to increase with age was observed in the HG and DMX with a significant age versus diagnosis interaction observed in these nuclei where binding decreased with age in controls but increased with age in SIDS cases. In addition, no differences in NK1R binding were observed between premature and term SIDS infants, potentially explaining the differential age-related pattern in NK1R expression observed between SIDS cases and controls. NK1R binding in premature SIDS infants was significantly lower than in premature controls infants in the ROB, GC and PGCL, which are recognized as key autonomic and respiratory control nuclei within the medullary homeostatic network. However NK1R binding was lower only in the NTS in term SIDS infants compared to term controls. These observations indicate that prematurity is, at least in part, driving the observations of altered NK1R binding in this study. A caveat to these observations is that only n=3 premature infants are included in the control cohort; nevertheless the magnitude of the difference in
binding between premature controls and premature SIDS infants was greater than 50% in each of the nuclei, indicating that the observation is robust despite the small sample size of the premature control cohort. The limited number of premature control cases may also explain why we did not observe more widespread absolute differences in NK1R binding in SIDS cases compared to controls. Animal models have shown that juveniles are more sensitive to SP and its modulation of respiratory activity during the early postnatal period particularly when challenged by hypoxia (Shvarev and Lagercrantz, 2006, Yamamoto and Lagercrantz, 1985). Therefore SIDS infants and premature SIDS infants in particular may be at risk of respiratory failure given they exhibited abnormally decreased NK1R expression in key medullary nuclei during the early postnatal period. Taken together, these observations indicate that the pathogenesis of altered NK1R expression in SIDS originates during gestation and further supports the idea that SIDS is a complex underlying developmental disorder with a prenatal etiology.

3.4.3 Significant sexual dimorphism in NK1R binding in the medulla and implications for SIDS

The second striking feature of this study was the observation of a significant sexual dimorphism in NK1R binding within medullary nuclei. Male controls exhibited higher binding compared to female controls while no differences in binding were observed between male and female SIDS cases. Moreover, male SIDS cases displayed lower binding compared to male controls but no comparable differences were observed between female SIDS and female controls. Higher binding in male controls in the present study was likely responsible for driving an increase in the mean binding density when compared to SIDS cases. Normally males have a reduced ventilatory response to hypoxia and a longer post hypoxic recovery compared to females in experimental models (Holley et al., 2012, Garcia et al., 2013a) and sexual dimorphisms have been reported in a number of neurotransmitter systems (Nishizawa et al., 1997, Galanopoulou, 2005, Curtis et al., 2005). In addition, significantly decreased 5HT1A receptor binding has been reported in SIDS males compared to SIDS females (Paterson et al., 2006a), although this observation has yet to be replicated. Significant differences in binding between the sexes are consistent with observations across all analyses performed in the present study where SIDS males but not SIDS females exhibited significantly lower levels of NK1R binding when compared with controls. Lower binding in male SIDS combined with the relatively greater proportion of male to female SIDS (3:2) compared to controls (1:1) is likely responsible for reducing the mean binding density observed in SIDS cases. Indeed the triple risk model for SIDS recognizes male sex as an intrinsic risk factor, with mortality rates showing male predominance (3:2 ratio) (Blair et al., 2006, Kinney and Thach, 2009b). Accordingly, our observations provide further neurochemical evidence that may assist in understanding the increased vulnerability of male infants to SIDS.
3.5 CONCLUSION

In conclusion, this study has identified a subset of SIDS infants with a significant developmental abnormality of the SP/NK1R system, with altered NK1R binding in multiple nuclei intimately related to cardiorespiratory function, autonomic control, cerebellar circuitry and motor control within the medulla oblongata. These observations were influenced by prematurity and male sex, which may further explain the increased risk of SIDS in premature and male infants. Taken together, our observations support the hypothesis that abnormalities in a multi-neurotransmitter network underlie the pathogenesis of a subset of SIDS infants and that SIDS is a complex developmental disorder with a prenatal etiology.

Author contributions

Principal author (candidate) FMB conducted the experiments, analyzed and interpreted the data and wrote the manuscript. DSP assisted with interpretation of data and manuscript preparation. FMB, DSP, RWB and RV conceived the study. RWB, RV, HK and JRD contributed to manuscript preparation.
Chapter 4. SIDS vs. Controls: Medullary 5-HT study
Serotonin abnormalities in the medullary serotonergic network in a South Australia SIDS cohort

Fiona M. Bright1,2, Roger W. Byard1, Robert Vink3, David S. Paterson2

1Discipline of Anatomy and Pathology, Adelaide Medical School, University of Adelaide, Adelaide, SA, Australia
2Department of Pathology, Boston Children’s Hospital and Harvard Medical School, Boston, MA, USA
3Sansom Institute for Health Research, University of South Australia, Adelaide, SA, Australia

Abstract

Serotonin (5-HT) neurons in the medulla project extensively to key autonomic and respiratory nuclei in the brainstem and spinal cord, regulating critical homeostatic functions. Multiple abnormalities in markers of 5-HT function have been reported in the medullary 5-HT network in SIDS cases by several investigators. These observations informed the hypothesis that at least a subset of SIDS cases is caused by deficits in 5-HT function that result in impaired homeostatic responses to potentially life-threatening events during sleep. In 2006, Paterson and colleagues reported an increased number and density of immature 5-HT neurons in the medullary 5-HT network in a cohort of SIDS cases from the San Diego SIDS Database (Paterson et al., 2006). To date, however, this observation has not been replicated in an independent dataset. The objective of this study was to determine if the number and density of 5-HT neurons in the medullary 5-HT network is altered in an independent cohort of SIDS cases from South Australia. Formalin-fixed paraffin-embedded medullae from infants dying from SIDS (n=41) and non-SIDS infant deaths (n=28) were obtained from Forensic Science South Australia over the period 1999-2006. Sections of medulla were immunostained for tryptophan hydroxylase 2 (THP2) using the PH8 antibody to identify 5-HT positive neurons, followed by quantitative 5-HT neuron cell counting using the Neurolucida computer based method. Compared to controls, we observed significant differences in the number, density and morphological subtypes of 5-HT neurons in SIDS cases. Principally 5-HT neuron number and density were significantly higher in SIDS cases across all sub-regions of the caudal, mid and rostral medulla. Furthermore paradoxically higher proportions of 5-HT neurons of both immature and mature morphology were observed in SIDS cases, which varied depending on the medullary sub-region and level of medulla analyzed. This study further supports the hypothesis that medullary 5-HT dysfunction contributes to the pathogenesis of a subset of SIDS victims and provides additional evidence suggesting that 5-HT neuron abnormalities in SIDS may be more complex in conjunction with developmental abnormalities.

Subject terms: Serotonin, medulla oblongata, sudden infant death syndrome, neurons
4.1. INTRODUCTION

Sudden infant death syndrome (SIDS) is the sudden unexpected death of an infant less than 1 year of age that cannot be explained after a thorough investigation is conducted, including a complete autopsy, examination of the death scene, and a review of the clinical history (Krous, 2010). SIDS is the leading cause of death in infants (0-12 months) in Australia and the developed world. There is substantial evidence for multiple neural mechanisms contributing to the fatal event in SIDS, however the most compelling and reproducible research to date has focused on the hypothesis that SIDS is due to a developmental disorder of medullary serotonergic (5-HT) and related neurotransmitter systems within respiratory and autonomic brainstem mediated pathways. It is postulated that these abnormalities develop prenatally but are not uncovered until the postnatal period where there is heightened vulnerability of the infant to harmful stimuli such as hypoxia (Harper and Kinney, 2010, Kinney et al., 2009a, Kinney et al., 1992). 5-HT abnormalities have been reported across multiple separate and individual SIDS data-sets from varying ethnic, social and cultural backgrounds (Panigrahy et al., 2000a, Kinney et al., 2003, Duncan et al., 2010, Paterson et al., 2006a) involve raphe, extra-raphe, and ventral (arcuate) populations of the brainstem containing 5-HT neurons and their projection sites, such as the dorsal motor nucleus of the vagus and the nucleus of the solitary tract (Kinney et al., 2011). Abnormalities identified include alterations in 5HT receptor binding patterns (5HT1A and 5HT2A receptors) (Panigrahy et al., 2000a, Ozawa and Okado, 2002, Machaalani et al., 2009, Paterson et al., 2006a, Kinney, 2005), reduced brainstem levels of 5-HT and tryptophan hydroxylase 2 (TPH2) (the rate limiting enzyme regulating 5-HT synthesis) (Duncan et al., 2010), decreased binding to 5HT transporter relative to 5HT cell density (Paterson et al., 2006a), increased 5HT cell number and density of 5HT neurons, morphological immaturity of 5HT neurons (Paterson et al., 2006a) and reductions in the level of the 14-3-3 signal transduction family of proteins in regions of the medulla oblongata critically involved in the regulation of homeostatic function (Broadbelt et al., 2012). Given the complex role of 5-HT within the medulla, associated abnormalities have been identified as likely responsible for impaired reflexes and responses of critical autonomic respiratory defence mechanisms to exogenous stressors such as hypoxia (Harper and Kinney, 2010, Panigrahy et al., 2000, Kinney, 2009b).

While 5-HT abnormalities are the most reproducible observations of neurotransmitter abnormalities within the medullary network in SIDS research to date, replication and verification of these studies in separate independent cohorts of SIDS cases is crucial for a greater understanding of the mechanisms underlying neurotransmitter abnormalities and to facilitate future research into development of potential biomarkers for infants who may be at increased risk of SIDS. Hence this study is the first to attempt to replicate and corroborate in an independent dataset observations by Paterson et al. (2006), which reported an increased number and density of immature 5-HT neurons in the medullary 5-HT network in cohort of SIDS cases from the San Diego SIDS Database in the USA.
4.2. METHODS AND MATERIALS

4.2.1 Clinical Database

Formalin-fixed paraffin-embedded human brainstem medullae from 69 infant cases dying from either SIDS (n=41) or causes other than SIDS (n=28) were obtained from FSSA. Human tissue research approval was granted by South Australia Pathology and the University of Adelaide Human ethics committees. Cases were dated from 1999-2006 with all cases having had autopsies performed in South Australia by forensic pathologists at FSSA. Autopsy information and associated case notes were reviewed by senior forensic pathologists at FSSA with expertise in SIDS and infant pathology. SIDS cases were classified according to the San Diego definition. The non-SIDS cohort had full autopsy case reviews using a digital database, with causes of death diagnosed at autopsy that were then sub-classified into categories for the current study: Acute (19) and Asphyxia (9) controls respectively. Acute cases were defined as healthy infants who died suddenly and in whom a complete autopsy established a known cause of death. Acute deaths in this cohort included infection (8), trauma (4), drowning (3), congenital abnormalities (1) and combination of illnesses (3). Where a case was classified due to asphyxia there was a definite diagnosis based on the history, circumstances and autopsy findings. All cases in the study cohort had post-mortem intervals less than 72 hours.

Recognized risk factors for SIDS in each case were divided into two categories: 1) ‘abnormality’ risks including factors that may increase the probability of an infant having an underlying vulnerability or adverse prenatal exposure; and 2) ‘stressors’ including environmental or physical factors that impinge on the vulnerable infant during the critical period of postnatal development potentially challenging homeostatic function. These risk factors were determined for each SIDS and control case by review of the digital autopsy database. Race or ethnicity of each SIDS infants was also determined to establish whether there are differences in SIDS rates amongst different populations in the South Australian cohort that may influence observations.

4.2.2 Determination of number, morphology and density of 5-HT neurons in caudal, mid and rostral medulla

Immunohistochemistry was performed for tryptophan hydroxylase 2 (TPH2), a marker of 5-HT neurons, on 5µm formalin fixed paraffin embedded sections of infant brainstem medulla with the PH8 antibody (MAB5278 mouse monoclonal, Merck Millipore, USA) using previous protocols (Paterson et al., 2006a). Positively stained TPH2 neurons were counted at standardized regions of the caudal, mid and rostral medulla which had previously been rigorously defined based on the atlas of Olszewski and Baxter by Kinney et al (Olszewski, 1954, Kinney et al., 2007). In the current study these defined medullary regions were used as a guide and sub-classified into five levels: caudal medulla Level 4 and Level 5; mid-rostral medulla Level 6-6/7; and rostral medulla Level 7/8. The caudal medulla corresponds to Plate XI, mid
medulla corresponds to Plate XII and rostral medulla corresponds to Plate XIV according to the atlas of Olszewski and Baxter. One examiner blinded to the diagnoses of all cases manually counted immunopositive neurons marked with different graphic symbols and labels according to strict guidelines using the Neurolucida (64bit, MBF Bioscience) computer-based method. Neurons were only recorded as immunolabeled cell bodies if they were intensely stained and morphologically identifiable as immunopositive neurons belonging to one of five morphological cell types previously defined by Kinney et al. 2007 including granular, fusiform, pyramidal and multipolar, with immunopositive cells not able to be morphologically classified labelled as ‘other’ (Fig 4.1). The perimeter of each section was traced to determine the area (mm$^2$) for total cell density measurements. The distribution of total immunoreactive cells within the entire section and then specifically within the Raphe and Extra Raphe sub regions were counted, in addition to total number of cells by morphology within the entire section and regionally specific. Two sections per medullary level were counted twice by the same examiner and the mean cell count value used for statistical analysis. Review of both immunopositive neuron cell counts and classification of specific brainstem levels were performed by a second examiner, also blinded to cause of death diagnoses, with expertise in the methodology to ensure accuracy and consistency.

4.2.3. Statistical analysis

Analysis of covariance (ANCOVA) was used to examine mean differences in cell count, area and density measurements across medullary levels by diagnosis group with data adjusted for sex, post-mortem interval (PMI), and postconceptional age. Pairwise p-values for SIDS vs. each of the other cause of death groups were also analyzed and the p-values unadjusted for multiple comparisons. In all cases p<0.05 was considered statistically significant. There were no statistical differences between acute and asphyxia controls, therefore these groups could be combined for subsequent analysis against the SIDS cohort.

4.3. RESULTS

4.3.1 Clinicopathological data

SIDS cohort ages ranged from 1.1 to 51.4 postnatal weeks, with an average of 17.7 ± 9.8 weeks, compared to combined non-SIDS cases (acute and asphyxia) with an average age of 25.5 ± 19.3 postnatal weeks, ranging from 1 to 62.6 weeks. When combined, non-SIDS control cases trended towards being older than SIDS cases (p=0.077). However statistical analysis of age by individual diagnoses did not reveal a significant difference between SIDS and acute controls (p=0.203) or SIDS and asphyxia (p=0.125). There were no significant differences in age evident between non-SIDS groups (p=0.742). PMI was <72 hours in all cohorts, mean PMI for SIDS cohort was 25.05 ± 16.18 hours ranging from 6 to 71 hours compared to combined non-SIDS cases with mean 29.04 ± 11.97 ranging from 7 to 53 hours. There were no significant differences in PMI between SIDS and combined controls (p=0.244), or when analyzed by individual control diagnosis and there were no significant PMI differences between non-SIDS cohorts. Sex was analyzed by
level and diagnoses. The SIDS cohort comprised of 25 male and 16 female infants; acute: 12 male, 7 female; asphyxia: 6 male, 3 female. There were no significant differences in total 5-HT cell count between male and female SIDS cases at each level analyzed. There were also no significant differences in male and female SIDS cases compared to male and female non-SIDS groups analyzed by level (Table 4.1).

4.3.2 Risk Factors identified in SIDS cohort

The 41 SIDS cases comprised 25 male (61%) and 16 female (39%) infants from varying races and ethnicities. Information regarding race was available for 37 SIDS cases with 32 Caucasian (86%) and five Aboriginal (14%). Birth information was available for 28 SIDS cases, five were premature (18%) and 23 recorded as having term birth (82%). All 41 SIDS cases were identified as being exposed to at least one risk factor or stressor. Thirty-six cases (88%) were subject to at least one abnormality risk factor, 35 cases (85%) exposed to at least one stressor at the time of death and all cases were subject to at least 1 factor from both categories. Fourteen of the 41 SIDS cases were identified as being subject to a prenatal exposure including the mother smoking (seven cases; 17%), other members of the household smoking (two cases; 5%), mother abusing alcohol (one case; 2%) or mother abusing illicit drugs (eight cases; 20%) such as opiates, cannabis and amphetamines. There was also one case where the mother admitted to regular petrol sniffing (2%). Eleven SIDS cases were bed sharing with one or more adult/s or other children at the time of death (27%). Fifteen cases were found supine (37%) and 22 (54%) were found in an adverse sleeping position, 19 prone (46%) and 3 side (7%). One case was found face up (2%), 12 face down (30%), 5 to the right (12%), and four to the left (10%). Twenty-four cases (59%) were reported as having experienced symptoms or illness recently one month prior to death. Symptoms or illness described included cold and respiratory symptoms (19 cases; 46%), vomiting (6 cases; 15%) and diarrhoea (4 cases; 10%). Other symptoms included one case with a reported apnoeic episode previously after feeding, one case with abdominal pain and one case where the infant was described as having a croaky voice (Table 4.1). Comparison of 5-HT neuron count, density and morphology with age, sex and PMI showed no significant differences between SIDS and controls. Similarly no associations were observed for other risk factors identified.
Fig 4.1. The four morphological subtypes of 5-HT expressing neurons within human infant medullary 5-HT network (Kinney et al., 2007), stained for TPH2 with PH8 antibody. (A) Granular, considered the most immature, are small to round with a large nucleus and one to two thick cytoplasmic processes. (B) Pyramidal, large triangular shaped cytoplasm with thick processes extending from each point. (C) Fusiform, medium spindle shaped with two processes extending from each pole. (D) Multipolar, large oval cytoplasm with multiple processes extending outwards. Scale bar 10µm.
Table 4.1. Epidemiologic and Clinicopathological data for SIDS and control cases

<table>
<thead>
<tr>
<th>CASE</th>
<th>PMI</th>
<th>AGE</th>
<th>SEX</th>
<th>RACE</th>
<th>PREM</th>
<th>PRENATAL EXPOSURE</th>
<th>ABNORMALITY RISK</th>
<th>POSITION WHEN FOUND</th>
<th>POSITION HEAD WHEN FOUND</th>
<th>BED SHARING</th>
<th>DEATH SITE</th>
<th>SYMPTOMS/ILLNESS PRIOR TO DEATH</th>
<th>TOTAL RISKS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>13.86</td>
<td>M</td>
<td>Caucasian</td>
<td>No</td>
<td>Mother smoking, opiate use</td>
<td>Supine Face up No Na</td>
<td>Yes, sniffs, recent colic</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>30.71</td>
<td>M</td>
<td>Caucasian</td>
<td>No</td>
<td>NO</td>
<td>Supine To right No Cot</td>
<td>Yes, minor cold/cough, vomiting,</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>16.57</td>
<td>M</td>
<td>Caucasian</td>
<td>No</td>
<td>NO</td>
<td>Prone Face down No Adult bed</td>
<td>Cold, snifles</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>28</td>
<td>20.86</td>
<td>M</td>
<td>Caucasian</td>
<td>No</td>
<td>NO</td>
<td>Side To right Yes Adult bed</td>
<td>No</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>26</td>
<td>13.71</td>
<td>M</td>
<td>Caucasian</td>
<td>No</td>
<td>Cannabis use</td>
<td>Prone Face down No Cot</td>
<td>No</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>44</td>
<td>6.43</td>
<td>M</td>
<td>Caucasian</td>
<td>No</td>
<td>NO</td>
<td>Prone Face down No Cot</td>
<td>No</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>21</td>
<td>28.57</td>
<td>M</td>
<td>Caucasian</td>
<td>No</td>
<td>NO</td>
<td>Supine Na No Cot</td>
<td>Vomit on sheet at death</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>34</td>
<td>21.71</td>
<td>M</td>
<td>Caucasian</td>
<td>No</td>
<td>NO</td>
<td>Prone Na No Adult bed</td>
<td>Cold, cough</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>6</td>
<td>33</td>
<td>M</td>
<td>Caucasian</td>
<td>No</td>
<td>NO</td>
<td>Prone Face down No Cot</td>
<td>No</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>7</td>
<td>8.14</td>
<td>M</td>
<td>Caucasian</td>
<td>No</td>
<td>NO</td>
<td>Supine Na No Cot</td>
<td>No</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>26</td>
<td>14.86</td>
<td>M</td>
<td>Caucasian</td>
<td>No</td>
<td>Mother smoking, cannabis use</td>
<td>Prone To right No Pram</td>
<td>Slight cold, sniffs, cough, wheezing, vomiting</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>9</td>
<td>9.14</td>
<td>M</td>
<td>Caucasian</td>
<td>No</td>
<td>NO</td>
<td>Prone Face down No Cot</td>
<td>No</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>21</td>
<td>13.57</td>
<td>F</td>
<td>Caucasian</td>
<td>No</td>
<td>NO</td>
<td>Supine To left No Cot</td>
<td>Sniffles</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>6</td>
<td>25.71</td>
<td>F</td>
<td>Caucasian</td>
<td>No</td>
<td>Mother Smoking, cannabis use</td>
<td>Prone Face down Yes Mattress on floor</td>
<td>Cold, sniffs, cough, fever</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>21</td>
<td>23.86</td>
<td>F</td>
<td>Caucasian</td>
<td>No</td>
<td>Mother Smoking, cannabis use</td>
<td>Prone Face down No Cot</td>
<td>No</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>22</td>
<td>24.86</td>
<td>F</td>
<td>Caucasian</td>
<td>No</td>
<td>Mother smoking</td>
<td>Na Na No Pram</td>
<td>Cough, history of reflux</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>20</td>
<td>23.43</td>
<td>F</td>
<td>Caucasian</td>
<td>No</td>
<td>NO</td>
<td>Prone Face down No Mattress on floor</td>
<td>Cough, cold month prior</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>7</td>
<td>8.43</td>
<td>F</td>
<td>Caucasian</td>
<td>No</td>
<td>NO</td>
<td>Supine To left Yes Adult bed</td>
<td>Cold, sniffles</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>28</td>
<td>11.29</td>
<td>F</td>
<td>Caucasian</td>
<td>No</td>
<td>Cannabis use</td>
<td>Supine Na Yes Adult bed</td>
<td>Cough, diahorrea, ears yellow fluid</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>34</td>
<td>20.43</td>
<td>M</td>
<td>Aboriginal</td>
<td>No</td>
<td>NO</td>
<td>Supine Na Yes Adult bed</td>
<td>No</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>17</td>
<td>15</td>
<td>M</td>
<td>Aboriginal</td>
<td>No</td>
<td>NO</td>
<td>Na Na Yes Adult bed</td>
<td>No</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>21</td>
<td>18.29</td>
<td>F</td>
<td>Aboriginal</td>
<td>No</td>
<td>Possible petrol sniffing</td>
<td>Side Na Yes Mattress on floor</td>
<td>Cough</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sex</td>
<td>Aboriginal</td>
<td>Other member household smoking</td>
<td>Supine</td>
<td>Prone</td>
<td>Prone</td>
<td>Prone</td>
<td>Supine</td>
<td>Mattress on floor</td>
<td>Mattress on floor</td>
<td>Cold, snuffles, cough few days prior</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>-----</td>
<td>------------</td>
<td>-----------------------------</td>
<td>-------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>-------</td>
<td>------------------</td>
<td>------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>23</td>
<td>49</td>
<td>20</td>
<td>F</td>
<td>Aboriginal</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Supine</td>
<td>Mattress on floor</td>
<td>Cold, snuffles, cough few days prior</td>
</tr>
<tr>
<td>24</td>
<td>6</td>
<td>10</td>
<td>F</td>
<td>NA</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Supine</td>
<td>Mattress on floor</td>
<td>Cold, snuffles, cough few days prior</td>
</tr>
<tr>
<td>25</td>
<td>45</td>
<td>27.86</td>
<td>F</td>
<td>NA</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Prone</td>
<td>Mattress on floor</td>
<td>Cold, snuffles, cough few days prior</td>
</tr>
<tr>
<td>26</td>
<td>24</td>
<td>1.14</td>
<td>F</td>
<td>NA</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Na</td>
<td>Mattress on floor</td>
<td>Cold, snuffles, cough few days prior</td>
</tr>
<tr>
<td>27</td>
<td>27</td>
<td>10.29</td>
<td>M</td>
<td>Caucasian</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Supine</td>
<td>Bassinette</td>
<td>Cold, snuffles, cough few days prior</td>
</tr>
<tr>
<td>28</td>
<td>24</td>
<td>10.29</td>
<td>M</td>
<td>Caucasian</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Prone</td>
<td>Cot</td>
<td>Cold, snuffles, cough few days prior</td>
</tr>
<tr>
<td>29</td>
<td>60</td>
<td>25.43</td>
<td>M</td>
<td>Caucasian</td>
<td>Na</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Prone</td>
<td>Mattress on floor</td>
<td>Cold, snuffles, cough few days prior</td>
</tr>
<tr>
<td>30</td>
<td>20</td>
<td>28.43</td>
<td>F</td>
<td>Caucasian</td>
<td>Na</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Prone</td>
<td>Cot</td>
<td>Cold, snuffles, cough few days prior</td>
</tr>
<tr>
<td>31</td>
<td>71</td>
<td>5.57</td>
<td>F</td>
<td>NA</td>
<td>Na</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Supine</td>
<td>Bassinette</td>
<td>Cold, snuffles, cough few days prior</td>
</tr>
<tr>
<td>32</td>
<td>18</td>
<td>17.14</td>
<td>F</td>
<td>Caucasian</td>
<td>Na</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Prone</td>
<td>Cot</td>
<td>Cold, snuffles, cough few days prior</td>
</tr>
<tr>
<td>33</td>
<td>22</td>
<td>23.43</td>
<td>M</td>
<td>Caucasian</td>
<td>Na</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Side</td>
<td>Cot</td>
<td>Cold, snuffles, cough few days prior</td>
</tr>
<tr>
<td>34</td>
<td>21</td>
<td>15.29</td>
<td>M</td>
<td>Caucasian</td>
<td>Na</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Prone</td>
<td>Cot</td>
<td>Cold, snuffles, cough few days prior</td>
</tr>
<tr>
<td>35</td>
<td>27</td>
<td>7.71</td>
<td>M</td>
<td>Caucasian</td>
<td>Na</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Supine</td>
<td>Cot</td>
<td>Cold, snuffles, cough few days prior</td>
</tr>
<tr>
<td>36</td>
<td>27</td>
<td>13</td>
<td>M</td>
<td>Caucasian</td>
<td>Na</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Na</td>
<td>Bassinette</td>
<td>Sniffles, mucus in nose prior</td>
</tr>
<tr>
<td>37</td>
<td>27</td>
<td>13</td>
<td>M</td>
<td>Caucasian</td>
<td>Na</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Supine</td>
<td>Bassinette</td>
<td>Cold, snuffles, cough few days prior</td>
</tr>
<tr>
<td>38</td>
<td>27</td>
<td>13</td>
<td>M</td>
<td>Caucasian</td>
<td>Na</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Prone</td>
<td>Cot</td>
<td>Cold, snuffles, cough few days prior</td>
</tr>
<tr>
<td>39</td>
<td>27</td>
<td>13</td>
<td>M</td>
<td>Caucasian</td>
<td>Na</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Prone</td>
<td>Cot</td>
<td>Cold, snuffles, cough few days prior</td>
</tr>
<tr>
<td>40</td>
<td>60</td>
<td>6.71</td>
<td>F</td>
<td>Aboriginal</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Prone</td>
<td>Mattress on floor</td>
<td>Cold, snuffles, cough few days prior</td>
</tr>
<tr>
<td>41</td>
<td>53</td>
<td>51.43</td>
<td>M</td>
<td>Caucasian</td>
<td>Na</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Supine</td>
<td>Mattress on floor</td>
<td>Cold, snuffles, cough few days prior</td>
</tr>
<tr>
<td>Controls n=28</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------</td>
<td>------------------------------</td>
<td>------------------------------</td>
<td>------------------------------</td>
<td>------------------------------</td>
<td>------------------------------</td>
<td>------------------------------</td>
<td>------------------------------</td>
<td>------------------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>47</td>
<td>M</td>
<td>Caucasian</td>
<td>No</td>
<td>NR</td>
<td>Nr</td>
<td>Prone</td>
<td>Face up</td>
<td>Na</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>28</td>
<td>M</td>
<td>Na</td>
<td>Na</td>
<td>NO</td>
<td>Supine</td>
<td>To left</td>
<td>Alone</td>
<td>Bassinette</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>53</td>
<td>M</td>
<td>Caucasian</td>
<td>No</td>
<td>NO</td>
<td>Supine</td>
<td>Prone</td>
<td>Face down</td>
<td>Alone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>38</td>
<td>M</td>
<td>Na</td>
<td>Na</td>
<td>YES opiate &amp; amphetamine use</td>
<td>Prone</td>
<td>Face down</td>
<td>Alone</td>
<td>Bassinette</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>39</td>
<td>M</td>
<td>Asian</td>
<td>Na</td>
<td>NA</td>
<td>Prone</td>
<td>Face down</td>
<td>Alone</td>
<td>Mattress</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>48</td>
<td>M</td>
<td>Caucasian</td>
<td>No</td>
<td>YES opiate use</td>
<td>Supine</td>
<td>Prone</td>
<td>Face down</td>
<td>Alone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>31</td>
<td>M</td>
<td>Caucasian</td>
<td>No</td>
<td>NA</td>
<td>Supine</td>
<td>Face up</td>
<td>Na</td>
<td>Bassinette</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>16</td>
<td>M</td>
<td>Caucasian</td>
<td>No</td>
<td>NA</td>
<td>Nr</td>
<td>Nr</td>
<td>Nr</td>
<td>Nr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>36</td>
<td>M</td>
<td>Na</td>
<td>Na</td>
<td>NA</td>
<td>Nr</td>
<td>Nr</td>
<td>Nr</td>
<td>Nr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>17</td>
<td>M</td>
<td>Na</td>
<td>Na</td>
<td>NA</td>
<td>Na</td>
<td>Na</td>
<td>Alone</td>
<td>Cot</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>27</td>
<td>M</td>
<td>Na</td>
<td>Na</td>
<td>NA</td>
<td>Nr</td>
<td>Nr</td>
<td>Nr</td>
<td>Nr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>38</td>
<td>M</td>
<td>Na</td>
<td>Na</td>
<td>NA</td>
<td>Nr</td>
<td>Nr</td>
<td>Nr</td>
<td>Nr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>24</td>
<td>F</td>
<td>Na</td>
<td>Na</td>
<td>NA</td>
<td>Nr</td>
<td>Nr</td>
<td>Nr</td>
<td>Nr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>20</td>
<td>F</td>
<td>Caucasian</td>
<td>No</td>
<td>NO</td>
<td>Nr</td>
<td>Nr</td>
<td>Nr</td>
<td>Nr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>27</td>
<td>F</td>
<td>Aboriginal</td>
<td>Yes</td>
<td>YES alcohol abuse</td>
<td>Na</td>
<td>Na</td>
<td>Co sleeping</td>
<td>Adult bed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>18</td>
<td>F</td>
<td>Caucasian</td>
<td>Yes</td>
<td>NA</td>
<td>Na</td>
<td>Na</td>
<td>Alone</td>
<td>Pram</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>58</td>
<td>19</td>
<td>F</td>
<td>Caucasian</td>
<td>Yes</td>
<td>Mother &amp; family members smoking</td>
<td>Nr</td>
<td>Nr</td>
<td>Nr</td>
<td>Nr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>59</td>
<td>40</td>
<td>F</td>
<td>Na</td>
<td>Na</td>
<td>NA</td>
<td>Nr</td>
<td>Nr</td>
<td>Nr</td>
<td>Nr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>37</td>
<td>F</td>
<td>Na</td>
<td>Na</td>
<td>NA</td>
<td>Nr</td>
<td>Nr</td>
<td>Nr</td>
<td>Nr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>33</td>
<td>F</td>
<td>Na</td>
<td>Na</td>
<td>NA</td>
<td>Na</td>
<td>Na</td>
<td>Na</td>
<td>Na</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>62</td>
<td>26</td>
<td>M</td>
<td>Na</td>
<td>Na</td>
<td>Mother smoking, unidentified substance abuse</td>
<td>Side</td>
<td>Na</td>
<td>Alone</td>
<td>Between couch and mattress</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>63</td>
<td>46</td>
<td>M</td>
<td>Caucasian</td>
<td>No</td>
<td>NO</td>
<td>Side</td>
<td>To right</td>
<td>Alone</td>
<td>Mattress on the floor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>7</td>
<td>M</td>
<td>Na</td>
<td>Na</td>
<td>NA</td>
<td>Na</td>
<td>Na</td>
<td>Co sleeping</td>
<td>Found on floor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>21</td>
<td>M</td>
<td>Caucasian</td>
<td>Yes</td>
<td>NO</td>
<td>Na</td>
<td>Na</td>
<td>Na</td>
<td>Na</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>26</td>
<td>F</td>
<td>Na</td>
<td>Na</td>
<td>NA</td>
<td>Prone</td>
<td>Face down</td>
<td>Alone</td>
<td>Cot</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>24</td>
<td>F</td>
<td>Caucasian</td>
<td>No</td>
<td>Mother smoking</td>
<td>Prone</td>
<td>Face down</td>
<td>Alone</td>
<td>Inflatable bed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>7</td>
<td>M</td>
<td>Caucasian</td>
<td>No</td>
<td>NO</td>
<td>Prone</td>
<td>Face down</td>
<td>Alone</td>
<td>Cot</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>69</td>
<td>20</td>
<td>F</td>
<td>Caucasian</td>
<td>No</td>
<td>NO</td>
<td>Prone</td>
<td>To left</td>
<td>Alone</td>
<td>Couch</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NA: information not available, Nr: not relevant, M: Male, F: Female
4.3.3 All medullary levels combined

Compared to combined controls, SIDS cases showed a significantly higher combined total number of 5-HT neurons for all sub-regions (p=0.008) and a significantly higher number of neurons within the raphe (p=0.024) and extra raphe nuclei (p=0.010). A trend toward significance was observed in total density of 5-HT neurons, with higher density in SIDS cases (p=0.086). This trend for higher density of 5-HT neurons in SIDS was evident in the raphe and extra raphe sub-regions. Total area combined for all sub-regions across all medullary levels was significantly higher in SIDS (p=0.029). No significant effect of PCA, sex or PMI was observed across analyses for all levels combined (Fig 4.2, Table 4.2).

Table 4.2. 5-HT neuron number, density and morphology in SIDS vs. combined controls across all medullary levels and sub nuclei.

<table>
<thead>
<tr>
<th></th>
<th>SIDS Adj mean (±SE)</th>
<th>Combined controls Adj mean (±SE)</th>
<th>p-value</th>
<th>PCA</th>
<th>SEX</th>
<th>PMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined Total</td>
<td>105.91 (7.87)</td>
<td>70.20 (10.10)</td>
<td>0.008</td>
<td>0.466</td>
<td>0.388</td>
<td>0.401</td>
</tr>
<tr>
<td>Raphe Total</td>
<td>66.00 (6.06)</td>
<td>43.01 (7.78)</td>
<td>0.024</td>
<td>0.345</td>
<td>0.273</td>
<td>0.681</td>
</tr>
<tr>
<td>Extra Raphe Total</td>
<td>39.92 (2.89)</td>
<td>27.19 (3.71)</td>
<td>0.01</td>
<td>0.995</td>
<td>0.891</td>
<td>0.151</td>
</tr>
<tr>
<td>Density Total</td>
<td>0.53 (0.03)</td>
<td>0.45 (0.04)</td>
<td>0.086</td>
<td>0.348</td>
<td>0.395</td>
<td>0.805</td>
</tr>
<tr>
<td>Density Raphe</td>
<td>0.32 (0.02)</td>
<td>0.27 (0.03)</td>
<td>0.141</td>
<td>0.924</td>
<td>0.869</td>
<td>0.563</td>
</tr>
<tr>
<td>Density Extra Raphe</td>
<td>0.21 (0.02)</td>
<td>0.18 (0.02)</td>
<td>0.279</td>
<td>0.102</td>
<td>0.141</td>
<td>0.746</td>
</tr>
<tr>
<td>Area Total, mm²</td>
<td>204.62 (14.05)</td>
<td>153.07 (18.03)</td>
<td>0.029</td>
<td>0.174</td>
<td>0.132</td>
<td>0.463</td>
</tr>
</tbody>
</table>
Fig 4.2. Mean 5-HT neuron count and density adjusted for sex, PMI and PCA across all medullary levels (caudal to rostral) in SIDS vs. controls. Compared to controls, 5-HT neuron count was significantly higher across all levels (A), and within the raphe (C) and extra raphe (D) sub-regions in SIDS. There was a trend toward higher density of 5-HT neurons within the medullary 5-HT network in SIDS (B). ** = p < 0.01; * = p < 0.05.
4.3.4 Caudal medulla L4 and L5

No significant differences were observed in the caudal L4 medulla between SIDS and non-SIDS controls. In the caudal medulla L5, the combined total number of 5-HT neurons for all sub-regions was higher in SIDS (p=0.058). SIDS cases had a higher proportion of fusiform neurons (p=0.057) and significantly more pyramidal neurons (p=0.018). No significant differences were observed in the raphe sub-region at this level. However in the extra raphe nuclei, SIDS cases had a significantly higher number of 5HT neurons (p=0.016)(Fig 4.3A, Fig 4.4), primarily of granular (p=0.033), fusiform (p=0.041) and pyramidal (p=0.043) morphology. While there were no significant differences in density at this level, a trend for higher total density and total density of 5-HT neurons within the extra raphe sub-region was observed in SIDS cases. Although not statistically significant, a trend for higher total area was observed in SIDS cases (p=0.054)(Table 4.3, Fig 4.3A, Fig 4.4).

Table 4.3. 5-HT neuron number, density and morphology in SIDS vs. control in caudal L5 medulla.

<table>
<thead>
<tr>
<th></th>
<th>SIDS Adj. mean (SE)</th>
<th>CONTROLS Adj. mean (SE)</th>
<th>P value</th>
<th>PCA</th>
<th>SEX</th>
<th>PMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined Total</td>
<td>45.66 (3.31)</td>
<td>34.40 (4.43)</td>
<td>0.058</td>
<td>0.53</td>
<td>0.111</td>
<td>0.12</td>
</tr>
<tr>
<td>Raphe Total</td>
<td>24.64 (3.11)</td>
<td>20.60 (4.16)</td>
<td>0.452</td>
<td>0.792</td>
<td>0.068</td>
<td>0.171</td>
</tr>
<tr>
<td>Extra Raphe Total</td>
<td>21.02 (1.61)</td>
<td>13.80 (2.16)</td>
<td>0.016</td>
<td>0.423</td>
<td>0.819</td>
<td>0.607</td>
</tr>
<tr>
<td>Extra Raphe Granular</td>
<td>14.02 (1.12)</td>
<td>9.67 (1.51)</td>
<td>0.033</td>
<td>0.351</td>
<td>0.406</td>
<td>0.717</td>
</tr>
<tr>
<td>Extra Raphe Fusiform</td>
<td>4.48 (0.55)</td>
<td>2.42 (0.74)</td>
<td>0.041</td>
<td>0.536</td>
<td>0.076</td>
<td>0.496</td>
</tr>
<tr>
<td>Extra Raphe Pyramidal</td>
<td>0.71 (0.16)</td>
<td>0.13 (0.21)</td>
<td>0.043</td>
<td>0.613</td>
<td>0.919</td>
<td>0.473</td>
</tr>
<tr>
<td>Fusiform Total</td>
<td>10.05 (1.12)</td>
<td>6.23 (1.49)</td>
<td>0.057</td>
<td>0.919</td>
<td>0.044</td>
<td>0.197</td>
</tr>
<tr>
<td>Pyramidal Total</td>
<td>1.54 (0.24)</td>
<td>0.52 (0.32)</td>
<td>0.018</td>
<td>0.298</td>
<td>0.179</td>
<td>0.737</td>
</tr>
<tr>
<td>Density Total</td>
<td>0.42 (0.03)</td>
<td>0.38 (0.04)</td>
<td>0.459</td>
<td>0.132</td>
<td>0.71</td>
<td>0.209</td>
</tr>
<tr>
<td>Density Raphe</td>
<td>0.22 (0.03)</td>
<td>0.22 (0.04)</td>
<td>0.899</td>
<td>0.442</td>
<td>0.22</td>
<td>0.203</td>
</tr>
<tr>
<td>Density Extra Raphe</td>
<td>0.20 (0.02)</td>
<td>0.16 (0.03)</td>
<td>0.29</td>
<td>0.149</td>
<td>0.374</td>
<td>0.627</td>
</tr>
<tr>
<td>Area Total, mm²</td>
<td>110.32 (3.09)</td>
<td>99.57 (4.15)</td>
<td>0.054</td>
<td>0.107</td>
<td>0.07</td>
<td>0.928</td>
</tr>
</tbody>
</table>
4.3.5 Mid-Rostral medulla L6-6/7

At the mid rostral level, SIDS cases had a significantly higher combined total number of 5-HT neurons (p=0.026) compared to combined controls. Specifically, the total number of granular (p=0.037) and multipolar neurons (p=0.005) was significantly higher in SIDS cases. In the raphe sub-region SIDS cases had a higher total number of 5-HT neurons (p=0.074)(Fig 4.3B, Fig 4.5), specifically a significantly higher number of multipolar neurons (p=0.040). A trend toward a higher number of granular neurons in the raphe nuclei was observed in SIDS. In the extra raphe sub-region SIDS cases again had a significantly higher number of multipolar neurons (p=0.003). SIDS cases had a significantly higher total density of 5-HT neurons (p=0.034), principally a higher density of granular neurons (p=0.028). In the raphe sub region, SIDS cases had a significantly greater density of 5-HT neurons (p=0.019), specifically of granular morphology (p=0.028) (Table 4.4, Fig 4.3B).

Table 4.4. 5-HT neuron number, density and morphology in SIDS vs. controls in the mid-rostral medulla.

<table>
<thead>
<tr>
<th></th>
<th>SIDS Adj. mean (SE)</th>
<th>CONTROLS Adj. mean (SE)</th>
<th>P value</th>
<th>PCA</th>
<th>SEX</th>
<th>PMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined Total</td>
<td>68.76 (4.24)</td>
<td>46.35 (7.95)</td>
<td>0.026</td>
<td>0.259</td>
<td>0.722</td>
<td>0.721</td>
</tr>
<tr>
<td>Raphe Total</td>
<td>43.30 (4.05)</td>
<td>26.73 (7.59)</td>
<td>0.074</td>
<td>0.541</td>
<td>0.589</td>
<td>0.241</td>
</tr>
<tr>
<td>Extra Raphe Total</td>
<td>25.47 (2.19)</td>
<td>19.62 (4.10)</td>
<td>0.23</td>
<td>0.334</td>
<td>0.801</td>
<td>0.154</td>
</tr>
<tr>
<td>Granular Total</td>
<td>46.79 (3.02)</td>
<td>32.06 (5.65)</td>
<td>0.037</td>
<td>0.193</td>
<td>0.564</td>
<td>0.571</td>
</tr>
<tr>
<td>Multipolar Total</td>
<td>3.32 (0.40)</td>
<td>0.52 (0.75)</td>
<td>0.005</td>
<td>0.597</td>
<td>0.398</td>
<td>0.604</td>
</tr>
<tr>
<td>Raphe Granular</td>
<td>30.08 (2.96)</td>
<td>19.16 (5.55)</td>
<td>0.105</td>
<td>0.379</td>
<td>0.309</td>
<td>0.66</td>
</tr>
<tr>
<td>Raphe Multipolar</td>
<td>1.92 (0.33)</td>
<td>0.36 (0.61)</td>
<td>0.04</td>
<td>0.795</td>
<td>0.426</td>
<td>0.189</td>
</tr>
<tr>
<td>Extra Raphe Multipolar</td>
<td>1.40 (0.16)</td>
<td>0.16 (0.30)</td>
<td>0.003</td>
<td>0.431</td>
<td>0.687</td>
<td>0.34</td>
</tr>
<tr>
<td>Density Total</td>
<td>0.62 (0.04)</td>
<td>0.41 (0.08)</td>
<td>0.034</td>
<td>0.819</td>
<td>0.389</td>
<td>0.698</td>
</tr>
<tr>
<td>Density Raphe</td>
<td>0.38 (0.03)</td>
<td>0.22 (0.05)</td>
<td>0.019</td>
<td>0.588</td>
<td>0.666</td>
<td>0.251</td>
</tr>
<tr>
<td>Raphe granular density</td>
<td>0.27 (0.02)</td>
<td>0.16 (0.04)</td>
<td>0.028</td>
<td>0.961</td>
<td>0.756</td>
<td>0.733</td>
</tr>
<tr>
<td>Area Total, mm²</td>
<td>115.58 (6.43)</td>
<td>115.04 (12.06)</td>
<td>0.969</td>
<td>0.34</td>
<td>0.026</td>
<td>0.881</td>
</tr>
</tbody>
</table>
4.3.6 Rostral medulla L7-7/8

In the rostral medulla, SIDS cases showed a significantly higher number of combined total 5-HT neurons (p=0.032) specifically granular neurons (p=0.022) compared to controls. In the raphe nuclei SIDS cases had a significantly higher total number of 5-HT neurons (p=0.016), specifically of granular morphology (p=0.026) (Fig 4.3C, Fig 4.5). A higher number of multipolar neurons within the raphe were also observed (p=0.073). There were no significant differences in the extra raphe at this medullary level. Although not statistically significant, there was a trend for higher total density and density of neurons in the raphe in SIDS compared to controls. Total area was higher in SIDS (p=0.073) (Table 4.5, Fig 4.3C, Fig 4.5).

**Table 4.5.** 5-HT neuron number, density and morphology in SIDS vs. controls in rostral medulla.

<table>
<thead>
<tr>
<th></th>
<th><strong>SIDS</strong> Adj. mean (SE)</th>
<th><strong>CONTROLS</strong> Adj. mean (SE)</th>
<th><strong>P value</strong></th>
<th><strong>PCA</strong></th>
<th><strong>SEX</strong></th>
<th><strong>PMI</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined Total</td>
<td>89.63 (5.46)</td>
<td>68.33 (7.12)</td>
<td>0.032</td>
<td>0.543</td>
<td>0.401</td>
<td>0.496</td>
</tr>
<tr>
<td>Raphe Total</td>
<td>62.93 (4.25)</td>
<td>43.97 (5.54)</td>
<td>0.016</td>
<td>0.886</td>
<td>0.248</td>
<td>0.877</td>
</tr>
<tr>
<td>Extra Raphe Total</td>
<td>26.71 (2.36)</td>
<td>24.36 (3.08)</td>
<td>0.561</td>
<td>0.094</td>
<td>0.898</td>
<td>0.095</td>
</tr>
<tr>
<td>Granular Total</td>
<td>60.77 (4.12)</td>
<td>43.47 (5.37)</td>
<td>0.022</td>
<td>0.63</td>
<td>0.378</td>
<td>0.932</td>
</tr>
<tr>
<td>Raphe Granular</td>
<td>41.41 (3.32)</td>
<td>27.97 (4.33)</td>
<td>0.026</td>
<td>0.948</td>
<td>0.216</td>
<td>0.605</td>
</tr>
<tr>
<td>Raphe Multipolar</td>
<td>2.32 (0.32)</td>
<td>1.30 (0.42)</td>
<td>0.073</td>
<td>0.89</td>
<td>0.351</td>
<td>0.872</td>
</tr>
<tr>
<td>Density Total</td>
<td>0.67 (0.05)</td>
<td>0.61 (0.06)</td>
<td>0.446</td>
<td>0.542</td>
<td>0.423</td>
<td>0.434</td>
</tr>
<tr>
<td>Density Raphe</td>
<td>0.47 (0.04)</td>
<td>0.40 (0.05)</td>
<td>0.263</td>
<td>0.904</td>
<td>0.769</td>
<td>0.997</td>
</tr>
<tr>
<td>Area Total, mm²</td>
<td>139.23 (6.99)</td>
<td>114.77 (9.11)</td>
<td>0.051</td>
<td>0.677</td>
<td><strong>0.064</strong></td>
<td>0.98</td>
</tr>
</tbody>
</table>
**Fig 4.3.** Distribution of (A) Caudal-medullary, (B) Mid-Rostral medullary and (C) Rostral medullary serotonergic (5-HT) neurons in an infant dying from sudden infant death syndrome (SIDS case) and an infant with acute death from a cause other than SIDS (control), plotted using Neurolucida version 6.02.2 (Microbrightfield Inc., Williston, VT). There are qualitatively more 5-HT neurons in the SIDS case, particularly in the midline raphe sub-nuclei in the mid to rostral medulla (B & C) than in the control and more 5-HT neurons in the extra raphe sub-nuclei in SIDS in the Caudal medulla (A) compared to the control. Blue symbols= raphe nuclei, green symbols= extra raphe nuclei.
Fig 4.4. Transverse tissue sections of caudal extra raphe in SIDS versus control infant medulla. Compared to controls, SIDS cases had significantly higher numbers of 5-HT neurons and a trend for higher density within the extra raphe nuclei at the caudal level (SUB), characterized by more neurons of granular, fusiform and pyramidal morphology. 20x mag, Scale= 100µm

Fig 4.5. Transverse tissue sections of mid-rostral midline raphe nuclei in SIDS versus control infant medulla. Compared to controls, SIDS cases had a significantly higher number and density of 5-HT neurons, principally within the raphe nuclei at, characterized by a greater proportion and density of neurons of granular morphology and a higher proportion of multipolar neurons compared to controls. 20x mag, Scale= 100µm
4.4 DISCUSSION

Medullary 5-HT neurons exert a major modulatory influence on nociceptive processing, motoneuron excitability and respiratory and autonomic control (Benarroch, 2014). This neuronal network has been previously implicated in respiratory failure and sudden death in SIDS (Paterson et al., 2006a), with reduced levels of tryptophan hydroxylase and alteration of 5HT receptors and transporter (SERT) binding having been reported (Panigrahy et al., 2000b, Kinney et al., 2003, Duncan et al., 2010). For the first time, this study has replicated the principal observations of Paterson et al (2006) in a separate independent SIDS cohort from South Australia. In the present study, 5-HT neuron abnormalities within the medulla were observed in SIDS cases despite risk factors associated with SIDS pathology including adverse sleep positions, co-sleeping and prenatal exposures. These abnormalities included significantly increased total 5-HT neuron number and density, significantly greater proportions and density of 5-HT neurons within the raphe and extra raphe sub-nuclei and significant differences in morphological subtypes of 5-HT neurons within these nuclei in SIDS cases compared to non-SIDS controls. While the present study provides further evidence of abnormal development of the medullary 5-HT system in a subset of SIDS cases, our observations did differ somewhat from the previous study and suggested that 5-HT neuron abnormalities in SIDS are likely to be more complex.

The profile of 5-HT neurons varied depending on the level of medulla analyzed (caudal, mid, rostral). In the caudal medulla differences between SIDS and controls were principally observed in the extra raphe nuclei. These included a significantly higher number and density of 5-HT neurons, specifically of granular, fusiform and pyramidal morphology in the extra raphe in SIDS cases. In contrast, the mid-rostral medulla showed differences across all nuclei sub regions including a significantly higher number and density of 5-HT neurons in the raphe nuclei, primarily granular and multipolar in morphology, and a trend for a higher number and density of neurons in the extra raphe. There was also a trend toward a higher number of granular neurons and a significantly greater proportion of multipolar neurons in the extra raphe in SIDS cases. Similarly in the rostral medulla, we again observed a significantly greater number and trend towards a higher density of 5-HT neurons within the raphe nuclei, consisting of a significantly greater proportion and density of granular neurons and a trend for higher number of multipolar neurons in SIDS cases compared to controls. However, in the extra raphe nuclei we did not observed any significant differences or trends between SIDS and controls at the rostral level.

Our observation of a significantly increased proportion of neurons of simple morphology i.e. granular neurons, are consistent with Paterson et al (2006) and support the concept of an underlying developmental disorder involving abnormal regulation of 5-HT neuron count and the potential delay and failure of differentiation and maturation of immature 5-HT neurons in SIDS. However in
contrast, we also observed a significant increase in the morphological subtypes of fusiform, pyramidal and multipolar neurons in SIDS cases. These neurons are considered more complex and mature than simple granular neurons (Kinney et al., 2007). Kinney et al (2007) reported that maturation of the normal infant brainstem into the postnatal period exhibited a shift in the ratio of 5-HT neuron subtypes with a significant decrease in the simple less mature granular neurons and an increase in more mature neurons. The authors speculated that granular neurons likely differentiate further into the more complex neuron subtypes across early human development in order to serve more mature functions in the medullary 5-HT network. Furthermore, that study showed that multipolar neurons, which are regarded as the most complex neuron subtype, were present early at mid-gestation as a small subpopulation and did not increase with infant maturation even as the physiological system becomes more complex during early life (Kinney et al., 2007). Our observations of a paradoxical increase in both mature and immature 5-HT neurons in the medullary 5-HT network in SIDS cases may suggest a more complex abnormality in conjunction with a maturational delay in neuron differentiation.

The variability in the 5-HT neuron profile across medullary levels and nuclei sub regions observed between SIDS and controls may reflect the different functional roles of the 5-HT network in the caudal versus rostral domains, given they are distinct in their anatomical location, developmental origins, functions and connectivity (Kinney et al., 2009a). Indeed animal studies have shown that 5-HT neuron subtypes, principally within the raphe, serve different specialized physiological functions and the genetically defined substructure of these distinct subtypes may execute these particular functions despite coexisting within the same anatomical nuclei (Brust et al., 2014, Gaspar and Lillesaar, 2012, Jensen et al., 2008). This would suggest that different subtypes of 5-HT neurons are associated with different disease vulnerabilities. Therefore abnormalities in 5-HT number, density and morphology observed in the present study may be indicative of the specific dysfunction of the 5-HT network associated with the pathogenesis of a subset of SIDS victims. This concept is supported in part by the observation that 5-HT neuron migration was not altered in SIDS given that the anatomical positioning of 5-HT neurons within component nuclei of the medullary 5-HT system was consistent across SIDS and controls, but rather the number and density of particular morphological subtypes of 5-HT neurons within the sub-nuclei at the different medullary levels were significantly altered in SIDS cases.

To address the implications of increased 5-HT neurons in the medulla in SIDS cases, together with abnormalities in other markers of 5-HT function, Paterson et al (2006) proposed three primary scenarios: 1) increased 5-HT neurons coupled with decreased 5-HT1A receptor binding and 5-HTT binding is suggestive that the synthesis and availability of 5-HT neurons and by extrapolation neuron firing is altered; 2) excess extracellular 5-HT resulting from increased 5-HT neurons results in a
compensatory decreased in 5-HT1A receptors; or 3) 5-HT neurons are overabundant in compensation for dysfunctional 5-HT synthesis, release or both resulting in deficient extracellular 5-HT. While each of these may in part explain the potential consequences of increased 5-HT neurons, future studies are required to address what potential molecular mechanisms are responsible for the auto-regulation of 5-HT maturation, number and morphology within critical medullary sites and what the consequences of alterations in this regulation might have on local circuit modulation. In addition, analysis of the genetic profile of 5-HT neuron subtypes to determine their function at specific medullary sites and assess what morphological subtypes are defective could contribute to an understanding of the underlying pathogenesis of SIDS. Finally, investigation of the potential relationship between SIDS risk factors and abnormal gene expression within the medullary 5-HT network would be beneficial, given that multiple SIDS studies including the present study, have identified numerous risks primarily associated with prenatal exposures, sex and adverse sleeping environments.

While analysis of risk factors did not reveal any significant differences in 5-HT neuron count or density in SIDS cases compared to controls, all SIDS cases were identified as being exposed to at least one risk factor or stressor with over half of infants found in an adverse sleeping position and had experienced symptoms or illness one month prior to death. There were also more male SIDS deaths compared to females. These risk factors identified align with the greater SIDS literature, further confirming these parameters as important aspects of SIDS investigations critical for fully understanding non-pathological parameters associated with these deaths.

We acknowledge that differences are to be expected between study cohorts given the epidemiologic and demographic profiles are different. Findings from the present studies may therefore reflect the distinct abnormal profile of the 5-HT system in the medulla of Australian SIDS victims. Nevertheless both datasets align within the same time period (mid 1990s- mid 2000s), utilized the same methods of analysis and standardized classification of medullary levels and causes of death. Therefore this study provides a snapshot of SIDS in Australia comparable to that of the USA, during a time of rigorous public health initiatives regarding safe sleep practices for infants in order for risk reduction of sudden death.
4.5 Conclusion

For the first time in a separate independent SIDS cohort, this study has replicated and corroborated the principal observations from Paterson et al (2006) supporting the hypothesis that the medullary 5-HT system develops abnormally in SIDS cases compared to controls and further strengthens the hypothesis that medullary 5-HT dysfunction contributes to the pathogenesis of a subset of SIDS victims. Moreover, our observations provide evidence for a more complex abnormality in 5-HT neuron dysfunction within the different caudal and rostral medullary 5-HT domains in SIDS cases that requires further investigation at the molecular and subcellular levels, in conjunction with investigation of the potential effects of SIDS risk factors on the mechanisms that underpin the medullary 5-HT network during development.

Author contributions

Principal author (candidate) FMB conducted the experiments, analyzed and interpreted the data and wrote the manuscript. DSP assisted with interpretation of data and manuscript preparation. FMB, and DSP conceived the study. RWB and RV contributed to manuscript preparation.
Chapter 5. General Discussion
5.1 Summary of major thesis outcomes

Chapter 2. Normative distribution investigation

This study showed the extensive distribution and interaction of SP and NK1R within the medullary 5-HT network in the normal (control) human infant during development. The study provided evidence of a greater importance of SP in the medulla during the early postnatal period and supported the potential role of SP and NK1R in the modulation of cardiorespiratory and autonomic control based on the widespread distribution of this system across multiple medullary sites. Finally, the study identified significantly higher NK1R binding in premature and male infants, which may explain the increased risk and mortality of paediatric brainstem disorders in these infants.

Chapter 3. SIDS vs. Controls investigation: Medullary SP/NK1R study

This study identified a subset of SIDS infants with a significant developmental abnormality of the SP/NK1R system and altered NK1R binding in multiple medullary nuclei intimately related to cardiorespiratory function and autonomic control. Observations were influenced by prematurity and sex which may potentially explain why there is an increased risk of SIDS in premature and male infants. Taken together, the outcomes of this study provided support for the hypothesis that abnormalities in a multi-neurotransmitter network underlie the pathogenesis of a subset of SIDS cases and that SIDS is a complex developmental disorder with a prenatal aetiology.

Chapter 4. SIDS vs. Controls investigation: Medullary 5-HT study

For the first time, this study replicated and corroborated in a separate independent cohort from South Australia the principal findings of previous SIDS research from the USA suggesting that a significant medullary 5-HT dysfunction in the form of altered cell number, density and morphology of 5-HT neurons is associated with SIDS. Our findings support the hypothesis that medullary 5-HT dysfunction contributes to the pathogenesis of a subset of SIDS cases, and moreover provides additional evidence of a more complex abnormality in medullary 5-HT neuron dysfunction in SIDS.
5.2 General discussion and future research directions

The present thesis consisted of three core interrelated studies providing a portrait of SIDS pathology over a period of sixteen years (1999-2015), in which advances in research practice and technology and the diagnosis and classification of SIDS cases have occurred. In an international collaborative effort, human infant brainstem tissue (SIDS and non-SIDS controls) and the corresponding digital autopsy files from the San Diego infant database (USA) and FSSA (Australia) were used to perform quantitative investigations of the underlying brainstem pathology associated with the pathogenesis of SIDS. Collectively the studies provide further evidence of significant abnormalities in the development and function of a multi-neurotransmitter homeostatic network in a subset of SIDS cases. In addition these studies substantiate the increased risk of premature and male infants to medullary neurotransmitter dysfunction in SIDS and highlight the need for further in depth investigation of these parameters in the future. This thesis emphasises the need for replication and validation of previously published neuropathological findings and the continued investigation of specific brainstem regions, nuclei, projection sites and neurochemical networks for a focused and comprehensive approach to SIDS research. Moreover the thesis outcomes provide a foundation for the development of promising future research directions, demonstrating that the use of human tissue in paediatric neuropathological research is imperative.

Human tissue SIDS research is associated with multiple limitations, however the collaboration undertaken in this thesis proved successful in overcoming many of these. A lack of consistency in the use of definitions to characterize SIDS cases continues to hinder research activity. While the research utilized two separate independent cohorts of human tissue from different epidemiologic and demographic backgrounds, stringent efforts were made to ensure consistency and validity for the comparison between databases primarily by using the same standardized definition of SIDS (San Diego definition) and the consistent use of methods in classifying appropriate controls into subgroups for comparison. A further limitation to SIDS research is access to appropriate human SIDS and control brain tissue, primarily due to an inability to gain consent for research practice and differences in the laws across jurisdictions associated with retaining human tissue for research purposes. An example of this was evident in this study, given that our collaborators in the USA do not have access to formalin fixed human infant SIDS and control tissue and similarly in South Australia we do not have access to fresh frozen human infant tissue. Therefore investigators are often restricted in the research tools and techniques available to them. However the joint laboratory collaboration facilitated combined fresh frozen and fixed tissue research that enabled both Australian and American investigators to utilise research methods and techniques that would otherwise not be possible.
All forms of human tissue research require appropriate controls for pathological comparison, however controls in paediatric research are not always obtainable and are often not suitable for use. Fortunately, a sufficient cohort of controls was available for analysis and proved to be invaluable to the research. Utilizing control cases, the normative distribution study provided the necessary foundation to investigate a potential role of SP in the pathogenesis of SIDS given its interaction with the medullary 5-HT network, dysfunction of which has been previously established in SIDS. The study also provided further support for a relationship between SP and 5-HT in nuclei intimately related to homeostatic control, thus promoting future research focused on determining the combined role of 5-HT and SP dysfunction in SIDS, which until now has been unexplored.

Analysis of key parameters associated with SIDS risk is essential for a comprehensive understanding of SIDS pathology. Unfortunately not all SIDS human tissue research utilizes this approach, which can make interpretation of data across studies and datasets difficult and can offset its translation into future research studies. In each of the present thesis studies analysis and adjustment of data was performed according to age, prematurity, sex and PMI. In doing so, this thesis identified significant effects of age, prematurity and sex associated with SP/NK1R expression and was able to exclude possible effects of these parameters in the 5-HT study. Furthermore the potential affects of PMI on human tissue were able to be ruled out in each study, which is particularly important given a key factor in post-mortem research is tissue quality and the ability to yield accurate results.

Future studies resulting from the outcomes of the present thesis may include investigating the relationship between 5-HT1A receptor binding (previously published by our collaborators) and the NK1R binding data from this thesis, given that the same cases from the same cohort (San Diego) were used in both studies. Furthermore, the current analysis of NK1R binding density in the medullary 5-HT network might be incorporated into an investigation of metabolites in the brains of SIDS infants, given that key metabolites may specifically or non-specifically be associated with 5-HT1A receptors and NK1R. Additional future research should also be targeted at the specific investigation of medullary nuclei, principally the raphe and its associated projections to the extra raphe (PBC), which have been consistently associated with abnormalities of neurotransmitter dysfunction in SIDS. Identification of these nuclei and confirmation of abnormalities associated with their function enables investigators to direct the focus of future research to specific medullary sites, providing a basis for the development of future animal studies to target key regions for investigation of the physiology associated with SIDS pathology. For example, current and ongoing transcriptome analysis of 5-HT neurons in the medullary raphe and extra raphe nuclei of genetically modified rodent models and human infant post-mortem tissue (SIDS and controls) is likely to be highly beneficial in understanding the molecular and subcellular mechanisms underpinning 5-HT and associated neuron abnormalities. Laser-capture microdissection and RNA-seq technology are...
currently being utilised to isolate neuron populations, including the excess of 5-HT neurons in the medullary raphe and extra raphe (as identified in SIDS vs. controls study B). Following individual isolation these neurons are able to be extracted for whole genome expression profiles, providing new insight into molecular pathways underlying 5-HT and brainstem dysfunction in SIDS. The combined observations from this thesis including the confirmation of 5-HT neuron dysfunction in medullary nuclei in Australian SIDS cases, the extensive interaction of 5-HT, SP and NK1R in the developing brainstem and absolute NK1R binding differences in SIDS cases within the medullary 5-HT system, provides further support for the continued targeting of specific medullary regions such as the raphe and extra raphe (PBC) nuclei and for transcriptome analysis of 5-HT neurons that also express other neurochemicals such as SP.

While successful outcomes have been achieved using post-mortem human SIDS and control brainstem tissue which will continue to be utilized in the future, the diagnosis of SIDS is one of exclusion of other potential causes at autopsy and it is not possible to identify SIDS prior to death or analyze the events that lead to failure of brainstem mechanisms. Therefore moving forward, a key feature of SIDS research should aim to bridge the gap between human SIDS post-mortem pathology and juvenile animal physiology studies. This will enable investigation and extrapolation of the potential physiology that precedes post-mortem SIDS pathology, for a comprehensive multidisciplinary approach to further understanding the pathogenesis of SIDS.

5.3 Concluding remarks

Unfortunately, despite extensive research to date there remains no cure for SIDS, no universally accepted definition or theory and no way of identifying infants that may be at increased risk of sudden death. However the outcomes of this thesis identify that in order to overcome the limitations associated with SIDS, human tissue research, collaborative research activity, integrated multidisciplinary involvement and a commitment to pursuing well-formulated scientific investigation are fundamental for successful future outcomes in SIDS research. In addition to establishing advances in diagnostic tools and research technology, biological credibility to public health campaigns and risk reduction initiatives, the ultimate goal is to determine and understand the underlying causes and pathogenesis of the syndrome, to facilitate development of screening and prevention methods and ultimately to eradicate SIDS.
BIBLIOGRAPHY


