

ADAPTIVE IMMUNE INFLUENCES ON THE BRAIN AND BEHAVIOUR

**THE INFLUENCE OF THE ADAPTIVE IMMUNE SYSTEM ON BEHAVIOUR,
BEHAVIOURAL SYSTEMS, AND FUNCTIONAL NEUROANATOMY**

By:

**KELLY C. RILETT,
B.H.Sc. (Honours), M.Sc.**

A Thesis

Submitted to the School of Graduate Studies in partial
fulfillment of the requirements for the degree

Doctor of Philosophy

McMaster University
© Copyright by Kelly C. Rilett, July 2013

DOCTOR OF PHILOSOPHY (2013)

MCMASTER UNIVERSITY

(Neuroscience)

Hamilton, Ontario, Canada

TITLE: The influence of the adaptive immune system on behaviour,
molecular biology, and functional neuroanatomy

AUTHOR: Kelly C. Rilett, B.H.Sc. (Honours; McMaster University),
M.Sc. (McMaster University)

SUPERVISOR: Dr. Jane A. Foster, Ph.D.

NO. OF PAGES: xvi, 166

ABSTRACT

Immune-brain communication has important influences on stress circuitry and stress-related behaviours. Adaptive immune deficiency through loss of lymphocytes or an absence of gut bacteria has been linked to anxiety behaviours and stress responsiveness. In these models, there is a common deficit of T lymphocytes leading to the central hypothesis that T lymphocytes influence stress responsiveness and stress-related behaviours. This project considers the effects of T lymphocyte deficiency on anxiety and fear related behaviours as well as stress responsiveness in the hypothalamic pituitary adrenal (HPA) axis. Mice lacking T lymphocytes through knockout of the T cell receptor (TCR) β and δ chains, and B lymphocytes through knockout of the immunoglobulin M μ chain, were obtained and compared to C57BL/6 control mice. Activity, exploration, anxiety, fear and spatial learning tests were employed. Separately, gene expression was assessed for genes related to stress circuitry following chronic restraint stress. Additionally, lipopolysaccharide was used to determine the stress response to an innate immune challenge that was previously shown to elicit an exaggerated stress response in mice lacking Class I Major Histocompatibility Complex (MHC) and CD8⁺ T lymphocytes. It was found that mice lacking T lymphocytes, but not B lymphocytes, have reduced anxiety-like behaviour but an increased fear response. *TCR β ^{-/-} δ ^{-/-}* mice also had altered expression of components of the HPA axis, serotonergic receptors and NMDA receptor subunits indicating an altered response to chronic stress. Finally, *TCR β ^{-/-} δ ^{-/-}* mice do not display an exaggerated stress response to an innate immune

challenge suggesting a central role for Class I MHC in the stress response that is not due to the CD8⁺ T lymphocyte deficiency that accompanies the functional loss of Class I MHC. These studies reflect an important role for T lymphocytes specifically in the development of the stress system and stress-related behaviours and enables a deeper understanding of neuroimmune influences on stress.

ACKNOWLEDGMENTS

First I would like to thank my mentor and supervisor, Dr. Jane Foster. Throughout my graduate career Jane has offered support, advice, and compassion whenever needed. Above that, I would like thank Jane for believing in me, often more than I believed in myself. That will stay with me.

I have also been very fortunate to have supportive committee members. Thank you to Dr. Claudio Soares for career advice, Dr. Henry Szechman for extra support during the preparation of this thesis, Dr. Elyanne Ratcliffe for encouragement during the final stages of my Ph.D. and Dr. Ali Ashkar who has offered methodological advice.

To Robyn MacKenzie, who taught me many of the protocols within, thank you for your technological help. More importantly, thank you for the friendship that has developed. Additionally, I would like to thank my past labmates Dr. Karen-Anne McVey Neufeld and Dr. Michelle Sidor for enduring encouragement. To Sufian Odeh, I am grateful to have had a thesis companion to help motivate me when I needed it most. A special thanks to Jonathan Lai, who has been tremendously understanding regarding shared projects in the laboratory and who I have shared many ideas with over the past four years.

I would also like to thank my whole family for just about everything. I have known nothing but love, support and encouragement from each and every one of you. Thank you to my Mom for enriching my education and to my Dad for encouraging a curious mind. Undoubtedly this helped direct me down the scientific path that I have taken.

ABBREVIATIONS

5-HT	5-Hydroxytryptamine (Serotonin)
ACTH	Adrenocorticotrophic Releasing Hormone
ANOVA	Analysis of Variance
BLA	Basolateral Amygdala
BNST	Bed Nucleus of the Stria Terminalis
CeA	Central Amygdala
CNS	Central Nervous System
CON	Control
CORT	Corticosterone
CRH	Corticotropin Releasing Hormone
CRH-BP	Corticotropin Releasing Hormone- Binding Protein
CRHR	Corticotropin Releasing Hormone Receptor
CS	Cued Stimulus
DG	Dentate Granule layer of the Hippocampus
EDTA	Ethylenediaminetetraacetic Acid
EIA	Enzyme-Linked Immunoabsorbant Assay
EPM	Elevated Plus Maze
Er β	Estrogen Receptor β
FC	Fear Conditioning
GABA	γ -Aminobutyric Acid
GF	Germ Free
GR	Glucocorticoid Receptor
HPA	Hypothalamic Pituitary Adrenal
IL	Interleukin
i.p.	Intraperitoneal
KO	Knock Out
LA	Lateral Amygdala
LD	Light/Dark Test
LPS	Lipopolysaccharide
MDD	Major Depression Disorder
MHC	Major Histocompatibility Complex
MR	Mineralocorticoid Receptor
MRI	Magnetic Resonance Imaging
mRNA	messenger Ribonucleic Acid
MWM	Morris Water Maze
NMDA	N-methyl-D-Aspartate
NR	NMDA Receptor
OF	Open Field
PAG	Periaqueductal Gray
PNPP	Para-Nitrophenylphosphate
PSD	Post-Synaptic Density
PVN	Paraventricular Nucleus of the Hypothalamus
PTSD	Post-Traumatic Stress Disorder
RAG	Recombinase Activating Gene

Rgs	Regulator of G Protein Signaling
RS	Restraint Stress
SAL	Saline
SCID	Severe Combined Immunodeficiency
SE	Standard Error
SSC	Saline-Sodium Citrate
TAP	Transporter Associated Protein
TCR	T Cell Receptor
TNF	Tumor Necrosis Factor
T _{regs}	T Regulatory Cell
UCN	Urocortin
VMH	Ventral Medial Hypothalamus
WT	Wild Type
$\beta 2M$	$\beta 2$ -Microglobulin
μMT	Immunoglobulin M μ chain

TABLE OF CONTENTS

ABSTRACT.....	iv
ACKNOWLEDGEMENTS.....	vi
ABBREVIATIONS.....	vii
TABLE OF CONTENTS.....	x
LIST OF FIGURES.....	xiii
LIST OF TABLES.....	xvi
DECLARATION OF ACADEMIC ACHIEVEMENT.....	xvii

TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION	1
1.1. Immune-Brain Communication	1
1.2. Hypothalamic Pituitary Adrenal (HPA) Axis	3
1.3. Immunity and Stress	4
1.3.1. Innate Immune System and the HPA Axis	4
1.3.2. Adaptive Immunity and the HPA Axis	5
1.4. Neuroimmunology and Psychiatric Illnesses	7
1.5. Mouse Models of Behaviour	8
1.5.1. Approach-Avoidance Models of Anxiety-Like Behaviour	8
1.5.2. Learning Behaviour Paradigms	11
1.6. Neuroimmunology and Behaviour	12
1.6.1. Behaviour and Adaptive Immune Deficiency	12
1.6.2. Novel Roles for Immune Molecules in Brain Function	14
1.6.3. Class I MHC	15
1.7. Sexual Dimorphisms in Behaviour	17
1.8. Neurotransmitter Systems and Behaviour	18
1.8.1. Serotonergic Influences on Anxiety	18
1.8.2. Glutamatergic Signaling and Anxiety	21
1.8.3. Stress Signaling and Anxiety	23
1.9. Specific Aims and Hypotheses	25
 CHAPTER 2. METHODS	 28
2.1. Behavioural Phenotyping of Immune Deficient Mice	29
2.2. Expression of Neurotransmitter Receptor mRNA and Plasma Corticosterone Levels Following Repeated Restraint Stress	37
2.3. Innate Immune Response in <i>TCRβ^{-/-}δ^{-/-} Mice</i>	40

CHAPTER 3. RESULTS	42
3.1. Overview of Experiment #1	42
3.2. Results of Experiment #1	44
3.2.1. Behaviour of <i>TCRβ</i> ^{-/-δ} Mice	44
3.2.2. Behaviour of <i>μMT</i> Mice	54
3.3. Summary of Experiment #1	61
3.4. Overview of Experiment #2	63
3.5. Results of Experiment #2	64
3.6. Summary of Experiment #2	98
3.7. Overview of Experiment #3	100
3.8. Results of Experiment #3	101
3.9. Summary of Experiment #3	107
 CHAPTER 4. DISCUSSION	 108
4.1. Summary of Findings	108
4.2. The Adaptive Immune System Influences Anxiety-Like Behaviour	110
4.3. Loss of Sexually Dimorphic Behaviour in Immune Compromised Mice	114
4.4. The Adaptive Immune System and Learning Behaviours	118
4.5. Basal Differences in HPA Axis Regulation in Adaptive Immune Deficient Mice	121
4.6. Differences in the Expression of Stress-Related Genes in Adaptive Immune Deficient Mice	128

4.7. Adaptive Immune Deficiency and Stress Reactivity	129
4.8. T Lymphocyte Deficiency is Associated with Loss of Sex Differences in Stress Reactivity	131
4.9. Adaptive Immune Deficiency and Acute Stress Reactivity	134
4.10. Glutamatergic System	136
4.11. Serotonergic System	138
4.12. T Lymphocytes Could Influence the Brain and Behaviour During Development	141
4.13. Conclusion	143
4.14. Future Directions	145
REFERENCES	147

LIST OF FIGURES

Figure 1: Experimental Design	30
Figure 2: Zone Maps for Open Field	32
Figure 3: Zone Map for the Elevated Plus Maze	34
Figure 4: Open Field Test Behaviour in T lymphocyte deficient mice	45
Figure 5: Light/Dark Test Behaviour in T lymphocyte deficient mice	47
Figure 6: Elevated Plus Maze Behaviour in T lymphocyte deficient mice	49
Figure 7: Fear Learning in the Auditory Fear Conditioning Test in T lymphocyte deficient mice	51
Figure 8: Spatial Learning in the Morris Water Maze in T lymphocyte deficient mice	53
Figure 9: Open Field Test Behaviour in B lymphocyte deficient mice	55
Figure 10: Light/Dark Test Behaviour in B lymphocyte deficient mice	56
Figure 11: EPM Behaviour in B lymphocyte deficient mice	59
Figure 12: Fear Learning in the Auditory Fear Conditioning Test in B lymphocyte deficient mice	60
Figure 13: Spatial Learning in the Morris Water Maze in B lymphocyte deficient mice	65
Figure 14: Representative Film Autoradiographs of CRH mRNA Expression in the Bed Nucleus of the Stria Terminalis	68
Figure 15: Representative Film Autoradiographs of CRH mRNA Expression in the Paraventricular Nucleus of the Hypothalamus	69
Figure 16: Representative Film Autoradiographs of CRH mRNA Expression in the Central Amygdala	70
Figure 17: Representative Film Autoradiographs of CRHR1 mRNA Expression in the Hippocampus	72
Figure 18: Representative Film Autoradiographs of CRHR2 mRNA Expression in the Hippocampus	73

Figure 19: Representative Film Autoradiographs of GR mRNA Expression in the Hippocampus	75
Figure 20: Representative Film Autoradiographs of MR mRNA Expression in the Hippocampus	76
Figure 21: Representative Film Autoradiographs of 5-HT1A mRNA Expression in the Hippocampus	79
Figure 22: Representative Film Autoradiographs of 5-HT2A mRNA Expression in the Hippocampus	80
Figure 23: Representative Film Autoradiographs of 5-HT1A mRNA Expression in the Amygdala	81
Figure 24: Representative Film Autoradiographs of 5-HT2C mRNA Expression in the Amygdala	82
Figure 25: Representative Film Autoradiographs of NR1 mRNA Expression in the Hippocampus	85
Figure 26: Representative Film Autoradiographs of NR2A mRNA Expression in the Hippocampus	86
Figure 27: Representative Film Autoradiographs of NR2B mRNA Expression in the Hippocampus	87
Figure 28: Representative Film Autoradiographs of NR1 mRNA Expression in the Central Amygdala	91
Figure 29: Representative Film Autoradiographs of NR2A mRNA Expression in the Central Amygdala	92
Figure 30: Representative Film Autoradiographs of NR2B mRNA Expression in the Central Amygdala	93
Figure 31: Representative Film Autoradiographs of NR1 mRNA Expression in the Amygdala	95
Figure 32: Representative Film Autoradiographs of NR2A mRNA Expression in the Amygdala	96
Figure 33: Representative Film Autoradiographs of NR2B mRNA Expression in the Amygdala	97
Figure 34: Habituation to the Open Field Test in Mice Deficient of T Lymphocytes	102

Figure 35: The Effects of LPS Challenge on Behaviour of T Lymphocyte Deficient Mice in the Open Field	103
Figure 36: Plasma Corticosterone Levels Following LPS Challenge	105

LIST OF TABLES

Table 1: mRNA expression of stress-related genes in C57Bl/6 and <i>TCRβ</i> ^{-/-} <i>δ</i> ^{-/-} mice	67
Table 2: mRNA expression for serotonin receptor subunit genes in wild type (WT) and T cell receptor knock out (TCR) mice in amygdala and hippocampal subregions	78
Table 3: mRNA expression profile for NMDA receptor (NR) subunit genes in wild type (WT) and T cell receptor knock out mice (<i>TCRβ</i> ^{-/-} <i>δ</i> ^{-/-}) in hippocampal subregions	84
Table 4: mRNA expression profile for NMDA receptor (NR) subunit genes in wild type (WT) and T cell receptor knock out mice (<i>TCRβ</i> ^{-/-} <i>δ</i> ^{-/-}) in amygdala subregions	89

DECLARATION OF ACADEMIC ACHIEVEMENT

The author gratefully acknowledges the contributions made by individuals collaborating on this project. First, Robyn MacKenzie split the responsibilities for running the first set of behavioural experiments on *TCR β -/- δ -/-* mice with the author and provided training opportunities for the author to learn these protocols. Dr. Karen-Anne McVey Neufeld did much of the restraint stress protocol and performed tissue collection with the author. Camilla Halgren helped with handling and weights in the *μ MT-/-* and LPS experiments. All subsequent behavioural testing was conducted by the author including the tests of open field habituation, response to LPS, and B Cell deficient mice.

All tissue processing and tests of gene expression were conducted by the author. Radioimmunoassay measurements of corticosterone in the restraint stress experiments were conducted by Marg Cootes. Blood collection from the LPS experiments was performed by Dr. Jane Foster and ELISA measurements of corticosterone in the LPS experiments were conducted by Robyn MacKenzie.

All data acquisition, analysis, and interpretation of the data from the above experiments were performed by the author as part of the thesis requirements.

CHAPTER 1. INTRODUCTION

1.1. Immune-Brain Communication

Historically the brain and the immune system were studied separately and it was widely believed that the immune system and its related signaling did not exist within the healthy central nervous system (CNS). However, in the last few decades the field of neuroimmunology has expanded considerably and the importance of immune-brain communication is increasing. Immune functioning has been linked to mood, behaviour, and neurologic or psychiatric illnesses including Alzheimer's Disease, Multiple Sclerosis, Major Depressive Disorder and anxiety disorders. The first insight that immune functioning influenced behaviour began with the discovery of sickness behaviours.

The collective symptoms of lethargy, weakness, malaise, listlessness, and disinterest in surroundings, food and drink are known as sickness behaviours (Hart, 1988) and are an adaptive response to pathological infection. This kind of response results in protective behaviours of the organism such as seeking warmer temperatures and resting in order to conserve energy for the febrile response (Kluger, 1986). It was proposed that the immune system could not induce sickness behaviours independently of the CNS, therefore, cytokines such as interleukin (IL-1) would need to communicate with the CNS (Dantzer and Kelley, 1989). Support for this theory came from studies on humans that received

treatment with IL-2 or interferon (IFN) α treatment for cancer or Hepatitis C, in which it was found that IL-2 increased depressive symptoms including sadness, pessimism, suicide ideation, anorexia, loss of concentration and loss of sleep compared to patients who did not receive cytokine treatment (Capuron et al., 2003; Capuron et al., 2004). In preclinical rodent experiments, it has been demonstrated reproducibly that peripheral IL-1 β cytokine injection activates the hypothalamic pituitary adrenal (HPA) axis (Sapolsky et al., 1987; Propes and Johnson, 1997; Wang and Dunn, 1999), induces sickness behaviours (Kluger, 1986; Hart, 1988; Bluthé et al., 1995; Propes and Johnson, 1997; Swiergiel and Dunn, 2007) and neuronal activation within the CNS (Ericsson et al., 1995; Herkenham et al., 1998). Furthermore, peripheral IL-1 β or lipopolysaccharide (LPS) results in an innate immune response centrally that mirrors the peripheral immune response. Centrally, innate immune challenge results in an upregulation of c-fos, immediate early gene, at the blood brain barrier initially and then the signal propagates inward to the parenchyma (Herkenham et al., 1998). Even at low doses, LPS injection leads to IL-1 β and Tumor Necrosis Factor (TNF)- α mRNA production along barrier regions of the CNS as well as I κ B α mRNA expression indicating a possible mechanism for immune signaling to the CNS (Quan et al., 1999). These early studies in neuroimmunology demonstrated clearly that a peripheral immune response results in a central innate immune response that has an impact on the brain function and behaviour.

1.2. Hypothalamic Pituitary Adrenal (HPA) Axis

The circuitry of the HPA axis, sometimes referred to simply as “stress axis,” as originally described by Hans Selye has been well established. Briefly, when an animal is confronted with a stressor, corticotropin releasing hormone (CRH) is released from the paraventricular nucleus (PVN) of the hypothalamus into the hypothalamo-hypophyseal portal system where it is transported to the anterior pituitary. Within the anterior pituitary, CRH stimulates the release of adrenocorticotrophic hormone (ACTH), which in turn is transported through the blood to the adrenal glands. In the adrenal glands, ACTH stimulates the release of glucocorticoid stress hormone- cortisol for humans, corticosterone (CORT) for non-humans. A negative feedback loop is formed via the inhibitory effects of CORT through glucocorticoid receptors and melanocorticoid receptors within the hippocampus resulting in decreased production and secretion of CRH (Turnbull and Rivier, 1999; Kellendonk et al., 2002).

Increasingly, HPA axis dysregulation is considered to have a strong influence on the development of anxiety-like behaviours in rodents (Landgraf and Wigger, 2002; Jakovcicski et al., 2011) and mood disorders in humans (Coryell et al., 2006). This finding includes reduced salivary cortisol levels at baseline in adults with anxiety disorders (Hek et al., 2013), increased cortisol awakening response at the beginning of internalizing disorders (Ruttle et al., 2011) but reduced longitudinal cortisol in children with symptoms of internalizing disorders

(Badanes et al., 2011; Ruttle et al., 2011) and depression (Merali et al., 2004; Coryell et al., 2006; Jakovcecski et al., 2011). Current reviews indicate that patients with depressive symptoms have often been found to have significantly higher levels of plasma CORT (Zoccola and Dickerson, 2012) than healthy controls. Furthermore, central HPA axis dysregulation in the form of increased mRNA expression of CRH has also been observed in the post-mortem brains of suicide victims (Merali et al., 2004). Importantly, correction of HPA axis dysregulation through pharmaceutical intervention lead to symptom improvements in patients with depression (Wilkinson and Goodyer, 2011). These findings suggest a role for the HPA axis on the development of trait behaviours.

1.3. Immunity and Stress

1.3.1. Innate Immune System and the HPA Axis

There is considerable crosstalk between the HPA axis and the immune system that forms a negative feedback loop. Peripheral administration of inflammatory cytokines such as IL-1 (Besedovsky and del Rey, 1987; Besedovsky and del Rey, 1989; Matta et al., 1993; Brady et al., 1994; Ebisui et al., 1994; Melik Parsadaniantz et al., 1994) and TNF α (Tracey et al., 1987; Bernardini et al., 1990; Besedovsky et al., 1991; Ebisui et al., 1994) stimulate the production of CRH and the release of ACTH, and plasma CORT in a variety of animal models. Meanwhile, CORT is an immunosuppressant that prevents over

production of inflammatory molecules. The importance of this negative feedback between the immune system and the HPA axis was highlighted in studies of adrenalectomy. Rodents that have been adrenalectomized to prevent the production of CORT have increased production of proinflammatory cytokines such as IL-1, and TNF- α following immune challenge with LPS (Perretti et al., 1993; Goujon et al., 1996) and have increased risk of LPS induced mortality that is reversible with dexamethasone treatment (Butler et al., 1989). Thus, the immune system activates the HPA axis leading to secretion of the immunosuppressant CORT.

1.3.2. Adaptive Immunity and the HPA Axis

The adaptive immune system, that can recognize a large diversity of antigens and mount targeted immune responses, consists of T and B lymphocytes. B lymphocytes can produce antibodies against specific pathogens and form the humoral immune response to extracellular pathogens. This response may or may not involve effector CD4⁺ T helper cells, also a component of humoral immunity, depending on the antigen. Effector CD8⁺ T lymphocytes are responsible for the adaptive cellular immune response that targets intracellular pathogens. Both CD4⁺ and CD8⁺ T lymphocytes can also exist as T regulatory cells (T_{regs}) which are involved in immune tolerance to self-antigens. In addition to CD4⁺ and CD8⁺ T lymphocytes, which contain α and β chain subunits

for the T Cell Receptor (TCR), there are also $\gamma\delta$ T lymphocytes, though the function of these cells is less understood.

While crosstalk between cytokines and HPA axis has been extensively reported, less is known about the role of adaptive immunity and HPA axis regulation. There is some evidence that T lymphocytes are involved in HPA axis activity. T lymphocyte function and profile have a strong influence on the regulation of the HPA axis. Increased T cell proliferative capacity has been correlated with an exaggerated CORT response to stress in male rats that are highly vulnerable to stress (Stiller et al., 2011b). In mice challenged with staphylococcal enterotoxin B, an immune challenge that induces a strong CD4⁺ T cell response, it was found that CRH mRNA expression is upregulated in both the PVN and the central amygdala (CeA; (Kusnecov et al., 1999). Furthermore, athymic “nude” mice, that lack T cells, have elevated plasma ACTH but normal CORT basally. To determine if these results were the consequence of adrenal failure, ACTH was used as a challenge both *in vivo* and *in vitro*. ACTH induced significantly less CORT both in athymic mice, an effect that was normalized by reconstituting athymic mice with splenocytes from wild type mice (Gaillard et al., 1998). The reconstitution of athymic mice confirms that it is the presence T cells rather than an endocrine function of the thymus that affects the regulation of the HPA axis in these animals.

1.4. Neuroimmunology and Psychiatric Illnesses

Adaptive immune dysfunction has been linked to psychiatric illness (Maxeiner et al., 2009). While most of the literature on T lymphocytes and psychiatry focuses on major depression disorder (MDD), T lymphocytes have also been linked to anxiety disorders. In a comparison of patients with combined MDD and panic disorder and panic disorder patients who do not suffer from MDD, T lymphocyte numbers were elevated in the depression free panic disorder cohort (Andreoli et al., 1992). Furthermore, the ratio of CD4⁺ to CD8⁺ T lymphocytes was positively correlated with the severity of anxiety symptoms in MDD patients (Charles et al., 1992) indicating that the balance of subtypes is relevant to psychiatry.

Providing further support for links between the adaptive immune system and anxiety behaviours is the evidence of alterations to the immune system by anxiolytic medication. Certain benzodiazepines, one of the most successful classes of anxiolytic medications, interfere with T lymphocyte proliferation in response to mitogen (Ramseier et al., 1993) while others boost cellular immunity (Jirillo et al., 1993). Additionally, some antipsychotic medications reduce cellular immunity (Descotes et al., 1985). These studies indicate that some treatments for anxiety may already be targeting the immune system in addition to central targets. Use of a preclinical animal model of behaviour and adaptive immune deficiency clarifies the role of the adaptive immune system in behavioural

development, which is less established than the behavioural influence of innate immune components.

1.5. Mouse Models of Behaviour

1.5.1. Approach-Avoidance Models of Anxiety-like Behaviour

Rodent anxiety-related behaviour tools utilize knowledge of approach and avoidance tendencies to create environments in which the animal can explore novel environments (approach behaviour) or avoid stressful environments (Montgomery, 1955b, a; Holmes, 2001). Mice are naturally exploratory but avoid potentially dangerous areas such as brightly lit or exposed environments (Montgomery, 1955a; Mizukawa et al., 1989; Berdel et al., 1997). Three standard tests of anxiety that are constructed using this knowledge are the open field test, light/dark test (Bourin and Hascoet, 2003), and elevated plus maze (EPM; (Handley and Mithani, 1984; Lapiz-Bluhm et al., 2008).

In the open field test, the center of the chamber is considered the aversive zone. Anxiety-like behaviour is assessed through quantification of time spent in the periphery and the center of the open field. A mouse is considered to be less anxious if it spends more time in the center of the open field (Miller et al., 2010). In this test, exploration is measured by quantifying the distance traveled (Crawley, 1985).

The light/dark test consists of a large rectangular clear Plexiglas box that is divided by a smaller black Plexiglas box. The black box contains a door facing

the light chamber so that the mouse can move freely between the light and dark chambers. The aversive zone within the light/dark test is the light chamber and increased time spent in the light chamber is indicative of a reduced anxiety-like phenotype (Crawley and Goodwin, 1980; Chaouloff et al., 1997). Additionally, exploratory behaviour can be measured by quantifying the number of transitions between chambers the animal makes and the amount of rearing behaviour within the test (Crawley and Goodwin, 1980).

Finally, the EPM is a t-shaped test in which there are two raised opposing open arms and, at a right angle to each arm, two opposing closed arms. The closed arms have 38 cm high black Plexiglas walls creating a dark, enclosed environment; whereas the open arms do not have walls and are the aversive zone. Anxiety-like behaviour is measured as time spent and entries into the open arms (Pellow and File, 1986; Trullas and Skolnick, 1993; Rodgers and Johnson, 1995). Head dips and intersection time (Rodgers and Johnson, 1995) are considered to be exploratory/risk assessment behaviours. Closed arm distance and entries are measured as activity (Rodgers and Johnson, 1995).

These tests have been validated pharmacologically and statistically. Anxiolytic medications, such as benzodiazepines, have been shown to result in decreased preference to remain in the periphery, called “thigmotaxis,” in the open field (Choleris et al., 2001), increased time spent and entries into the open arms of the EPM (Gentsch et al., 1987; Chaouloff et al., 1997) and increased time in the light chamber of light/dark box test (Belzung et al., 1987; Lepicard et al.,

2000). Conversely, administration of inverse agonists of the benzodiazepine receptor decreased the amount of time spent in the light chamber of the light/dark box test (Belzung et al., 1987). In the elevated plus maze, known anxiogenic drugs such as yohimbine, phenylenetetrazole, caffeine, and amphetamine resulted in reduced exploration of the open arms (Pellow et al., 1985; Pellow and File, 1986). Statistically, principal component analysis (PCA) has been used to determine which outcomes of these tests correlate. Outcome measures that are sensitive to anxiolytic treatment (Pellow and File, 1986; Gentsch et al., 1987; Lepicard et al., 2000) such as time spent in the open arms of the EPM and time in the light chamber of the light/dark box test, factor together. Additionally, closed arm entries factors with total open field locomotion in PCA and is considered a measure of locomotor activity (Ramos et al., 1997). However, it should be noted that PCA on the open field test, light/dark box test, and elevated zero maze test revealed that locomotor activity in the open field strongly correlates with the anxiety-like measure of open arm time in the elevated zero maze (Milner and Crabbe, 2008). It is therefore important to include a battery of behaviour tests as reproducing anxiety-like phenotypes across multiple tests strengthens the interpretation of the results. Furthermore, it has been demonstrated that these tests should be staggered a minimum of one week to avoid inter-test effects and should be conducted in order from least stressful to most stressful (Crawley, 2008).

1.5.2. Learning Behaviour Paradigms

Two common learning paradigms that are used to assess cognition in rodents include the auditory fear conditioning test and the Morris Water Maze. The auditory fear conditioning test is used to examine both cued and contextual associative learning. The premise is that on the training day animals learn to associate a cued stimulus, a tone, with a noxious stimulus, a mild foot shock (Boddez et al., 2012). Cued learning is assessed by testing the response to the cued stimulus in a different context while contextual learning is assessed by examining the behaviour of the animals placed in the same context as the training day. In response to the foot shock, mice exhibit freezing behaviour. Mice that exhibit increased percent immobility, or “freezing behaviour,” during the contextual test or following the tone during the cued test are considered to have an increased fear response.

The Morris Water Maze (MWM) is a visuospatial learning test in which visual cues are placed in four quadrants around a pool of water and mice are placed in the pool and allowed to swim until they locate a submerged platform (Morris, 1981). Upon locating the platform, mice are removed from the pool and put back into their home cage between trials. Each day, four swim trials are conducted, one beginning from each of the four quadrants, and the average time to reach the platform is analyzed. Improved spatial learning is observed as reduced latency to reach the platform. Additionally, during a probe trial, the platform is removed and the time spent exploring the quadrant in which the

platform was previously located is recorded (Paylor et al., 1994). Finally, a reversal trial is conducted in which the platform is moved to a different quadrant of the pool and the time to reach the platform is recorded (Russig et al., 2003).

1.6. Neuroimmunology and Behaviour

1.6.1. Behaviour and Adaptive Immune Deficiency

Researchers in psychiatry and behavioural neuroscience are increasingly recognizing the importance of the adaptive immune system in the development of behaviour. Adaptive immune function can influence anxiety-like behaviours. Specifically, lymphocytes have reproducibly been shown to influence the development of anxiety-like behaviours (Oliveira-Dos-Santos et al., 2000; Cushman et al., 2003). Animals lacking recombinaase activating gene (RAG)-1, and consequently lacking all B and T lymphocytes, have reduced fearfulness and increased activity in the elevated plus maze and open field (Cushman et al., 2003). It has also been shown that regulators of G protein signaling (rgs2) knock out animals, with impaired T lymphocyte activation, have increased anxiety-like behaviour in the light/dark preference test (Oliveira-Dos-Santos et al., 2000). Work in our laboratory and others has demonstrated that germ-free mice that lack all gut microbiota, and have an undeveloped adaptive immune system, have reduced trait anxiety (Heijtz et al., 2011b; Neufeld et al., 2011b; Clarke et al., 2012). In addition, we have examined anxiety-like behaviours in mice lacking

CD8⁺ T lymphocytes due to knockout of β 2M and TAP. Here it was shown that knockout of β 2M and TAP results in reduced activity within the open field in females as well as reduced activity in the EPM in the male population.

Additionally, risk assessment behaviours were altered in both male and female β 2M^{-/-}TAP^{-/-} mice observed as increased time spent in the intersection of the EPM and increased poke arounds. However, it was noticeable that β 2M^{-/-}TAP^{-/-} did not exhibit reduced anxiety-like behaviour (Sankar et al., 2012b).

While these models have differences in behaviour and construct, we believe that the changes in anxiety-like behaviour are driven by T lymphocytes as this is the common factor in each of the above models. It is clear from these studies that the adaptive immune system has an influence on anxiety-like behaviours but the contributions of T lymphocytes compared to B lymphocytes cannot be inferred from these studies.

The influence of the adaptive immune system on spatial learning behaviour has also been examined by other groups using the MWM task. Severe combined immunodeficient (SCID) mice, lacking the recombinae activating gene (RAG) 1 and 2, as well as athymic “nude” mice demonstrated increased latency to reach the submerged platform. However, this effect was reversible in the athymic mice upon replenishment of T cells (Kipnis et al., 2004). Furthering the evidence of T cells influencing the CNS, this group also showed reduced neurogenesis in the hippocampus of SCID mice (Ziv et al., 2006). As the MWM is a hippocampus-dependent task, this work provides strong evidence that T cells

are required for learning and a mechanism is proposed. However, as RAG1 is expressed in the CNS (Kim et al., 2003), it cannot be discounted that a central role for RAG1 is involved. Also, the thymus is not solely purposed for production of T cells; therefore, it is important to reproduce these findings in a model of T cell deficiency without these confounding factors.

1.6.2. Novel Roles for Immune Molecules in Brain Function

Recently, non-immunological roles for immune components have come into focus. Microglia in particular, which are considered to be the immune cell of the brain, have been demonstrated to engulf and eliminate synapses during development (Paolicelli et al., 2011). This synaptic pruning is essential for normal brain development (Miyamoto et al., 2013) and microglial dysfunction has been correlated with neurodevelopmental disorders such as autism and Rett syndrome (Garay and McAllister, 2010; Frick et al., 2013). In addition to neuronal roles of immune cells, several immune molecules have been located at neuronal synapses and linked to synaptogenesis including Class I Major Histocompatibility Complex (MHC; (Goddard et al., 2007). These findings underscore the importance of immune molecules within the brain and indicate that further investigation into the neuronal functions of immune molecules within the CNS is warranted. Of particular relevance to stress and behaviour is Class I MHC (discussed below).

1.6.3. *Class I MHC*

Class I MHC, expressed on nearly every mammalian cell type (Janeway et al., 2005), has long been known to have important immunological functions that include the presentation of peptide fragments to CD8⁺ cytotoxic T lymphocytes (Flajnik and Kasahara, 2001). The binding cleft in which the peptide fragments are presented includes the α 1-3 subunits of class I MHC in association with β 2-Microglobulin (β 2M; Flajnik and Kasahara, 2001). Assembly of these subunits occurs within the endoplasmic reticulum. Transporter associated with antigen processing (TAP) transfers peptide fragments from the cytoplasm into the endoplasmic reticulum. The peptide fragment bound to class I MHC can then be transported to the cell surface where presentation to cytotoxic T lymphocytes may occur. Loss of class I MHC can be achieved through double knockout of β 2M and TAP (Ljunggren et al., 1995).

Relatively recently, class I MHC has been linked to neurological functions. This was unexpected as it had previously been thought that neurons were among the small group of cells that do not express class I MHC at all (Boulanger, 2009). Namely, blockade of activity-dependent neuronal plasticity has been shown to reduce class I MHC expression in the visual system during development (Corriveau et al., 1998). This surprising discovery led to further experiments that discovered expression of class I MHC throughout the developing brain and in key regions of the adult brain including regions that remain plastic throughout life such as the hippocampus and olfactory bulb (Corriveau et al., 1998; Linda et al.,

1999; Huh et al., 2000; Oliveira et al., 2004). Moreover, expression of class I MHC protein has been found to overlap extensively with post-synaptic density (PSD)-95, a protein found on the post-synaptic cleft of neuronal synapses (Goddard et al., 2007). In mice lacking the $\beta 2M$ and TAP genes ($\beta 2M^{-/-}TAP^{-/-}$), and consequently lacking class I MHC, presynaptic bouton size increased and mini excitatory postsynaptic potentials increased in frequency (Goddard et al., 2007). These discoveries indicate that class I MHC is not restricted to immunological functions within the CNS and is involved in neuronal plasticity.

Previous work has also demonstrated a link between neuronal class I MHC and the HPA axis stress reactivity to an innate immune challenge (Foster et al., 2002). To further examine the role of class I MHC on the innate immune response, our laboratory challenged $\beta 2M^{-/-}TAP^{-/-}$ mice with LPS. Following injection of LPS or saline mice were placed immediately into the open field. In WT females, there was a normal sickness response observed as a decrease in distance traveled in the LPS group compared to saline controls. However, in the $\beta 2M^{-/-}TAP^{-/-}$ females, the saline group showed a sickness response that was comparable to the LPS group suggesting a behavioural stress response to the injection alone. This conclusion was corroborated with plasma CORT analysis 2 hours after the injection that revealed elevated CORT in saline treated $\beta 2M^{-/-}TAP^{-/-}$ females that was comparable to LPS treated $\beta 2M^{-/-}TAP^{-/-}$ females. Sickness behaviour in males was similar, with both WT and $\beta 2M^{-/-}TAP^{-/-}$ males exhibiting decreased activity in the open field following LPS injection. Sex

differences in stress responsiveness have been reported in rat models of innate immune challenge (Seale et al., 2004; Pitychoutis et al., 2009) and in humans following exposure to LPS (van Eijk et al., 2007). Gonadectomy increased the basal corticosterone (CORT) levels as well as CORT levels following LPS exposure in male rats but decreased the CORT response to LPS in females (Seale et al., 2004) suggesting that HPA axis activity and the stress response to an innate immune challenge is tightly regulated by sex hormones.

HPA axis activity also differs between men and women. Beginning as early as adolescence, females are more responsive to stressors and exhibit increased HPA axis responsiveness (Oldehinkel and Bouma, 2011). It has also been demonstrated in humans that females have a heightened stress response to LPS challenge (van Eijk et al., 2007). Sex differences in stress reactivity have been linked to differences in the prevalence of stress related psychiatric conditions including double the prevalence of depression in women compared to men (Weissman and Olfson, 1995), and post-traumatic stress disorder. Due to sex differences in stress responsiveness and stress-related illness, the experiments within this thesis include both sexes.

1.7. Sexual Dimorphisms in Behaviour

Sexual dimorphisms are observed in the presentation of anxiety-like behaviour (Palanza, 2001; Voikar et al., 2001; An et al., 2011). For example in the EPM, DBA/2 males exhibited increased head dips and closed arm entries

compared to females (Rodgers and Cole, 1993). However, these differences were not found in the TK1 strain of mouse indicating that sexually dimorphic behaviour may not be conserved across mouse strains or may not always be observed in different laboratories. Additionally, it has been proposed that different tests of anxiety-like behaviour could vary in sensitivity between sexes. As reviewed by Palanza (2001), the EPM is less sensitive to anxiety-like behaviours in females while the Vogel conflict test, in which the rodent receives a minor shock for drinking water following a deprivation period, is more sensitive to females (Palanza, 2001). Furthermore, it has long been known that females exhibit increased ambulation in the open field test, a finding that has been discovered in multiple strains over a long history of open field testing (reviewed by (Archer, 1975). However, variability between strains has been found in this outcome measure as well as higher activity in both male and female C57BL/6J mice compared to BALB/cJ mice (An et al., 2011). It is therefore worthwhile to explore the relationship between anxiety-like behaviour and the immune system in both sexes.

1.8. Neurotransmitter Systems and Behaviour

1.8.1. Serotonergic influences on anxiety

5-Hydroxytryptamine (serotonin) is a pleiotropic neuromodulator that has been studied extensively for its relation to anxiety and anxiety disorders. Clinical

studies have largely focused on the 5-HT transporter (5-HTT) that transports serotonin out of the synaptic cleft. Notably, there is a polymorphism of the 5-HTT gene that leads to decreased ability to transport 5-HT out of the synapse and is affiliated with increased risk of anxiety disorders (Kenna et al., 2012).

Interestingly, anxiety disorders are often treated with serotonergic reuptake inhibitors (SSRIs; (Koen and Stein, 2011) which have been shown by *in vivo* microdialysis to increase serotonin availability in the CNS (Bel and Artigas, 1993; Fuller, 1994; Ortega et al., 2013) leading to the hypothesis that serotonin was anxiolytic. However, evidence that the function of serotonin depends on receptor subtype ratio, receptor subtype action, location within the CNS, time course of serotonin release, and interactions with other systems (Lowry et al., 2005) suggests a more nuanced system in which receptors are of high importance.

Serotonin receptors are predominantly G protein coupled receptors that fit into one of 7 serotonergic receptor families (5-HT₁₋₇) with several varieties within many families: 5-HT_{1A-B}, 5-HT_{1D-F}, 5-HT_{3A-B}, 5-HT_{2A-C}, 5-HT_{5A-B} (Nichols and Nichols, 2008). Of particular importance to anxiety behaviours are serotonergic receptors 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} within the limbic system (Lowry et al., 2005), which have also been examined preclinically. Disruption of 5-HT_{1A} receptor mRNA expression in the amygdala reduced the amount of time spent in the center of the open field and the open arms of the EPM representing an increased anxiety-like behavioural phenotype (Li et al., 2012). Mice lacking the 5-HT_{1A} receptor through gene knockout also exhibited increased anxiety-like

behaviour in the open field and EPM (Ramboz et al., 1998; Gross et al., 2002) supporting earlier notions that activation of the 5-HT_{1A} receptor has anxiolytic effects. Using conditional knockout mice in which 5-HT_{1A} receptor expression was restored only in the hippocampus and cortex, it was demonstrated that the anxiety-like phenotype could be normalized to the phenotype of WT mice (Gross et al., 2002).

In contrast, serotonergic receptor 5-HT_{2C} has been shown to have anxiogenic effects. Li *et al.* 2012, used injection of adenovirus in the amygdala, particularly the basolateral region, to induce overexpression of the 5-HT_{2C} receptor. These animals spent less time in the center of the open field and open arms of the EPM (Li et al., 2012). Conversely, 5-HT_{2C} receptor knockout mice exhibit reduced anxiety-like behaviour across several tests including the open field and elevated zero maze (Heisler et al., 2007). Finally, the 5-HT_{2A} receptor, which is expressed in the cortex, ventral striatum, hippocampus and amygdala (Millan, 2003), has also been implicated in anxiety-related behaviour. Mice deficient of 5-HT_{2A} receptor through genetic knockout (*htr2a*^{-/-}) exhibit reduced anxiety-like behaviours in the open field test, light/dark test, EPM, and novelty suppressed feeding test suggesting that activation of 5-HT_{2A} receptor is anxiogenic (Weisstaub et al., 2006). In the same study, it was demonstrated that *htr2a*^{-/-} mice behave normally in both a cued and contextual fear learning test indicating that hippocampal and amygdala functioning were intact. This led the authors to hypothesize that restoration of 5-HT_{2A} receptor signaling in the cortex

could rescue the behavioural phenotype. As the *htr2a*^{-/-} mouse was generated by inserting lox-P sites surrounding the *htr2a* gene they were able to remove the inserted sequence and restore cortical expression of *htr2a*. These animals behaved similarly to WT control animals providing additional evidence that 5-HT_{2A} receptor activation within the cortex is anxiogenic. Collectively, these studies indicate that the effect of serotonin on anxiety-like behaviour depends on which receptor subtypes are activated and where they are located in the CNS.

1.8.2. Glutamatergic Signaling and Anxiety

The N-methyl-D-aspartate (NMDA) receptor channel is a heterotetramer that contains NR1 and three variable additional subunits (Dingledine et al., 1999), the affinities of which are determined by subunit composition (Laurie and Seeburg, 1994). There are seven known NMDA receptor subunits in mice including NR1, NR2A-D, and NR3A-B (Dingledine et al., 1999). While the gene expression of NMDA receptor subunits is better established in rats, immunoblot analyses of NMDA receptor subunits NR1 and NR2A-D expression in rat, mouse, human, rabbit and frog suggests that these subunits are evolutionarily conserved and have similar expression across species (Laurie and Seeburg, 1994). In rats, NR1 is ubiquitously expressed throughout the brain while the NR2 subunits are spatially and temporally regulated (Monyer et al., 1994). The NR2A and NR2D subunits are highly expressed early in life (Scheetz and Constantine-Paton, 1994). The ratio of NR2A to NR2B increases during development and NR2B is

known to be involved in synaptic plasticity during development and in learning memory (Cull-Candy et al., 2001). Altered expression of NMDA receptor subunits within the hippocampus has also been linked to anxiety-like behaviour. Mice lacking the NR2a receptor subunit show reduced anxiety-like behaviours in three standard tests (Boyce-Rustay and Holmes, 2006b). Knockouts of the NR1 and NR2b genes in cells of the dentate granule layer of the hippocampus are also associated with reduced anxiety-like behaviour (Niewoehner et al., 2007; von Engelhardt et al., 2008). Manipulations of NMDA receptors also affect behaviour. Blocking the activation of NR2b has been shown to be anxiogenic (Delawary et al., 2010). Pharmacologically, NMDA receptor antagonists result in reduced exploration of the open arms in the EPM (Gatch et al., 1999; Kotlinska and Biala, 1999).

In our laboratory, work with germ-free mice showed that reduced anxiety-related behaviour in the EPM was associated with reduced expression of the NR2B subunit of the NMDA receptor in the central amygdala (Neufeld et al., 2011b). This observation is consistent with an emerging role for NMDA-related glutamate transmission in anxiety-related behaviours (Gatch et al., 1999; Kotlinska and Biala, 1999; Boyce-Rustay and Holmes, 2006a; Grova et al., 2008). Administration of the polycyclic aromatic hydrocarbon benzo[a]pyrene resulted in reduced anxiety-like behaviour in the EPM that was associated with alterations in expression of NMDA subunit mRNAs in the hippocampus, hypothalamus, and cerebellum (Grova et al., 2008). Molecular genetic

approaches also link NMDA receptors to anxiety-related behaviour as NR2A knockout mice show reduced anxiety-like behaviours in the EPM, the light/dark test, and the novel open field (Boyce-Rustay and Holmes, 2006a). These studies establish a link between NMDA receptor modulation and anxiety-related behaviour; however, the role of immune-brain crosstalk in these instances is not clear.

1.8.3. Stress Signaling and Anxiety

Central stress circuitry includes the hippocampus, hypothalamus, and pituitary adrenal axis. Signaling is mediated by CRH and the related CRH receptors CRHR1 and CRHR2, as well as glucocorticoids and related receptors glucocorticoid receptor (GR) and mineralocorticoid receptor (MR; (Turnbull and Rivier, 1999). Many studies have examined changes in stress signaling systems in rodents. Here some key studies related to anxiety-like behaviour are reviewed. Mice lacking CRHR1 display anxiolytic behaviours and are hypo-responsive to restraint stress (Weiser et al., 2008; Weiser et al., 2010). To differentiate the roles of the HPA axis from the limbic system, Cre/loxP conditional knockout mice for CRHR1 have been generated in which CRHR1 is absent from the anterior forebrain and limbic structures while remaining intact in the pituitary. These animals show an anxiolytic phenotype in the light/dark test and EPM and are hyper-responsive to restraint stress (RS) with significantly higher ACTH and CORT levels following restraint stress (RS) (Muller et al., 2003).

CRHR2 has been studied both in the central nervous system (CNS) and in the periphery with varying results. Centrally CRHR2 is expressed in stress-related regions of the CNS including the hippocampus (our data), lateral septum (Bale et al., 2000) PVN and ventralmedial hypothalamus (VMH) (Chalmers et al., 1995). Animals that lack CRHR2 have been shown to have an anxiogenic phenotype in the open field, light/dark test and EPM (Bale et al., 2000; Kishimoto et al., 2000) and were hyperresponsive to RS treatment (Coste et al., 2000). Stress hyperresponsiveness was observed as increased secretion of ACTH and CORT following RS (Preil et al., 2001). CRHR2 KO mice had a more robust increase of ACTH following RS but also had a more rapid decline of ACTH levels. The kinetics of CORT levels following RS were altered in CRHR2 KO mice too with the elevated CORT lasting longer (Coste et al., 2000). It is therefore suggested that CRHR1 mediates the initiation of the stress response while CRHR2 mediates the late-phase stress response and recovery from restraint stress. Notably, there is no difference between WT and CRHR2 KO mice for expression of CRHR1, CRH, or urocortin (UCN) indicating that these effects are in fact mediated by CRHR2 (Coste et al., 2000).

It is theorized that sex steroids may have a regulatory role within the HPA axis. In support of this concept, prenatal stress differentially influences the expression of CRH related mRNAs in the CNS. In females, prenatal stress leads to increased CRH within the PVN and CeA and decreases the expression of CRHR1 in the PVN. Meanwhile, CRH binding protein (CRH-BP), which has an

inhibitory effect of pituitary secretion of ACTH, is decreased in both males and females (Zohar and Weinstock, 2011). Additionally, sex differences were noted in CRHR1 and CRHR2 KO mice. In female CRHR2 KO, there was a more rapid peak and subsequent decline in ACTH secretion following restraint stress that was absent in males (Preil et al., 2001). Furthermore, in females CRH levels in the PVN were decreased by ovariectomy but restored with injections of estrogen (Roy et al., 1999). In stark contrast, androgens have an inhibitory effect on expression of CRH in the PVN (Haas and George, 1988). Administration of androgens increased CRHR2 expression in the hippocampus, hypothalamus, and lateral septum as well as its binding capacity (Weiser et al., 2008). Due to the reciprocal connections between the lateral septum and the PVN, both of which have high expression of CRHR2, it has been suggested that CRHR2 activation mediates the duration of the stress response (Jacobson and Sapolsky, 1991).

1.9. Specific Aims and Hypotheses

Central Hypothesis: T lymphocytes influence stress responsiveness and the development of stress-related behaviour.

Hypothesis 1: Loss of T lymphocytes will result in changes to anxiety-like and fear-related behaviour.

This thesis aims to provide a novel look at how components of the adaptive immune system influence anxiety- and fear-related behaviour. Here we explore this relationship using both male and female *TCR β ^{-/-} δ ^{-/-}* mice that lack functional T lymphocytes (Mombaerts et al., 1994), as well as male and female *μ MT* mice lacking B lymphocytes through knockout of the μ chain of Immunoglobulin M (Kitamura et al., 1991). Previous studies on mice with adaptive immune deficiencies has been conducted only in males (Cushman et al., 2003) or only in females (Neufeld et al., 2011a). Due to known sex differences in behaviour (Palanza, 2001; Voikar et al., 2001), and sex differences observed in our previous experiments on *β 2M^{-/-}TAP^{-/-}* mice (Sankar et al., 2012b), both sexes were included in all studies and multiple tests of anxiety were employed. To avoid strain differences in behaviour (Moore et al., 2011), control animals were selected to have the same genetic background as the knockout mice in each experiment.

Hypothesis 2: Loss of T lymphocytes will result in altered gene expression within stress related systems.

Our laboratory has previously demonstrated that germ-free mice, despite having exhibited reduced anxiety-like behaviours, have elevated stress

responsiveness following chronic restraint stress. *TCR β ^{-/-} δ ^{-/-}* mice also display reduced anxiety-like behaviour but have an increased fear response. We hypothesized that the behavioural outcomes on stress, fear, and anxiety-like behaviour in germ-free mice were due to an underdeveloped adaptive immune deficiency. To determine stress responsiveness in mice lacking T lymphocytes, *TCR β ^{-/-} δ ^{-/-}* mice were subjected to chronic restraint stress and gene expression analyses were conducted within the CNS to determine the effects of this treatment on neuronal systems related to stress, anxiety and fear.

Hypothesis 3: Neuronal expression of Class I MHC is an essential component of the stress response.

TCR β ^{-/-} δ ^{-/-} mice were exposed to an innate immune challenge in the form of an LPS injection. Previously, *β 2M^{-/-}TAP^{-/-}* mice were injected with either LPS or saline as the vehicle control and the stress response to the saline injection was comparable to the reaction to the LPS injection (Sankar et al., 2012b). These results suggested an increased stress response in *β 2M^{-/-}TAP^{-/-}* mice. As *β 2M^{-/-}TAP^{-/-}* mice lack CD8⁺ T lymphocytes as well as neuronal Class I MHC, it was not possible to determine if the effects were due to immune deficiency or loss of neuronal Class I MHC. By repeating the innate immune challenge experiment in *TCR β ^{-/-} δ ^{-/-}* mice we aimed to determine the influence, if any, of peripheral T lymphocytes on the stress response to LPS challenge.

CHAPTER 2. METHODS

Animals. $TCR\beta^{-/-}\delta^{-/-}$ mice, characterized by a loss of T cell receptor (TCR) function by double knock-out of the β and δ chains (Mombaerts et al., 1994), were obtained from Dr. Andrew McPherson at McMaster University. These animals were on a C57BL/6 background and a breeding colony was maintained in the Central Animal Facility at McMaster University. Age- and sex-matched wild type (WT) control mice were obtained from Taconic. Animals were housed in a ventilated rack room with a 12 h light/dark cycle from 5 AM to 5 PM with *ad libitum* access to food and water. All experimental procedures were approved by the Animal Research Ethics Board of McMaster University and were in accordance with the guidelines of the Canadian Council on Animal Care.

Behavioural Testing. Male (n=26) and female (n=21) offspring from 12 litters of mice for each genotype ($TCR\beta^{-/-}\delta^{-/-}$ and WT) were included in behavioural tests. Male and female Immunoglobulin M μ chain knockout mice, that lacked mature B lymphocytes due to arrested development at the pre-B-lymphocyte stage (Kitamura et al., 1991), as well as age and sex matched C57BL/6 controls (WT) were obtained from Jackson Laboratories (n=12 per sex per genotype).

Stress Reactivity. A separate cohort of mice was used to examine the effects of chronic restraint stress on gene expression within central stress related systems. $TCR\beta^{-/-}\delta^{-/-}$ males (n=8 per treatment) and females (n=8 per treatment) and age

matched WT males (n=9 per treatment) and females (n=10 per treatment) were included in the gene expression analyses.

Immune and stress reactivity experiments. Nine week old $TCR\beta^{-/-}\delta^{-/-}$ mice and C57Bl/6 wild type control mice were obtained from the Central Animal Facility and Charles River respectively. For the immune challenge experiment, $TCR\beta^{-/-}\delta^{-/-}$ males (12 Saline (SAL), 13 lipopolysaccharide (LPS)) and females (14 SAL, 13 LPS) as well as WT males (12 SAL, 12 LPS) and females (14 SAL, 14 LPS) were used.

2.1. Behavioural Phenotyping of Immune Deficient Mice.

The timeline for behaviour tests in both $TCR\beta^{-/-}\delta^{-/-}$ and μMT mice is shown in Figure 1. $TCR\beta^{-/-}\delta^{-/-}$ and age- and sex-matched C57Bl/6 mice arrived at 9 weeks of age. Mice were handled for 2 min a day, 3 days a week beginning on week 14. Behaviour tests began at 16 weeks of age. All tests were staggered one week to avoid interactions between tests. Tests included: open field, light/dark test, elevated plus maze, auditory fear conditioning, and the Morris Water Maze. The μMT mice arrived at 8 weeks of age were handled for 2 min per day, 3 days per week beginning at week 9. Open field, light/dark test, elevated plus maze, and auditory fear conditioning were conducted in one week intervals beginning at 10 weeks of age.

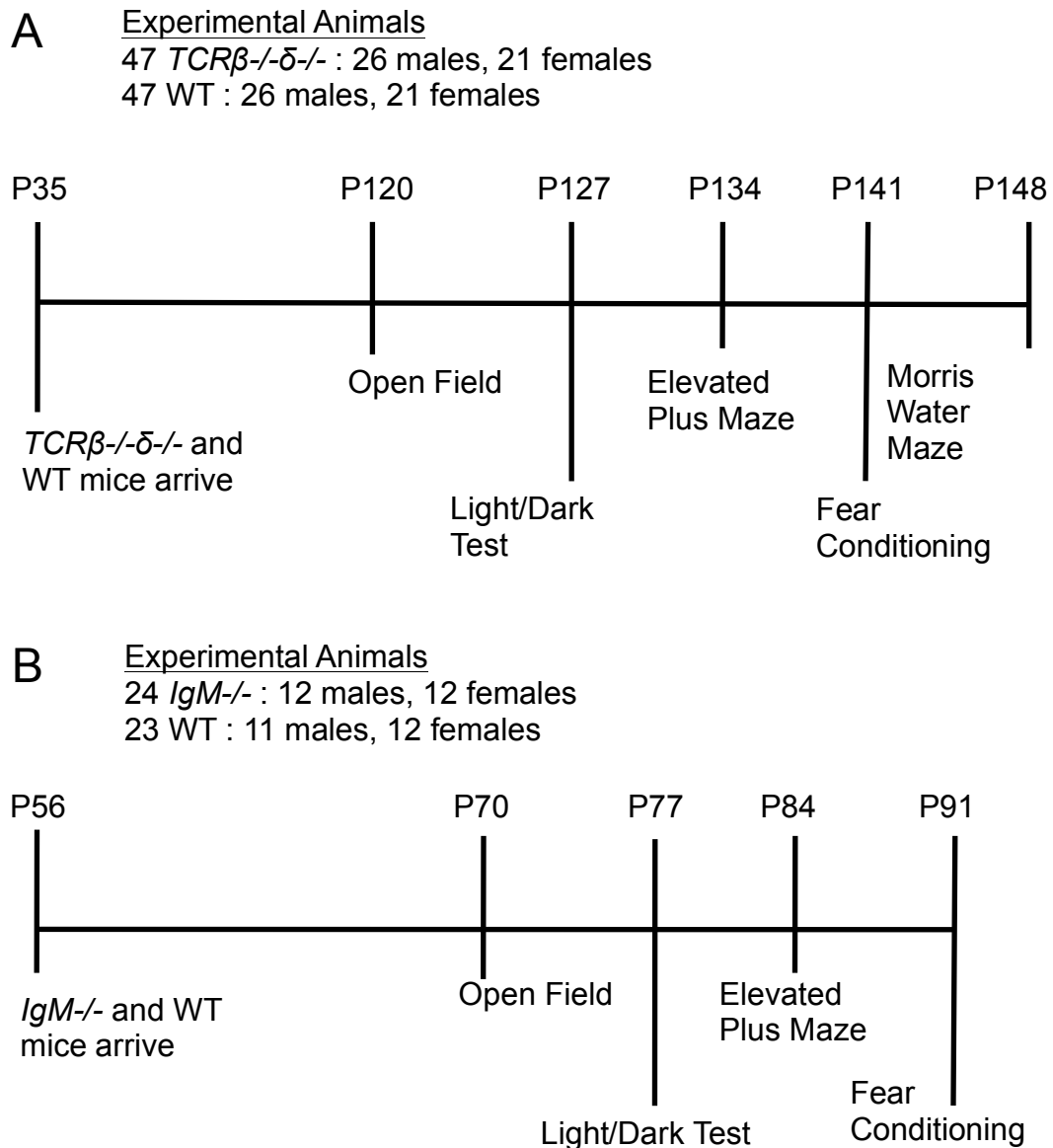


Figure 1. Experimental Design. In the first behaviour study (A), $TCR\beta^{-/-}\delta^{-/-}$ and WT mice were included. In the second study (B), $IgM^{-/-}$ and WT mice were included. In both cohorts, open field, light/dark, elevated plus maze and fear conditioning tests were conducted with a one week interval between all tests. Additionally, the Morris Water Maze was conducted on the first cohort. Postnatal age (P) is shown across the top of each panel.

Handling & Habituation. Beginning one week prior to testing, mice were handled for 2 min, 3 times per week. Once per week the mice were weighed and moved from the housing room to the behaviour suite for handling and habituation, and then returned to the housing room.

Open Field. Behaviour in the open field was measured using the Kinderscientific Smart Cage Rack System consisting of a 24 cm wide x 45 cm long x 24 cm high cage rack system, with 22 infrared beams (7 X & 15 Y) and a rearing option (22 additional beams). The zone map is presented in Figure 2A. Mice were transported to a non-colony room for behavioural testing and habituated to the room for 1 h prior to the start of the test. Mice were placed in the Plexiglas cage (6 cages allow 6 mice to be tested at one time) and left to explore for 60 min. Behaviours were recorded with a computer, using Kinderscientific Motor Monitor software. Following testing animals were immediately returned to their home cage and returned to the housing room.

Light/Dark. The light/dark test was administered using the Kinderscientific Smart Cage Rack System. For this test a black box insert was placed at one end of the Cage Rack System (zone map in Figure 2B). This Plexiglas insert contained an opening for the mouse to move from the dark chamber to the light chamber. Mice were transported to the non-colony room for testing and habituated for 30 min. Mice were placed in the light side of the chamber and left to explore the

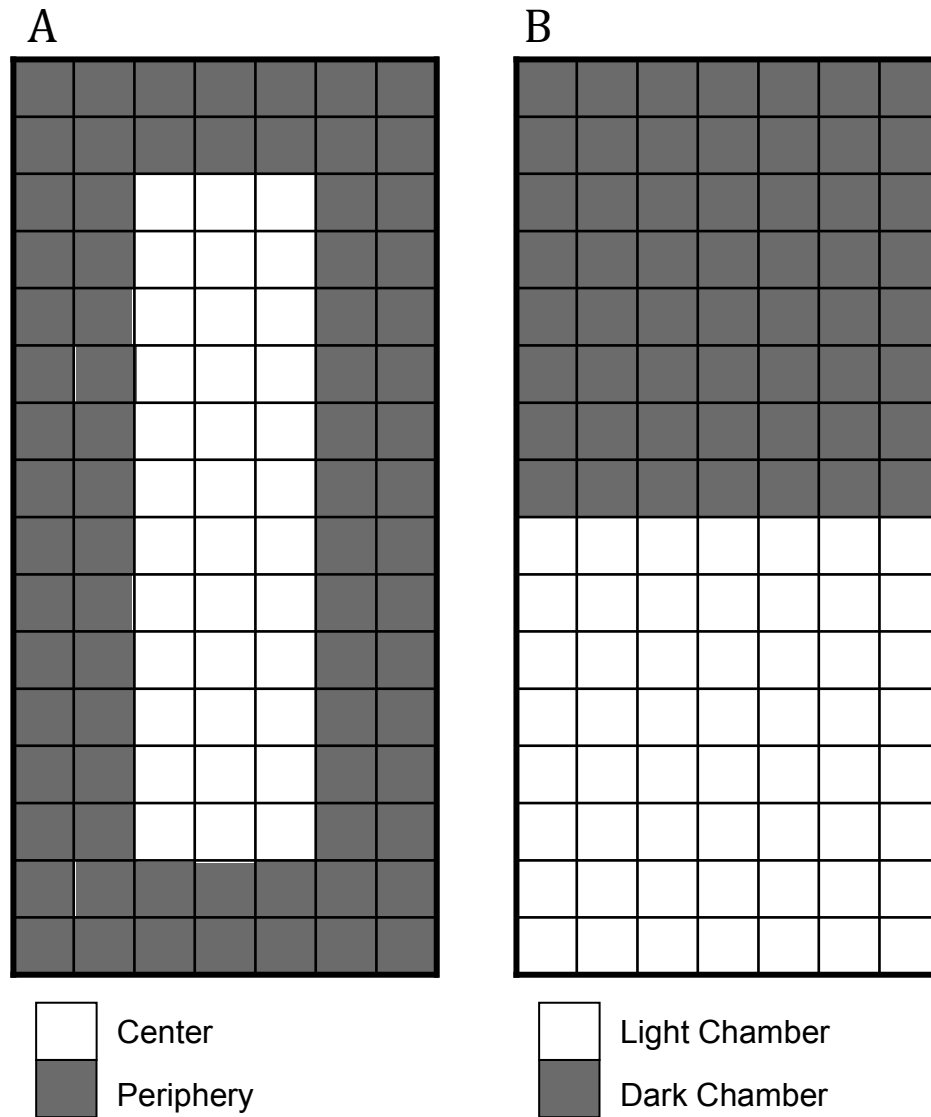


Figure 2. *Zone Maps for the Open Field (A) and Light/Dark Test (B).* Each test uses the Kinderscientific Smart Cage Rack System (dimensions: 24 cm wide x 46 cm long). Horizontal activity was recorded with 15 lengthwise and 7 widthwise infrared beams.

apparatus for 10 min. Behaviour was recorded using Kinderscientific Motor Monitor software. Following testing mice were immediately returned to their home cage and returned to the housing room.

Elevated Plus Maze (EPM). This device is a '+' shaped maze, elevated 66 cm above the ground with four perpendicular arms (35.5 cm long, 5 cm wide); two opposing arms are surrounded by 15.2 cm high dark walls (closed arms), while the two open arms are devoid of walls. Mice were transported to the non-colony room and habituated for 30 min. Each mouse was placed in the center of the maze (5 cm × 5 cm area) and its behaviour was recorded for 5 min. Movement throughout the maze was recorded using Kinderscientific Motor Monitor software and analyzed with a modified zone map previously validated in our laboratory (Figure 3B; (Sidor et al., 2010a). Following testing, mice were immediately returned to their home cage and returned to the housing room.

Auditory Fear Conditioning. Day 1. Mice were transported from the colony room to the behaviour suite and allowed to habituate for 30 min prior to testing. Mice were placed into Plexiglas chambers and allowed to explore for 5 min. After 135 s, a 65 dB tone was given for 30 s accompanied by a foot shock for the last 2 s of the tone.

Day 2. Cued learning was tested 24 h later. Mice were transported to the non-colony room 30 min prior to testing. Each mouse was placed into a modified test

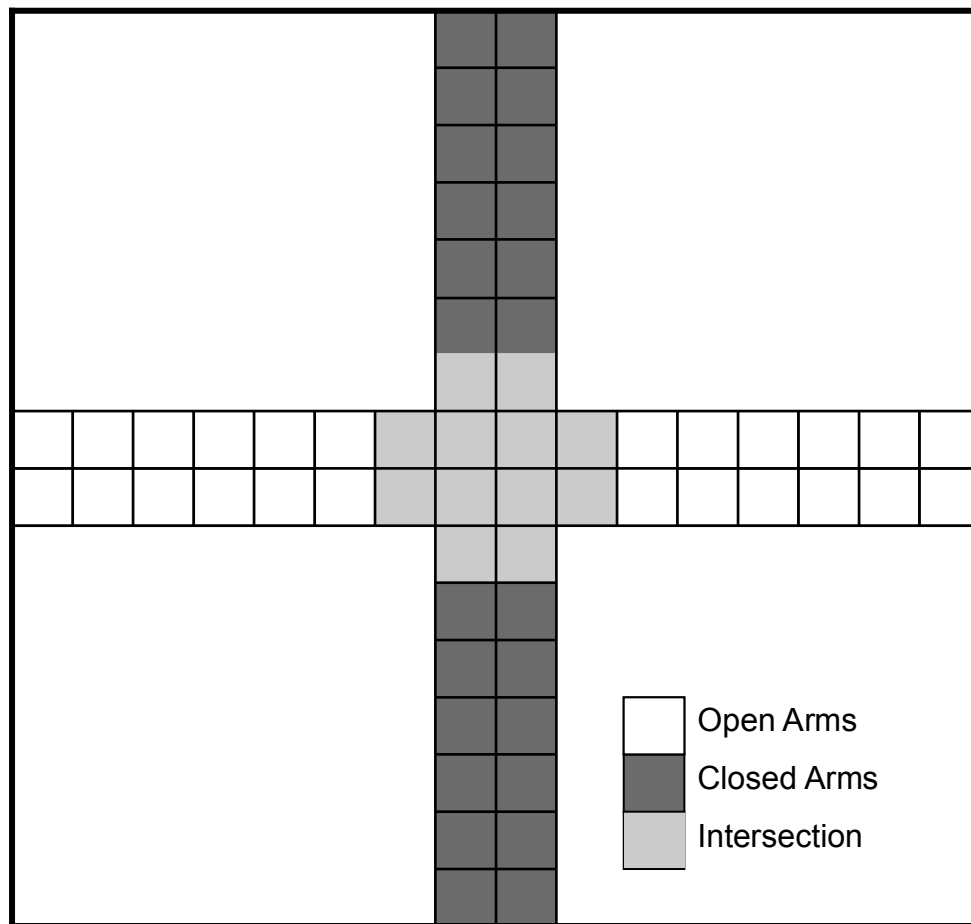


Figure 3. *Zone Map for the Elevated Plus Maze.* This “+” shaped device consists of 2 closed arms (35 cm long, surrounded by 15 cm high black Plexiglas walls) and, perpendicular to these, 2 open arms (35 cm long, no walls). Infrared beams connected to a computer record movements in each zone depicted above.

apparatus, the wire grid was covered with a black Plexiglas sheet and a black square insert was placed inside the chamber to provide a novel context compared to the conditioning trial on day 1. Behaviour was recorded for 5 min, and mice were presented with a 30 s tone at 135 s without the foot shock. Mice were returned to the home cage and returned to the housing room for one hour. One hour later, contextual learning was tested by returning each mouse to the same contextual apparatus as day 1 and freezing behavior was recorded for 5 min using Kinderscientific Motor Monitor software.

Morris Water Maze. The Morris Maze consists of a circular pool, 175 cm in diameter, with a submerged platform in one quadrant. Spatial clues included differing walls in the behavioural room as well as 4 different black shapes that were put on the sides of the pool. A platform was submerged in one quadrant of the pool. Mice were transported to the behaviour suite 30 min prior to testing for habituation. On day 1 only, a visible pylon was placed on the platform. Each mouse underwent 4 trials in the pool per day, each one starting in a different quadrant of the pool (north-N, south-S, east-E, west-W). Mice were placed in the pool and behavior was recorded using live-tracking program in Ethovision. Each trial was stopped when the mouse climbed onto the platform. The maximum time allowed to search for the platform was 2 min. After 2 min, if the mouse did not find the platform, the trial was stopped and the mouse was placed on the platform for 30 s and then returned to its home cage. In the acquisition trials on days 2 to

5, mice were again allowed up to 2 minutes to find the submerged platform. On day 6, a probe trial was conducted in which the platform was removed and mice explored the pool for 2 min. On day 7, a reversal trial was conducted in which the platform was returned to the pool, but in the opposite quadrant from the acquisition test and mice were allowed up to 2 min to find the platform. Time to reach the platform and time in the platform quadrant were recorded using the live tracking option of Ethovision.

Data Analysis of Behaviour. Outcome measures in the open field test included distance travelled, number of rearing events, and time spent in the center of the chamber. Distance traveled was analyzed over time with 5 min intervals using two-way repeat measures analysis of variance (ANOVA). Rearing and center time were collapsed across the 60 minutes of the test and were compared using two-way ANOVAs. All outcome measures from the light/dark test and EPM were analyzed using two-way ANOVAs. Outcome measures in the light/dark box include: time in the light chamber, transitions between chambers, and rearing throughout the test. The EPM was used to examine activity (entries into and distance traveled in the closed arms) and anxiety-like behaviour (time in open arms and open arm entries) and risk assessment behaviour (intersection time and head dips). The distance traveled by mice in the fear conditioning test on day 1 was analyzed with two-way repeat measures ANOVAs, as was percent immobility observed during the cued trial. The total percent immobility observed

in the contextual fear conditioning trial was analyzed with a two-way ANOVA. Two-way repeat measures ANOVAs were also used to analyze the latency to reach the platform in the Morris Water Maze during the cued, acquisition, and reversal trials. However, a separate two-way ANOVA was conducted on the data from the probe trial. Post-hoc tests were performed using Bonferroni and pair-wise t-tests as appropriate. All data are presented as the mean \pm the standard error (SE). Statistical significance was set at $p < 0.05$.

2.2. Expression of Neurotransmitter Receptor mRNA and Plasma Corticosterone Levels Following Repeated Restraint Stress.

Restraint Stress. At 8 weeks age, mice were divided into unstressed (CON) and restraint stressed (RS) groups that were balanced for genotype and sex. Mice were housed singly for one week prior to beginning stress procedures. For RS, mice were placed in wire mesh restrainers in their home cage for 2 hours per day in the morning for 9 days while CON animals were left unrestrained in their home cage but without food or water for the 2 hours. On the tenth day tissue and blood collection followed 1 hour RS.

Tissue Collection & Sectioning. Mice were killed by decapitation. Blood and brains were collected immediately. Blood was collected into tubes containing EDTA and processed to obtain plasma that was stored at -70°C . Brains were

collected and frozen in 2-methylbutane at -60°C and stored at -70° C until sectioning. Tissue was sectioned coronally at 12 µm at the levels of nucleus accumbens bed nucleus of the stria terminalis (BNST; Bregma 0.26), paraventricular nucleus of the hypothalamus (PVN with central amygdala (CeA); Bregma -0.58), and dorsal hippocampus (Hip; with lateral (LA) and basolateral amygdala (BLA); Bregma -1.70mm). (Paxinos G, 2001). Sectioned tissue was stored at -35°C.

Radioimmunoassay. Samples were centrifuged at 3000 RPM for 15 minutes at 4°C. Plasma was aliquoted, frozen on dry ice and stored at -70°C. Corticosterone (CORT) was measured in duplicate samples by using a standard radioimmunoassay kit from MP Biomedicals (Solon, ON).

In Situ Hybridization. Tissue sections were processed as previously reported (Foster et al., 2002; Kolozsi et al., 2009; Neufeld et al., 2011b). Ribrobes were generously provided by Dr. James Herman from the University of Kentucky (CRH; (Figueiredo et al., 2003), Dr. Ronald M. Evans from The Salk Institute for Biological Studies (MR; (Arriza et al., 1987), Dr. Keith R. Yamamoto from the University of California, San Francisco (GR; (Miesfeld et al., 1984), and Dr. Pat Levitt from Vanderbilt University (5-HT1A; (Bonnin et al., 2006). Anti-sense probes for NMDA subunits NR1, NR2A and NR2B (Neufeld et al., 2011b) and serotonergic receptors 5-HT2A and 5-HT2C (Sidor et al., 2010b) were generated

in our laboratory and transcribed from linearized plasmids with α -³⁵S-UTP (specific activity >1,000 Ci/mmol) and T7 polymerase, T3 polymerase, or SP6 polymerase (NR1). Tissue sections were hybridized overnight at 55°C in a hybridization mix containing 500,000 CPM/ section of riboprobe diluted in hybridization buffer. To reduce non-specific binding, sections were treated with RNase in RNase buffer for 30 m, slides were put through a series of 1 hour high stringency washes of 2 X SSC at 50°C, and then 0.2 X SSC at 55°C and 60°C. Slides were dehydrated with ethanol and air dried before being placed in cassettes for autoradiography.

Autoradiography. Slides and a ¹⁴C control slide with known radioactivity (American Radiochemicals; St. Louis, MO) were placed in X-ray cassettes and apposed to film for 20-120 hours. Expression of mRNA for each probe was quantified using NIH Image analysis software (<http://rst.info.nih.gov/nih-image>) on a Macintosh computer connected to a Qiacam digital camera (Quorum Technologies; Guelph, ON). The Rodbard curve was applied to the ¹⁴C and used to convert the light transmittance (optical density) to disintegrations per minute.

Data Analysis. Data collected from *in situ* hybridization and corticosterone analyses were analyzed using three-way ANOVAs with genotype, sex, and restraint stress treatment as factors. Independently, pairwise t-tests were

conducted to assess group differences. All data are presented as the mean \pm the standard error. Statistical significance was set at $*p < 0.05$.

2.3. Innate Immune Response in $TCR\beta^{-/-}\delta^{-/-}$ Mice.

Open Field Test. The open field test was conducted weekly for 3 weeks during the active period (dark cycle). Mice were first put into the open field at 12 weeks of age for 30 minutes to examine behaviour in the novel open field test. One week later, the animals were retested for 30 minutes to determine between test habituation.

Lipopolysaccharide (LPS) Injections. At week 14, mice were administered LPS (1 mg/kg; Sigma, Oakville, ON) in 5 μ l/g of 0.9% saline. Control animals were injected with an equivalent volume of saline (SAL) i.p. immediately prior to the 30 min open field test.

Tissue Collection. Two hours after the injection of LPS or saline, animals were killed and their blood was collected into tubes containing 50 μ L of 50 mM EDTA. Immediately, tubes were gently inverted several times and stored on ice. Samples were then centrifuged for 15 min and plasma was transferred to 1.5 mL tubes, frozen on dry ice. Samples were stored at -70°C until used for corticosterone analysis.

Corticosterone EIA. Corticosterone Enzyme Immunoassay from Enzo Life Sciences (Farmingdale, NY) was used to measure the plasma corticosterone (CORT) levels. Briefly, samples were prepared by adding 1 μ L of steroid displacement reagent and transferring 5 μ L of the sample into 2.45 mL assay buffer on the EIA plate. Then 50 μ L each of blue conjugate and yellow antibody were added into each well, the plate was sealed, and placed on a shaker for 2 hours at 500 rpm. Following 3 washes, 200 μ L of Para-nitrophenylphosphate (pNPP) substrate solution for colourimetric detection was added to each well and incubated at room temperature. Then 50 μ L of Stop solution was added to every well and the plate was read immediately to ascertain optical density. These values were compared to standard wells containing known dilutions to determine the ng/mL plasma CORT levels.

Data Analysis. The open field data collected from the novel and habituation trials were analyzed together in a two-way repeat measures ANOVA with Bonferroni corrected post-hoc tests. Additional two-way repeat measures ANOVAs were conducted to analyze the LPS trial. Plasma corticosterone was analyzed using a three-way ANOVA. Post-hoc tests were performed with Bonferroni corrections and pair-wise t-tests as appropriate. All data are presented as the mean \pm the SE. Statistical significance was set at $p < 0.05$.

CHAPTER 3. RESULTS

Experiment #1

3.1. Overview of Experiment 1

Researchers in psychiatry and behavioural neuroscience are increasingly recognizing the importance of the adaptive immune system in behaviour. Specifically, lymphocytes have been shown to influence anxiety-like behaviours in mice (Oliveira-Dos-Santos et al., 2000; Cushman et al., 2003). Genetic knockout of recombinaise activating gene 1 (*RAG1*) in mice results in a deficit of mature T and B lymphocytes; *RAG1*^{-/-} mice show reduced anxiety-like behaviour in the elevated plus maze (EPM) (Cushman et al., 2003). Our laboratory has shown that germ-free mice, which have reduced mature T and B lymphocytes, also exhibit reduced anxiety-like behaviours in the EPM compared to conventionally housed mice (Neufeld et al., 2011b). Heijtz *et al.* have also reported reduced anxiety-like behaviour in germ-free mice (Heijtz et al., 2011a). Given the known immune deficits in *RAG1*^{-/-} and germ-free mice (Cebra, 1999; Macpherson and Uhr, 2004), *we hypothesized that loss of T lymphocytes will result in changes to anxiety-like and fear-related behaviours.* To test this, we conducted open field, light/dark, elevated plus maze (EPM), auditory fear conditioning, and Morris Water Maze (MWM) behavioural tests in mice that lack T cell receptor (TCR) β and δ chains and therefore lack functional or mature T cells

(Mombaerts et al., 1994; Macpherson et al., 2000). Additionally, we conducted open field, light/dark test, EPM, and the auditory fear conditioning tests in mice lacking the IgM μ chain ($\mu MT^{-/-}$), leading to a functional loss of B cells, to independently differentiate the effects of T cell deficiency and B cell deficiency. Also, many previous studies on adaptive immunity and behaviour were conducted only in males (Cushman, 2003) or only in females (Neufeld, 2010). Due to known sex differences in these tests (Palanza, 2001; Voikar et al., 2001), both sexes were included in all studies and multiple tests of anxiety were employed.

3.2. Results of Experiment 1

3.2.1. Behaviour of $TCR\beta^{-/-}\delta^{-/-}$ Mice

Open Field. Outcome measures in the open field were generated by automated software (Motor Monitor, Kinder Scientific) and included total distance traveled and rearing as indices of activity, habituation, and exploratory behaviour (Yaguchi et al.; Choleris et al., 2001; Brenes et al., 2009). Time spent in the center of the chamber was used as an index of anxiety-like behaviour (Brenes et al., 2009). WT and $TCR\beta^{-/-}\delta^{-/-}$ mice showed similar within test habituation of activity revealed as a reduction in distance travelled over the 60 min period. With respect to distance travelled by WT and $TCR\beta^{-/-}\delta^{-/-}$ mice, there was a main effect of genotype (Fig. 4A; $F_{(1,576)}=36.1$, $p<0.0001$) and a main effect of time ($F_{(11,576)}=40.1$, $p<0.0001$), where $TCR\beta^{-/-}\delta^{-/-}$ males had significantly higher distance travelled in the last 20 minutes of the test compared to WT males (Bonferroni post-hoc test, $p<0.05$); however, there was no difference observed between $TCR\beta^{-/-}\delta^{-/-}$ and WT females (Fig. 4B). With regard to total distance traveled during the 60 min open field test, an interaction effect between sex and genotype was observed ($F_{(1,90)}=7.03$, $p=0.0095$). Bonferroni post-hoc testing revealed that this difference was between WT mice with females (2977 ± 127.7 cm) traveling a significantly greater distance than males (2560 ± 92.2 cm). This is in accordance with previous literature demonstrating increased locomotor activity in females in the open field (Palanza, 2001; Voikar et al., 2001). Interestingly, this sex difference was not observed in $TCR\beta^{-/-}\delta^{-/-}$ mice (females, 2653 ± 146.1

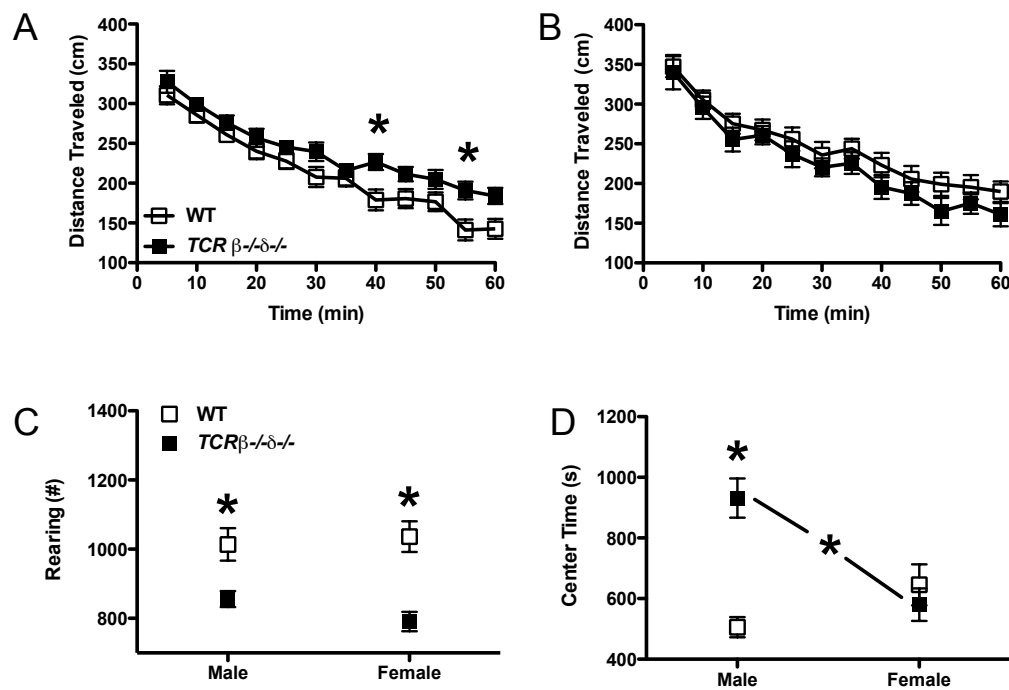


Figure 4. $TCR \beta^{-/-}\delta^{-/-}$ and WT mice were tested in the open field test for activity, exploration and anxiety-like behaviour. $TCR \beta^{-/-}\delta^{-/-}$ males (A) but not females (B) showed a significant increase in locomotor activity shown here as distance traveled compared to WT mice. Reduced total rearing (over the 60 min) was observed in both male and female $TCR \beta^{-/-}\delta^{-/-}$ (C) mice compared to WT mice. $TCR \beta^{-/-}\delta^{-/-}$ male mice spent significantly more time in the center of the open field compared to WT male mice (D). In contrast, there were no differences in time spent in the center observed between $TCR \beta^{-/-}\delta^{-/-}$ females and WT mice. * $p < 0.05$.

cm; males, 2829 ± 86.0 cm) indicating a loss of behavioural sex differences. A main effect of genotype was found with regard to total rearing in the open field (Figure 4C. $F_{(1,90)}=29.08$, $p=0.0001$). In addition, a main effect of genotype (Figure 4D. $F_{(1,576)}=10.29$, $p=0.0019$) as well as a genotype by sex interaction effect ($F_{(1,576)}=19.17$, $p<0.0001$) were observed for time spent in the center of the test chamber. Bonferroni post-hoc testing revealed that $TCR\beta^{-/-}\delta^{-/-}$ males spent more time in the center of the test chamber compared to WT males ($p<0.05$), an effect that was not observed in females ($p>0.05$). Interestingly, while there were no differences between sexes in total time spent in the center of the open field in the WT mice, an interaction effect between genotype and sex was observed ($F_{(1,90)}=19.17$, $p<0.0001$). Bonferroni post-hoc testing revealed a significant increase in time spent in the center of the chamber by $TCR\beta^{-/-}\delta^{-/-}$ males compared to $TCR\beta^{-/-}\delta^{-/-}$ females (* $p<0.05$).

Light/Dark Box. In the light/dark box, outcome measures included time in the light chamber as an index of anxiety, and transitions and rearing as exploratory measures (Bourin and Hascoët, 2003). A main effect of genotype was observed for time spent in the light chamber of the light/dark test (Figure 5A. $F_{(1,90)}=7.7$, $p=0.0067$). Separate analyses of males and females using the student's t-test statistic demonstrate that females ($t=2.59$, $df=40$, $p=0.014$) but not males ($t=1.43$, $df=50$, $p=0.16$) spent more time in the light chamber. There was also a significant effect of genotype with regard to rearing behaviour (Figure 5B. $F_{(1,89)}=55.1$,

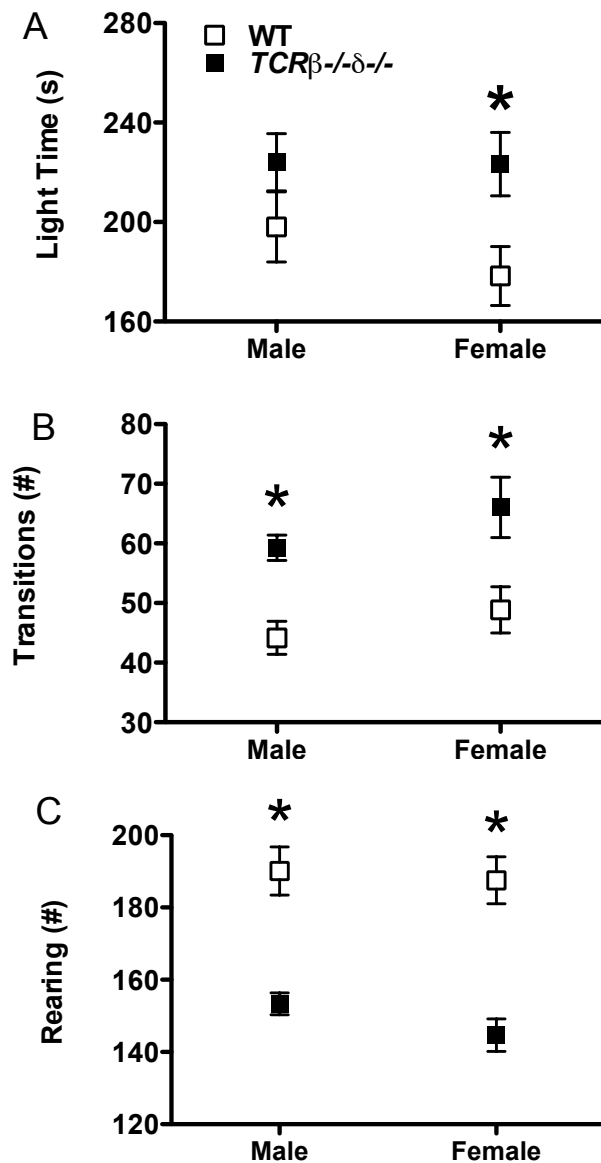


Figure 5. Anxiety-like behaviour and exploration were measured in the light/dark test. Compared to WT controls, $TCR\beta^{-/-}\delta^{-/-}$ females, but not males, spent significantly more time in the light chamber (A). Additionally, both male and female $TCR\beta^{-/-}\delta^{-/-}$ mice exhibited less rearing behaviour (B) but transitioned between the light and dark chambers more than WT mice (C). * $p < 0.05$

$p < 0.0001$) in which rearing behaviour was reduced in $TCR\beta^{-/-}\delta^{-/-}$ mice. A main effect of genotype was also observed in transitions (Figure 5C. $F_{(1,90)} = 21.8$, $p < 0.0001$), however in contrast to the rearing data, transitions between the chambers, also a measure of exploratory behaviour, were elevated in the $TCR\beta^{-/-}\delta^{-/-}$ mice relative to their WT controls.

Elevated Plus Maze. Anxiety-like behaviour was assessed in the EPM by examining the amount of time spent in the open arms as well as entries into the open arms. A main effect of genotype was observed in which $TCR\beta^{-/-}\delta^{-/-}$ mice displayed a significant increase in the amount of time spent in the open arm compared to WT mice (Fig 6A; $F_{(1,90)} = 27.33$, $p < 0.0001$). With regard to entries into the open arm (Figure 6B), both a main effect of genotype ($F_{(1,90)} = 16.79$, $p < 0.0001$) and an interaction effect between sex and genotype ($F_{(1,90)} = 6.7$, $p = 0.011$) were observed. Bonferroni post-hoc testing revealed an increased number of open arm entries in $TCR\beta^{-/-}\delta^{-/-}$ female mice compared to WT females (* $p < 0.05$). Head dips were used as an indicator of risk assessment behaviour (Rodgers and Cole, 1993; Rodgers and Dalvi, 1997). There was a main effect of genotype on head dipping behaviour (Figure 6C. $F_{(1,90)} = 31.54$, $p < 0.0001$) and an interaction effect between genotype and sex ($F_{(1,90)} = 6.7$, $p = 0.011$). Bonferroni post-hoc testing revealed that increased head dipping behaviour was in $TCR\beta^{-/-}\delta^{-/-}$ males (* $p < 0.05$), not $TCR\beta^{-/-}\delta^{-/-}$ females. A genotype effect (Figure 6D. $F_{(1,90)} = 10.1$, $p = 0.002$) as well as an interaction between genotype and sex

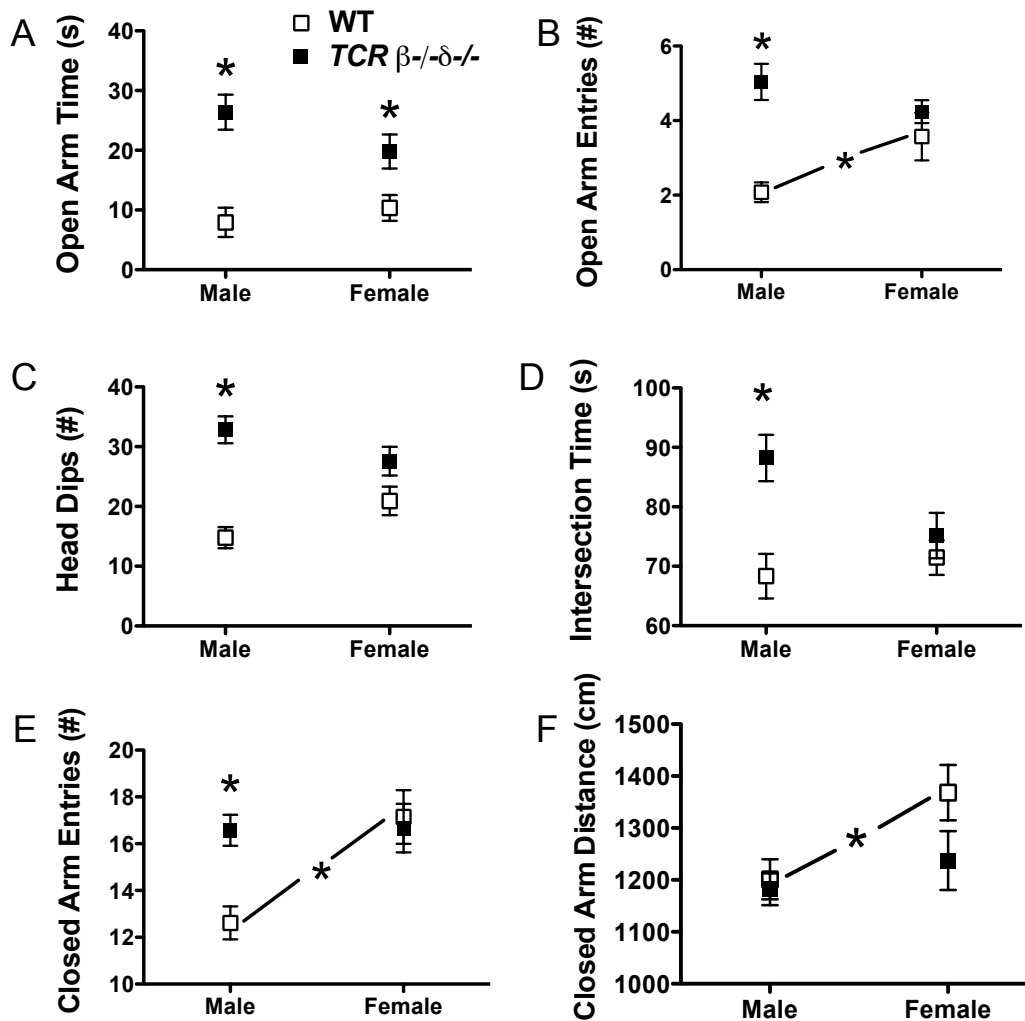


Figure 6. The elevated plus maze (EPM) was used to assess anxiety-like behaviour, risk assessment, and activity. *TCR β-/-δ-/-* males and females spent increased time in the open arms of the EPM (A) indicative of reduced anxiety-like behaviour. Male *TCR β-/-δ-/-* mice entered the open arms more (B) and had increased head dips (C) compared to male WT mice. *TCR β-/-δ-/-* males spent significantly less time in the closed arms than WT males and *TCR β-/-δ-/-* females (D). In the closed arms, WT but not *TCR β-/-δ-/-* males had fewer entries (E) and traveled less (F) than WT females. The sexually dimorphic behaviour seen in WT mice was absent in the *TCR β-/-δ-/-* mice except for closed arm time. *p<0.05

($F_{(1,90)}=4.7$, $p=0.032$) were found for the time spent in the center of the EPM with $TCR\beta^{-/-}\delta^{-/-}$ males spending more time in the center than WT males and $TCR\beta^{-/-}\delta^{-/-}$ females ($*p<0.05$). Activity in the EPM was measured as number of entries into the closed arms (Rodgers and Dalvi, 1997) as well as distance travelled in the closed arms. Similar to the findings of increased activity in WT females compared to WT males observed in the open field, WT females exhibited increased activity in the EPM. With regard to entries into the closed arms (Fig. 6E) a main effect of sex ($F_{(1,90)}=6.9$, $p=0.010$) and an interaction effect between genotype and sex ($F_{(1,90)}=6.36$, $p=0.013$) were found. Bonferroni post-hoc testing revealed that there were more entries into the closed arms in WT females compared to WT males ($*p<0.05$). Additionally, $TCR\beta^{-/-}\delta^{-/-}$ males entered the closed arm more often than WT males (Bonferroni post-hoc test, $*p<0.05$). Interestingly, $TCR\beta^{-/-}\delta^{-/-}$ mice did not display the sex differences observed in WT mice with regard to closed arm entries. Finally, a main effect of sex was also found in distance travelled in the closed arms (Fig. 6F. $F_{(1,90)}=6.3$, $p=0.014$).

Fear Conditioning. Activity in the fear conditioning apparatus during the conditioning trial on day 1 is shown in Fig. 7A for male mice and Fig. 7B for female mice. All mice responded to the presentation of the tone at 135 s with increased activity and responded to the footshock by freezing and reduced activity.

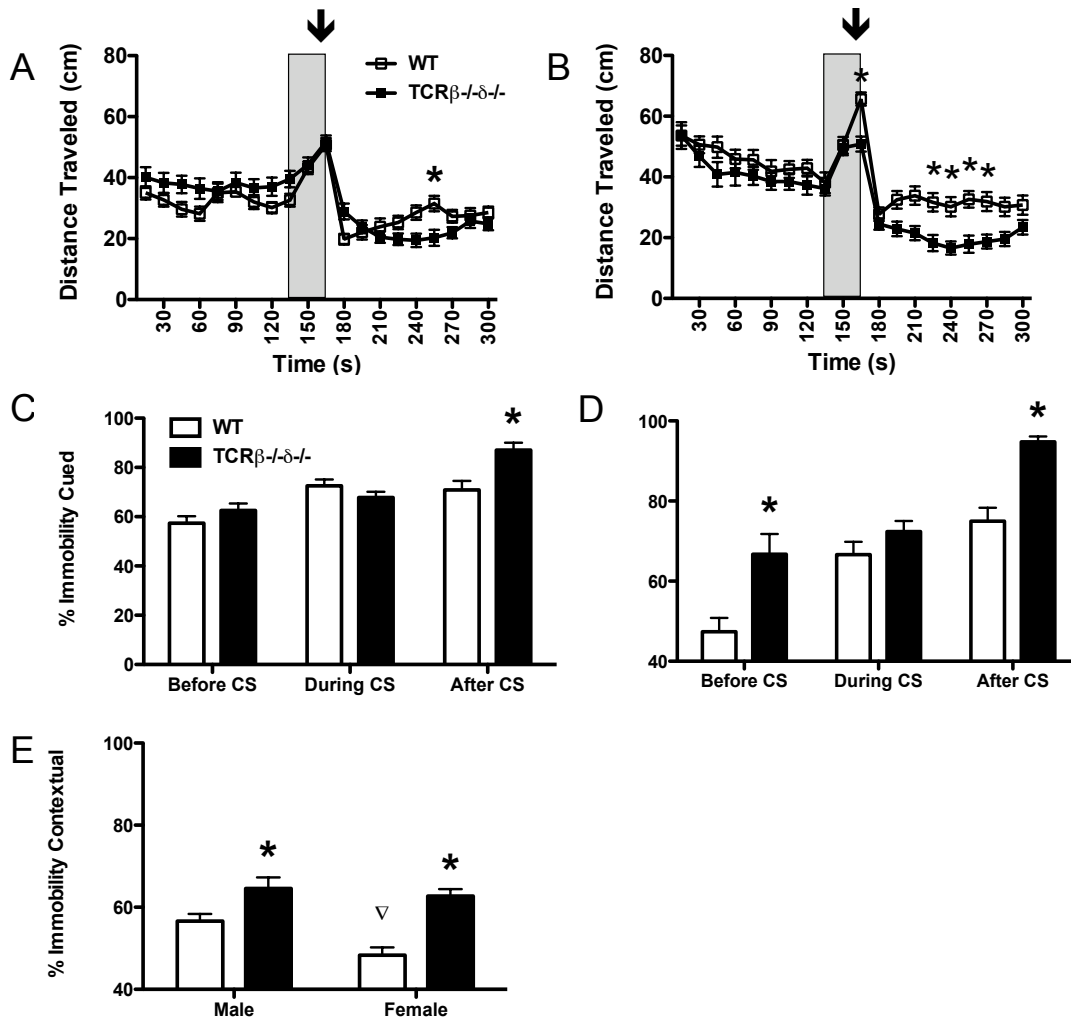


Figure 7. Fear learning was assessed with the auditory fear conditioning test. The conditioning trial on Day 1 is shown for male (A) and female (B) mice as distance traveled during the test. A 30 second tone (gray rectangle) was given and a 2 second foot shock was delivered during the last 2 seconds of the tone (↓). Cued fear conditioning, tested 24 h later, is shown as % immobility, for males and females separately in panels C and D, respectively. *TCR* $\beta^{-/-}\delta^{-/-}$ males and females had increased immobility following the CS. Panel E shows increased immobility in *TCR* $\beta^{-/-}\delta^{-/-}$ mice in the contextual fear learning trial. Additionally, WT males had significantly higher immobility than WT females. * $p < 0.05$ between *TCR* $\beta^{-/-}\delta^{-/-}$ and WT; $\nabla p < 0.05$ between males and females

On day 2 of fear conditioning, learning was assessed by freezing behaviour (percent time immobile) for both cued and contextual fear learning paradigms (Paylor et al., 1994). Figure 7C depicts cued learning in male mice and the analysis revealed a main effect of genotype ($F_{(1,100)}=4.2$, $p=0.046$) and an interaction effect between genotype and interval ($F_{(2,100)}=7.03$, $p=.0014$). Bonferroni post-hoc comparisons revealed that $TCR\beta^{-/-}\delta^{-/-}$ male mice had increased freezing in the 30 s interval following the tone compared to WT male mice (* $p<0.05$). A main effect of genotype (Figure 7D; $F_{(1,80)}=21.07$, $p<0.0001$) and an interaction effect between genotype and interval ($F_{(2,80)}=4.3$, $p=0.017$) were also found in the comparison of $TCR\beta^{-/-}\delta^{-/-}$ and WT females. However, in this case, Bonferroni post-hoc testing revealed increased freezing behaviour in $TCR\beta^{-/-}\delta^{-/-}$ female mice in the 30 s intervals both before and after the CS compared to WT female mice (* $p<0.05$).

In the contextual learning test a main effect of genotype was observed (Fig. 7E; $F_{(1,90)}=41.41$, $p<0.0001$) in which $TCR\beta^{-/-}\delta^{-/-}$ mice exhibited increased freezing behaviour compared to WT. In addition to the effect of genotype, a main effect of sex was found ($F_{(1,90)}=5.98$, $p=0.016$) in which males exhibited increased freezing behaviour compared to females.

Morris Water Maze. Spatial learning was measured with the Morris water maze (MWM). Figures 8A and 8B depict the performance of male and female mice, respectively, within the MWM during the cued, acquisition, and reversal trials.

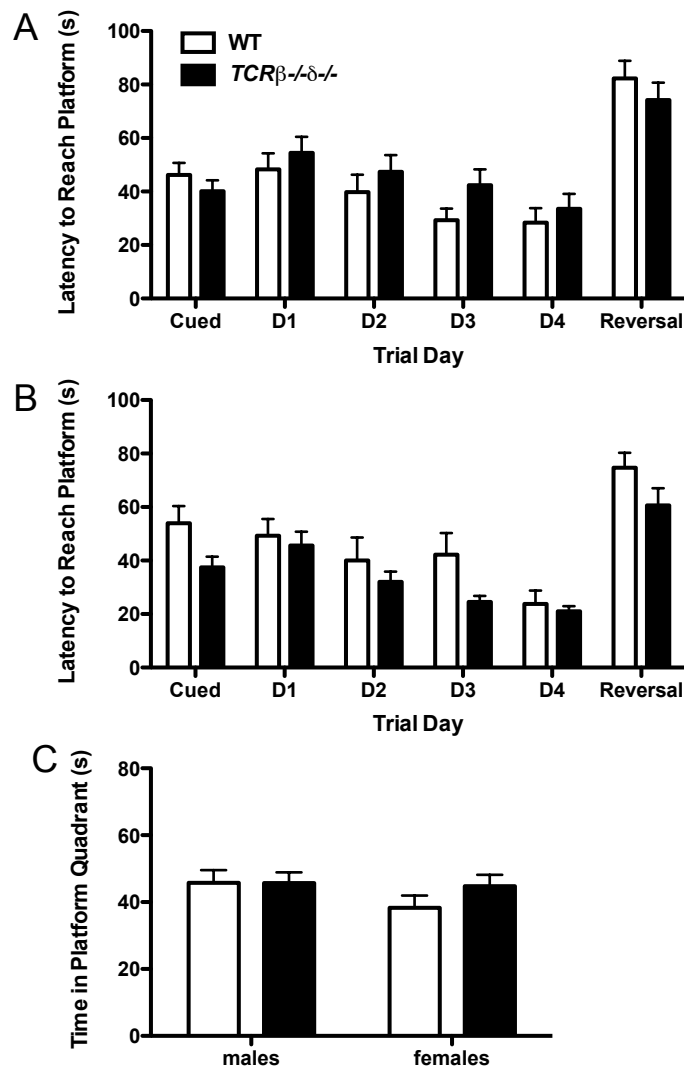


Figure 8. The Morris Water Maze was used to analyze spatial learning. *TCR β*^{-/-}*δ*^{-/-} males (A) and females (B) were recorded for latency to reach the submerged platform and values were averaged across 4 trials per day. Values are shown for a cued trial, in which the platform was visibly marked, acquisition days (D) 1-4, and a reversal trial in which the platform was moved to the opposite side of the maze. Additionally, a probe trial (C) was conducted in which mice were allowed to explore the pool for 2 minutes. Time spent in the platform quadrant was recorded. No differences were observed between *TCR β*^{-/-}*δ*^{-/-} and WT mice in any of the trials. *p*>0.05

There were no main effects of genotype or sex on latency to reach the platform for any of these groups. Additionally, analysis was conducted on a probe trial, which is the most salient trial for spatial learning in this test, and there were no significant effects of genotype or sex (Fig. 8C).

3.2.2. Behaviour of μMT Mice.

Open Field. The $\mu MT^{-/-}$ mice did not show significant differences in open field behaviours compared to WT mice including total distance travelled (Figure. 9A), rearing events (Figure. 9B), or time spent in the center (Figure. 9C); however, WT females traveled a greater total distance than WT males ($t=2.59$, $df=21$, $*p=0.017$).

Light/Dark Test. There was no main effect of genotype between $\mu MT^{-/-}$ and WT males (Figure. 10A) or females (Figure. 10B) for the measure of time spent in the light chamber. Similarly, there were no effects of genotype for rearing behaviour in either males (Figure. 10C) or females (Figure. 10D). Finally, analysis of transition events also did not reveal any effects of genotype for males (Figure. 10E) or females (Figure. 10F).

EPM. There were no differences between $\mu MT^{-/-}$ males or females compared to sex matched WT controls in the amount of time spent in the open arms (Figure.

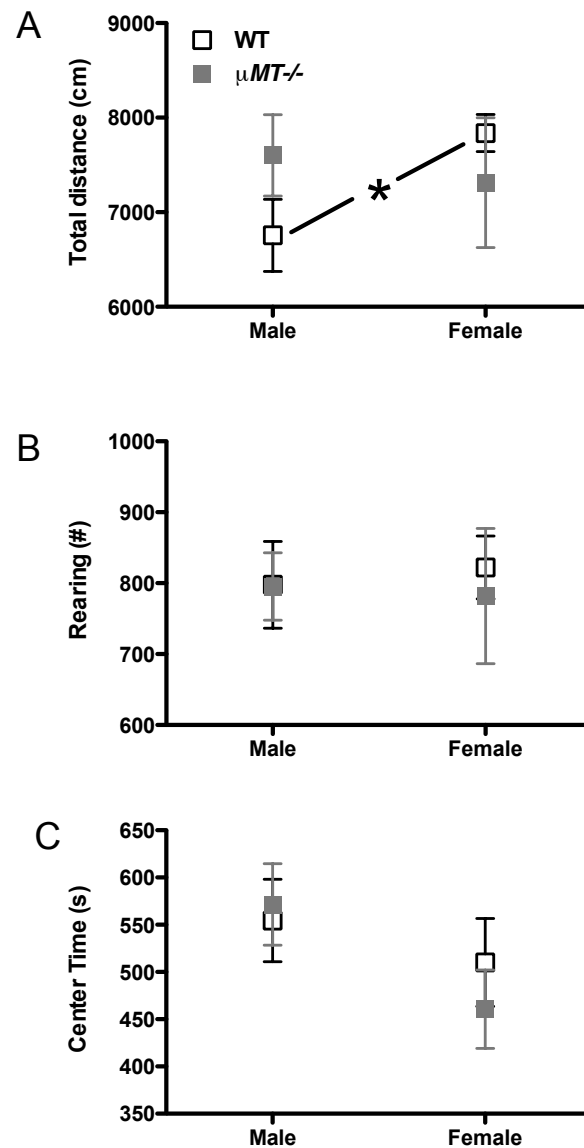


Figure 9. In the open field test, no differences were observed between $\mu MT^{-/-}$ and WT control mice in total distance travelled (A), rearing behaviour (B), or time spent in the center of the chamber (C). There was a sex effect in the WT mice in which WT females traveled a greater distance than WT males (A). * $p < 0.05$

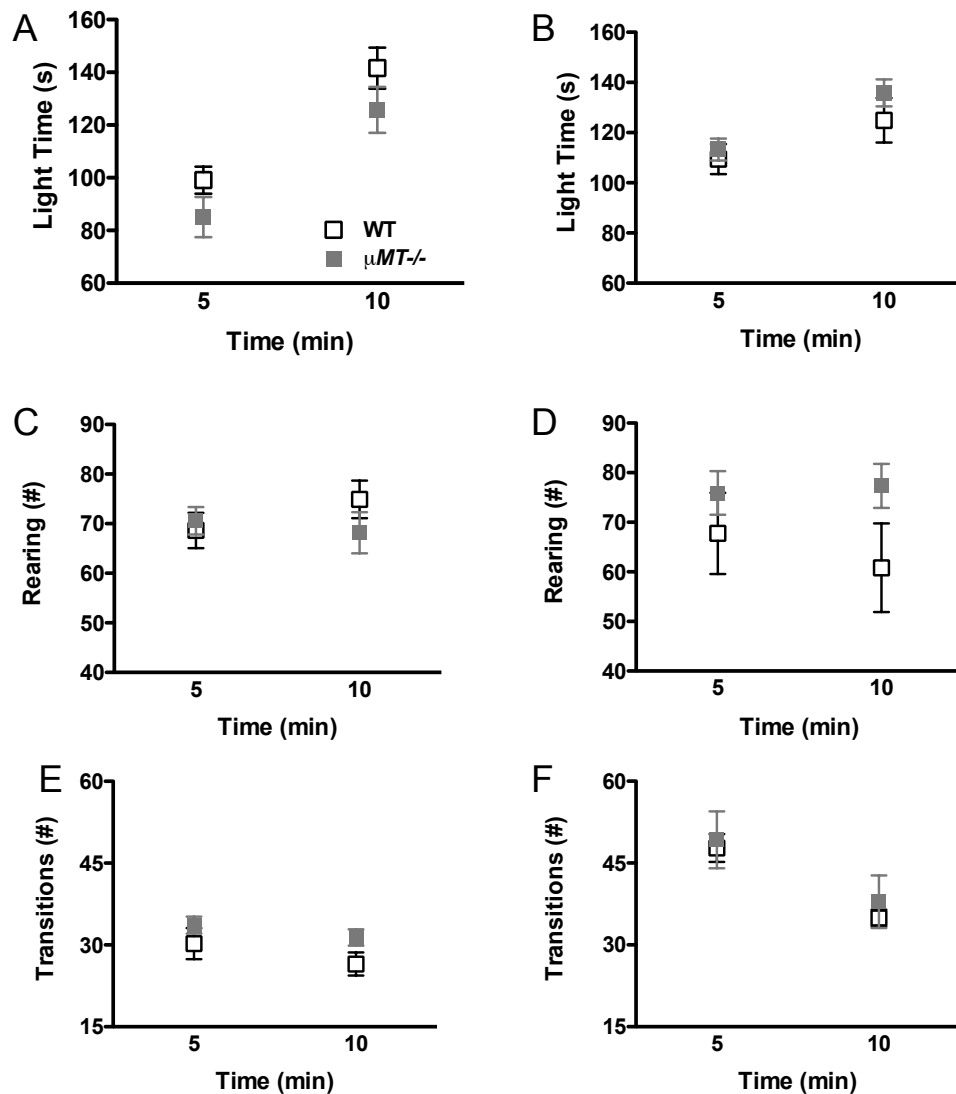


Figure 10. In the light/dark test there were no main effects of genotype on time spent in the light chamber in males (A) or females (B). No differences were found between $\mu MT^{-/-}$ and WT males in rearing behaviour (C); however, $\mu MT^{-/-}$ females exhibited more rearing behaviour than WT females during the last 5 minutes of the test (D; ■ $p < 0.05$). No main effects of genotype or sex were observed for transitions between the light and dark chambers in males (E) or females (F).

11A), or in the number of entries into the open arms (Figure 11B). Additionally, no differences were observed in the amount of head dips over the side of the EPM (Figure. 11C) or time spent in the center intersection (Figure. 11D). There was a main effect of genotype for both the distance traveled within the closed arms (Figure. 11E; $F_{(1,43)}=4.2$), and the entries into (Figure. 11F; $F_{(1,43)}=4.2$, $p=0.047$), in which $\mu MT^{-/-}$ mice entered the closed arms fewer times and traveled less distance than WT mice. An independent t-test was conducted for closed arm distance for females and statistical significance was not observed ($t=2.07$, $df=22$, $p=0.0502$). Similarly, an independent t-test was conducted for closed arm entries for males and again statistical significance was not reached ($t=2.06$, $df=21$, $p=0.052$).

Fear Conditioning. On day 1 of this task the $\mu MT^{-/-}$ male mice exhibited fewer movements than the WT mice during the first 30 seconds (Bonferroni post-hoc test, $*p<0.05$) but otherwise behaved the same during the cued stimulus (CS) and following the footshock (Figure 12A. $F_{(1,171)}=23.35$, $p=0.0009$). A main effect of genotype on day 1 was also observed in the females (Figure 12B. $F_{(1,171)}=190$, $p=0.0425$) but Bonferroni post-hoc testing again revealed that the difference in activity was only observable prior to the CS ($*p<0.05$).

On day 2, in the cued learning tests, no main effects of genotype or sex for freezing behaviour were observed in the 30 s intervals prior to, during, and after the CS in either males (Figure. 12C) or females (Figure. 12D). Furthermore, there

were no effects of genotype or sex (Figure. 12E) with respect to performance in the contextual fear learning test.

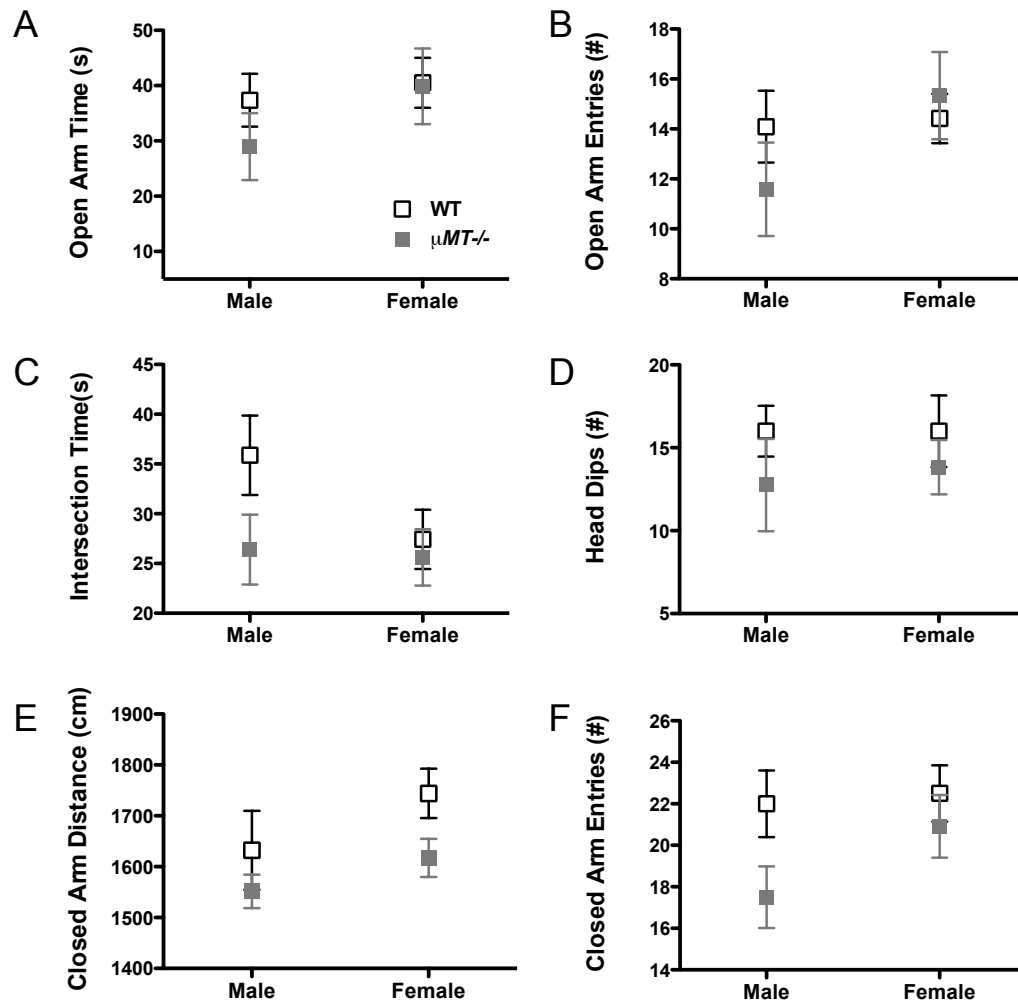


Figure 11. In the EPM, there were no observable differences between $\mu MT^{-/-}$ and WT mice with regards to time spent (A) or entries into (B) the open arms, head dips over the edge of the EPM (C), time spent in the center, or time spent in the intersection (D). There was a main effect of genotype on distance traveled in the closed arms (E) and entries into the closed arms (F). However, independent pairwise t-tests did not reveal differences between $\mu MT^{-/-}$ and WT mice.

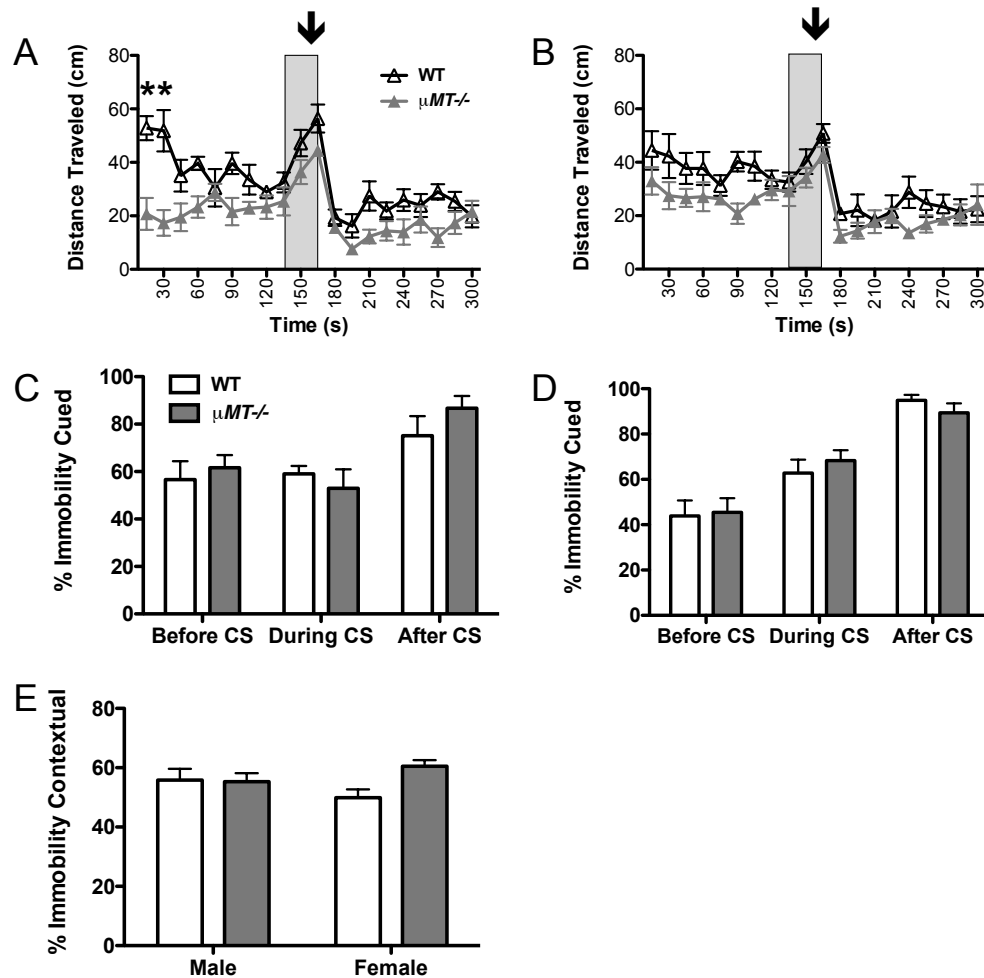


Figure 12. Fear learning was assessed with the auditory fear conditioning test. The conditioning trial on Day 1 is shown for male (A) and female (B) mice as distance traveled during the test. A 30 second tone (gray rectangle) was given and a 2 second foot shock was delivered during the last 2 seconds of the tone (↓). Cued fear conditioning, tested 24 h later, is shown as % immobility, for males and females separately in panels C and D, respectively. Male (A) and female (B) $\mu MT^{-/-}$ mice show normal responses to the CS and footshock. There were no effects of genotype observed in the cued (C, males; D females) or contextual learning tests (E).

Summary of Experiment #1

The results presented here suggest that T cells play a pivotal role in the development of anxiety-like behaviours. Mice deficient of T cells, $\text{TCR}\beta^{-/-}\delta^{-/-}$ mice, demonstrated reduced anxiety-like behaviour in the open field (increased time in the center), light/dark test (increased time in the light chamber), and the EPM (increased entries into and time spent in the open arms). Additionally, $\text{TCR}\beta^{-/-}\delta^{-/-}$ mice exhibited an increased fear response (increased immobility) in the auditory fear conditioning test. Notably, the reduced anxiety-like phenotype presented differently in males and females. These findings are consistent with previous reports suggesting a role for the adaptive immune system in anxiety-like behaviour (Cushman et al., 2003). We also demonstrated a loss of sex differences in $\text{TCR}\beta^{-/-}\delta^{-/-}$ mice for behaviours that were normally sexually dimorphic in WT mice such as increased activity in females (Gray, 1971; Archer, 1975; Johnston and File, 1991; Voikar et al., 2001), however determining the precise roles for various sex-specific factors will require additional study. It is important to note that our data suggest that altered peripheral immune function is a key contributor to changes in anxiety-like and exploratory behaviour suggesting that further exploration of the link between T cells and anxiety may facilitate the development of immune-based manipulations of behaviour with potential therapeutic applications.

Interestingly, there was no main effect of genotype in the $\mu\text{MT}^{-/-}$ mice for any of the aforementioned anxiety-like behaviours or fear response, though $\mu\text{MT}^{-/-}$

MT^{-/-} females did spend significantly more time in the light chamber than *MT*^{-/-} males and there were effects of genotype on activity measures in the EPM including distance traveled and entries into the closed arms. These results support the hypothesis that it is the loss of T lymphocytes that results in behavioural changes for anxiety-like and fear-related behaviours in mouse models of adaptive immune deficiency.

Experiment #2

CNS Gene Expression Analyses of Neurotransmitter Systems and the HPA Axis.

3.4. Overview of Experiment #2

Stress reactivity was examined in $TCR\beta^{-/-}\delta^{-/-}$ mice in response to repeated restraint stress. Adaptive immune functioning has previously been shown to influence the stress axis. For example, male rats with increased T cell proliferative capacity have an exaggerated CORT response to stress (Stiller et al., 2011a). In male mice, an immune challenge leading to a strong CD4⁺ T cell response was shown to induce higher expression of CRH mRNA in the PVN and CeA (Kusnecov et al., 1999). With respect to adaptive immune deficiency, female athymic nude mice have elevated plasma ACTH (Gaillard et al., 1998). However, each of these studies linking adaptive immune function to stress was conducted in only one sex.

In studies of stress reactivity examining differences between males and females it has been shown that sex is an important factor to consider. In mice, acute restraint stress results in a higher CORT response in female compared to male mice. However, this effect is eliminated in gonadectomized females (Goel and Bale, 2010). In humans, adult males compared to females have been shown to have elevated CORT and ACTH in response to social stress, including public speaking and mental arithmetic (Kirschbaum et al., 1999; Lovallo et al., 2010).

Given these reported differences between males and females in stress reactivity, both sexes were included in these experiments.

To examine the influence of T lymphocytes, we exposed wild type (WT – C57Bl/6) mice and $TCR\beta^{-/-}\delta^{-/-}$ mice of both sexes to repeated daily restraint stress (RS). Following 10 days of RS we examined HPA axis activation by measuring plasma corticosterone levels and stress-related CNS gene expression in unstressed and stressed mice. Overall, these studies provide important insights into the role of immune compromise in the development of sex differences in central stress circuitry and stress reactivity.

3.5. Results of Experiment 2

Stress Response. HPA axis activation was observed following repeat restraint stress (RS) using radioimmunoassay to determine plasma corticosterone (CORT) levels. Univariate analysis with treatment, genotype, and sex as fixed factors was used to analyze the results (Figure 13). Main effects of both sex ($F_{(1,64)}=4.07$, $p=0.048$) and RS treatment ($F_{(1,64)}=105.5$, $p<0.001$) were observed.

Independently, pairwise t-tests were conducted to examine differences between groups. In all groups, RS treatment lead to increased plasma CORT levels compared to sex- and genotype-matched CON mice: WT females ($t=6.92$, $df=16$, $p<0.001$), WT males ($t=3.70$, $df=13$, $p=0.003$), $TCR\beta^{-/-}\delta^{-/-}$ females ($t=5.81$, $df=14$, $p<0.001$), and $TCR\beta^{-/-}\delta^{-/-}$ males ($t=5.17$, $df=14$, $p<0.001$). Within the CON

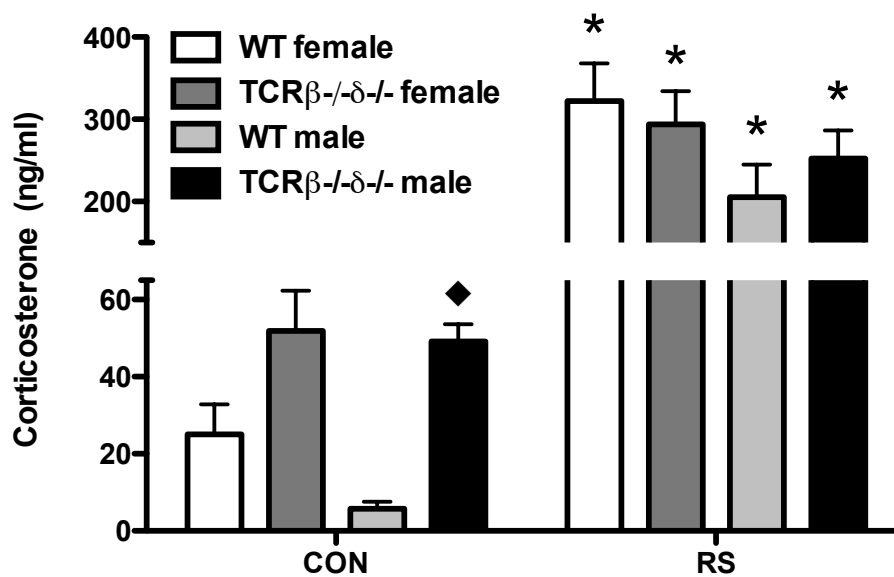


Figure 13. Corticosterone (CORT) was measured in plasma samples collected immediately following restraint stress (RS) treatment on the tenth day of RS treatment. TCR $\beta^{-/-}\delta^{-/-}$ males had a significantly higher level of plasma CORT compared to WT males in the CON group ($\blacklozenge p < 0.05$). RS treatment led to increased plasma CORT in all groups compared to sex and genotype matched CON mice ($*p < 0.05$).

group, $TCR\beta^{-/-}\delta^{-/-}$ males had significantly higher levels of plasma CORT than CON WT males ($t=8.94$, $df=12$, $p<0.001$) suggesting HPA axis dysregulation at baseline. In females, the mean plasma CORT level was twice as high in $TCR\beta^{-/-}\delta^{-/-}$ mice compared to WT mice; however, this difference was not statistically significant. No genotype differences were observed in response to RS.

Central Stress Circuitry. Central stress circuitry was investigated by measuring mRNA expression levels of stress related genes using *in situ* hybridization. CRH mRNA expression was found in the bed nucleus of the stria terminalis (BNST; Figure 14), the paraventricular nucleus of the hypothalamus (PVN; Figure 15), and central amygdala (CeA; Figure 16). The gene expression results for CRH mRNA are presented in Table 1. Univariate analysis with treatment, genotype, and sex as fixed factors revealed changes in stress-related gene expression. There were main effects of genotype ($F_{(1,67)}=4.19$, $p=0.045$) as well as treatment on the expression of CRH mRNA in the PVN ($F_{(1,67)}=22.9$, $p<0.001$). Additionally, there was an interaction between treatment and genotype in the PVN ($F_{(1,67)}=5.21$, $p=0.026$). In the CeA, there was a main effect of RS treatment on CRH mRNA expression ($F_{(1,67)}=7.38$, $p=0.009$). There were no main effects of genotype, sex, or RS treatment on CRH mRNA expression in the BNST.

Pairwise t-tests were used to assess differences between groups. RS treatment resulted in a central elevation of CRH mRNA in both male and female

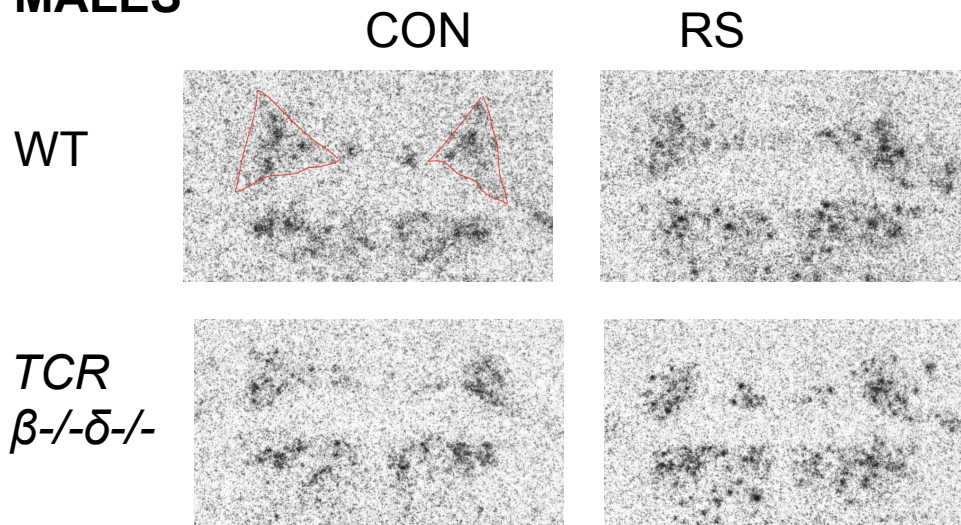
Table 1: mRNA expression of stress-related genes in C57Bl/6 and *TCRβ*^{-/-}*δ*^{-/-} mice

mRNA	Brain Region	WT		TCR	
		CON	RS	CON	RS
Males					
BNST	CRH	259.8 ± 31.7	266.8 ± 29.1	261.0 ± 34.3	310.9 ± 32.4
PVN	CRH	1641.6 ± 89.0	1812.4 ± 200.4	1452.4 ± 85.0	2379.6 ± 278.3 ^b
CeA	CRH	302.8 ± 24.3	354.6 ± 32.6	254.8 ± 18.6	345.3 ± 29.4 ^b
CA1	CRHR1	261.6 ± 69.4	416.7 ± 123.1	264.8 ± 133.9	345.0 ± 78.9
	CRHR2	94.6 ± 9.4 ^c	182.9 ± 41.9*	107.9 ± 23.7	137.1 ± 23.8
	GR	763.3 ± 96.1	584.3 ± 135.3	714.2 ± 122.2	754.7 ± 84.6
	MR	2173.3 ± 356.7	2110.2 ± 387.2 ^d	1724.5 ± 296.8	1982.8 ± 245.2
CA3	CRHR1	142.2 ± 16.1	179.2 ± 18.7	125.3 ± 16.1	162.8 ± 13.0
	GR	347.5 ± 45.6	288.7 ± 61.2	329.4 ± 57.8	337.8 ± 34.5
	MR	1779.7 ± 297.8	1700.6 ± 351.3 ^d	1274.0 ± 199.6	1517.3 ± 199.7
DG	CRHR1	109.9 ± 13.6	131.0 ± 15.9	97.0 ± 16.1	126.2 ± 12.9
	CRHR2	146.7 ± 11.5 ^c	244.3 ± 39.2*	164.9 ± 23.0	202.6 ± 22.9
	GR	697.4± 92.2	532.2 ± 119.2	658.5 ± 116.5	690.3 ± 68.9
	MR	2260.9± 376.7	2109.1 ± 386.1 ^d	1776.2 ± 263.1	2032.3 ± 315.2
Females					
BNST	CRH	215.4 ± 29.2	239.0 ± 21.4	249.7 ± 24.5	296.6 ± 22.1
PVN	CRH	1585.1 ± 78.4	1993.8 ± 108.5*	1719.7 ± 94.9	2427.9 ± 299.8 ^b
CeA	CRH	296.5 ± 26.4	296.3 ± 30.0	251.3 ± 18.5	313.7 ± 24.1
CA1	CRHR1	240.8 ± 64.4	273.8 ± 51.4	352.6 ± 136.4	362.6 ± 121.4
	CRHR2	152.5 ± 23.5	100.7 ± 14.0	109.0 ± 29.9	191.5 ± 39.0 ^a
	GR	584.4 ± 117.4	682.7 ± 117.8	639.5 ± 108.0	718.8 ± 182.7
	MR	1499.4 ± 317.5	1163.3 ± 151.5	1563.5 ± 265.6	1673.7 ± 234.0
CA3	CRHR1	142.2 ± 12.7	154.4 ± 11.2	138.3 ± 19.5	152.8 ± 16.7
	GR	274.2 ± 62.2	314.7 ± 58.8	294.6 ± 51.2	380.6 ± 105.5
	MR	1256.7 ± 263.7	920.3 ± 115.9	1247.4 ± 221.8	1290.3 ± 183.9
DG	CRHR1	110.9 ± 11.0	114.8 ± 10.6	112.9 ± 19.6	122.3 ± 17.7
	CRHR2	209.7 ± 22.2	161.5 ± 20.9	176.5 ± 41.1	272.8 ± 37.1 ^a
	GR	509.0 ± 102.4	640.7 ± 111.6	565.2 ± 87.2	711.65 ± 199.4
	MR	1447.5 ± 294.3	1095.8 ± 111.3	1728.0 ± 318.2	1719.2 ± 238.2 ^a

Corticotropin releasing hormone (CRH) mRNA expression levels were measured in the bed Nucleus of the stria terminalis (BNST), paraventricular nucleus of the hypothalamus (PVN), and central amygdala (CeA). CRH Receptor 1 (CRHR1), and glucocorticoid receptor (GR) mRNA levels were measured in the CA1, CA3 and dentate granule layer (DG). CRHR2 and mineralocorticoid receptor (MR) mRNA expression levels were measured in the CA1 and DG subregions of the hippocampus. Data is shown as the mean ± standard error. Significant differences were determined using t-test ($p < 0.05$).

* significant ($p < 0.05$) compared to WT CON; ^a significant ($p < 0.05$) compared to WT RS; ^b significant ($p < 0.05$) compared to TCR CON; ^c significant ($p < 0.05$) compared to WT CON F; ^d significant ($p < 0.05$) compared to WT RS F

MALES



FEMALES

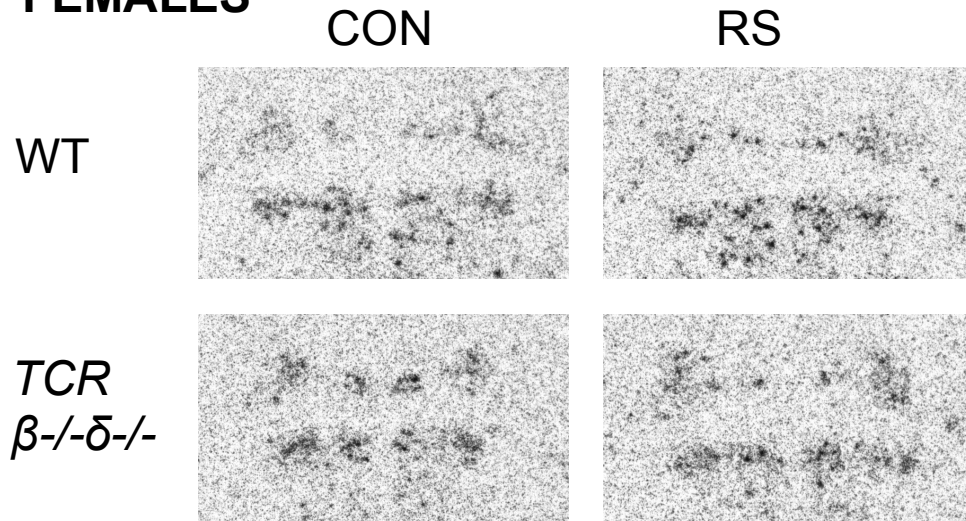


Figure 14. Representative film autoradiographs show corticotrophin releasing hormone (CRH) mRNA expression in the bed nucleus of the stria terminalis (BNST; upper regions were measured as outlined in red; Bregma 0.26) in WT and *TCR* $\beta^{-/-}\delta^{-/-}$, males and females, and unstressed (CON and restraint stressed (RS) mice. No genotype, sex, or treatment differences in CRH mRNA expression were observed.

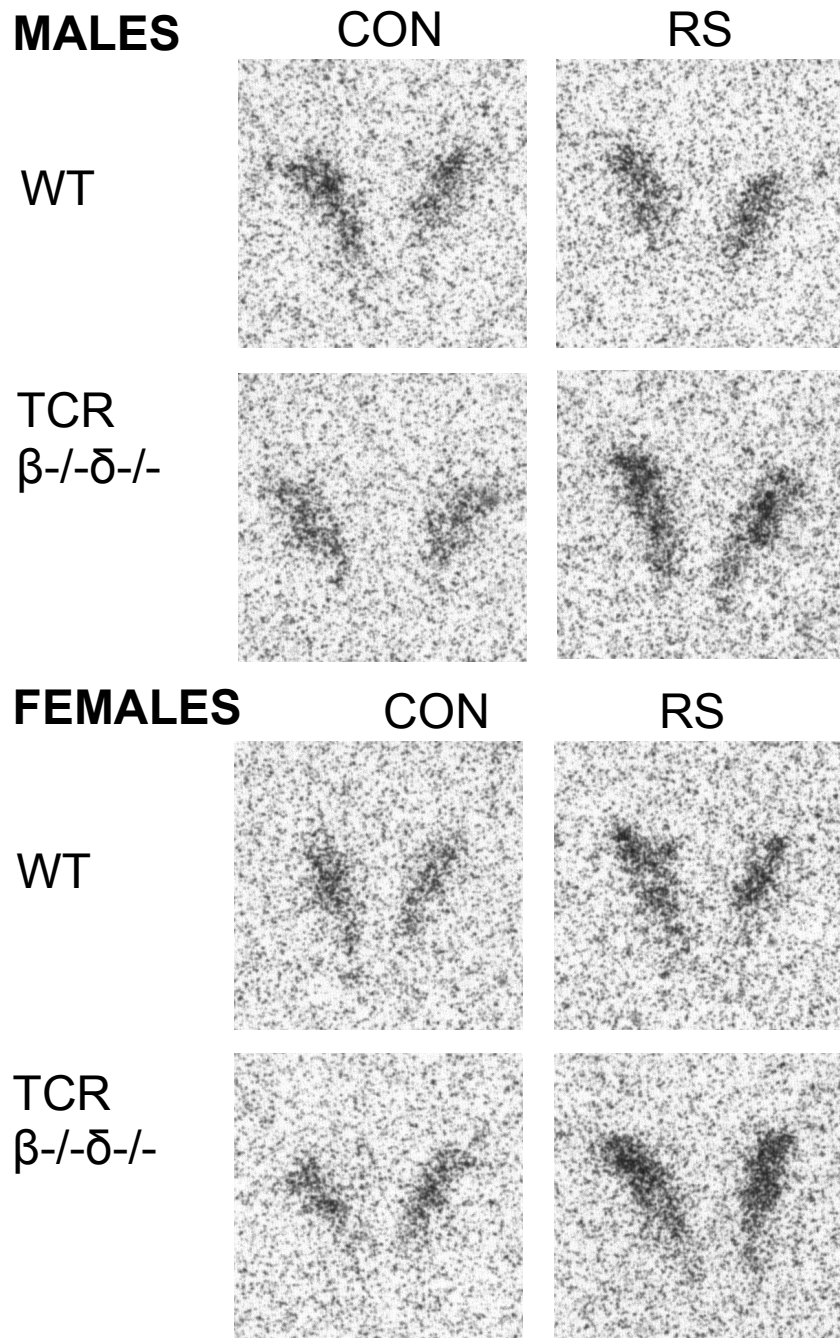


Figure 15. Representative film autoradiographs show corticotropin releasing hormone (CRH) mRNA expression in the paraventricular nucleus of the hypothalamus (PVN; Bregma -0.58) in WT and *TCRβ^{-/-}δ^{-/-}*, males and females, and unstressed (CON) and restraint stressed (RS) mice. RS led to increased mRNA expression in WT females, and *TCRβ^{-/-}δ^{-/-}* males and females compared to genotype-matched CON.

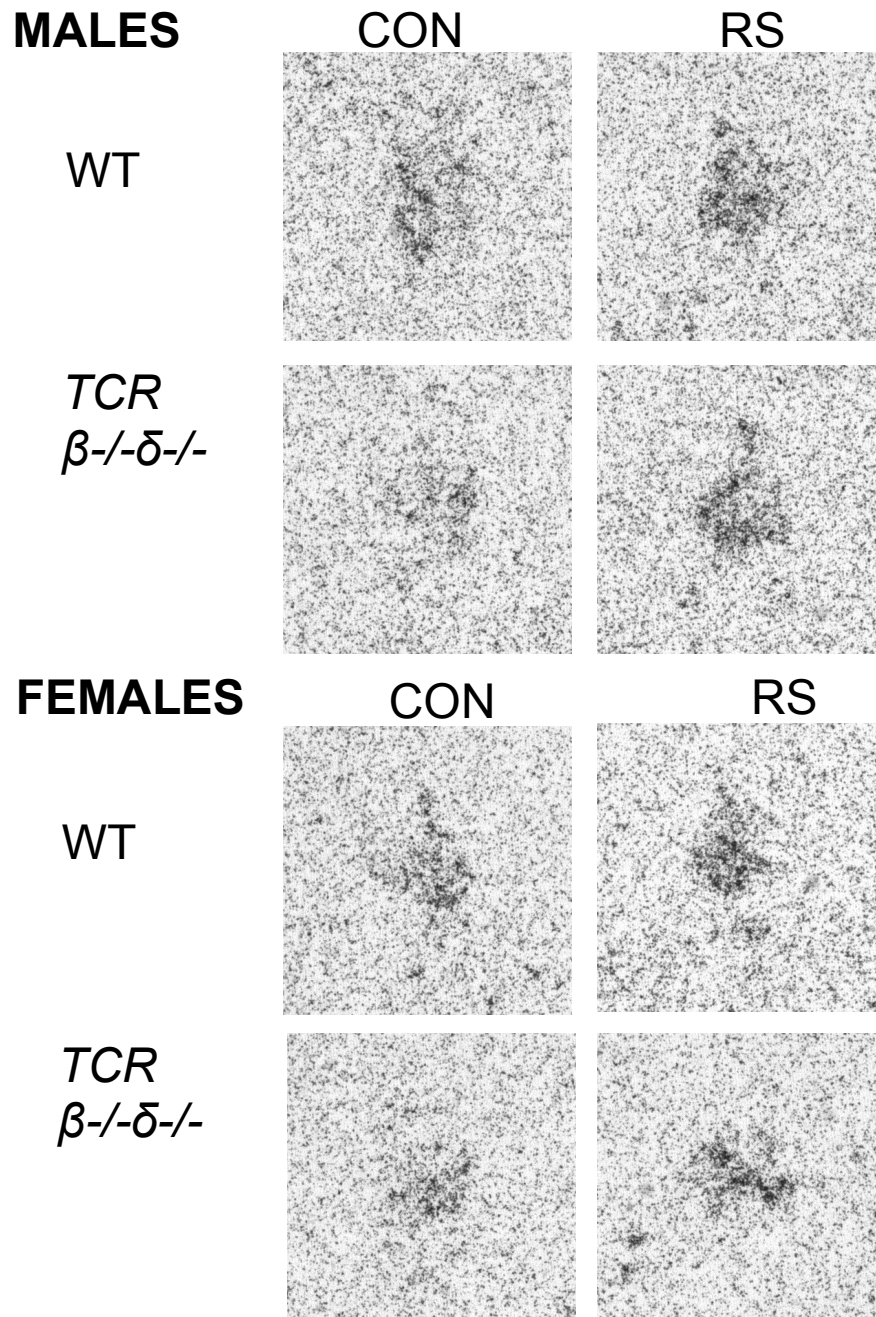


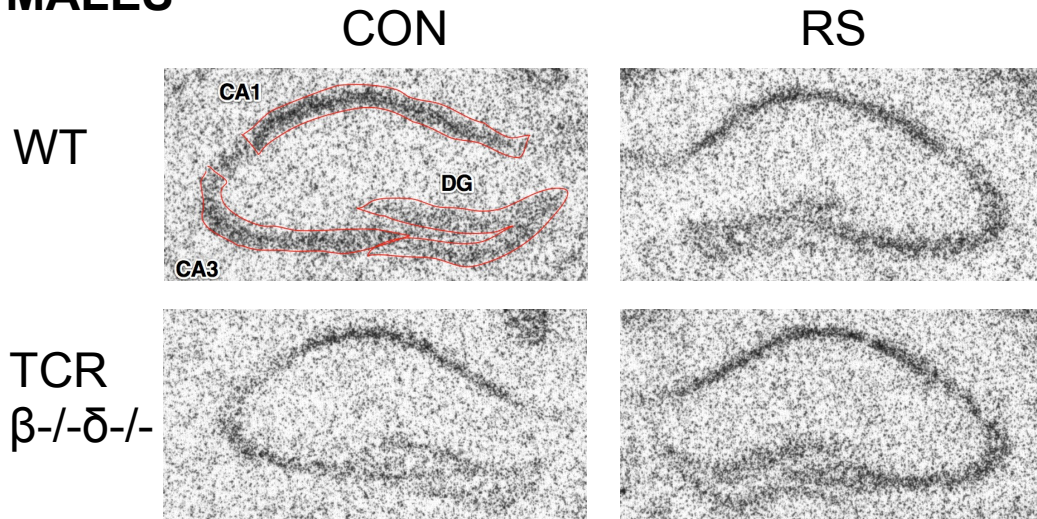
Figure 16. Representative film autoradiographs show corticotropin releasing hormone (CRH) mRNA expression in the central amygdala (CeA; Bregma - 0.58) in WT and *TCRβ-/-δ-/-*, males and females, and unstressed (CON) and restraint stressed (RS) mice. RS treatment led to increased expression of CRH mRNA in the CeA in *TCRβ-/-δ-/-* males but not female *TCRβ-/-δ-/-* or WT mice.

TCRβ^{-/-δ} mice but in WT mice, CRH was elevated only in females. RS treatment resulted in elevated expression of CRH mRNA in the PVN of WT females compared to WT CON females ($t=3.05$, $df=18$, $p=0.007$). RS treatment also led to increased CRH mRNA expression in the PVN of both male ($t=3.19$, $df=14$, $p=0.007$) and female ($t=2.39$, $df=13$, $p=0.033$) *TCRβ*^{-/-δ} mice compared to sex-matched *TCRβ*^{-/-δ} CON mice. Male RS *TCRβ*^{-/-δ} mice expressed increased CRH mRNA in the CeA compared to CON *TCRβ*^{-/-δ} males ($t=2.60$, $df=14$, $p=0.021$).

CRH receptors 1 (CRHR1) and 2 (CRHR2) mRNAs were measured in the hippocampus. CRHR1 expression was observed throughout the hippocampus (Figure 17) while CRHR2 expression was measured within the CA1 and dentate gyrus (DG) regions of the hippocampus (Figure 18). Univariate analysis with treatment, genotype, and sex as fixed factors revealed a main effect of treatment on CRHR1 mRNA expression in the CA3 region ($F_{(1,66)}=5.33$, $p=0.024$). With regard to CRHR2 mRNA expression, univariate analysis revealed a main effect of treatment in the CA1 ($F_{(1,66)}=4.01$, $p=0.05$) as well as a 3-way interaction between genotype, sex and treatment ($F_{(1,66)}=6.83$, $p=0.011$). In the DG, there was a main effect of treatment ($F_{(1,66)}=5.42$, $p=0.023$) and a 3-way interaction between genotype, sex and treatment ($F_{(1,66)}=6.75$, $p=0.012$).

Expression of CRH receptors CRHR1 and CRHR2 were also analyzed using pairwise t-tests. No differences were observed between groups for CRHR1 mRNA expression. Stress-related changes in gene expression were observed for

MALES



FEMALES

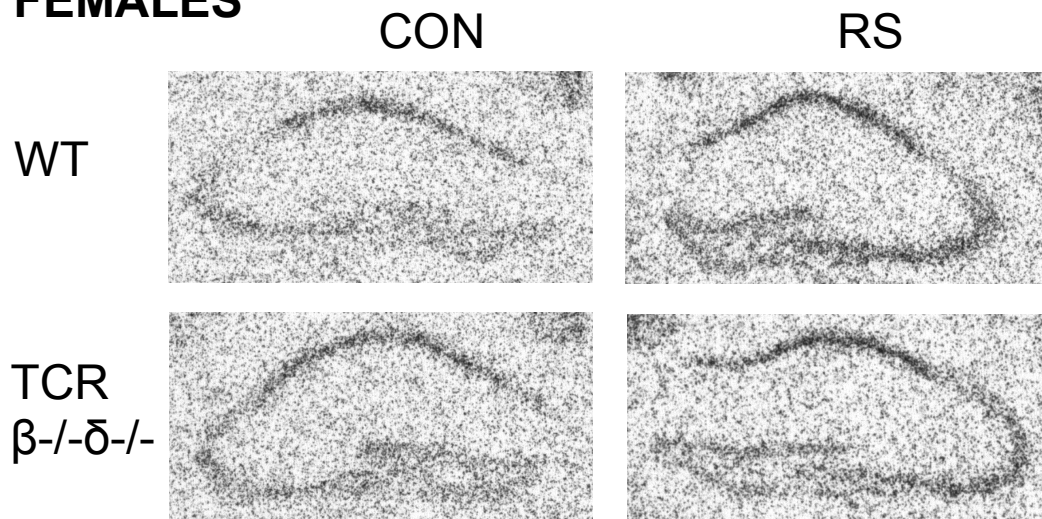
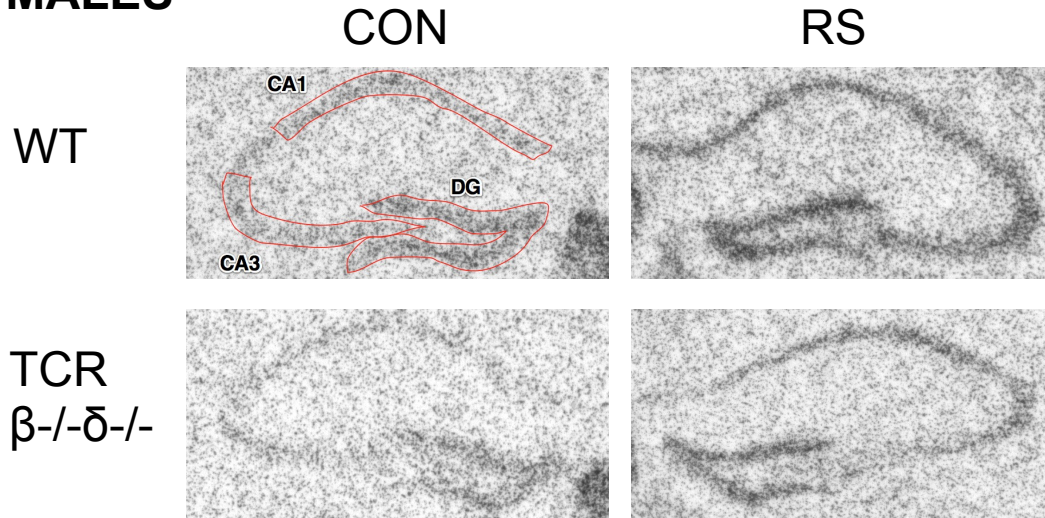


Figure 17. Representative film autoradiographs show corticotropin releasing hormone receptor 1 (CRHR1) mRNA expression in the hippocampal subregions CA1, CA3, and dentate gyrus (DG; Bregma – 1.70) in WT and *TCR $\beta^{-/-}\delta^{-/-}$* , males and females, and unstressed (CON) and restraint stressed (RS) mice. Target regions are outlined in red. No significant differences of sex, genotype, or treatment were observed.

MALES



FEMALES

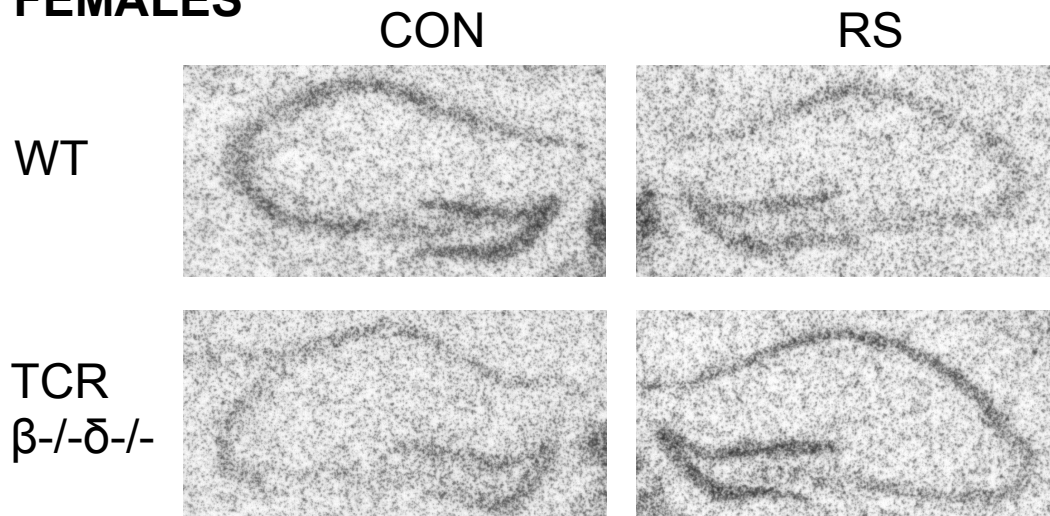
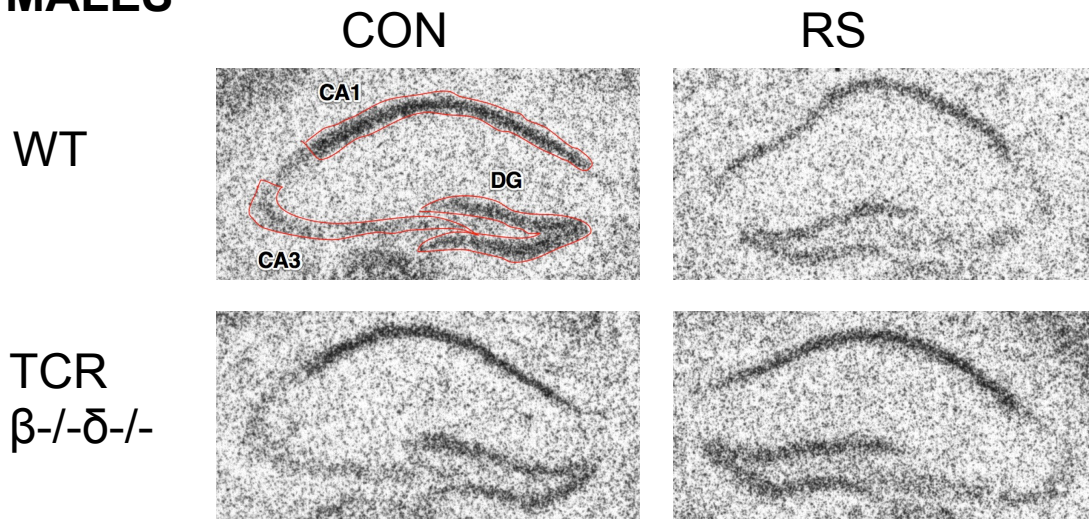


Figure 18. Representative film autoradiographs show corticotropin releasing hormone receptor 2 (CRHR2) mRNA expression in the hippocampal subregions CA1 and dentate gyrus (DG; Bregma -1.70) in WT and *TCR $\beta^{-/-}\delta^{-/-}$* , males and females, and unstressed (CON) and restraint stressed (RS) mice. Target regions are outlined in red. RS treatment led to increased expression of CRHR2 mRNA in WT males compared to CON males as well as RS *TCR $\beta^{-/-}\delta^{-/-}$* females compared to RS WT females in both the CA1 and DG. Additionally, CON WT females expressed higher levels of CRHR2 than CON WT males in the CA1 and DG regions of the hippocampus.

CRHR2 where expression of CRHR2 mRNA was upregulated in RS WT males compared to CON WT males in the CA1 ($t=2.18$, $df=14$, $p=0.046$) and DG ($t=2.52$, $df=15$, $p=0.024$). Sex and genotype differences in stress response were evident as there were no differences between RS and CON WT females but there was significantly increased CRHR2 mRNA expression in RS *TCR β ^{-/-} δ ^{-/-}* females compared to RS WT females in both the CA1 ($t=2.61$, $df=14$, $p=0.020$) and the DG ($t=2.84$, $df=14$, $p=0.013$). Sexual dimorphism was observed in WT mice for CRHR2 mRNA expression, CON WT females expressed significantly more CRHR2 mRNA in the CA1 ($t=2.20$, $df=17$, $p=0.042$) and DG ($t=2.44$, $df=17$, $p=0.026$) compared to CON WT males. This sex difference was not observed in the *TCR β ^{-/-} δ ^{-/-}* mice indicating a loss of sexually dimorphic expression of CRHR2 mRNA.

Corticosterone receptors GR (Figure 19) and MR (Figure 20) were both strongly expressed throughout the hippocampus. The GR was expressed ubiquitously throughout the brain while the MR, at the level of the hippocampus, was only expressed within the hippocampus. Univariate analysis using genotype, sex and RS treatment as fixed factors did not reveal any main effects on the expression of GR mRNA expression in the hippocampus. There was a main effect of sex on the expression of MR mRNA in the CA1 ($F_{(1,65)}=6.42$, $p=0.014$), CA3 ($F_{(1,65)}=5.24$, $p=0.026$), and DG ($F_{(1,65)}=6.70$, $p=0.012$) subregions of the hippocampus.

MALES



FEMALES

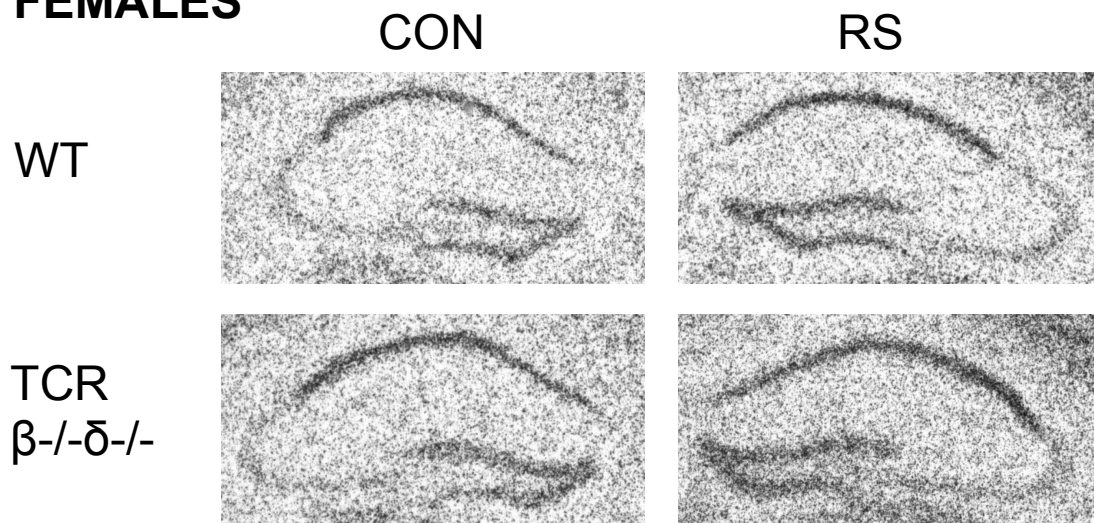


Figure 19. Representative film autoradiographs show glucocorticoid receptor (GR) mRNA expression in the CA1, CA3, and DG subregions of the hippocampus (Hip; Bregma -1.70) in WT and *TCR* $\beta^{-/-}\delta^{-/-}$, males and females, and unstressed (CON) and restraint stressed (RS) mice. Target regions are outlined in red. No significant differences were observed.

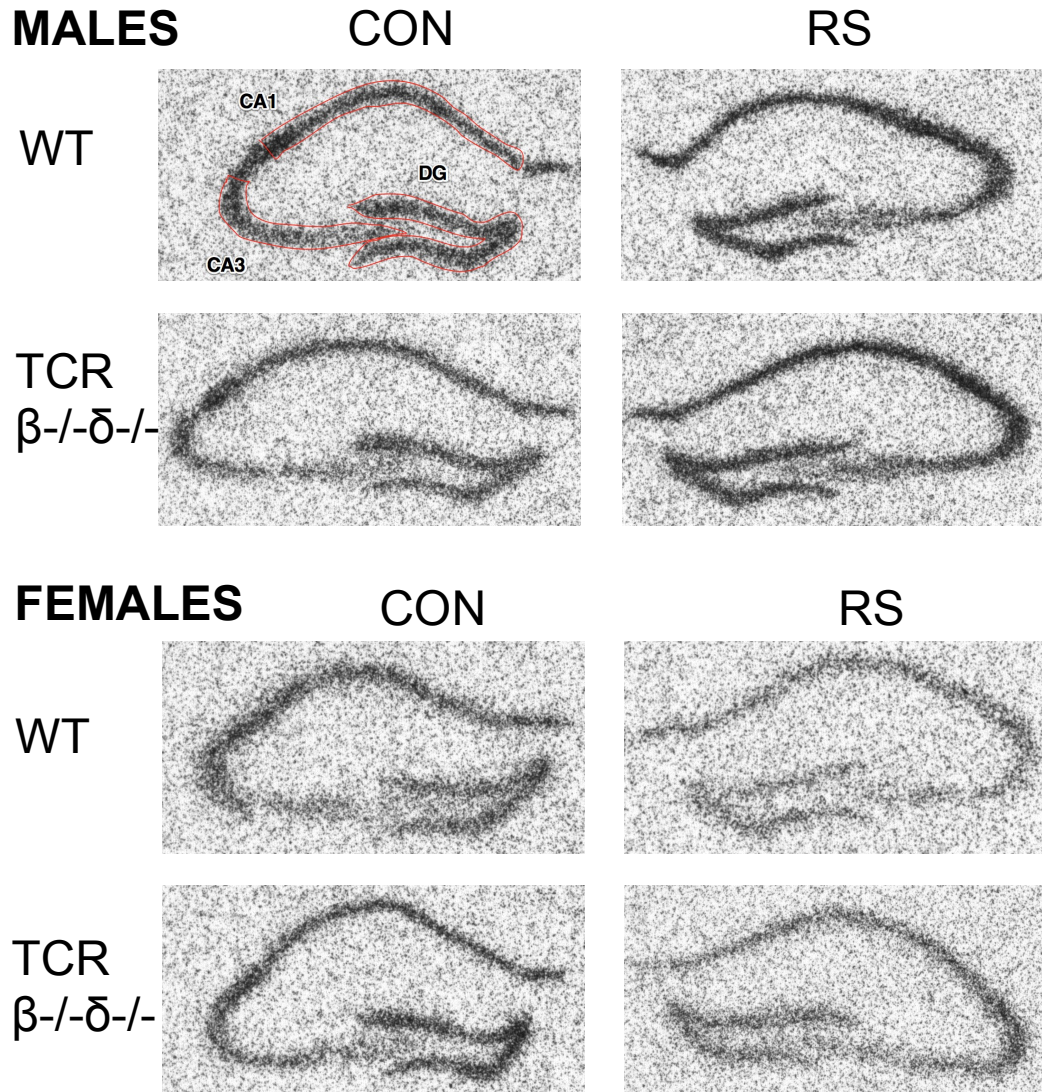


Figure 20. Representative film autoradiographs show mineralocorticoid receptor (MR) mRNA expression in the CA1, CA3, and DG subregions of the hippocampus (Bregma, -1.70) in WT and *TCR $\beta^{-/-}\delta^{-/-}$* , males and females, and unstressed (CON) and restraint stressed (RS) mice. Target regions are outlined in red. RS treated WT males expressed significantly higher levels of MR mRNA throughout the hippocampus. Additionally RS *TCR $\beta^{-/-}\delta^{-/-}$* females expressed significantly higher levels of MR in the DG than RS WT females.

Pairwise comparisons of MR mRNA expression revealed increased MR mRNA in RS treated males compared to RS WT females in the CA1 ($t=2.57$, $df=15$, $p=0.021$), CA3 ($t=2.43$, $df=15$, $p=0.028$), and DG ($t=2.93$, $df=15$, $p=0.010$) regions of the hippocampus. There were no differences between RS $TCR\beta^{-/-}\delta^{-/-}$ males and RS $TCR\beta^{-/-}\delta^{-/-}$ females, again indicating a loss of sex differences with loss of functional T lymphocytes. There was, however, increased MR mRNA expression in RS $TCR\beta^{-/-}\delta^{-/-}$ females compared to RS WT females in the DG ($t=2.62$, $df=15$, $p=0.019$) indicating altered sensitivity to the effects of stress.

Serotonergic Receptors. Gene expression of serotonergic receptors 5-HT1A (Figure 21) and 5-HT2A (Figure 22) mRNAs was examined in the hippocampus while expression of 5-HT1A (Figure 23) and 5-HT2C (Figure 24) mRNAs was studied in the amygdala. The densitometric results of these experiments are presented in Table 2. Univariate analysis with treatment, genotype, and sex as fixed factors revealed a main effect of genotype in 5-HT2C mRNA expression in the BLA ($F_{(1,64)}=6.05$, $p=0.017$). No main effects of genotype, sex, or treatment were observed on the expression of 5-HT1A or 5-HT2A mRNAs.

Pairwise comparisons were made using t-tests. In males, $TCR\beta^{-/-}\delta^{-/-}$ mice exposed to RS displayed differences in 5-HT2A and 5-HT2C mRNA expression. Sexually dimorphic expression of 5HT2A was observed, where WT males express higher levels of 5-HT2A mRNA in the CA1 than WT females ($t=2.20$,

Table 2: mRNA expression for serotonin receptor subunit genes in wild type (WT) and T cell receptor knock out (TCR) mice in amygdala and hippocampal subregions

		WT		TCR	
Brain Region	mRNA	CON	RS	CON	RS
Males					
CA1	5HT1A	658.7 ± 54.5	741.9 ± 62.3	688.0 ± 76.1	682.5 ± 61.9
	5HT2A	114.8 ± 9.1 ^c	123.2 ± 10.7	104.0 ± 5.5	99.0 ± 3.7 ^a
CA3	5HT1A	217.6 ± 23.6	236.2 ± 21.9	217.0 ± 28.0	213.9 ± 23.1
	5HT2A	88.2 ± 4.2	96.8 ± 6.6	87.1 ± 3.9	80.7 ± 2.9 ^a
DG	5HT1A	376.2 ± 47.6	396.9 ± 40.1	358.0 ± 52.8	326.6 ± 41.4
	5HT2A	75.5 ± 2.8	75.4 ± 5.2	70.6 ± 3.6	69.1 ± 3.5
LA	5HT1A	136.5 ± 14.8	143.4 ± 12.3	137.3 ± 15.3	142.6 ± 14.8
	5HT2C	657.6 ± 32.1	598.2 ± 32.4	655.7 ± 32.1	634.1 ± 44.3
BLA	5HT1A	144.6 ± 16.3	160.3 ± 15.9	144.9 ± 17.8	144.1 ± 12.7
	5HT2C	504.5 ± 31.8	433.4 ± 50.9	558.3 ± 22.2	590.9 ± 47.1 ^a
Females					
CA1	5HT1A	562.2 ± 43.6	678.5 ± 44.7	683.5 ± 55.0	721.8 ± 68.5
	5HT2A	92.3 ± 4.8	121.6 ± 4.7*	104.0 ± 6.3	107.9 ± 7.8
CA3	5HT1A	208.4 ± 21.0	233.5 ± 15.2	217.0 ± 14.8	226.4 ± 24.7
	5HT2A	79.1 ± 3.5	94.9 ± 4.6*	84.4 ± 6.2	89.3 ± 4.5
DG	5HT1A	314.5 ± 37.7	341.2 ± 22.5	347.9 ± 38.0	368.2 ± 48.5
	5HT2A	69.5 ± 3.2	77.4 ± 3.3	70.7 ± 5.0	76.4 ± 4.5
LA	5HT1A	135.7 ± 13.4	141.6 ± 7.9	132.1 ± 10.5	151.1 ± 12.4
	5HT2C	618.2 ± 40.1	595.6 ± 36.8	604.8 ± 22.5	646.6 ± 66.6
BLA	5HT1A	133.7 ± 11.1	152.7 ± 6.6	141.9 ± 11.4	150.2 ± 7.7
	5HT2C	525.5 ± 39.5	500.4 ± 44.9	562.3 ± 41.5	565.3 ± 76.5

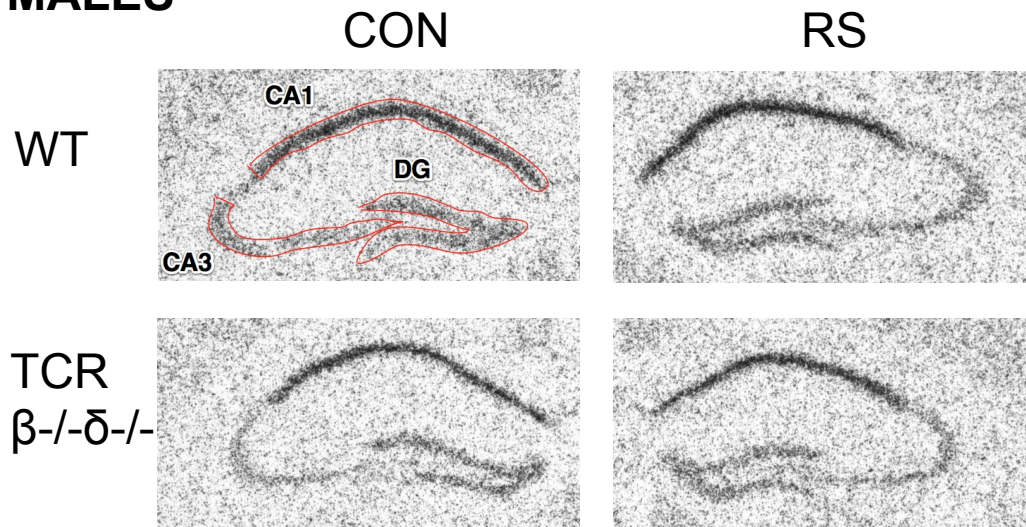
Serotonin Receptors 5-HT1A and 5-HT2A mRNA levels were measured in subregions of the hippocampus – CA1, CA3 and dentate granule layer (DG). 5-HT1A and 5-HT2C mRNA levels were measured in the lateral amygdala (LA) and basolateral amygdala (BLA). Data is shown as the mean ± standard error. Significant differences were determined using t-test ($p < 0.05$) and bolded text indicates that a significant difference was observed.

*significant ($p < 0.05$) compared to WT CON

^a significant ($p < 0.05$) compared to WT RS

^c significant ($p < 0.05$) compared to WT CON F

MALES



FEMALES

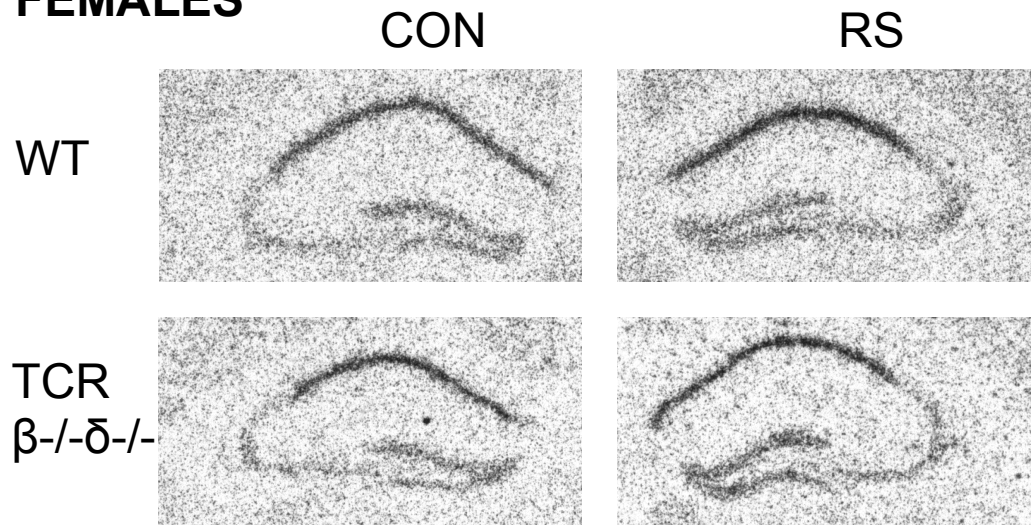


Figure 21. Representative film autoradiographs show 5-HT_{1A} receptor mRNA expression in the Hippocampus (Hip) in WT and *TCR* $\beta^{-/-}\delta^{-/-}$, males and females, and unstressed (CON) and restraint stressed (RS) mice. Target regions are outlined in red. No significant differences were observed.

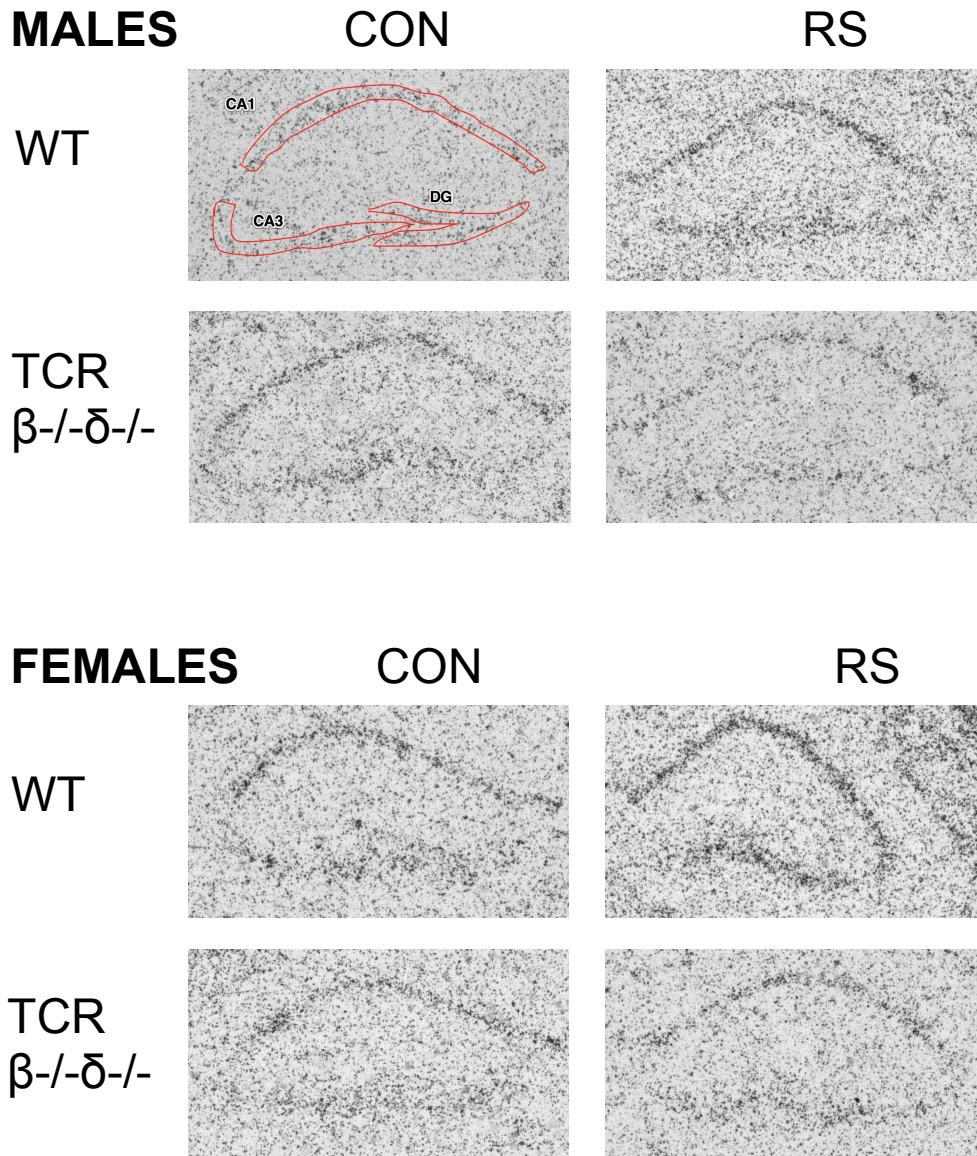


Figure 22. Representative film autoradiographs show 5-HT_{2A} receptor mRNA expression in the Hippocampus (Hip) in WT and *TCR* $\beta^{-/-}\delta^{-/-}$, males and females, and unstressed (CON) and restraint stressed (RS) mice. Target regions are outlined in red. Expression of 5-HT_{2A} receptor was elevated in RS *TCR* $\beta^{-/-}\delta^{-/-}$ males compared to RS WT males, and an RS WT females compared to CON WT females. 5-HT_{2A} receptor expression was also higher in the CA1 region of the Hip in CON WT males compared to CON WT females.

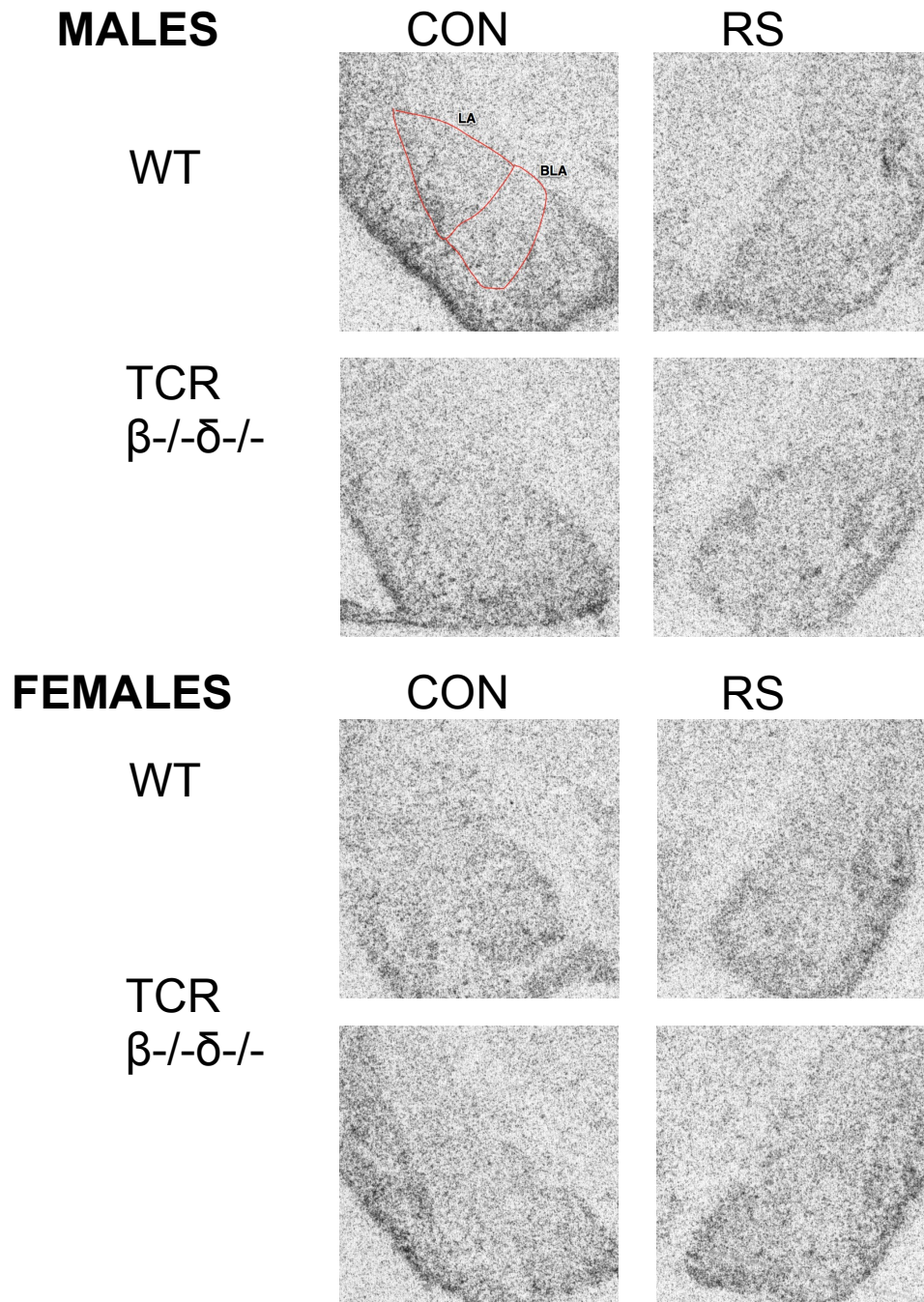


Figure 23. Representative film autoradiographs show 5-HT_{1A} receptor mRNA expression in the lateral amygdala (LA) and basolateral amygdala (BLA) in WT and *TCR* $\beta^{-/-}\delta^{-/-}$, males and females, and unstressed (CON) and restraint stressed (RS) mice. Target regions are outlined in red. No significant differences were observed.

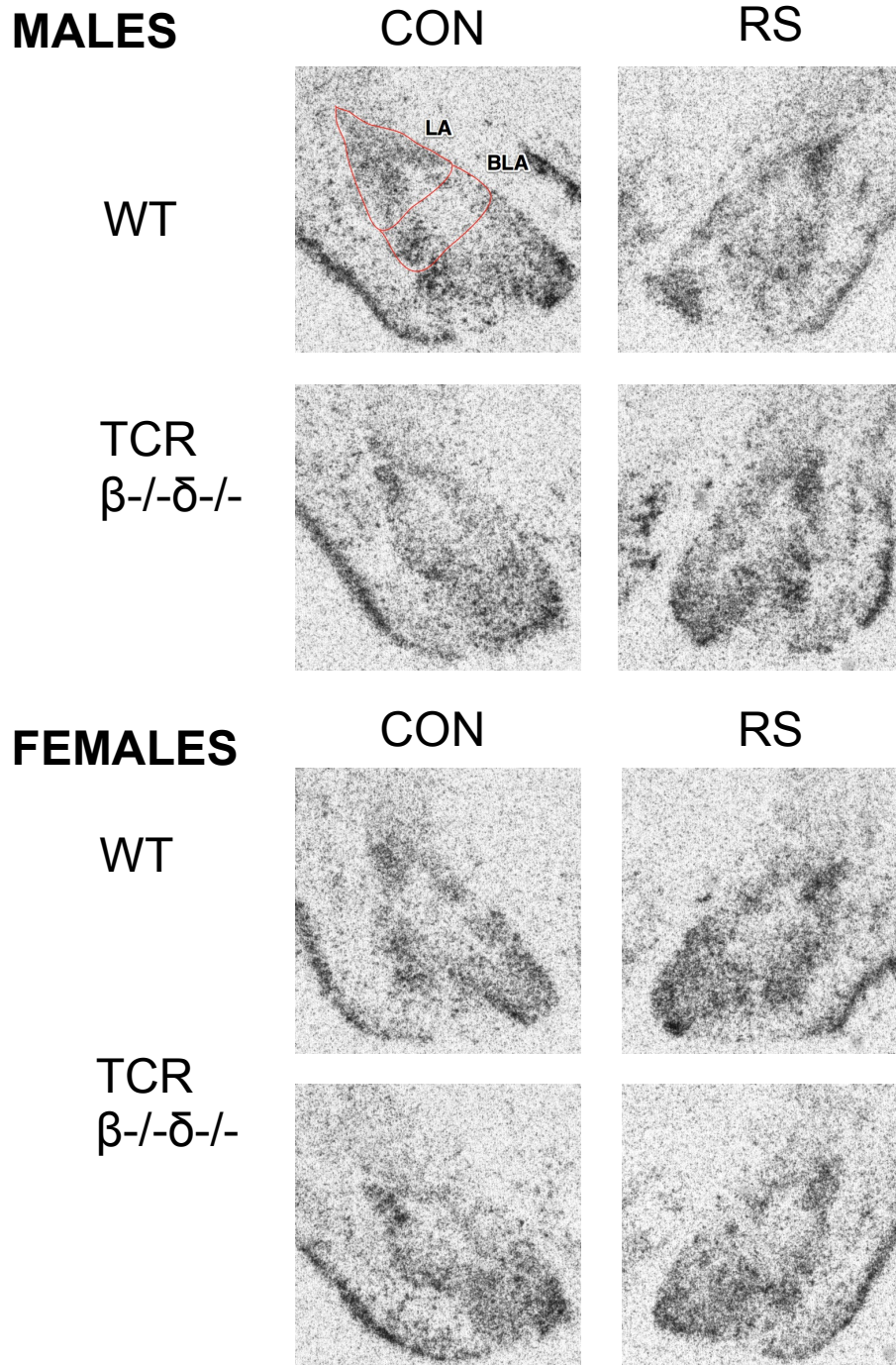


Figure 24. Representative film autoradiographs show 5-HT_{2C} receptor mRNA expression in the lateral amygdala (LA) and basolateral amygdala (BLA) in WT and *TCR* $\beta^{-/-}\delta^{-/-}$, males and females, and unstressed (CON) and restraint stressed (RS) mice. Target regions are outlined in red. RS *TCR* $\beta^{-/-}\delta^{-/-}$ males expressed higher levels of 5-HT_{2C} receptor mRNA in the BLA compared to RS WT males.

df=16, $p=0.043$). In contrast, no sex differences were observed within the *TCR β* ^{-/-} mice. In addition, RS treatment lead to increased expression of 5-HT2A mRNA in WT females compared to CON WT females in the CA1 ($t=4.34$, df=15, $p=0.001$) and CA3 ($t=2.76$, df=15, $p=0.015$) regions of the hippocampus.

Conversely, RS treated *TCR β* ^{-/-} males expressed significantly less 5-HT2A mRNA in the CA1 ($t=2.25$, df=15, $p=0.040$) and CA3 ($t=2.33$, df=15, $p=0.034$) regions compared to RS WT males. Additionally, 5-HT2C mRNA expression was elevated in RS *TCR β* ^{-/-} compared to RS WT males ($t=2.27$, df=14, $p=0.039$).

Glutamatergic NMDA Receptors. NMDA receptor (NR) subunits NR1, NR2A, and NR2B mRNA expression were analyzed in the hippocampus (Table 3) and amygdala (Table 4). Each of these mRNAs was expressed differentially throughout the hippocampus and the amygdala. Representative images of hippocampal expression of NR1, NR2A, and NR2B are provided in Figure 25, Figure 26, and Figure 27, respectively. Univariate analysis with treatment, genotype, and sex as fixed factors did not reveal any main effects of genotype, sex or RS treatment on the expression of NR1 in the hippocampus or the amygdala. In the DG there was a main effect of genotype ($F_{(1,64)}=22.8$, $p<0.001$) on the expression of NR2A mRNA. There were no effects of genotype, sex or RS on NR2B mRNA expression in the hippocampus.

Pairwise comparisons were made independently using t-tests. In the hippocampus, no differences were observed in the expression of either NR1 or

Table 3: mRNA expression profile for NMDA receptor (NR) subunit genes in wild type (WT and T cell receptor knock out mice (*TCR β -/- δ -/-*) in hippocampal subregions

		WT		<i>TCRβ-/-δ-/-</i>	
Brain Region	mRNA	CON	RS	CON	RS
Males					
CA1	NR1	1492.5 ± 110.1	1747.4 ± 287.5	1558.3 ± 157.0	1357.0 ± 135.0
	NR2A	4432.2 ± 40.6	4483.5 ± 115.2	4234.6 ± 107.7	4413.5 ± 86.4
	NR2B	5323.3 ± 342.3	5684.4 ± 295.9	5348.1 ± 388.9	5864.5 ± 311.0
CA3	NR1	1546.3 ± 105.2	1791.3 ± 286.1	1471.1 ± 131.2	1280.4 ± 128.1
	NR2A	3078.4 ± 64.2	3071.0 ± 63.5	2960.1 ± 80.7	3070.1 ± 76.8
	NR2B	3758.1 ± 276.5	3924.8 ± 234.3	3793.1 ± 317.3	4062.1 ± 172.1
DG	NR1	1483.4 ± 109.1	1734.9 ± 285.9	1541.8 ± 149.0	1346.8 ± 134.0
	NR2A	3849.2 ± 87.1	3984.8 ± 71.6	4136.6 ± 109.3	4419.4 ± 123.6 ^a
	NR2B	3730.5 ± 272.8	3827.3 ± 254.4	3894.6 ± 332.5	4240.9 ± 227.1
Females					
CA1	NR1	1446.9 ± 105.2	1352.7 ± 133.0	1391.8 ± 100.4	1529.3 ± 101.5
	NR2A	4338.7 ± 133.8	4440.1 ± 97.2	4280.4 ± 171.4	4313.7 ± 155.7
	NR2B	5226.3 ± 229.9	4964.5 ± 361.6	5337.2 ± 375.3	5212.5 ± 568.6
CA3	NR1	1481.8± 108.4	1312.0 ± 135.0	1388.8 ± 125.2	1424.0 ± 117.1
	NR2A	2969.0 ± 83.7	3107.3 ± 56.3	2944.2 ± 52.8	3075.2 ± 155.6
	NR2B	3723.4 ± 209.5	3434.6 ± 226.1	4037.8 ± 245.4	3828.7 ± 431.8
DG	NR1	1427.2 ± 106.0	1307.6 ± 135.6	1339.8 ± 86.6	1467.7 ± 128.6
	NR2A	3791.2 ± 11.5	3906.3 ± 76.5	4259.4 ± 174.6*	4278.3 ± 171.0 ^a
	NR2B	3622.7 ± 168.8	3379.1 ± 279.4	4007.7 ± 288.6	3755.3 ± 396.9

NR subunits NR1, NR2A, and NR2B mRNA levels were measured in subregions of the hippocampus – CA1, CA3 and dentate granule layer (DG). Data is shown as the mean \pm standard error. Significant differences were determined using t-test ($p < 0.05$). Bolded text indicates significant differences.

* significant ($p < 0.05$) compared to WT CON

^a significant ($p < 0.05$) compared to WT RS

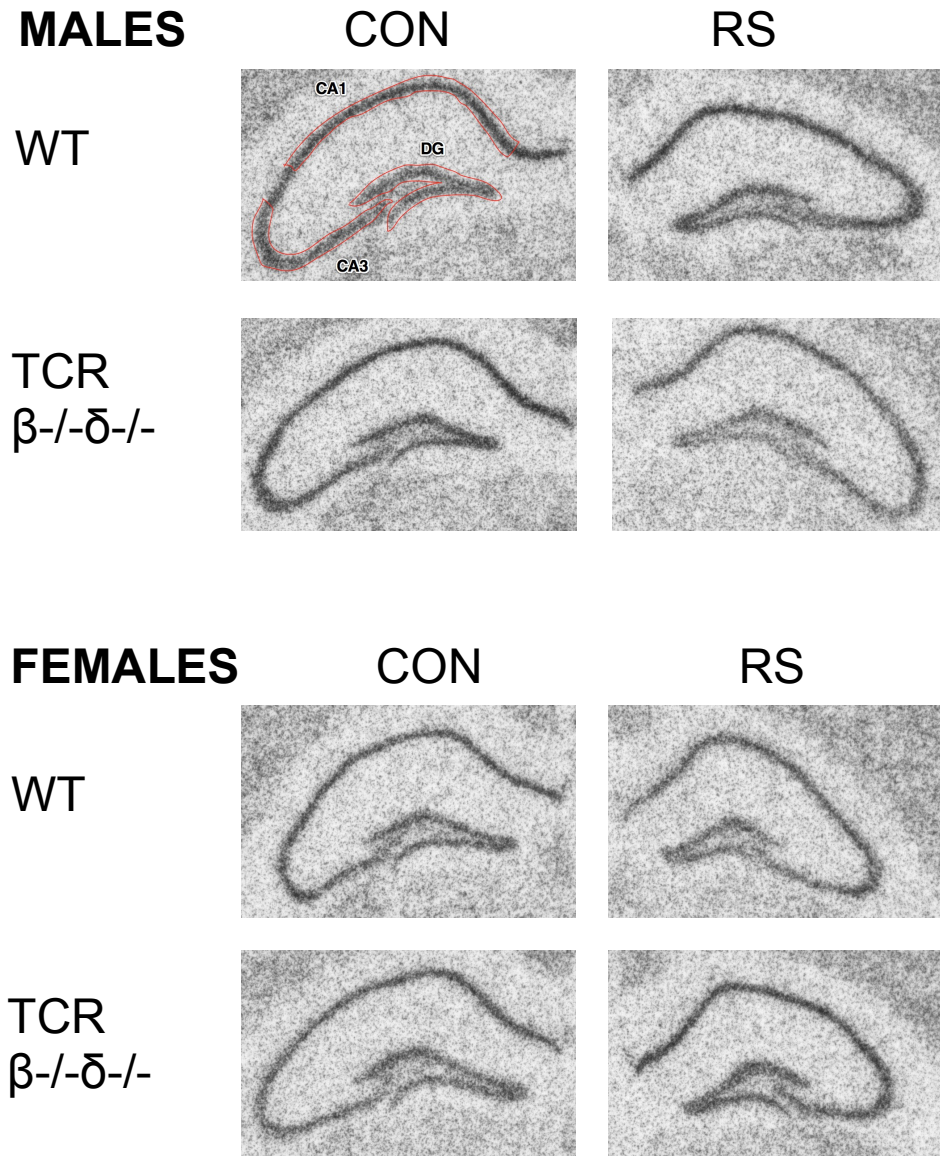


Figure 25. Representative film autoradiographs show NMDA receptor subunit NR1 mRNA expression in the hippocampus (Hip) in WT and *TCR $\beta^{-/-}\delta^{-/-}$* , males and females, and unstressed (CON) and restraint stressed (RS) mice. Target regions are outlined in red. No significant differences were observed.

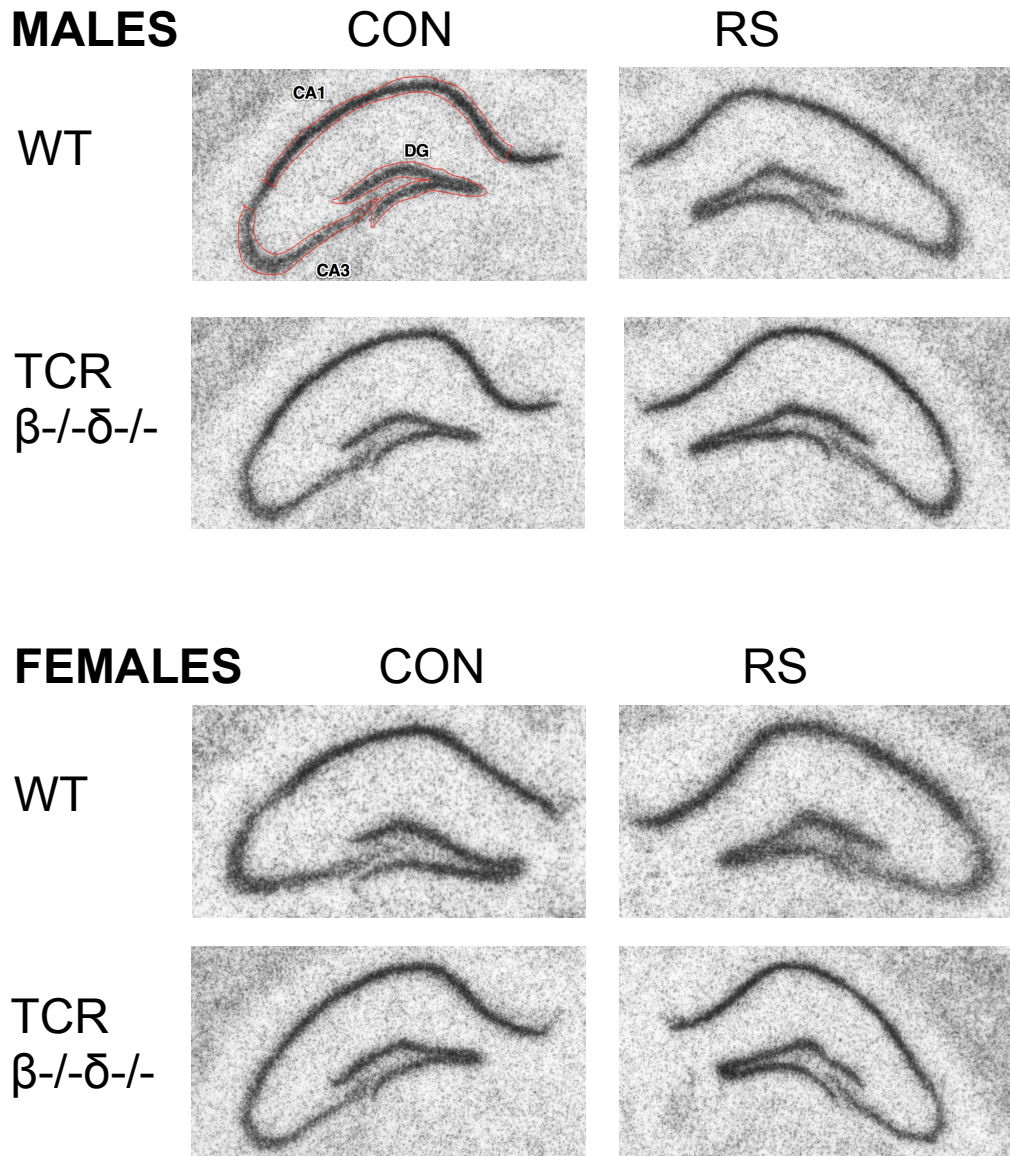


Figure 26. Representative film autoradiographs show NMDA receptor subunit NR2A mRNA expression in the hippocampus (Hip) in WT and *TCR $\beta^{-/-}\delta^{-/-}$* , males and females, and unstressed (CON) and restraint stressed (RS) mice. Target regions are outlined in red. The NR2A subunit mRNA expression in the dentate granule (DG) layer of the Hip was elevated in RS *TCR $\beta^{-/-}\delta^{-/-}$* males and females compared to CON *TCR $\beta^{-/-}\delta^{-/-}$* males and females, respectively. CON *TCR $\beta^{-/-}\delta^{-/-}$* females also expressed higher levels of the NR2A subunit in the DG compared to CON WT females.

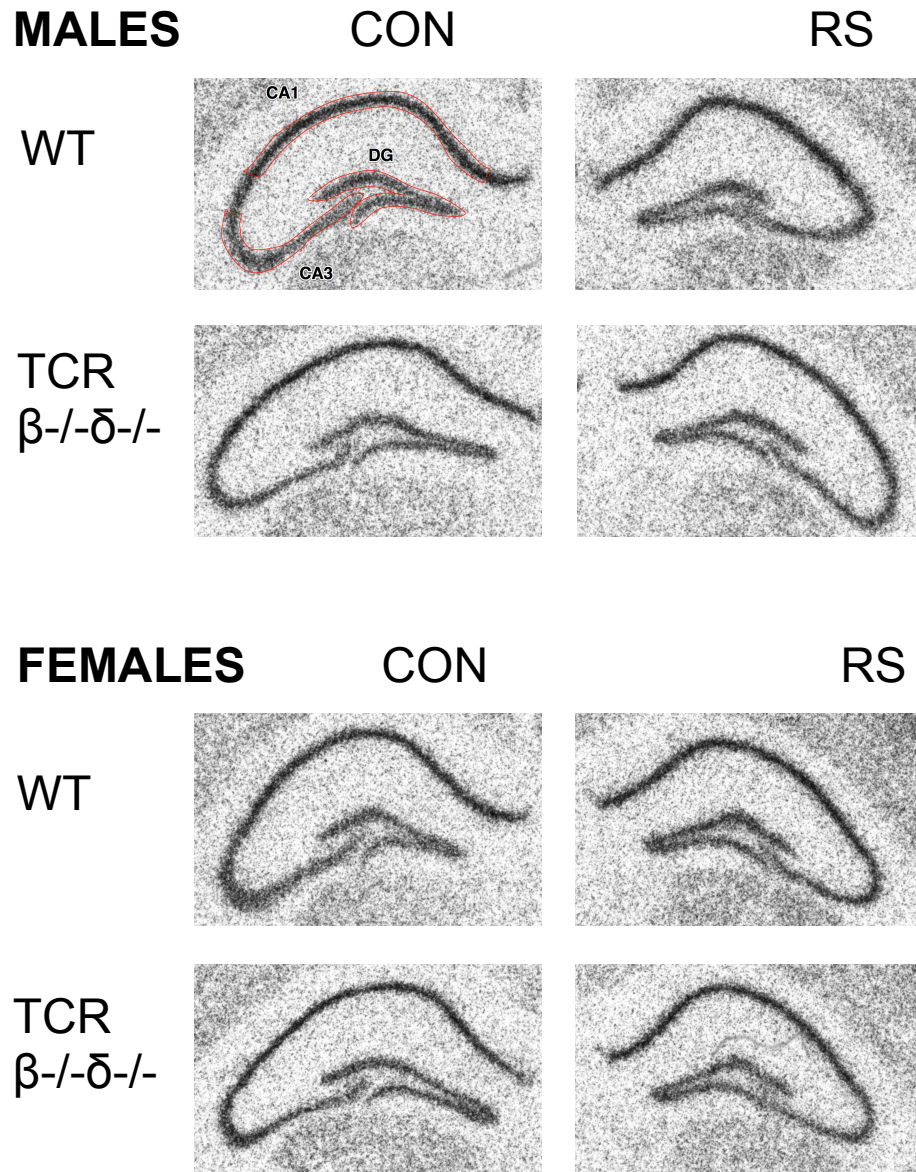


Figure 27. Representative film autoradiographs show NMDA receptor subunit NR2B mRNA expression in the hippocampus (Hip) in WT and *TCR $\beta^{-/-}\delta^{-/-}$* , males and females, and unstressed (CON) and restraint stressed (RS) mice. Target regions are outlined in red. No significant differences were observed.

NR2B mRNA in the CA1, CA3, and DG regions. Within the CON females, *TCRβ*^{-/-δ} mice expressed significantly higher levels of NR2A mRNA in the DG compared to WT females ($t=2.36$, $df=14$, $p=0.034$). RS *TCRβ*^{-/-δ} females ($t=2.28$, $df=14$, $p=0.039$) and RS *TCRβ*^{-/-δ} males ($t=2.94$, $df=15$, $p=0.010$) expressed significantly higher NR2A mRNA expression in the DG than did sex-matched RS WT mice.

Representative images of NR1 (Figure 28), NR2A (Figure 29), and NR2B (Figure 30) mRNA expression in the CeA are provided. In the CeA, there was a main effect of treatment ($F_{(1,64)}=15.9$, $p<0.001$) and a 3-way interaction between genotype, sex and treatment was observed ($F_{(1,65)}=8.89$, $p=0.004$). Pairwise comparisons revealed that NR2A mRNA ($t=3.35$, $df=15$, $p=0.004$) and NR2B mRNA ($t=3.09$, $df=15$, $p=0.007$) expression were elevated in the CeA of CON *TCRβ*^{-/-δ} females compared to CON WT females, though no differences were observed in the expression of NR1 mRNA in this region. Furthermore, RS treated WT females had increased expression of NR2A mRNA compared to CON WT females ($t=8.94$, $df=17$, $p<0.001$). Interestingly, *TCRβ*^{-/-δ} females exposed to RS expressed less NR2A mRNA in the CeA compared to RS treated WT females ($t=2.70$, $df=14$, $p=0.017$) and thus expressed levels of NR2A more similar to the WT CON females. The expression of NR2B mRNA in the CeA of RS treated *TCRβ*^{-/-δ} females was similar to WT CON at a level significantly lower than CON *TCRβ*^{-/-δ} females ($t=2.40$, $df=12$, $p=0.033$). In males however, there was

Table 4: mRNA expression profile for NMDA receptor (NR) subunit genes in wild type (WT and T cell receptor knock out mice (*TCRβ*^{-/-}*δ*^{-/-}) in amygdala subregions

		WT		TCRβ-/-δ-/-	
Brain Region	mRNA	CON	RS	CON	RS
Males					
CeA	NR1	460.8 ± 31.0	481.4 ± 39.1	479.9 ± 19.7	483.8 ± 28.5
	NR2A	464.8 ± 22.3	470.0 ± 6.6 ^d	452.3 ± 17.2	502.1 ± 27.4
	NR2B	1196.6 ± 74.6	1105.3 ± 36.2	1184.5 ± 53.6	1254.8 ± 42.7 ^{af}
LA	NR1	633.5 ± 30.7	803.7 ± 99.9	668.1 ± 72.1	547.7 ± 52.7 ^a
	NR2A	808.6 ± 27.1 ^c	827.9 ± 61.8	770.4 ± 16.5	846.4 ± 66.9
	NR2B	2579.2 ± 230.4	2292.1 ± 178.8	2634.1 ± 249.7	2981.3 ± 253.2 ^a
BLA	NR1	600.2 ± 31.9	789.3 ± 105.6 ^d	673.7 ± 75.9	514.9 ± 52.9 ^a
	NR2A	809.0 ± 37.8	853.7 ± 39.3	792.2 ± 21.9	787.5 ± 25.0
	NR2B	2423.9 ± 219.0	2054.2 ± 155.4	2350.6 ± 171.6	3002.0 ± 356.8 ^a
Females					
CeA	NR1	412.3 ± 24.8	473.1 ± 30.4	464.5 ± 23.7	477.9 ± 21.3
	NR2A	432.5 ± 5.2	536.7 ± 10.0 [*]	479.3 ± 13.7 [*]	496.3 ± 9.7 ^a
	NR2B	1048.1 ± 48.1	1121.3 ± 47.2	1392.2 ± 105.1 [*]	1078.7 ± 51.7 ^b
LA	NR1	618.1 ± 26.8	566.4 ± 68.2	645.1 ± 49.5	579.3 ± 59.3
	NR2A	701.6 ± 37.7	797.6 ± 19.9 [*]	768.0 ± 27.3	773.0 ± 28.5
	NR2B	2490.0 ± 197.8	2336.4 ± 185.4	2759.1 ± 429.4	2592.9 ± 290.3
BLA	NR1	596.5 ± 30.4	526.6 ± 59.9	653.4 ± 50.3	573.7 ± 58.6
	NR2A	769.7 ± 16.9	797.4 ± 23.0	804.1 ± 54.9	849.6 ± 61.9
	NR2B	2628.4 ± 284.8	2329.1 ± 125.2	2246.6 ± 297.9	2330.4 ± 291.5

NR subunits NR1, NR2A, and NR2B mRNA levels were measured in subregions of the amygdala- Central Amygdala (CeA), Lateral Amygdala (LA) and Basolateral Amygdala (BLA). Data is shown as the mean ± standard error. Significant differences were determined using t-test (p<0.05). Bolded text indicates significant differences.

* significant (p<0.05) compared to WT CON

^a significant (p<0.05) compared to WT RS

^b significant (p<0.05) compared to TCR CON

^c significant (p<0.05) compared to WT CON F

^d significant (p<0.05) compared to WT RS F

^f significant (p<0.05) compared to TCR RS F

increased NR2B mRNA expression in RS treated *TCRβ*^{-/-δ} mice over RS treated WT mice ($t=2.69$, $df=13$, $p=0.019$). No main effects of genotype, RS treatment, or sex were observed in NR1 or NR2A mRNA expression in the LA or BLA shown in Figure 31 and Figure 32, respectively. With regard to NR2B mRNA expression (Figure 33), there was a main effect of genotype ($F_{(1,64)}=6.1$, $p=0.016$) and a 3-way interaction of genotype by sex by treatment in the CeA ($F_{(1,63)}=9.6$, $p<0.003$). Additionally, there was a genotype by treatment effect on NR2B mRNA expression in the BLA ($F_{(1,57)}=4.2$, $p=0.046$).

Pairwise comparisons revealed sex and genotype differences in the expression of NR1 mRNA between groups. In the RS group, WT males had significantly higher expression of NR1 in the BLA ($t=2.28$, $df=16$, $p=0.037$) than did RS treated WT females. Comparatively, no sex differences were observed within the *TCRβ*^{-/-δ} mice. However, RS treated *TCRβ*^{-/-δ} mice did have altered expression of NR1 mRNA compared to RS treated WT mice. Compared to RS treated WT males, RS treated *TCRβ*^{-/-δ} males had significantly lower expression of NR1 in the LA ($t=2.17$, $df=13$, $p=0.049$) and BLA ($t=2.22$, $df=13$, $p=0.045$).

Sex differences were observed in pairwise comparisons of the WT mice that were not found in the *TCRβ*^{-/-δ} mice in NMDA receptor expression. In the WT CON group, males expressed increased NR2A mRNA in the LA compared to WT CON females ($t=2.25$, $df=15$, $p=0.040$). In the RS group, WT males had significantly

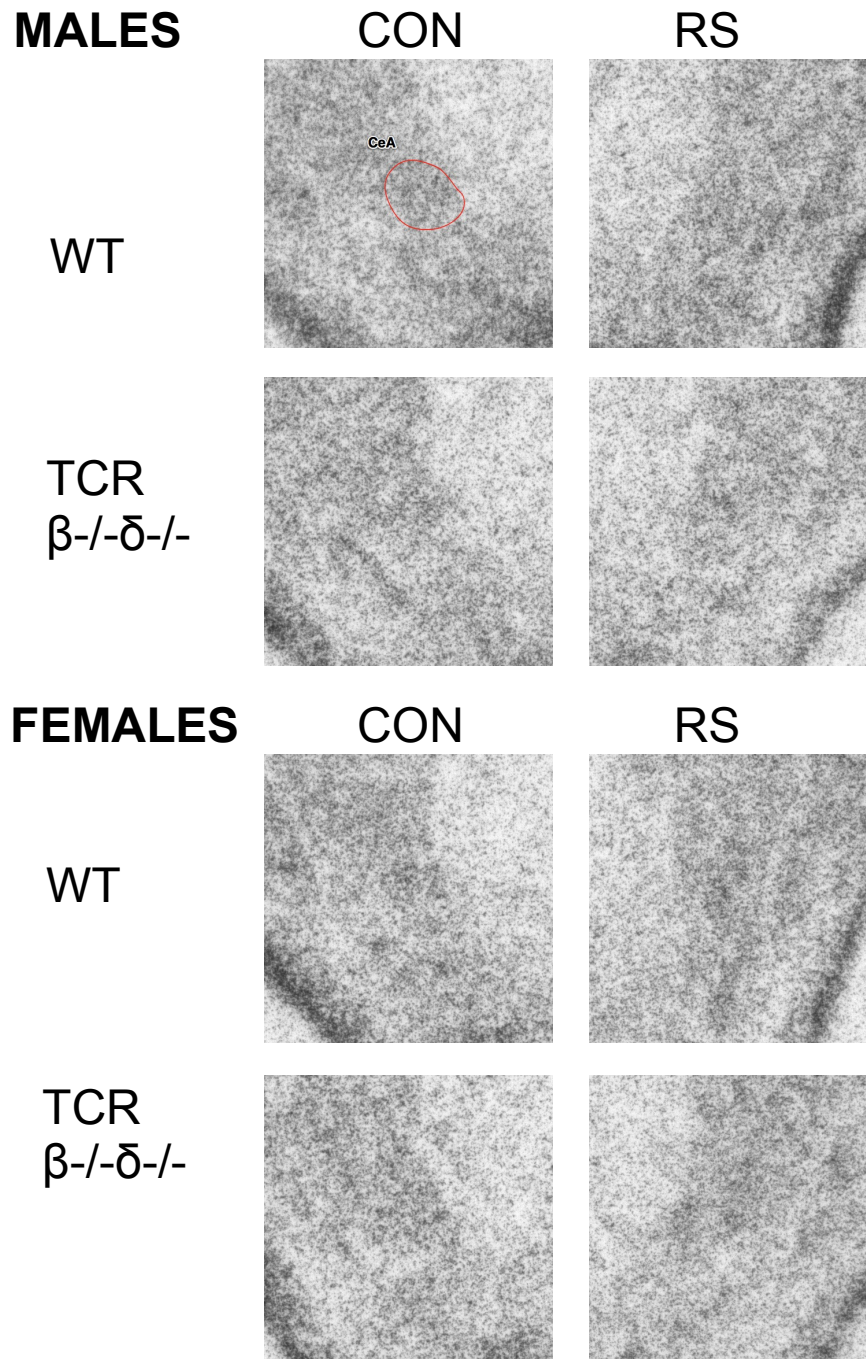


Figure 28. Representative film autoradiographs show NMDA receptor subunit NR1 mRNA expression in the central amygdala (CeA) in WT and *TCR $\beta^{-/-}\delta^{-/-}$* , males and females, and unstressed (CON) and restraint stressed (RS) mice. The CeA is outlined in red. No significant differences were observed.

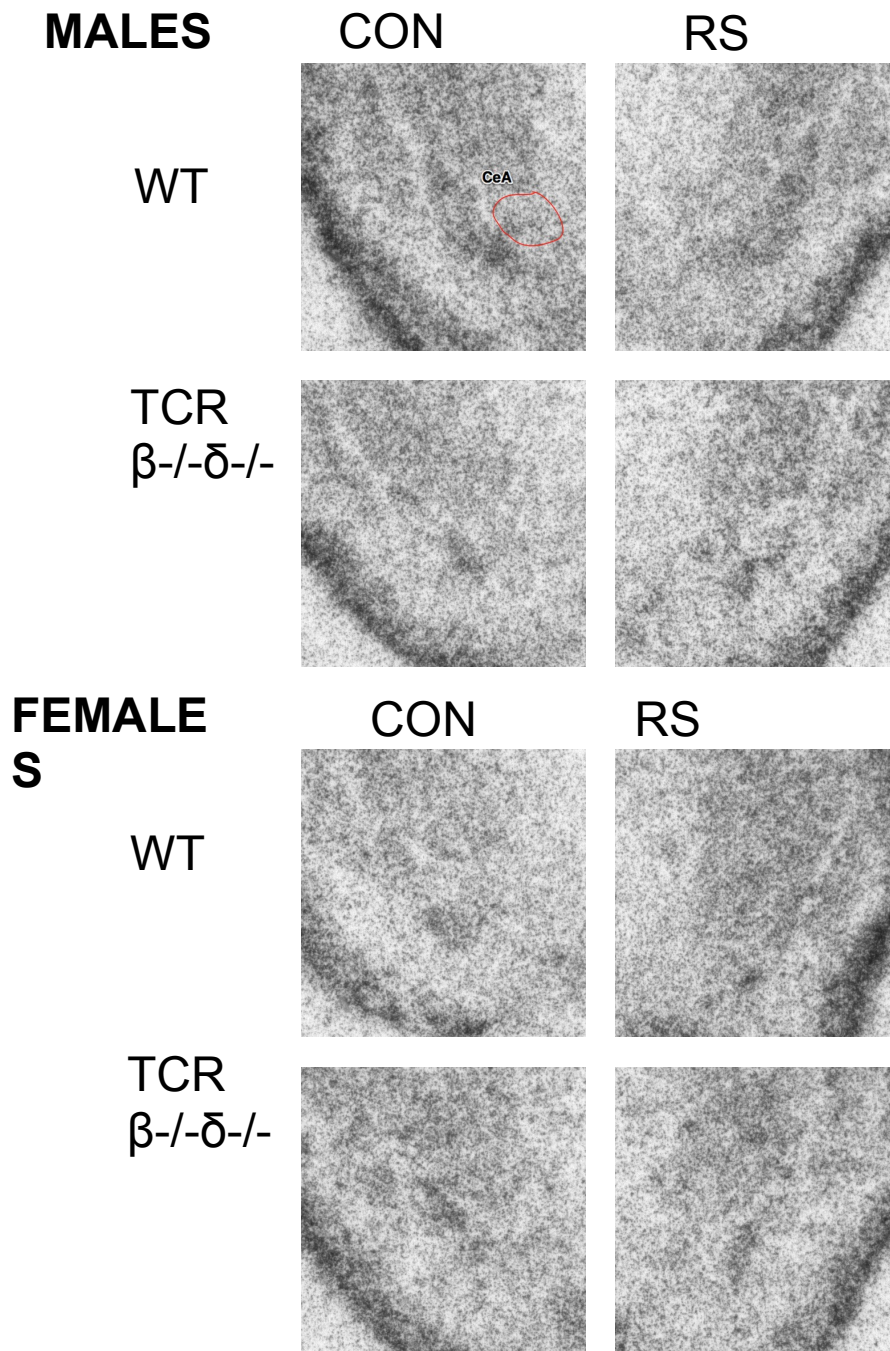


Figure 29. Representative film autoradiographs show NMDA receptor subunit NR2A mRNA expression in the central amygdala (CeA) in WT and *TCRβ-/-δ-/-*, males and females, and unstressed (CON) and restraint stressed (RS) mice. The CeA is outlined in red. The NR2A subunit mRNA expression was elevated in the CeA of RS WT and RS *TCRβ-/-δ-/-* females compared to CON WT and CON *TCRβ-/-δ-/-*, respectively. CON *TCRβ-/-δ-/-* females had higher expression for NR2A subunit mRNA than CON WT females. RS WT males expressed significantly less NR2A subunit mRNA compared to RS WT females.

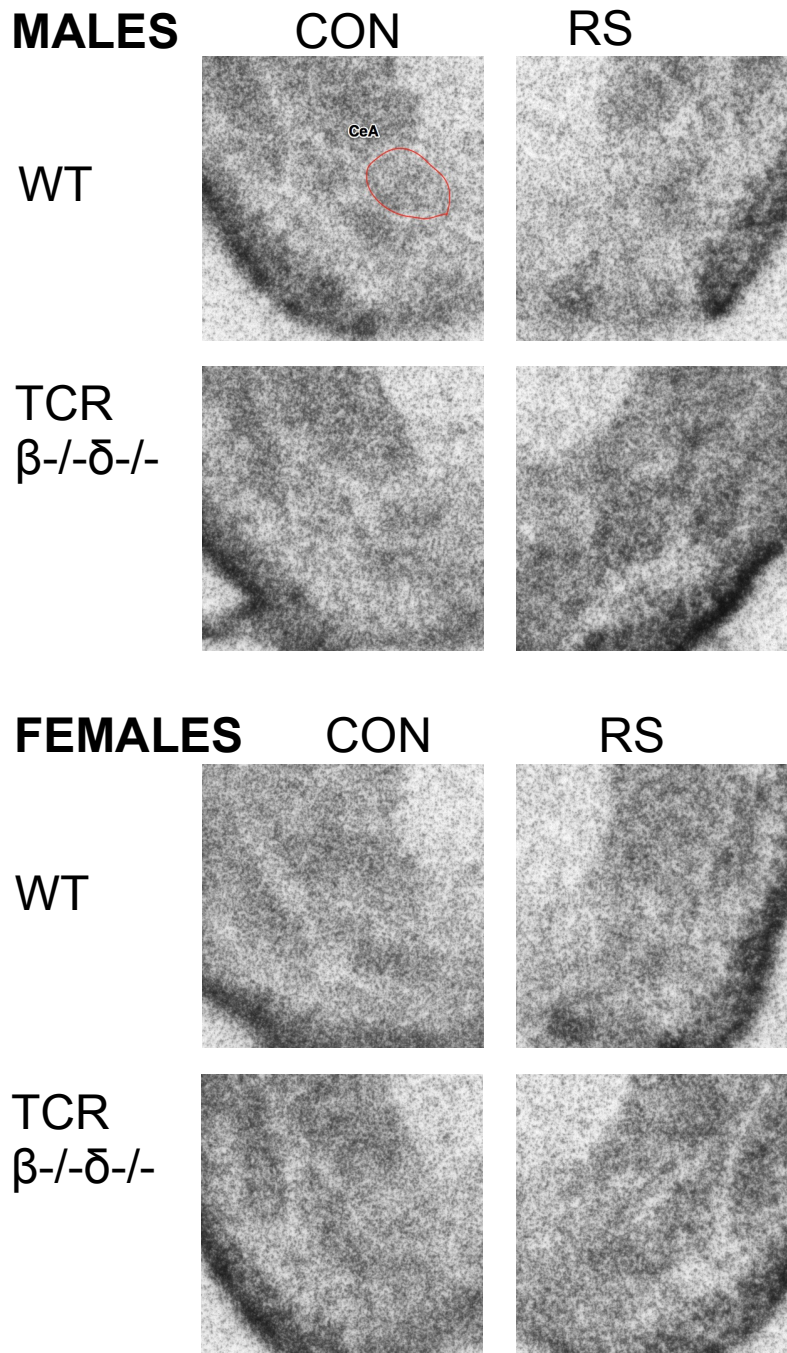


Figure 30. Representative film autoradiographs show NMDA receptor subunit NR2B mRNA expression in the central amygdala (CeA) in WT and *TCR $\beta^{-/-}\delta^{-/-}$* , males and females, and unstressed (CON) and restraint stressed (RS) mice. The CeA is outlined in red. In females, NR2B subunit mRNA expression was significantly higher in CON *TCR $\beta^{-/-}\delta^{-/-}$* compared to CON WT, but significantly lower in RS *TCR $\beta^{-/-}\delta^{-/-}$* compared to CON *TCR $\beta^{-/-}\delta^{-/-}$* . Additionally, RS *TCR $\beta^{-/-}\delta^{-/-}$* males expressed significantly higher levels of NR2B subunit compared to both RS WT males and RS *TCR $\beta^{-/-}\delta^{-/-}$* females.

lower expression of NR2A in the CeA than WT females ($t=5.26$, $df=16$, $p<0.001$), but no differences were observed between RS $TCR\beta^{-/-}\delta^{-/-}$ males and females. NR2A mRNA expression was also different between genotypes and treatment in the CeA. RS treated WT females expressed higher levels of NR2A mRNA expression in the LA ($t=2.32$, $df=17$, $p=0.033$) compared to CON WT females. This difference was not observed in WT males or $TCR\beta^{-/-}\delta^{-/-}$ mice. Finally, RS treated WT females expressed significantly higher levels of NR2A mRNA compared to CON WT females in both the CeA ($t=8.942$, $df=17$, $p<0.001$) and the LA ($t=2.321$, $df=17$, $p=0.033$). CON $TCR\beta^{-/-}\delta^{-/-}$ females expressed significantly higher levels of NR2A than CON WT females ($t=3.094$, $df=13$, $p=0.004$). In contrast, the RS treated $TCR\beta^{-/-}\delta^{-/-}$ females expressed significantly less NR2A mRNA in the CeA than RS treated WT females ($t=2.697$, $df=14$, $p=0.017$).

NR2B expression was altered in $TCR\beta^{-/-}\delta^{-/-}$ females basally in the CeA. CON $TCR\beta^{-/-}\delta^{-/-}$ females expressed significantly more NR2B mRNA in the CeA than CON WT females ($t=3.094$, $df=15$, $p=0.007$). Interestingly, NR2B mRNA expression was significantly lower in RS treated $TCR\beta^{-/-}\delta^{-/-}$ females compared to CON $TCR\beta^{-/-}\delta^{-/-}$ females ($t=2.405$, $df=12$, $p=0.033$). Conversely, NR2B expression in the CeA was elevated in RS treated $TCR\beta^{-/-}\delta^{-/-}$ males compared to both RS treated WT males ($t=2.691$, $df=13$, $p=0.019$) as well as RS treated $TCR\beta^{-/-}\delta^{-/-}$ females ($t=2.652$, $df=11$, $p=0.022$). Finally, NR2B mRNA expression was also elevated in RS treated $TCR\beta^{-/-}\delta^{-/-}$ males compared to RS treated WT males in the LA ($t=2.27$, $df=11$, $p=0.044$) and BLA ($t=2.57$, $df=11$, $p=0.026$).

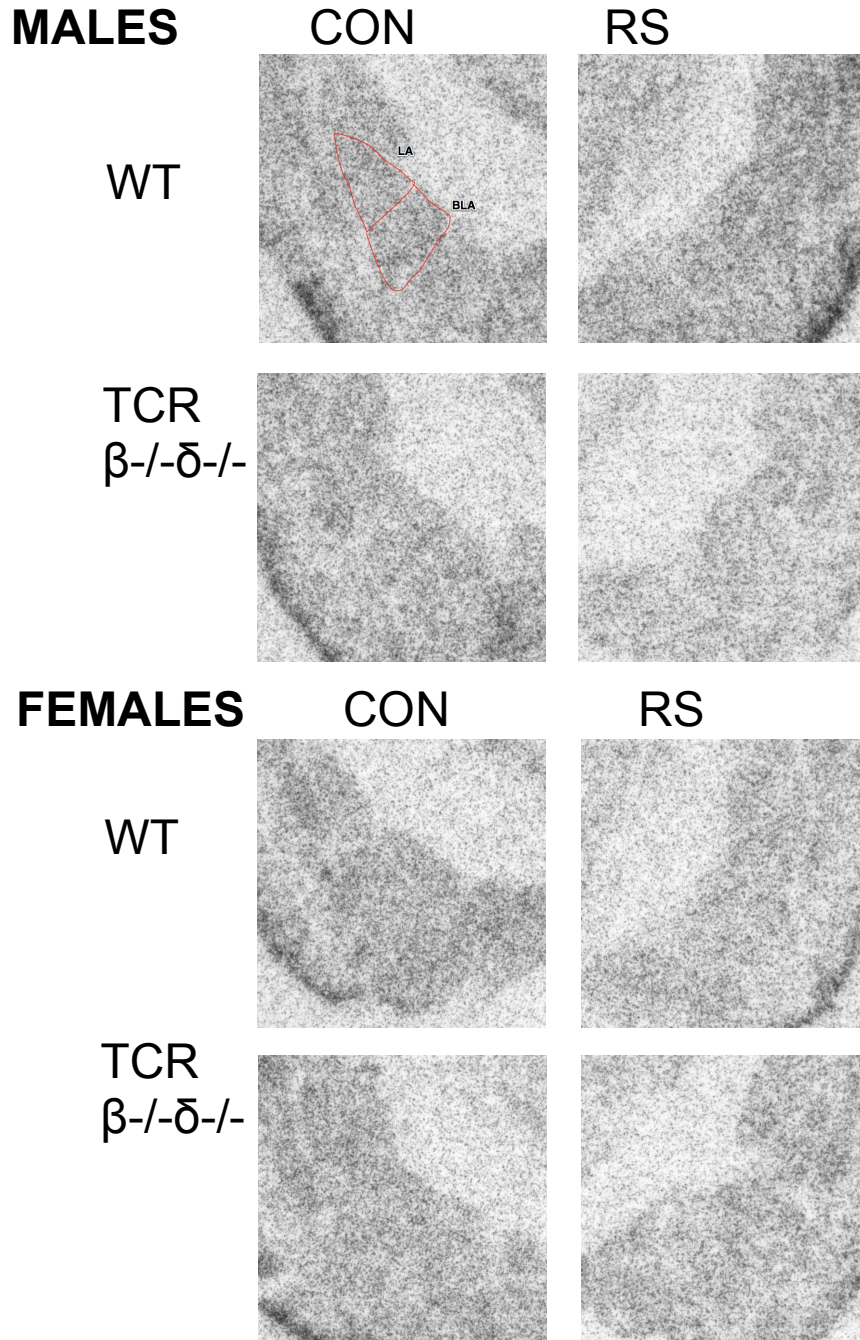


Figure 31. Representative film autoradiographs show NMDA receptor subunit NR1 mRNA expression in the lateral amygdala (LA) and basolateral amygdala (BLA) in WT and *TCRβ-/-δ-/-*, males and females, and unstressed (CON) and restraint stressed (RS) mice. Target regions are outlined in red. In the LA and BLA, RS WT males expressed significantly higher levels of NR1 subunit mRNA than RS *TCRβ-/-δ-/-* males which expressed NR1 subunit mRNA at a level comparable to the CON mice. RS WT males also had higher expression of NR1 receptor subunit mRNA compared to RS WT females in the BLA.

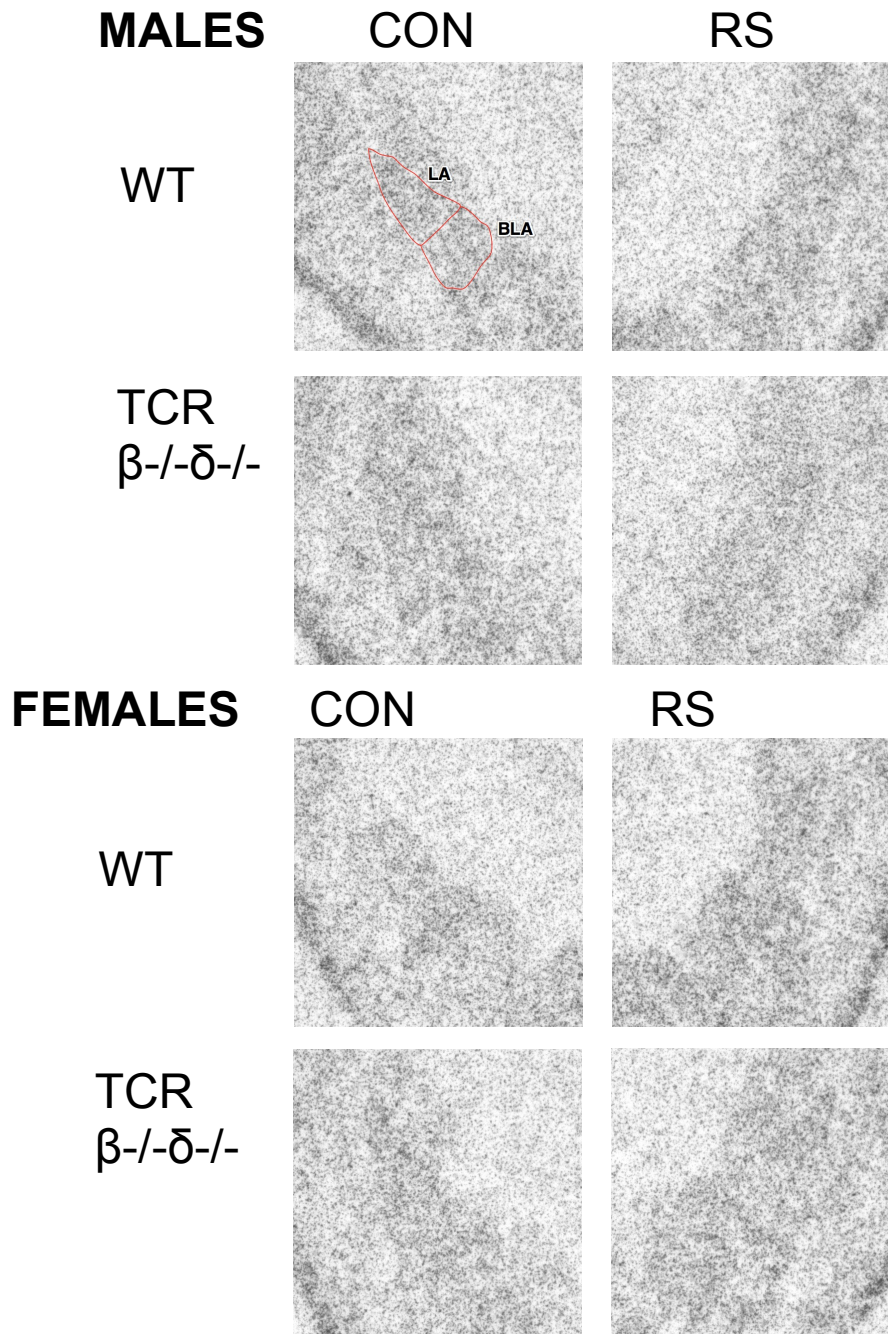


Figure 32. Representative film autoradiographs show NMDA receptor subunit NR2A mRNA expression in the lateral amygdala (LA) and basolateral amygdala (BLA) in WT and *TCRβ-/-δ-/-*, males and females, and unstressed (CON) and restraint stressed (RS) mice. Target regions are outlined in red. CON WT males expressed significantly higher levels of NR2A subunit mRNA compared to CON WT females in the LA. Additionally, RS treated WT females expressed higher levels of NR2A subunit mRNA than CON WT females in the LA.

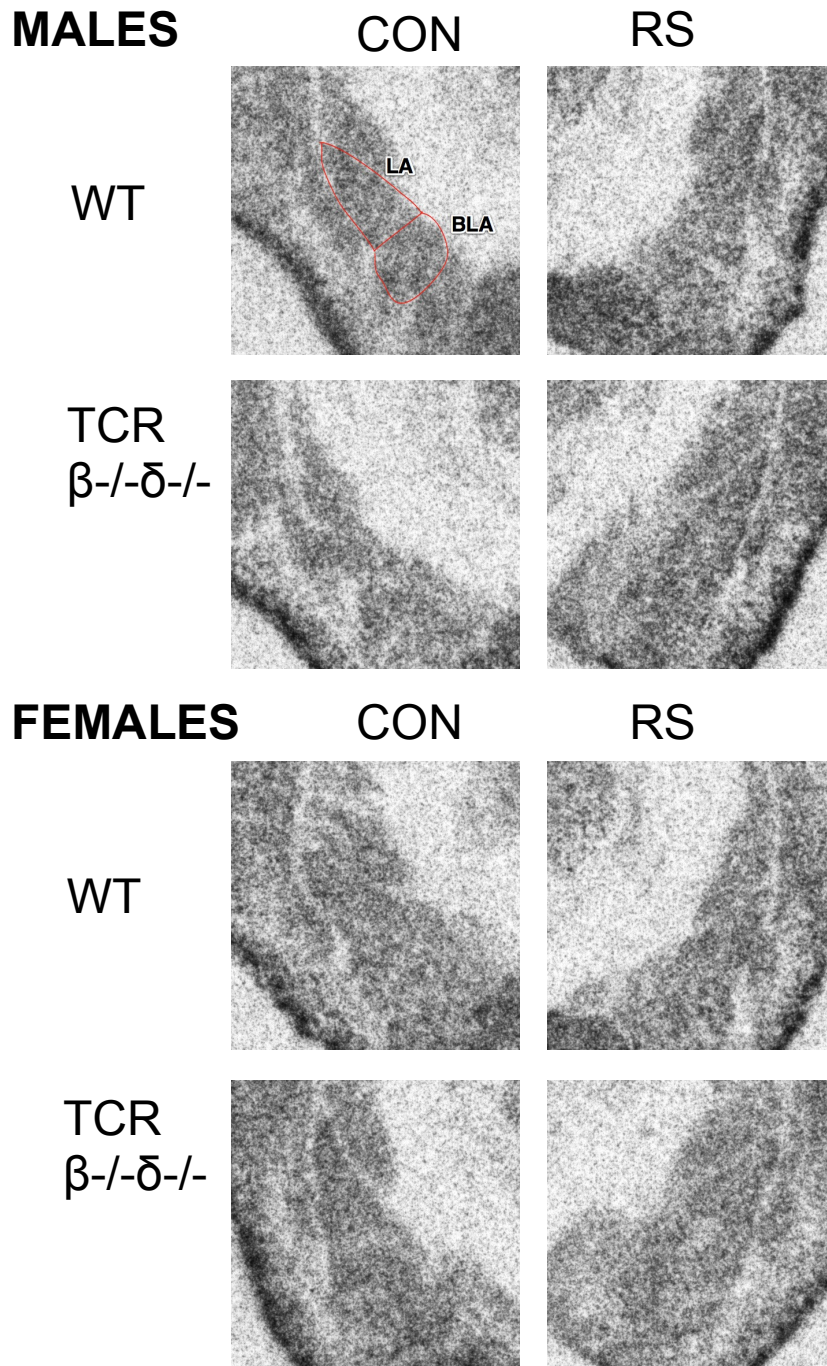


Figure 33. Representative film autoradiographs show NMDA receptor subunit NR2B mRNA expression in the lateral amygdala (LA) and basolateral amygdala (BLA) in WT and *TCR $\beta^{-/-}\delta^{-/-}$* , males and females, and unstressed (CON) and restraint stressed (RS) mice. Target regions are outlined in red. In RS mice, *TCR $\beta^{-/-}\delta^{-/-}$* males expressed higher levels of NR2B subunit mRNA in the LA and BLA. No differences were observed in females.

3.6. Summary of Experiment #2

This experiment was conducted to assess the response of $TCR\beta^{-/-}\delta^{-/-}$ mice to RS. Peripheral changes in plasma CORT as well as CNS changes in the expression of genes that have been linked to stress reactivity was assessed including HPA axis gene expression, serotonergic and NMDA receptors. Basally, $TCR\beta^{-/-}\delta^{-/-}$ males had higher levels of plasma CORT than WT males. In females, the mean plasma CORT level of $TCR\beta^{-/-}\delta^{-/-}$ mice was double the mean plasma CORT level of WT mice; however, this did not reach statistical significance. As expected, all groups had higher plasma CORT following RS demonstrating normal stress reactivity to RS in the periphery.

Differences in the expression of central stress related genes in may reflect behavioural vulnerabilities and predispositions. Both male and female $TCR\beta^{-/-}\delta^{-/-}$ mice were susceptible to the effects of RS but not with respect to the same genes. This is particularly interesting as $TCR\beta^{-/-}\delta^{-/-}$ males and females displayed a reduced anxiety-like phenotype but there were differences in how that phenotype was expressed in the open field, light/dark box, and EPM.

Notably, there was also a loss of sexually dimorphic expression of several genes in both the CON and RS conditions in $TCR\beta^{-/-}\delta^{-/-}$ mice compared to WT mice. This is particularly relevant in the context of the loss of sexually dimorphic behaviours we observed in the aforementioned behaviour tests. Loss of sexually dimorphic gene expression was observed with serotonergic receptor mRNAs,

NMDA receptor mRNAs, glucocorticoid receptor MR mRNA, and CRHR2 mRNA suggesting that T lymphocytes could influence sex differences in behaviour through regulation of stress related genes in the CNS.

Experiment #3

Innate Immune Response in $TCR\beta^{-/-}\delta^{-/-}$ mice

3.7. Overview of Experiment #3

Upregulation of neuronal Class I MHC expression following immune challenge with LPS, both centrally and peripherally, has been reported suggesting that Class I MHC mediates the stress response (Foster et al., 2002). Subsequently, work in our laboratory (Sankar et al., 2012) demonstrated that functional loss of Class I MHC through knockout of β 2-Microglobulin (β 2M) and Transporter associated with Antigen Processing (TAP) resulted in reduced activity and increased risk assessment behaviour. Following injection of lipopolysaccharide (LPS), there was a decrease in distance traveled in the open field in the LPS group compared to saline-injected controls in WT mice. However, in the β 2M $^{-/-}$ TAP $^{-/-}$ mice, saline injection also led to decreased activity compared to the controls that did not receive an injection suggestive of a behavioural stress response to the injection alone. Consistent with this behavioural effect, the plasma concentration of CORT in saline treated β 2M $^{-/-}$ TAP $^{-/-}$ females was similar to the concentration of CORT found in the LPS treated mice. Neuronal class I MHC expression has been found developmentally and established to be important to neuronal plasticity and synaptic pruning (Huh et al., 2000; Furgeaud et al., 2010). However, due to an additional deficit of CD8 $^{+}$ T

lymphocytes in $\beta 2M^{-/-}TAP^{-/-}$ mice, it was unclear if the altered stress response to innate immune challenge was due to loss of neuronal Class I MHC or CD8+ T lymphocytes.

The $TCR\beta^{-/-}\delta^{-/-}$ mice are an ideal candidate to differentiate the role of neuronal class I MHC expression and the role of adaptive immune deficiency. $TCR\beta^{-/-}\delta^{-/-}$ mice lack functional T cells but in contrast to the class I MHC genes, the β and δ chains of the T Cell Receptor are not expressed in the CNS. Mice were exposed to the open field test weekly for 3 weeks. On the final trial, mice were injected with saline or LPS and blood samples were collected 2 hours later to examine plasma CORT levels.

3.8. Results of Experiment #3

Open Field. $TCR\beta^{-/-}\delta^{-/-}$ and WT mice were tested in the open field at 12 and 13 w of age to examine within and between test habituation. In both the novel (week 12) and habituation (week 13) tests, an effect of genotype was observed. While distance traveled decreased over the 30 min test in all groups, $TCR\beta^{-/-}\delta^{-/-}$ mice traveled greater distances compared to WT mice across the 30 minutes of testing (Bonferroni post-hoc test, $*p < 0.05$) in comparison to both WT males (Figure. 34A. $F_{(1,230)} = 29.77$, $p < 0.0001$) and WT females (Figure. 34B. $F_{(1,250)} = 40.5$, $p < 0.0001$). At 13 weeks of age, the open field was conducted again to examine habituation to the test. Again, a main effect of genotype was observed in which $TCR\beta^{-/-}\delta^{-/-}$ males (Figure. 34A. $F_{(1,230)} = 9.2$, $p = 0.004$) and females (Figure. 34B. $F_{(1,250)} = 7.2$,

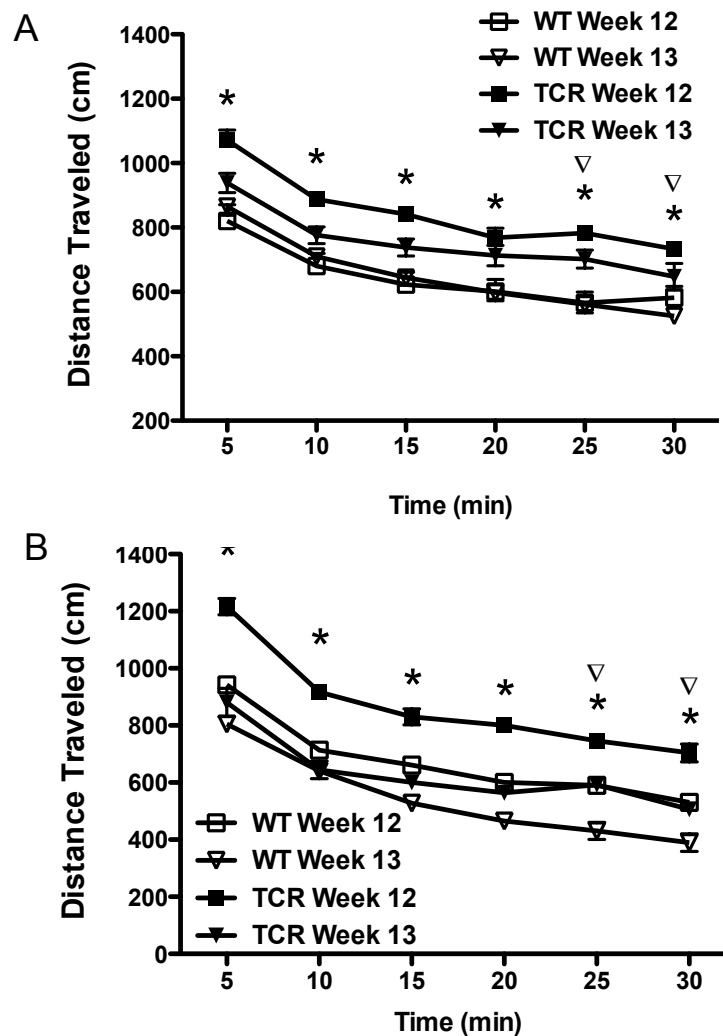


Figure 34. The open field test was conducted at 12 and 13 weeks of age to assess habituation. *TCR β-/-δ-/-* males (A) and females (B) showed significantly higher locomotor activity shown here as distance traveled compared to WT mice both in Week 12 (novel open field test) and Week 13 (habituation, repeat test). A greater reduction in distance traveled was observed between Week 12 and Week 13 in *TCR β-/-δ-/-* mice of both sexes than in WT mice, though both genotypes traveled less distance in Week 13. *p<0.05 WT vs *TCR β-/-δ-/-* WK12; ∇p<0.05 WT vs *TCR β-/-δ-/-* WK13; ■p<0.05 WT WK12 vs WT WK13; ◆p<0.05 *TCR β-/-δ-/-* WK12 vs *TCR β-/-δ-/-* WK13

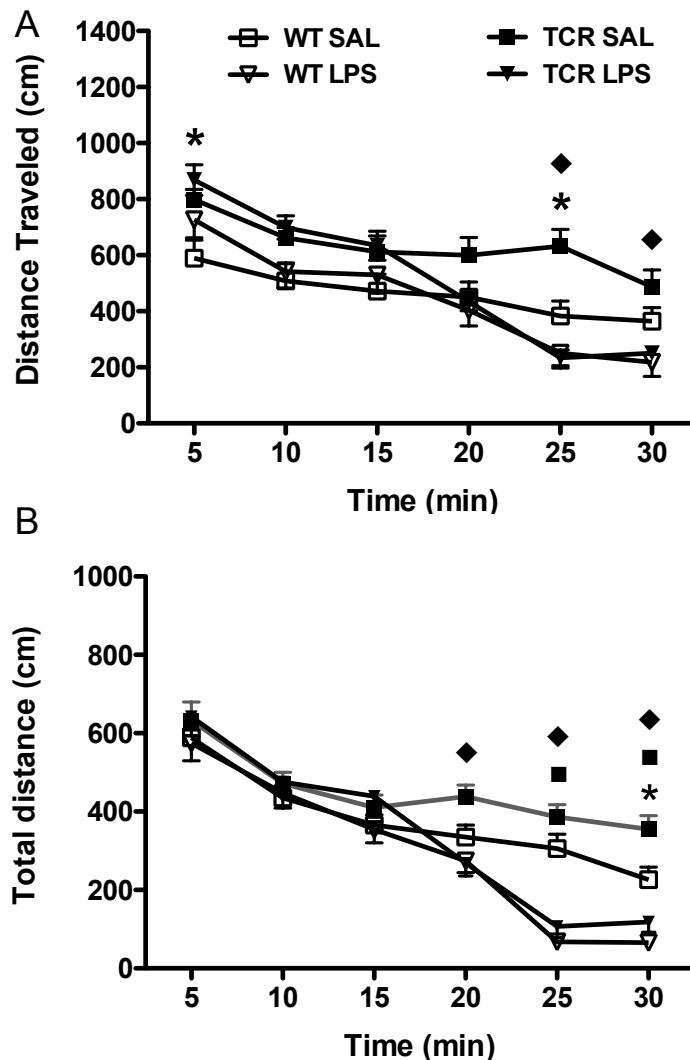


Figure 35. The open field test was conducted immediately following intraperitoneal injection of 1 mg/kg lipopolysaccharide (LPS) or an equivalent volume of saline (SAL). *TCR* $\beta^{-/-}\delta^{-/-}$ males (A) showed significantly higher locomotor activity than WT males in the SAL group (* $p < 0.05$). Additionally, LPS treated *TCR* $\beta^{-/-}\delta^{-/-}$ (◆ $p < 0.05$) but not WT mice traveled less distance following LPS. In females, LPS treatment lead to reduced distance traveled in both WT (■ $p < 0.05$) and *TCR* $\beta^{-/-}\delta^{-/-}$ (◆ $p < 0.05$) mice compared to genotype matched SAL controls. *TCR* $\beta^{-/-}\delta^{-/-}$ SAL mice also traveled a greater distance than WT SAL (* $p < 0.05$).

$p=0.0097$) traveled a greater distance than their age- and sex-matched WT controls ($\nabla p<0.05$).

The innate immune response of $TCR\beta^{-/-}\delta^{-/-}$ mice was examined by injecting mice with either LPS or saline followed by open field testing for activity changes. The results are depicted for males (Figure. 35A) and females (Figure. 35B). An interaction effect between group and time was found in males ($F_{(15,225)}=6.4$, $p<0.0001$). Bonferroni post-hoc testing revealed that male $TCR\beta^{-/-}\delta^{-/-}$ SAL mice travelled greater distances than the WT saline males ($*p<0.05$). Also, LPS treated $TCR\beta^{-/-}\delta^{-/-}$ males traveled less distance than saline treated $TCR\beta^{-/-}\delta^{-/-}$ males ($wp<0.05$). In females, there was a main effect of group ($F_{(3,250)}=6.6$, $p=0.0008$) and an interaction effect between group and time ($F_{(15,250)}=8.8$, $p<0.0001$). Bonferroni post-hoc testing revealed decreased distance traveled following LPS injection in both WT ($\blacksquare p<0.05$) and $TCR\beta^{-/-}\delta^{-/-}$ females ($up<0.05$).

Corticosterone (CORT) Analysis. CORT levels were assessed 2 hours following injection of LPS or saline (Figure 36). As expected, there was a main effect of LPS injection on the plasma levels of CORT ($F_{(1,99)}=45.75$, $p<0.001$) with elevated CORT levels observed in LPS treated mice. Additionally, there was a main effect of sex with higher levels of plasma CORT in females ($F_{(1,99)}=9.69$, $p=0.002$). No interaction effects between genotype, sex or LPS treatment were observed.

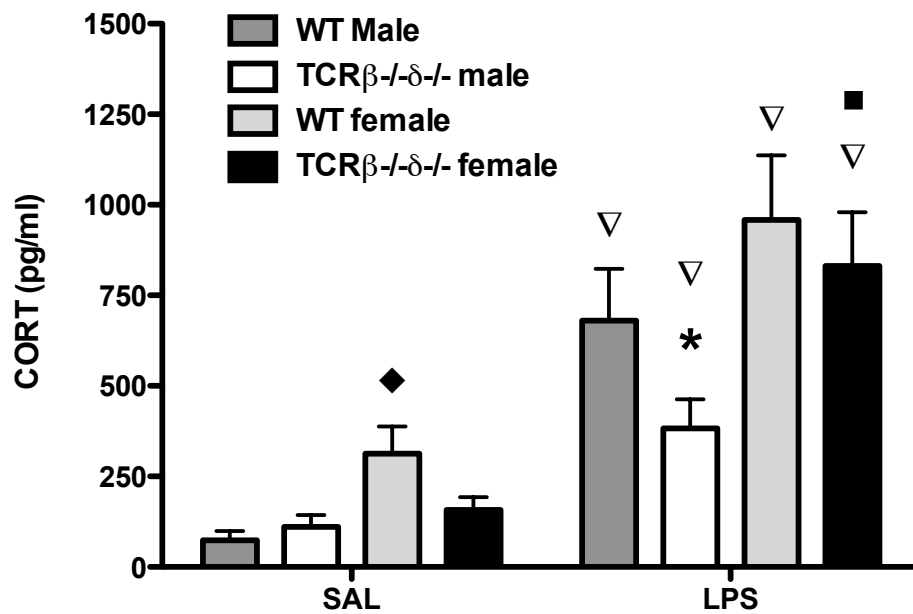


Figure 36. WT females had significantly higher plasma CORT compared to WT males (◆ $p<0.05$) in the saline treated group, though no effects of genotype were observed. In the LPS treated mice there was a significant elevation in plasma CORT over saline treated ($\nabla p<0.05$) mice 2 hours after injection in all groups except *TCR* β ^{-/-} δ ^{-/-} males which had significantly less plasma CORT than LPS treated WT males (* $p<0.05$). RS *TCR* β ^{-/-} δ ^{-/-} females had significantly higher plasma CORT than RS *TCR* β ^{-/-} δ ^{-/-} males (■ $p<0.05$).

Pairwise t-tests revealed additional differences between groups. Within the saline (SAL) treated control group, WT females had significantly elevated plasma CORT ($t=3.349$, $df=24$, $p=0.0027$). Additionally, in all groups treated with LPS, there was higher plasma CORT levels compared to sex- and genotype-matched SAL groups. This elevation reached statistical significance in all groups: WT males ($t=4.173$, $df=24$, $p<0.001$), WT females ($t=3.846$, $df=24$, $p<0.001$), $TCR\beta^{-/-}\delta^{-/-}$ males ($t=3.141$, $df=20$, $p=0.005$), and $TCR\beta^{-/-}\delta^{-/-}$ females ($t=4.167$, $df=21$, $p<0.001$). Finally, there was a sex difference present in $TCR\beta^{-/-}\delta^{-/-}$ mice that was not present in WT mice. LPS treated $TCR\beta^{-/-}\delta^{-/-}$ females had significantly higher plasma CORT than LPS treated $TCR\beta^{-/-}\delta^{-/-}$ males ($t=2.597$, $df=21$, $p=0.017$).

3.9. Summary of Experiment #3

TCRβ^{-/-δ} mice again demonstrated increased activity compared to WT control mice; however, they also exhibited normal habituation to repeated testing in the open field tests observed as a reduction in distance travelled at 13 weeks of age than at 12 weeks of age when they were first exposed to the paradigm. As expected, treatment with LPS led to reduced distance traveled in both WT and *TCRβ*^{-/-δ} males and females. Unlike the *β2M*^{-/-} *TAP*^{-/-} females, in which the response to LPS was indistinguishable from the saline response, LPS treatment in *TCRβ*^{-/-δ} females lead to reduced activity in comparison to the saline group. In addition to the behavioural differences between *TCRβ*^{-/-δ} and *β2M*^{-/-} *TAP*^{-/-} mice, plasma CORT levels in response to saline and LPS were different. Whereas *β2M*^{-/-} *TAP*^{-/-} females had equally high plasma CORT levels following LPS or saline injection, suggesting a stress response to the injection itself, *TCRβ*^{-/-δ} females had elevated plasma CORT from treatment with LPS compared to saline controls. These results support the interpretation that stress reactivity is mediated centrally by the presence of class I MHC in the brain rather than the adaptive immune deficits associated with these genetic knockout models.

CHAPTER 4. DISCUSSION

4.1. Summary of Findings

The influence of T lymphocytes on the brain and behaviour was explored using behavioural and molecular analyses. Specifically, anxiety and learning behaviours were assessed using standard tests of mouse anxiety-like behaviour, associative fear learning, and spatial learning. Additionally, stress responsiveness within mice lacking T lymphocytes was studied following repeated restraint stress and innate immune challenge. Alterations to gene expression profiles within anxiety, stress, and fear-related systems within the CNS were determined using *in situ* hybridization.

The use of mice that lack either T lymphocytes through the knockout of T cell receptor chains β and δ ($TCR\beta^{-/-}\delta^{-/-}$), as well as subsequent use of mice lacking only B lymphocytes through knockout of the immunoglobulin μ chain (μMT), enabled independent analyses of the influences of lymphocytes on behaviour. While there was slightly reduced activity behaviour in $\mu MT^{-/-}$ mice in the EPM, there were no differences in anxiety-like behaviour in $\mu MT^{-/-}$ mice. The absence of altered anxiety-like behaviour in $\mu MT^{-/-}$ mice and the presence of a reduced anxiety-like phenotype in $TCR\beta^{-/-}\delta^{-/-}$ mice supports the hypothesis that a deficit of T lymphocytes leads to a reduction in anxiety-like behaviour. We also observed an increased fear response in $TCR\beta^{-/-}\delta^{-/-}$ mice but no changes in spatial learning behaviour in the Morris Water Maze indicating that while $TCR\beta^{-/-}$

$\delta^{-/-}$ mice demonstrated increased fear in both cued and contextual learning tasks, they did not exhibit global learning deficits. Importantly, there was a loss of sex differences in anxiety-like and exploratory behaviour in $TCR\beta^{-/-}\delta^{-/-}$ mice suggesting that crosstalk between T lymphocytes and the brain influences the development of sex differences in behaviour.

Stress reactivity was examined in $TCR\beta^{-/-}\delta^{-/-}$ mice both following repeated restraint stress and an acute innate immune challenge. Analyses of plasma CORT levels of $TCR\beta^{-/-}\delta^{-/-}$ mice revealed elevated basal CORT indicative of HPA axis dysregulation. The plasma CORT levels in mice exposed to repeated stress were similar between $TCR\beta^{-/-}\delta^{-/-}$ and WT mice. In WT mice the acute stress response to lipopolysaccharide (LPS) injection led to elevated CORT levels in males and females. However, in $TCR\beta^{-/-}\delta^{-/-}$ mice, LPS treatment lead to elevated CORT in females compared to males demonstrating a sex by genotype interaction to acute stress. The behavioural response to LPS challenge was also examined within the open field test. One of the hallmarks of sickness behaviour in mice is reduced activity (Swiergiel and Dunn, 2007; Pitychoutis et al., 2009; Salazar et al., 2012). A reduction in distance traveled was observed in both WT and $TCR\beta^{-/-}\delta^{-/-}$ mice treated with LPS compared to those injected with saline indicating that both genotypes displayed a normal behavioural response to LPS. There were, however, differences between $TCR\beta^{-/-}\delta^{-/-}$ and WT mice as saline treated $TCR\beta^{-/-}\delta^{-/-}$ males had increased activity levels compared to saline treated WT males. This effect was independent of the stress response as it was

only observed within saline controls and confirms our earlier behavioural findings in which $TCR\beta^{-/-}\delta^{-/-}$ males traveled greater distance than WT males in the open field test. Therefore, $TCR\beta^{-/-}\delta^{-/-}$ mice exhibited a normal behavioural response to the acute stressor of LPS challenge.

While chronic stress in the form of repeated restraint stress did not lead to differences in plasma CORT between $TCR\beta^{-/-}\delta^{-/-}$ and WT, many changes were observed centrally in the expression of genes related to the stress circuitry, glutamatergic system, and serotonergic system indicating an altered vulnerability to long-term stressors. Furthermore, the effects of repeated restraint stress differed between males and females in WT and, to a lesser extent, $TCR\beta^{-/-}\delta^{-/-}$ mice, demonstrating different vulnerabilities to the effects of stress between the sexes. These results indicate that the adaptive immune system influences stress responsiveness and that sex differences may modify these immune influences.

4.2. The Adaptive Immune System Influences Anxiety-like Behaviour.

Our behavioural results provide evidence that loss of T lymphocytes led to reduced anxiety-like behaviour in mice. Moreover, this behavioural phenotype was observed across open field, light/dark, and EPM tests. These results add to the interpretation of behavioural phenotypes previously reported in other models of adaptive immune deficiency. Several groups have reported reduced anxiety-like behaviour in mice with adaptive immune deficits including studies examining $RAG1^{-/-}$ mice (Cushman et al., 2003), $rgs^{-/-}$ mice, and germ-free mice (Heijtz et

al.; Neufeld et al., 2011b; Clarke et al., 2012). Germ free (GF) mice have an underdeveloped adaptive immune system and, similar to $TCR\beta^{-/-}\delta^{-/-}$ mice, GF mice spent more time in the open arms of the EPM and entered these arms more frequently than specific pathogen free control mice indicative of reduced anxiety-like behaviour (Neufeld et al., 2011a). Immunocompromised $RAG1^{-/-}$ mice, which lack both T and B lymphocytes, exhibited increased activity observed as increased total movements during the open field test (Cushman et al., 2003). The outcome measure used to assess activity behaviour in $TCR\beta^{-/-}\delta^{-/-}$ mice was total distance traveled rather than total movements; however, the increased activity behaviour in $TCR\beta^{-/-}\delta^{-/-}$ male mice is in agreement with increased activity in $RAG1^{-/-}$ male mice. Cushman *et al.* (2003), also reported reduced anxiety-like behaviour in $RAG1^{-/-}$ mice indicated by increased entries into the center of the open field. Here we reported reduced anxiety-like behaviour in male $TCR\beta^{-/-}\delta^{-/-}$ mice in the open field defined as increased time spent in the center of the open field test. In contrast, mice lacking the gene for regulator of G protein signaling ($rgs2^{-/-}$), which leads to impaired T lymphocyte proliferation and IL-2 production in the presence of an immune stimulant, have an increased preference for the dark chamber of the light/dark box test over that of C57BL/6 WT controls, indicative of an increased anxiety-like behaviour phenotype (Oliveira-Dos-Santos et al., 2000). In our study, there were no significant differences between $TCR\beta^{-/-}\delta^{-/-}$ and WT mice for time spent in the dark chamber, although, it is notable that $TCR\beta^{-/-}\delta^{-/-}$ females spent significantly more time in the light chamber compared

to WT females. Although, there are many differences between these mice that could contribute to the different findings. Unlike *TCR β ^{-/-} δ ^{-/-}* mice that do not have any mature T lymphocytes, *rgs2^{-/-}* mice have normal levels of T lymphocytes basally (Oliveira-Dos-Santos et al., 2000). Furthermore, Oliveira-Dos-Santos *et al.* (2000), combined their data from male and female mice in the light/dark test, whereas we separated our data by sex for analysis and discovered an influence of T lymphocytes on time spent in the light chamber in female *TCR β ^{-/-} δ ^{-/-}* mice only. Finally, RGS is up-regulated within the CNS during events related to neural plasticity (Ingi et al., 1998) and *rgs2^{-/-}* mice have reduced spine density on neurons in the hippocampus (Kammermeier and Ikeda, 1999). Therefore, it is possible that the increased anxiety-like phenotype in *rgs2^{-/-}* mice was due to a non-immune neuronal function of RGS rather than an impairment of cellular immunity following an immune challenge.

Interestingly, despite the absence of the TCR β and δ chains in the CNS, a separate study of structural differences in the CNS of *TCR β ^{-/-} δ ^{-/-}* mice suggested that changes in brain structure may drive the behavioural phenotype reported within. *TCR β ^{-/-} δ ^{-/-}* mice were found to have sex specific alterations in limbic regions of the brain including the bed nucleus of the stria terminalis (BNST), amygdala, and periaqueductal gray (PAG). The BNST was larger in WT males compared to females but no difference was observed between *TCR β ^{-/-} δ ^{-/-}* females compared to males. The PAG was also smaller in WT females compared

to males, but was larger in *TCR β ^{-/-} δ ^{-/-}* females compared to males as well as WT females. Volumetric analysis of the amygdala also revealed a sex by genotype effect with increased volume in *TCR β ^{-/-} δ ^{-/-}* males compared to females. The BNST has neural projections to the PAG and this circuitry is associated with defensive behaviours (Holstege, 1992; Grupe et al., 2013) whereas the amygdala is more strongly associated with fear responses (LeDoux, 2000; Grupe et al., 2013). It is interesting that these limbic structures associated with emotional processing show sex by genotype interactions and it is likely that these differences contribute to the genotype and sex differences in behaviour that we observed in these studies.

Another important difference between mouse models of adaptive immune deficits is the extent of the immune deficiency. *RAG1^{-/-}* mice are deficient of both T and B lymphocytes, whereas *TCR β ^{-/-} δ ^{-/-}* mice lack only T lymphocytes. As such, the current work suggests a specific role for T lymphocytes in the development of anxiety-like behaviour. This interpretation is supported by the observation that *μ MT^{-/-}* mice, deficient of B lymphocytes did not show altered anxiety-like behaviour compared to WT mice. Interestingly, the reduced anxiety-like phenotype observed in *TCR β ^{-/-} δ ^{-/-}* mice was not observed in *β 2M^{-/-}TAP^{-/-}* mice. β 2M is a component of the class I major histocompatibility complex (MHC) binding cleft that allows for antigens to be presented to CD8⁺ T lymphocytes (Flajnik and Kasahara, 2001). The absence of β 2M and TAP subsequently results in loss of function of class I MHC (Ljunggren et al., 1990; Van Kaer et al., 1992)

and depleted CD8⁺ T lymphocytes (Ljunggren et al., 1995), whereas *TCR β ^{-/-} δ ^{-/-}* mice lack all subtypes of T lymphocytes. However, *β 2M^{-/-}TAP^{-/-}* mice displayed differences in behaviour in the open field and EPM test that indicated increased activity and exploration that was similar to the findings in *TCR β ^{-/-} δ ^{-/-}* mice.

Collectively, these studies suggest that the different subtypes of T lymphocytes have unique influences on behaviour.

4.3. Loss of Sexually Dimorphic Behaviour in Immune Compromised Mice.

A novel finding in this study and from a recently published study from our laboratory (Sankar et al., 2012) is the loss of sexually dimorphic behaviour in immune compromised mice. Because previous studies that reported on anxiety-like behaviour in immune compromised mice were conducted on one sex only, it is not possible to determine how universal our findings are in other immune compromised mouse models, yet it does further support the idea that sexual dimorphisms are an important consideration in studies of animal behaviour. In agreement with previous research, WT females were more active in the open field and the EPM tests than males (Palanza, 2001; Voikar et al., 2001). WT female mice also had reduced basal anxiety-like behaviour, which has previously been reported (Johnston and File, 1991; Rodgers and Cole, 1993; Voikar et al., 2001). However, sex differences were absent in both *TCR β ^{-/-} δ ^{-/-}* mice and *β 2M^{-/-}TAP^{-/-}* mice that lacked CD8⁺ T cells pointing to a role for T lymphocytes in the development of sexually dimorphic stress-related behaviour.

Previous studies on adaptive immune deficiency and behaviour also differ with respect to whether both sexes were included and separated in the analysis. For example, the study reporting reduced anxiety-like behaviour in *RAG1*^{-/-} mice only included males (Oliveira-Dos-Santos et al., 2000; Cushman et al., 2003) and the behavioural analyses on germ-free mice were only conducted on females (Neufeld et al., 2011a). Meanwhile, previous work with *rgs2*^{-/-} mice included both sexes in their studies but collapsed the data from male and female mice in their analysis of the light/dark box test (Oliveira-Dos-Santos et al., 2000). The experiments within this thesis clearly demonstrate sex differences in the manifestation of anxiety-like behaviours. In the open field, the reduced anxiety-like phenotype was only observed in *TCRβ*^{-/-δ}^{-/-} males whereas the reduced anxiety-like phenotype was exclusive to *TCRβ*^{-/-δ}^{-/-} females in the light/dark box test. Experiments with *β2M*^{-/-TAP}^{-/-} mice (Sankar et al., 2012) also found important sex differences in exploratory behaviour in the open field and EPM tests within the WT mice that were absent in *β2M*^{-/-TAP}^{-/-} mice. In experiments that use only one sex or that collapse the data across sexes, the loss of sexually dimorphic behaviour cannot be examined and it is therefore difficult to draw comparisons with the present experiments.

To date there is no clear understanding of the mechanisms underlying sexually dimorphic anxiety behaviour but our structural MRI scans of WT and *TCRβ*^{-/-δ}^{-/-} mice demonstrate neuroanatomical changes related to sex and genotype. Activity in the BNST region of the brain correlates with anxiety and

threat monitoring (Somerville et al., 2010) and is known to be much larger in males of several species including humans (Allen and Gorski, 1990) and rats (del Abril et al., 1987). Voxel-wise analysis showed that the BNST is larger in WT C57BL/6 male mice compared to females but there was no significant difference in BNST volume in *TCR β ^{-/-} δ ^{-/-}* mice. It is possible that a lack of sexual differentiation in the brain contributes to the loss of sexually dimorphic behaviour in models of adaptive immune deficiency.

There is also clinical evidence of sex differences in both immune function and psychiatric disorders. Sex differences in immune function are well established (Weinstein et al., 1984; Da Silva, 1999; De Leon-Nava et al., 2009) and contribute to gender differences in immune-related disorders such as infections and autoimmunity (Klein, 2000; Whitacre, 2001). Clinically this is an important consideration as sexual dimorphism influences the incidence, age of onset, and clinical presentation of mental illness (Andia et al., 1995; Kornstein et al., 1995; Szymanski et al., 1995; Hendrick et al., 2000; Klein and Corwin, 2002; Scheibe et al., 2003; Kawa et al., 2005; Robison et al., 2008; Vesga-Lopez et al., 2008); however determining the precise roles for various sex-specific factors has received limited attention. The findings here suggest that immune-brain crosstalk may be an important factor to the biological basis of sex differences in clinical psychiatry. In fact, there are known differences in immunity between the sexes and it is possible that these differences combine with endocrine effects to account for the sexually dimorphic behaviour observed. For example, endocrine-

immune interactions contribute to gender differences in the immune response to pathogens such as bacteria, viruses, and parasites leading to increased susceptibility to infectious disease in males (Klein, 2000). In contrast, females show increased risk of autoimmune disorders such as systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis (Whitacre, 2001). It is possible that sex differences in immunity are influenced by endocrine function as estrogens enhance the immune response in females and evidence suggests that estrogens may also modulate T cell trafficking, contributing to increased susceptibility to autoimmune processes in females (Mo et al., 2005). Understanding how immunological differences influence behaviour is needed to provide new insights into sex differences in the prevalence of anxiety disorders and differences in susceptibility to specific anxiety disorders between men and women.

Intriguingly, a role for altered immune function in the pathophysiology of anxiety disorders has been reported in clinical psychiatry. For example, patients with anxiety disorders and mild depression have increased numbers of CD8⁺ T lymphocytes and a decreased CD4⁺/CD8⁺ T lymphocyte ratio in blood samples at baseline (Atanackovic et al., 2004), while patients with panic disorder were found to have decreased CD8⁺ lymphocytes and an increased CD4⁺/CD8⁺ T lymphocyte ratio (Park et al., 2005). Women who suffer from post traumatic stress disorder (PTSD) also present with an altered T lymphocyte profile at baseline that includes increased CD4⁺ T lymphocytes and decreased CD8⁺ T

lymphocytes (Glover et al., 2005). While this profile did not change when the women were stressed, there was a strong correlation between CD4+/CD8+ T lymphocyte ratio and presence of PTSD symptoms. Importantly, these measures were observed in patients after diagnosis of psychiatric illness and thus do not indicate whether T lymphocyte profiles contribute to susceptibility to psychiatric illness or if these observations are the result of psychiatric illness. However, reduced anxiety-like behaviour in $TCR\beta^{-/-}\delta^{-/-}$ mice suggests that T lymphocytes can and do influence behaviours related to psychiatric illness.

4.4. The Adaptive Immune System and Learning Behaviours

Our results in the auditory fear conditioning test are consistent with previous studies. For example, we observed sex differences in the auditory fear conditioning test for contextual learning in which WT males exhibited increased freezing behaviour relative to WT females. This is in agreement with previous work in rats showing increased freezing behaviour in both sexes in a contextual fear learning test but with males significantly higher than females (Gresack et al., 2009). The increased fear response in $TCR\beta^{-/-}\delta^{-/-}$ mice is also in agreement with previous work on mice with immune deficiency. Male mice deficient of IL-1R1 ($IL1R1^{-/-}$) also freeze more during this test (Koo and Duman, 2009). In that study, $IL1R1^{-/-}$ mice also showed reduced anxiety-like behaviour in the light/dark test and EPM (Koo and Duman, 2009). These results are consistent with our findings of reduced anxiety and enhanced fear learning with immune deficiency. This is

surprising as it has been suggested that anxiety and fear behaviours are linked by shared neural circuitry. In support of this, mice bred based on high or low freezing behaviour in the fear conditioning test. The high freezing behaviour line also had increased anxiety-like behaviour in the open field and elevated zero maze tests, but spatial learning in the MWM was unaffected (Ponder et al., 2007). This indicates the link between anxiety and fear specifically rather than anxiety and general cognition. The discovery of reduced anxiety-like behaviour in the context of an increased fear response indicates that, while there may be some overlap in the neural circuitry, these behaviours can develop independently. Interestingly, voxel-wise analysis of the brain in $TCR\beta^{-/-}\delta^{-/-}$ mice revealed structural alterations in both the hippocampus and the amygdala. The hippocampus was smaller in both male and female $TCR\beta^{-/-}\delta^{-/-}$ mice while the amygdala was differentially altered in the sexes. The link between hippocampal volume and behaviour appears to be complicated. In humans, larger hippocampal volume correlates with improved ability to discriminate contexts associated with fear (Pohlack et al., 2012). Conversely, reduced hippocampal volume has been found in patients with PTSD (Bonne et al., 2008) and in mouse models of PTSD (Golub et al., 2011), suggesting that the hippocampus is protective in fear processing. Thus, the reduced hippocampal volume in $TCR\beta^{-/-}\delta^{-/-}$ mice may be contributing to the increased fear response in both sexes. Meanwhile, amygdala volume was increased in $TCR\beta^{-/-}\delta^{-/-}$ males but was decreased in $TCR\beta^{-/-}\delta^{-/-}$ females compared to sex-matched WT controls. As the

amygdala is a component of the limbic system, and amygdala volume (Baur et al., 2012) and activity (Stein et al., 2007) have previously been linked to emotional processing, it is possible that some of the sex-specific changes in anxiety-like and fear behaviour associated with adaptive immune deficiency are related to adaptive immune influences on limbic structures.

One finding previously reported as different in models of adaptive immune deficiency that we did not reproduce was altered spatial learning. Previous work demonstrated impaired learning in the Morris Water Maze (MWM) in RAG1/2 knockout mice (SCID) on a BALB/c/OLA background and nude mice on a BALB/c background (Kipnis et al., 2004). Furthermore, athymic “nude” mice reconstituted with T lymphocytes harvested from WT control mice exhibited improved spatial learning in the Morris Water Maze over nude mice that were not reconstituted. This impaired learning phenotype was not observed in our study with *TCR β -/- δ -/-* mice or in other unpublished work from our laboratory using nude mice on a CD1 genetic background, suggesting that the background strain contributes to the behavioural phenotype. Furthermore, RAG1 is expressed centrally in regions of high neuronal density, like the hippocampus and cerebellum (Chun et al., 1991). Consequently, it was proposed at the time that the effects on spatial learning were mediated by a neuronal function of RAG1. This idea is backed both by the absence of spatial learning deficits in *TCR β -/- δ -/-* mice and by a recent study that silenced the RAG1 gene by injecting a short hairpin RNA into the hippocampus and observing a loss of spatial learning

abilities (Fang et al., 2013). It should also be noted that Kipnis *et al.* (2004) examined SCID mice on a Balb/c background aged 8-12 weeks, whereas we conducted Morris Water Maze experiments on $TCR\beta^{-/-}\delta^{-/-}$ mice at 21 weeks of age on a C57BL/6 background. It is possible that spatial learning deficits would be observed if $TCR\beta^{-/-}\delta^{-/-}$ mice were tested earlier in development.

4.5. Basal Differences in HPA Axis Regulation in Adaptive Immune Deficient Mice

In the absence of any stress treatment, there were differences in basal corticosterone (CORT) between WT and $TCR\beta^{-/-}\delta^{-/-}$ mice indicating dysregulation of the HPA axis. In experiment 2, the control (CON) $TCR\beta^{-/-}\delta^{-/-}$ males had higher levels of plasma CORT than WT males. However, the results of these experiments do not indicate where the dysregulation occurs. Under normal circumstances glucocorticoid receptors regulate the HPA axis in that activation of mineralocorticoid receptor (MR) in the hippocampus mediates the basal tone of CORT while glucocorticoid receptor (GR) activation suppresses the HPA axis during a stress response (De Kloet et al., 1998). As MR has a much higher affinity for CORT compared with GR (Nishi and Kawata, 2006), GR is only activated when there are high levels of CORT during the peak diurnal phase or during exposure to a stressor (De Kloet et al., 1998). Prolonged periods of stress have been shown to decrease expression of GR in the CNS (Makino et al., 1995), thus diminishing the negative feedback of CORT leading to further HPA axis dysregulation (Landfield and Eldridge, 1994). It has been proposed that

dysregulation of the HPA axis as found in many psychiatric disorders, is the result of chronic stress on CORT and glucocorticoid receptors (De Kloet et al., 1998; Berardelli et al., 2013; Harris et al., 2013).

Despite the increased basal CORT, no differences in MR or GR mRNA expression were observed within the unstressed groups suggesting that basal HPA axis dysregulation does not occur via MR or GR feedback. Furthermore, no differences in corticotropin releasing hormone (CRH) mRNA expression were observed within the unstressed groups in the paraventricular nucleus of the hypothalamus (PVN) suggesting that dysregulation is not occurring at the level of the hypothalamus. It is possible that there is hypersecretion of ACTH from the pituitary in *TCRβ*^{-/-δ} mice or that ACTH receptor expression is elevated in the adrenal cortex. Furthermore, it is also possible that changes in negative feedback occurred but were not detected at the level of gene expression. We have observed volumetric changes in *TCRβ*^{-/-δ} mice in the hypothalamus and the amygdala suggesting that loss of T lymphocytes can lead to structural changes within the stress circuitry. In both the hypothalamus and amygdala, *TCRβ*^{-/-δ} males had increased volume over WT males while *TCRβ*^{-/-δ} females had reduced volume in comparison to sex-matched controls (Rilett et al., 2013). It is also possible that an examination of MR and GR expression at the level of protein would yield different results. Upon the binding of CORT to corticosterone receptors, the receptor-ligand complex is translocated into the cell nucleus where it regulates gene transcription (Nishi and Kawata, 2006). Whereas acute

treatment with CORT increases the expression of MR and GR within the hippocampus (Sarabdjitsingh et al., 2009), chronically elevated CORT results in reduced GR expression and higher tolerance to glucocorticoids (Makino et al., 1995). Additional experiments would be required to examine these possibilities. Interestingly, while the elevated basal CORT may appear at odds with the decreased anxiety-like behaviour reported in both *TCRβ*^{-/-}*δ*^{-/-} and germ-free mice (Neufeld et al., 2011b), our findings suggest that anxiety-like behaviours are not determined by HPA axis activity. Rather, the evidence indicates that immune function is coupled with anxiety, suggesting that anxiety phenotypes are influenced by immunological influences on brain structure and function.

It is also interesting that the elevated basal plasma CORT levels observed in the CON *TCRβ*^{-/-}*δ*^{-/-} male group, in which samples were collected in the morning, were not observed following immune challenge when samples were collected in the evening. Rather, CORT levels were elevated in saline (SAL) control WT females compared to SAL WT males and no difference was observed between male and female *TCRβ*^{-/-}*δ*^{-/-} mice. Plasma and salivary CORT levels are known to fluctuate with the circadian cycle (Atkinson and Waddell, 1997) with peak levels occurring within 1 hour of awakening in both rodents (Atkinson and Waddell, 1997), and humans (Wilhelm et al., 2007; Wolfram et al., 2011). As mice are nocturnal, this period occurs early in the dark cycle, rather than the light cycle. Plasma CORT then slowly decreases during the wake phase until reaching the daily minimum shortly into the light cycle (Atkinson and Waddell, 1997).

Accordingly, morning values reflect baseline CORT in mice during the sleep phase while evening values reflect CORT levels during the wake phase.

Therefore, the elevated plasma CORT in *TCRβ*^{-/-δ}^{-/-} mice during the morning indicates HPA axis dysregulation at baseline while the sex difference observed in SAL WT mice indicates a sex difference in plasma CORT during the wake phase. This seems to be in agreement with reports of increased CORT in women compared to men during day hours (Critchlow et al., 1963) and elevated CORT in unstressed female rats (Weinstock et al., 1998; Viau et al., 2005). Furthermore, the absence of increased plasma CORT in *TCRβ*^{-/-δ}^{-/-} females compared to *TCRβ*^{-/-δ}^{-/-} males once again demonstrates a loss of sex differences with adaptive immune deficiency. It would therefore be reasonable to hypothesize that T lymphocytes influence the sexually dimorphic circadian programming of the HPA axis by an increase of the daily minimum plasma CORT level in unstressed mice. However, it should be noted that the evening CORT measurements obtained in this study were taken after 1 hour in the open field test. Furthermore, as these measurements were taken at close approximations to the daily maximum and minimum rather than the exact times with respect to the light/dark cycle in which the mice were housed, the results of these experiments are not sufficient to reject or confirm the hypothesis that circadian programming is altered by adaptive immune deficiency. Additional study with several daily time points would be required to examine the circadian rhythm in *TCRβ*^{-/-δ}^{-/-} mice

A comprehensive analysis of the effects of circadian rhythm on behaviour has not been completed but the present experiments suggest that it would be an interesting future direction and an important factor to consider when comparing results between studies. The ability to detect behavioural differences in mice may depend on the time of day in which experiments were conducted. This has recently been found in a mouse model of bipolar disorder in which daytime, but not nighttime, optogenetic stimulation of the ventral tegmentum alters anxiety-like behaviour (Sidor et al., 2013). In our experiments, mice tested in the open field in the evening traveled more than twice the distance of mice tested in the morning, indicating that activity levels are highly sensitive to circadian rhythm. This, in turn, may influence the ability to detect other changes in behaviour that rely on activity.

T lymphocytes also follow a circadian rhythm is that is strongly linked to CORT levels in both humans (Lange et al., 2010) and mice (Kawate et al., 1981). In humans, T lymphocyte numbers are lowest in the morning shortly after waking when plasma CORT levels are highest, and both CD4⁺ and CD8⁺ naïve T lymphocytes are highest from late afternoon to early morning. When human T lymphocyte cultures were challenged with staphylococcus enterotoxin B or adenovirus, effector T lymphocyte numbers peak during the evening when CORT levels are lowest (Kirsch et al., 2012). In mice, a nocturnal species, peak levels of T and B lymphocytes were observed during the middle of the light phase which is the mouse equivalent of the less active phase with higher plasma CORT (Kawate et al., 1981; Bollinger et al., 2011). This relationship was abolished in mice that

underwent an adrenalectomy, thus confirming the relationship between circadian CORT levels and lymphocyte counts (Kawate et al., 1981). In the absence of an immune challenge, *ex vivo* T lymphocytes from humans follow circadian rhythms in the production of immune molecules including interferon- γ and CD40L (Bollinger et al., 2011), a component of the co-stimulatory signal between T lymphocytes and antigen presenting cells. The reduced anxiety-like and increased fear-related behaviours reported in *TCR β -/- δ -/-* mice were observed during the light phase when T lymphocytes are highest in WT mice (Kawate et al., 1981). Previous work in our laboratory suggests that there is an immune influence on developmental programming of behaviour. However, it cannot be ruled out that immune-brain communication with T lymphocytes is influencing behaviour at the time of the test, particularly when certain behaviours vary with the time of day. This could be examined directly using injection of anti-CD3 antibody to temporarily deplete T lymphocytes. If the effects of T lymphocyte depletion are developmental in nature, the injection should not affect the behavioural phenotype of mice. However, if CD3 injection resulted in a behavioural phenotype similar to that of the *TCR β -/- δ -/-* mice it would imply that crosstalk between T lymphocytes and the brain during the test is driving the observed phenotype and suggest that circadian fluctuations of T lymphocytes could contribute to circadian differences in behaviour.

An alternative explanation for the elevated CORT in saline injected WT females is that a sex difference exists causing WT females to react more strongly

to the stress of saline injection. An additional experiment comparing saline injection to no injection would be required to reject this possibility. Also, while the CORT levels of *TCR β -/- δ -/-* females were not significantly different from WT females, it is possible that we would have observed a different outcome had we controlled for phase of the estrous cycle. Menstrual cycle phase, and by extension sex hormones, have been linked to fluctuations in CORT. For example, compared to males, female Sprague-Dawley rats have lower ACTH during diestrous and metestrous phases and higher ACTH in the proestrous phase. In females, CORT is lowest during diestrous and metestrous compared to proestrous phases. However, in all phases females have higher CORT levels compared to males (Rhodes et al., 2002). Similarly, in Wistar rats it has been shown that while females have normal circadian rhythmicity, peak values double during the proestrous phase compared to the estrous phase which is similar to the CORT levels of males (Atkinson and Waddell, 1997). It has also been shown that activation of the estrogen receptor β (ER β) alters plasma CORT levels (Walf et al., 2009; Oyola et al., 2012). Mice lacking ER β have elevated levels of plasma CORT compared to C57Bl/6 WT controls (Walf et al., 2009). Administration of the ER β specific agonist, diarylpropionitrile, decreased plasma CORT and ACTH in WT but not ER β knockout mice (Oyola et al., 2012). Together these studies suggest that at least some of the variability in CORT levels observed in female mice could be due to variations in estrous cycle or variations in expression of estrogen receptors.

Interestingly, it is also possible that early life immune-brain signaling changes the trajectory of development of key stress circuitry. For example, in previous work in our laboratory, mice given postnatal injections of LPS showed developmental changes in the expression of serotonergic receptors (Sidor et al., 2010b) with accompanied sex-specific alterations to behaviour in adulthood with an increased anxiety-like phenotype in females and increased behavioural inhibition in males (Sidor, 2009). It is likely that crosstalk between the immune system and the serotonergic system is active before puberty, and possibly other systems are affected prior to puberty as well.

4.6. Differences in the Expression of Stress-Related Genes in Adaptive Immune Deficient Mice

In animal studies, the expression of MR and GR receptors (Jakovcecski et al., 2011) as well as CRH receptors in the CNS are linked to anxiety-like behaviour (Muller et al., 2003; Weiser et al., 2008; Weiser et al., 2010). This is of interest to models of adaptive immune deficiency as we and others have demonstrated reduced anxiety-like behaviour in mice with T lymphocyte deficiencies (Oliveira-Dos-Santos et al., 2000; Cushman et al., 2003; Neufeld et al., 2011b). With regard to CRH receptors, CRHR1 knockout mice display reduced anxiety-like behaviour (Muller et al., 2003), while CRHR2 knockout mice display increased anxiety-like behaviour (Weiser et al., 2008). As there were no differences in the expression of CRHR1 mRNA in *TCR β ^{-/-} δ ^{-/-}* mice, it is unlikely

that this receptor was involved in the establishment of the altered anxiety-like behaviour and stress response reported in $TCR\beta^{-/-}\delta^{-/-}$ mice within this thesis, although the expression of CRHR2 might have influenced other reported differences. For example, the anxiolytic influence of the CRHR2 receptor may have influenced entry behaviour into the open arms in the EPM as WT females entered more frequently than WT males and expressed significantly higher levels of CRHR2 mRNA in the hippocampus.

4.7. Adaptive Immune Deficiency and Stress Reactivity

We predicted that mice lacking T lymphocytes would have increased stress responsiveness to repeat restraint stress (RS) due to the similarities between $TCR\beta^{-/-}\delta^{-/-}$ and germ-free mice in anxiety phenotype and immune deficiency. Here we showed that both WT controls and $TCR\beta^{-/-}\delta^{-/-}$ mice have similarly elevated plasma CORT levels following stress. The absence of an exaggerated stress response following RS in $TCR\beta^{-/-}\delta^{-/-}$ mice suggests that altered stress reactivity in germ-free mice is not due to the adaptive immune deficiency they possess.

Centrally, $TCR\beta^{-/-}\delta^{-/-}$ mice display altered expression of stress related genes. In females, CRH mRNA was elevated in the PVN of RS treated WT and $TCR\beta^{-/-}\delta^{-/-}$ mice, indicating that both WT and $TCR\beta^{-/-}\delta^{-/-}$ females were susceptible to RS. Comparatively, in males only the $TCR\beta^{-/-}\delta^{-/-}$ RS treated mice had increased expression of CRH mRNA over genotype-matched controls. This

finding suggests that WT males were resilient to the effects of RS and demonstrates a loss of sex differences in response to restraint stress, again implicating T lymphocytes in the sexually dimorphic developmental programming of stress. Intriguingly, CON $TCR\beta^{-/-}\delta^{-/-}$ males expressed decreased CRH mRNA in the central amygdala (CeA) compared to RS $TCR\beta^{-/-}\delta^{-/-}$ males in which CRH levels were comparable to the other male groups. This finding is of interest because the CeA mediates the HPA axis. The CeA contains a high density of glucocorticoid receptors and is known to project to the medial parvocellular PVN (Gray et al., 1989). Therefore it can regulate the stress response. Elevated CORT exposure has been demonstrated to increase CRH mRNA expression in the CeA (Shepard et al., 2000, 2003) and to increase stress behaviour (Myers et al., 2005).

$TCR\beta^{-/-}\delta^{-/-}$ females also expressed increased CRHR2 mRNA following RS compared to RS treated WT females. In males, RS lead to CRHR2 mRNA upregulation in the hippocampus of WT, but not $TCR\beta^{-/-}\delta^{-/-}$ mice. Changes in the expression of CRHR2 mRNA are notable as it has previously been demonstrated that CRHR2 knockout mice are hyper-responsive to acute restraint stress with elevated CORT compared to WT controls after 10 minutes of restraint (Bale et al., 2000). While there were no differences in CORT between male and female, and WT and $TCR\beta^{-/-}\delta^{-/-}$ mice following 10 days of repeat restraint stress, the change in CRHR2 mRNA expression suggests that repeat restraint stress can impact the regulation of the HPA axis. It is thought that CRHR2 is involved in the

maintenance of the stress response as CRHR2 knockout mice recover heart rate and arterial pressure more rapidly after 10 minutes of restraint stress (Coste et al., 2000). It is therefore possible that changes in CRHR2 mRNA expression in our study reflect differences in coping mechanisms. This has previously been suggested by Liebsch *et al.*, (1999) who showed that infusion of CRHR2 antisense oligodeoxynucleotides into the lateral ventricle of rats reduces coping behaviours in the forced swim test (Liebsch et al., 1999). It would therefore be interesting to examine coping behaviours in our mice as this is the first report of altered CRHR2 mRNA expression in a study using repeat, rather than acute, restraint stress. Meanwhile, CRHR1 activation is important in the initiation of the stress response (Coste et al., 2000). The unaltered expression of CRHR1 mRNA in *TCR β ^{-/-} δ ^{-/-}* mice as well as elevated plasma CORT following RS suggests a normal ability to mount a stress response in T lymphocyte deficient mice.

4.8. T Lymphocyte Deficiency is Associated with Loss of Sex Differences in Stress Reactivity

A consistent finding across the behavioural and molecular studies on *TCR β ^{-/-} δ ^{-/-}* mice is a loss of sex differences. While no differences were observed in MR mRNA expression in *TCR β ^{-/-} δ ^{-/-}* mice, there were sex differences within the WT mice as MR mRNA expression was decreased throughout the hippocampus of RS WT female mice compared to RS WT male mice which expressed MR at levels similar to the other groups. As previously discussed,

differences in the expression of glucocorticoid receptors could reflect differences in vulnerability to various behaviours and psychiatric illnesses that men and women have different susceptibility to (Bao and Swaab, 2010).

There was also a loss of sexually dimorphic expression of CRHR2 mRNA in the $TCR\beta^{-/-}\delta^{-/-}$ RS group. CRHR2 mRNA was upregulated in the hippocampus of RS WT males but down-regulated in the hippocampus of RS WT females compared to sex-matched CON WT mice. No differences were observed in the $TCR\beta^{-/-}\delta^{-/-}$ mice. Previously in C57BL/6J mice it had been demonstrated that a single session of restraint stress for 30 minutes resulted in decreased CRHR1 and increased CRHR2 mRNA in the hippocampus (Greetfeld et al., 2009). The increased CRHR2 mRNA expression in WT males observed in our study agrees with those findings. However, as females were not included in the study by Greetfeld *et al.* (2009) and our results indicate an opposite effect in females, it is further evidence of the importance of distinguishing the sexes in studies of stress reactivity as well as considering how the immune system may be contributing to sex differences. This is also true in clinical studies of stress reactivity. For example, it is known that women are more susceptible to stress-related disorders including PTSD (Breslau, 2001) and depression (Nolen-Hoeksema, 2012), and that women usually respond more strongly to stressors (Bangasser and Valentino, 2012). Furthermore, the difference between CORT responses in men and women is exacerbated with age (Otte et al., 2005). As this has not been studied in preclinical models of stress reactivity, it is possible that studies on mice

in early adulthood, such as the results reported within this thesis, are under-representing sexually dimorphic responses to stress. Further studies including multiple ages would be required to assess this possibility.

The absence of change to CRHR1 mRNA expression in our mice compared to the reduced CRHR1 following acute restraint stress (Greetfeld et al., 2009) points to differences in the response to acute and chronic stress. This is supported by work in other laboratories in which chronically stressed rats expressed elevated CRH in the hypothalamus but expression of CRHR1 protein remained unaffected (Raone et al., 2007). It is therefore possible that long-term differences in stress reactivity are mediated through CRHR2 rather than CRHR1 and that opposing effects on CRHR2 expression between males and females contributes to the sexually dimorphic stress responses.

It is also possible that the absence of changes to CRHR2 expression in *TCRβ*^{-/-}*δ*^{-/-} males might reflect poor coping abilities to stress as the CRHR2 receptor mediates the slow response to stress and CRHR2 knockout mice have deficits in dearousal or coping behaviours (Liebsch et al., 1999; Coste et al., 2000) such as grooming (Spruijt et al., 1992). It is also possible that the kinetics of the stress response are altered in *TCRβ*^{-/-}*δ*^{-/-} male mice. Following urocortin induced stress, CRHR2 knockout mice display a normal early stress response, mediated by CRHR1, such as elevated ACTH and CORT and stress behaviours like hypophagia. However, these effects were shorter lived in CRHR2 knockout mice compared to WT suggesting that CRHR2 mediates the physical

manifestations of stress and maintenance of the stress response (Coste et al., 2000). However, further studies would be required to examine these possibilities.

4.9. Adaptive Immune Deficiency and Acute Stress Reactivity.

We have previously demonstrated differences in stress responsiveness to LPS challenge in $\beta 2M^{-/-}TAP^{-/-}$ mice that lack class I MHC (Sankar et al., 2012). $\beta 2M^{-/-}TAP^{-/-}$ mice have a stress response to intraperitoneal injection showing similar behavioural responses to both saline injection and LPS injection. It is known that class I MHC has neuronal functions within the CNS (Corriveau et al., 1998; Foster et al., 2002; Oliveira et al., 2004; Goddard et al., 2007) and it was suggested that class I MHC has a direct influence on the stress circuitry within the CNS (Sankar et al., 2012). However, as the $\beta 2M^{-/-}TAP^{-/-}$ mice lack CD8⁺ T lymphocytes as well as class I MHC, it is possible that adaptive immune deficits were responsible for the differences. By testing stress reactivity to LPS, the same innate immune challenge as $\beta 2M^{-/-}TAP^{-/-}$ mice, it was possible to examine the role, if any, of T lymphocytes on the acute stress response.

$TCR\beta^{-/-}\delta^{-/-}$ mice and WT controls were treated with LPS or saline (SAL) injections immediately prior to testing in the open field. LPS is known to activate the stress response resulting in elevated plasma ACTH and CORT levels (Newson et al., 2013) and to dose-dependently reduce activity behaviour in WT mice in the open field (Engeland et al., 2001; Juszczak et al., 2008; Sankar, et al., 2012; Tarr et al., 2012). Injection with LPS lead to decreased activity within

the open field test in both $TCR\beta^{-/-}\delta^{-/-}$ and WT mice compared to SAL injected mice. This response is typical following injection with LPS and indicates that the initiation of the stress response is intact in $TCR\beta^{-/-}\delta^{-/-}$ mice. Additionally, plasma CORT levels were measured 2 hours after injection and CORT was elevated in all groups treated with LPS. LPS treated $TCR\beta^{-/-}\delta^{-/-}$ males had significantly less CORT compared to LPS treated WT males and LPS treated $TCR\beta^{-/-}\delta^{-/-}$ females. As all LPS treated groups exhibited normal sickness behaviour in the open field test, it is clear that all mice responded to the LPS injection behaviourally but it is possible that there was dysregulation of the HPA axis that led to a reduced CORT response in $TCR\beta^{-/-}\delta^{-/-}$ males.

It is interesting to consider both the possibility of immune-brain crosstalk influencing the stress circuitry, and the idea that there are neuronal functions for immune molecules. For example, Class I MHC is expressed post-synaptically (Goddard et al., 2007) and electrophysiology experiments demonstrated changes to long-term potentiation and long-term depression in the hippocampus of $\beta 2M^{-/-}$ $TAP^{-/-}$ and $CD3\zeta$ knockout mice, which also lack class I MHC (Huh et al., 2000). Interestingly, no differences in neuron firing were reported in $RAG1^{-/-}$ mice (Huh et al., 2000) suggesting that the altered plasticity in $\beta 2M^{-/-}$ $TAP^{-/-}$ and $CD3\zeta$ knockout mice is mediated by class I MHC rather than general immune deficit. Furthermore, the results of the LPS experiment in $TCR\beta^{-/-}\delta^{-/-}$ mice support the conclusions of Sankar *et al.* (2012) that the absence of a neuronal function of class I MHC is responsible for the differences in stress reactivity rather than the

loss of CD8⁺ T lymphocytes (Sankar et al., 2012). However, the changes to chronic stress reactivity and central gene expression in systems related to stress behaviours (discussed below) suggests that T lymphocytes do influence the stress circuitry. Given that the β and δ chains of the TCR are not expressed centrally, it is likely that immune-brain crosstalk influences the wiring of stress related systems.

4.10. Glutamatergic System

It was anticipated that there would be dysregulation of N-methyl-D-aspartate (NMDA) receptor subunits in *TCR β ^{-/-} δ ^{-/-}* mice as they have been linked to alterations in anxiety and fear related behaviours. NMDA receptors in the basolateral amygdala (BLA) are known to be involved in synaptic transmission during fear expression (Campeau et al., 1992). NR1, the only subunit found in all NMDA receptors (Dingledine et al., 1999) was elevated in RS WT males compared to RS WT females. However, this was the only difference observed in NR1 expression. The subunit composition of the receptor also correlates with mouse behaviour. For example, in mice NR2A disruption leads to increased activity in the open field test (Miyamoto et al., 2001), anti-depressant-like behaviour in the forced swim and tail suspension tests (Boyce-Rustay and Holmes, 2006b; Taniguchi et al., 2009) and impaired fear learning in the auditory fear conditioning test (Kiyama et al., 1998; Nakazawa et al., 2006). NR2A knock-out mice have also displayed reduced anxiety-like behaviour in the open field,

light/dark test, and EPM (Boyce-Rustay and Holmes, 2006b). Interestingly, *TCRβ*^{-/-}*δ*^{-/-} female mice expressed higher levels of NR2A mRNA within the dentate gyrus (DG) region of the hippocampus and the CeA. In the context of reduced anxiety-like behaviour in *TCRβ*^{-/-}*δ*^{-/-} mice this seems unexpected. However, as auditory fear learning is disrupted in NR2A knockout mice (Moriya et al., 2000), it is possible that the increased expression of NR2A mRNA in *TCRβ*^{-/-}*δ*^{-/-} females contributes to the elevated fear response that was also observed in *TCRβ*^{-/-}*δ*^{-/-} mice. Meanwhile, the changes in anxiety-like behaviour may be explained elsewhere as many factors contribute to behavioural phenotypes. Additionally, *TCRβ*^{-/-}*δ*^{-/-} mice, both male and female, also had increased NR2A mRNA expression in the DG following RS treatment. This may reflect an increased sensitivity to the RS treatment and it would be interesting to examine the influence of T lymphocytes on behaviour under stressful conditions.

It is also likely that alterations in NR2B mRNA expression in the amygdala contributed to the changes in fear behaviour in *TCRβ*^{-/-}*δ*^{-/-} females. Previously, it was shown that if phosphorylation of the NR2B subunit of the NMDA receptor is inhibited through knock-in of a genetic mutation, both contextual and conditioned fear learning are inhibited (Nakazawa et al., 2006). Conversely, in CON *TCRβ*^{-/-}*δ*^{-/-} females, NR2B mRNA expression was elevated in the CeA compared to CON WT females and fear learning was enhanced. There was also elevated expression of NR2B in the CeA of *TCRβ*^{-/-}*δ*^{-/-} males but this was only observed within the RS cohort. This demonstrates that T lymphocytes have an influence on

the expression of NMDA receptor subunits and this may contribute to the anxiety and fear related behavioural phenotypes observed in $TCR\beta^{-/-}\delta^{-/-}$ mice. However, as many of the changes observed were only found in females, it may also reflect why the reduced anxiety-like phenotype in $TCR\beta^{-/-}\delta^{-/-}$ mice manifested differently between the sexes. Furthermore, the increased expression of NR2B mRNA in the CeA in stressed immunocompromised males but not females suggests that glutamatergic signaling in the amygdala contributes to sex-specific differences in stress vulnerability.

4.11. Serotonergic System

Expression of serotonergic receptors in the hippocampus and amygdala were subtly altered in male, but not female, $TCR\beta^{-/-}\delta^{-/-}$ mice. Furthermore, the differences in $TCR\beta^{-/-}\delta^{-/-}$ males were only observed within the RS treated cohort. The 5-HT_{2C} receptor, also thought to be anxiogenic (Li et al., 2012) was upregulated in the basolateral amygdala (BLA) of $TCR\beta^{-/-}\delta^{-/-}$ males. As the BLA primarily projects to other amygdala nuclei (Jankord and Herman, 2008), it is likely that any effects this may have on stress is mediated through additional brain structures.

While there were no basal differences in the expression of serotonin receptors between $TCR\beta^{-/-}\delta^{-/-}$ and WT mice, the expression of mRNA of 5-HT_{2A} and 5-HT_{2B} receptors was altered in $TCR\beta^{-/-}\delta^{-/-}$ mice following RS treatment. It is known that peripheral blockade of 5-HT_{2A} inhibits stress-induced hyperthermia

but whether this was due to activation of central or peripheral 5-HT_{2A} receptors was unclear (Beig et al., 2009). Another group that examined gamma-aminobutyric acid (GABA) release in the BLA of rats through serotonin discovered that the inhibitory effects of serotonin on stress were mediated via the 5-HT_{2A} receptor specifically (Jiang et al., 2009). Notably, this group also demonstrated that rats subjected to repeat restraint stress had impairments in 5-HT_{2A} signaling in the BLA, suggesting that it may be due to decreased expression of 5-HT_{2A}. Jiang *et al.* (2009) also noted that the impairment would lead to hyperexcitability of the amygdala and could be a factor in stress disorders. This idea is intriguing in the context of both elevated CRH mRNA expression in the PVN and CeA of RS *TCRβ*^{-/-}*δ*^{-/-} males and the reduced 5-HT_{2A} mRNA expression in the hippocampus of RS *TCRβ*^{-/-}*δ*^{-/-} males. It is therefore possible that loss of T lymphocytes leads to increased vulnerability to stress in males, observed as increased CRH mRNA expression, and that T lymphocytes have a protective role in chronic stress exposure.

In females, however, 5-HT_{2A} mRNA expression was increased in the CA1 and CA3 regions of the hippocampus in RS treated WT females compared to CON WT. This may reflect a coping mechanism for stress present only in females. First, however, it would be prudent to examine 5-HT_{2A} function at the level of protein using receptor binding assays or physiology experiments to confirm a functional alteration.

The expression of 5-HT_{2A} mRNA was also higher in the CA1 region of the hippocampus in CON WT males compared to CON WT females. This sex difference was absent in the *TCRβ*^{-/-}*δ*^{-/-} mice again demonstrating a loss of sexual differentiation. It should be noted that this was only found in the CA1 region of the hippocampus and much of the research to date involves genetic knockout of 5-HT_{2A} (Weisstaub et al., 2006), peripheral administration of agonists and antagonists (Beig et al., 2009), or focuses on the role of 5-HT_{2A} in the BLA (Jiang et al., 2009). Our results of alterations to 5-HT_{2A} expression in the hippocampus in the context of altered anxiety-like behaviour and stress responses suggest that the role of 5-HT_{2A} in the hippocampus is worth further exploration.

It is also worth noting that the absence of change in the expression of serotonergic receptors basally in *TCRβ*^{-/-}*δ*^{-/-} mice does not negate the possibility of a serotonergic influence on the altered anxiety-like phenotype that was observed. As previously mentioned, we found that the dorsal raphe nucleus was larger in WT males compared to WT females, which is opposite to the findings in *TCRβ*^{-/-}*δ*^{-/-} mice in which females had larger dorsal raphe nuclei (Rilett et al., 2013). Volumetric changes in the dorsal raphe nuclei of these mice could lead to functional changes within the CNS that were not detected at the level of gene expression of serotonergic receptors. Serotonergic neurons arise primarily from the raphe nuclei and the dorsal raphe projects heavily to limbic regions including the prefrontal cortex, amygdala, striatum, and hippocampus (Hensler, 2006). It is

possible that structural changes to the dorsal raphe affect serotonergic signaling to limbic structures that were not examined in *TCR β -/- δ -/-* mice. Further study on serotonin in models of adaptive immune deficiency is warranted to investigate this possibility.

4.12. T Lymphocytes Could Influence the Brain and Behaviour During Development.

One of the limitations of work with genetic knockout mice is that these experiments do not indicate when in development the changes occur. It is possible that the results observed in *TCR β -/- δ -/-* mice were due to the absence of T cells during early life development. This is particularly relevant because there is considerable overlap in the development of T lymphocytes and the central wiring of anxiety behaviour and, recently, anxiety has been discussed as a developmental disorder (Leonardo and Hen, 2008). In support of this claim, the onset of anxiety disorders in humans is typically during childhood and adolescence (Lewinsohn et al., 1998; Shear et al., 2005; Hefner and Holmes, 2007; Kessler et al., 2007). In mice, the neural structures related to anxiety are formed prenatally (Mizukawa et al., 1989; Berdel et al., 1997) but the wiring of anxiety remains plastic during early postnatal life (Mizukawa et al., 1989; Leonardo and Hen, 2008). High levels of neurogenesis and gliogenesis in the hippocampus, striatum, and cortex occur from postnatal day (P) 10-16, and proliferation declines from P17-P24 (Qiu et al., 2007). The functional connectivity

between the anatomical components of the anxiety circuitry also develops during early postnatal life. For example, the serotonergic system is involved in the wiring of anxiety from P15-P21. This was demonstrated in mice lacking the 5-HT_{1A} receptor resulting in increased anxiety-like behaviours. If the expression of 5-HT_{1A} was rescued by P15, a normal anxiety phenotype appeared; however, if 5-HT_{1A} was not rescued until P21, anxiogenic behaviour emerged (Gross et al., 2002). Both males and females appeared to have this same window for the development of anxiety circuitry from P15 to P21 (Sidor, 2009).

In agreement with this proposed period for the development of anxiety, major neurogenesis in the amygdala occurs between P10 and P20 in rodents (Bayer, 1980; Mizukawa et al., 1989; Berdel et al., 1997) confirming that crucial stages of limbic system development occur during early postnatal life. Anxiety behaviours emerge shortly following that period of neurogenesis (Klemenhagen et al., 2006; Hefner and Holmes, 2007; Kessler et al., 2007). Current literature suggests that the central wiring of anxiety overlaps with the period of humoral immune plasticity from P15 to P21.

Following birth, the thymus grows rapidly until reaching maximum size at P18. The adult thymus is much smaller and the export of T lymphocytes reaches a steady state at this stage (Dominguez-Gerpe and Rey-Mendez, 2003) that remains until 24 months of age in mice (Scollay et al., 1986). Prior to steady state, T lymphocytes undergo many developmental changes beginning at birth when fully mature T lymphocytes become apparent and are capable of mounting

a cellular, cytotoxic response to immune challenge (Dominguez-Gerpe and Rey-Mendez, 2003). T lymphocytes proliferate exponentially from P3-P6 and continue to slowly increase in number until P10 (Penit and Vasseur, 1989). In early postnatal life, the ratios of various subsets of T lymphocytes also change rapidly and frequently. At birth, CD8⁺ T lymphocytes predominate but this quickly switches to CD4⁺ cells (Vicente et al., 1998). Despite the presence of CD4⁺ T lymphocytes, both newborn and mice aged 10-15 days are unable to produce a strong humoral response. Instead, there is a bias toward immunotolerance and T regulatory cells (T_{regs}) early in life that switches to a bias toward defense around P20-P21 (Fagoaga et al., 2000). T_{regs} are the dominant CD4⁺ cells during early life until P20-21 when T helper cells become active and numerous. Importantly, these key stages of CD4⁺ T cell development occur during the third week of life when the anxiety circuitry is wired. Additionally, knockout of T lymphocytes results in reduced neurogenesis that is reversible by reconstitution with CD4⁺ cells (Huang et al., 2010). Thus, it is temporally plausible that CD4⁺ T cells, most likely T_{regs}, are driving the T cell influences on anxiety and the neurocircuitry thereof.

4.13. Conclusion

The results of this dissertation support the vastly increasing literature on the importance of immune-brain communication in brain development and behaviour. Here it was shown that loss of T cell receptor β and δ chains resulted

in reduced anxiety-like and increased exploratory behaviour in both males and females. The results are consistent with behavioural reports in other immune-compromised mice; however, in this study the effects of T and B lymphocytes were separately distinguished and indicate a specific role for T, rather than B, lymphocytes on the development of anxiety-like behaviour. Furthermore, changes to the expression of stress- and anxiety-related genes suggest that some of the consequences of adaptive immune deficiency are due to the influence of T lymphocytes on the central circuitry controlling anxiety behaviour. The discovery that repeated restraint stress led to different alterations in gene expression between WT and *TCR β -/- δ -/-* mice suggests that that immune-brain cross-talk, possibly during development, alters wiring and leads to changes in vulnerability to the effects of stress. There was also a loss of sexual dimorphisms in behaviour of immune compromised mice that is normally present in WT mice. The consistent loss of sex differences in *TCR β -/- δ -/-* mice across several behavioural and molecular parameters suggests that T lymphocytes are involved in sexual differentiation during development. Finally, the discovery that *TCR β -/- δ -/-* mice have a normal response to LPS injection demonstrates that, despite the long-term changes to the CNS following repeated restraint stress, their acute stress response was intact. Collectively these experiments indicate that T lymphocytes have a profound influence on the brain and behaviour.

4.14. Future Directions

The establishment of reduced anxiety-like behaviours in immune deficient knockout mice provides strong evidence that T lymphocytes affect behaviour. However, it remains unclear if these effects are induced by adaptive immune deficiency in adulthood or if there is a critical developmental window. Other influences on anxiety-like behaviour are known to have critical windows but that vary with the different influences. For example, the aforementioned period from P15-P21 in which conditional knockout of the 5-HT_{1A} receptor (Gross et al., 2002) or functional loss of the serotonin transporter (5-HTT) can influence the development of anxiety-like behaviours (Ansorge et al., 2004) differs from the critical window of germ-free mice. In germ-free mice, the altered anxiety phenotype can be rescued up to P28 by conventionalizing the germ-free mice through exposure to faeces of specific pathogen free control mice (McVey Neufeld, 2012). To examine a critical window for the influence of T lymphocytes on the development of anxiety-like behaviour in adulthood, it is possible to inject mice with antibodies against CD3 in order to deplete T lymphocytes; however, it would first be necessary to determine the required dose as this type of immune depletion has not yet been conducted during early life development.

Another limitation to this thesis that could be addressed by using antibody injections is that, at present, we do not know if certain subtypes of T lymphocytes influence anxiety or if different subtypes could have opposing effects on anxiety behaviour. CD4⁺CD25⁺ T regulatory cells (T_{regs}) are the predominant

subtype until P20-P21, indicating that $CD4^{+} T_{regs}$ cells are a prime candidate to influence anxiety. To assess the specificity of $CD4^{+}$ T cell influences on anxiety, an array of subtypes including $CD4^{+}$ could be depleted. If the anxiolytic phenotype in T cell deficient mice was specific to $CD4^{+}CD25^{+} T_{regs}$, depletion of other subtypes should not affect on behaviour. Use of anti-CD4, anti-CD8, and anti-CD25 antibodies could enable a more thorough investigation into which specific subtypes of T lymphocytes are involved.

If a developmental window exists in which T lymphocytes, or a subset of T lymphocytes, influence anxiety behaviour, additional experiments to reverse their effects would be necessary for confirmation. In such a case, T lymphocytes could be harvested from WT mice and used to reconstitute $TCR\beta^{-/-}\delta^{-/-}$ mice. Provided reconstitution occurs prior to the critical window, anxiety-like behaviour should develop comparably to WT mice.

Finally, given the loss of sex differences in both anxiety-like behaviour and the stress circuitry observed in $TCR\beta^{-/-}\delta^{-/-}$ mice, it would be interesting to examine sex hormones and their receptors in models of adaptive immune deficiency. Basally, it would be interesting to consider whether T lymphocytes influence circadian rhythm. Given that plasma CORT was elevated in $TCR\beta^{-/-}\delta^{-/-}$ mice in the morning, but not in the evening, suggests that the diurnal circadian cycle may have increased amplitude in mice lacking T lymphocytes. If correct, then it would be important to examine potential immune contributions to sleep disorders.

REFERENCES

- Allen LS, Gorski RA (1990) Sex difference in the bed nucleus of the stria terminalis of the human brain. *J Comp Neurol* 302:697-706.
- An XL, Zou JX, Wu RY, Yang Y, Tai FD, Zeng SY, Jia R, Zhang X, Liu EQ, Broders H (2011) Strain and sex differences in anxiety-like and social behaviors in C57BL/6J and BALB/cJ mice. *Exp Anim* 60:111-123.
- Andia AM, Zisook S, Heaton RK, Hesselink J, Jernigan T, Kuck J, Morganville J, Braff DL (1995) Gender differences in schizophrenia. *J ner ment dis* 183:522-528.
- Andreoli A, Keller SE, Rabaeus M, Zaugg L, Garrone G, Taban C (1992) Immunity, major depression, and panic disorder comorbidity. *Biol Psychiatry* 31:896-908.
- Ansorge MS, Zhou M, Lira A, Hen R, Gingrich JA (2004) Early-life blockade of the 5-HT transporter alters emotional behavior in adult mice. *Science* 306:879-881.
- Archer J (1975) Rodent sex differences in emotional and related behavior. *Behav Biol* 14:451-479.
- Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE, Evans RM (1987) Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. *Science* 237:268-275.
- Atanackovic D, Kroger H, Serke S, Deter H-C (2004) Immune parameters in patients with anxiety or depression during psychotherapy. *J Affect Dis* 81:201-209.
- Atkinson HC, Waddell BJ (1997) Circadian variation in basal plasma corticosterone and adrenocorticotropin in the rat: sexual dimorphism and changes across the estrous cycle. *Endocrinology* 138:3842-3848.
- Badanes LS, Watamura SE, Hankin BL (2011) Hypocortisolism as a potential marker of allostatic load in children: associations with family risk and internalizing disorders. *Dev Psychopathol* 23:881-896.
- Bale TL, Contarino A, Smith GW, Chan R, Gold LH, Sawchenko PE, Koob GF, Vale WW, Lee KF (2000) Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. *Nat Genet* 24:410-414.
- Bangasser DA, Valentino RJ (2012) Sex differences in molecular and cellular substrates of stress. *Cell Mol Neurobiol* 32:709-723.
- Bao AM, Swaab DF (2010) Sex differences in the brain, behavior, and neuropsychiatric disorders. *Neuroscientist* 16:550-565.
- Baur V, Hanggi J, Jancke L (2012) Volumetric associations between uncinate fasciculus, amygdala, and trait anxiety. *BMC Neurosci* 13:4.
- Bayer SA (1980) Quantitative 3H-thymidine radiographic analyses of neurogenesis in the rat amygdala. *J Comp Neurol* 194:845-875.

- Beig MI, Baumert M, Walker FR, Day TA, Nalivaiko E (2009) Blockade of 5-HT_{2A} receptors suppresses hyperthermic but not cardiovascular responses to psychosocial stress in rats. *Neuroscience* 159:1185-1191.
- Bel N, Artigas F (1993) Chronic treatment with fluvoxamine increases extracellular serotonin in frontal cortex but not in raphe nuclei. *Synapse* 15:243-245.
- Belzung C, Misslin R, Vogel E, Dodd RH, Chapouthier G (1987) Anxiogenic effects of methyl-beta-carboline-3-carboxylate in a light/dark choice situation. *Pharmacol Biochem Behav* 28:29-33.
- Berardelli R, Karamouzis I, D'Angelo V, Zichi C, Fussotto B, Giordano R, Ghigo E, Arvat E (2013) Role of mineralocorticoid receptors on the hypothalamus-pituitary-adrenal axis in humans. *Endocrine* 43:51-58.
- Berdel B, Morys J, Maciejewska B (1997) Neuronal changes in the basolateral complex during development of the amygdala of the rat. *Int J Dev Neurosci* 15:755-765.
- Bernardini R, Kamilaris TC, Calogero AE, Johnson EO, Gomez MT, Gold PW, Chrousos GP (1990) Interactions between tumor necrosis factor-alpha, hypothalamic corticotropin-releasing hormone, and adrenocorticotropin secretion in the rat. *Endocrinology* 126:2876-2881.
- Besedovsky H, del Rey A (1987) Neuroendocrine and metabolic responses induced by interleukin-1. *J Neurosci Res* 18:172-178.
- Besedovsky HO, del Rey A (1989) Mechanism of virus-induced stimulation of the hypothalamus-pituitary-adrenal axis. *J Steroid Biochem* 34:235-239.
- Besedovsky HO, del Rey A, Klusman I, Furukawa H, Monge Arditi G, Kabiersch A (1991) Cytokines as modulators of the hypothalamus-pituitary-adrenal axis. *J Steroid Biochem Mol Biol* 40:613-618.
- Bluthe RM, Beaudu C, Kelley KW, Dantzer R (1995) Differential effects of IL-1ra on sickness behavior and weight loss induced by IL-1 in rats. *Brain Res* 677:171-176.
- Boddez Y, Callaerts-Vegh Z, Vervliet B, Baeyens F, D'Hooge R, Hermans D, Beckers T (2012) Stimulus generalization and return of fear in C57BL/6J mice. *Front Behav Neurosci* 6:41.
- Bollinger T, Leutz A, Leliavski A, Skrum L, Kovac J, Bonacina L, Benedict C, Lange T, Westermann J, Oster H, Solbach W (2011) Circadian clocks in mouse and human CD4⁺ T cells. *PLoS One* 6:e29801.
- Bonne O, Vythilingam M, Inagaki M, Wood S, Neumeister A, Nugent AC, Snow J, Luckenbaugh DA, Bain EE, Drevets WC, Charney DS (2008) Reduced posterior hippocampal volume in posttraumatic stress disorder. *J Clin Psychiatry* 69:1087-1091.
- Bonnin A, Peng W, Hewlett W, Levitt P (2006) Expression mapping of 5-HT₁ serotonin receptor subtypes during fetal and early postnatal mouse forebrain development. *Neuroscience* 141:781-794.
- Boulanger LM (2009) Immune proteins in brain development and synaptic plasticity. *Neuron* 64:93-109.

- Bourin M, Hascoët M (2003) The mouse light/dark box test. *European Journal of Pharmacology* 463:55-65.
- Bourin M, Hascoët M (2003) The mouse light/dark box test. *Eur J Pharmacol* 463:55-65.
- Boyce-Rustay JM, Holmes A (2006a) Ethanol-related behaviors in mice lacking the NMDA receptor NR2A subunit. *Psychopharmacology (Berl)* 187:455-466.
- Boyce-Rustay JM, Holmes A (2006b) Genetic inactivation of the NMDA receptor NR2A subunit has anxiolytic- and antidepressant-like effects in mice. *Neuropsychopharmacology* 31:2405-2414.
- Brady LS, Lynn AB, Herkenham M, Gottesfeld Z (1994) Systemic interleukin-1 induces early and late patterns of c-fos mRNA expression in brain. *J Neurosci* 14:4951-4964.
- Brenes JC, Padilla M, Fornaguera J (2009) A detailed analysis of open-field habituation and behavioral and neurochemical antidepressant-like effects in postweaning enriched rats. *Behav Brain Res* 197:125-137.
- Breslau N (2001) The epidemiology of posttraumatic stress disorder: what is the extent of the problem? *J Clin Psychiatry* 62 Suppl 17:16-22.
- Butler LD, Layman NK, Riedl PE, Cain RL, Shellhaas J, Evans GF, Zuckerman SH (1989) Neuroendocrine regulation of in vivo cytokine production and effects: I. In vivo regulatory networks involving the neuroendocrine system, interleukin-1 and tumor necrosis factor-alpha. *J Neuroimmunol* 24:143-153.
- Campeau s, Miserendino MJD, Davis M (1992) Intra-amygdala infusion of the N-methyl-D-aspartate receptor antagonist AP5 blocks acquisition but not expression of fear-potentiated startle to an auditory conditioned stimulus. *Behav Neurosci* 111:1353-1360.
- Capuron L, Dantzer R, Miller AH (2003) [Neuro-immune interactions in psychopathology with the example of interferon-alpha-induced depression]. *J Soc Biol* 197:151-156.
- Capuron L, Ravaud A, Miller AH, Dantzer R (2004) Baseline mood and psychosocial characteristics of patients developing depressive symptoms during interleukin-2 and/or interferon-alpha cancer therapy. *Brain Behav Immun* 18:205-213.
- Cebra JJ (1999) Influences of microbiota on intestinal immune system development. *Am J Clin Nutr* 69:1046S-1051S.
- Chalmers DT, Lovenberg TW, DeSouza EB (1995) Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. *J Neurosci* 15:6340-6350.
- Chaouloff F, Durand M, Mormede P (1997) Anxiety- and activity-related effects of diazepam and chlordiazepoxide in the rat light/dark and dark/light tests. *Behav Brain Res* 85:27-35.

- Charles G, Machowski R, Brohee D, Wilmotte J, Kennes B (1992) Lymphocyte subsets in major depressive patients. Influence of anxiety and corticoadrenal overdrive. *Neuropsychobiology* 25:94-98.
- Choleris E, Thomas AW, Kavaliers M, Prato FS (2001) A detailed ethological analysis of the mouse open field test: effects of diazepam, chlordiazepoxide and an extremely low frequency pulsed magnetic field. *Neurosci Biobehav Rev* 25:235-260.
- Chun JJ, Schatz DG, Oettinger MA, Jaenisch R, Baltimore D (1991) The recombination activating gene-1 (RAG-1) transcript is present in the murine central nervous system. *Cell* 64:189-200.
- Clarke G, Grenham S, Scully P, Fitzgerald P, Moloney RD, Shanahan F, Dinan TG, Cryan JF (2012) The microbiome-gut brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Mol Psych*:1-8.
- Corriveau RA, Huh GS, Shatz CJ (1998) Regulation of class I MHC gene expression in the developing and mature CNS by neural activity. *Neuron* 21:505-520.
- Coryell W, Young E, Carroll B (2006) Hyperactivity of the hypothalamic-pituitary-adrenal axis and mortality in major depressive disorder. *Psychiatry Res* 142:99-104.
- Coste SC, Kesterson RA, Heldwein KA, Stevens SL, Heard AD, Hollis JH, Murray SE, Hill JK, Pantely GA, Hohimer AR, Hatton DC, Phillips TJ, Finn DA, Low MJ, Rittenberg MB, Stenzel P, Stenzel-Poore MP (2000) Abnormal adaptations to stress and impaired cardiovascular function in mice lacking corticotropin-releasing hormone receptor-2. *Nat Genet* 24:403-409.
- Crawley J, Goodwin F (1980) Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. *Pharmacology, Biochemistry, and Behavior* 13:167-170.
- Crawley JN (1985) Exploratory behavior models of anxiety in mice. *Neurosci Behav Rev* 9:37-44.
- Crawley JN (2008) Behavioral Phenotyping Strategies for Mutant Mice. *Neuron* 57:809-818.
- Critchlow V, Liebelt RA, Bar-Sela M, Mountcastle W, Lipscomb HS (1963) Sex differences in resting pituitary-adrenal function in the rat. *Am J Physiol* 205:807-815.
- Cull-Candy S, Brickley S, Farrant M (2001) NMDA receptor subunits: diversity, development and disease. *Curr Opin Neurobiol* 11:327-335.
- Cushman J, Lo J, Huang Z, Wasserfall C, Petitto JM (2003) Neurobehavioral changes resulting from recombinase activation gene 1 deletion. *Clin Diagn Lab Immunol* 10:13-18.
- Da Silva JA (1999) Sex hormones and glucocorticoids: interactions with the immune system. *Ann N Y Acad Sci* 876:102-117; discussion 117-108.

- Dantzer R, Kelley KW (1989) Stress and immunity: an integrated view of relationships between the brain and the immune system. *Life Science* 44:1995-2008.
- De Kloet ER, Vreugdenhil E, Oitzl MS, Joels M (1998) Brain corticosteroid receptor balance in health and disease. *Endocr Rev* 19:269-301.
- De Leon-Nava MA, Nava K, Soldevila G, Lopez-Griego L, Chavez-Rios JR, Vargas-Villavicencio JA, Morales-Montor J (2009) Immune sexual dimorphism: effect of gonadal steroids on the expression of cytokines, sex steroid receptors, and lymphocyte proliferation. *J Steroid Biochem Mol Biol* 113:57-64.
- del Abril A, Segovia S, Guillaumon A (1987) The bed nucleus of the stria terminalis in the rat: regional sex differences controlled by gonadal steroids early after birth. *Brain Res* 429:295-300.
- Delawary M, Tezuka T, Kiyama Y, Yokoyama K, Inoue T, Hattori S, Hashimoto R, Umemori H, Manabe T, Yamamoto T, Nakazawa T (2010) NMDAR2B tyrosine phosphorylation regulates anxiety-like behavior and CRF expression in the amygdala. *Mol Brain* 3:37.
- Descotes J, Tedone R, Evreux JC (1985) Different effects of psychotropic drugs on delayed hypersensitivity responses in mice. *J Neuroimmunol* 9:81-85.
- Dingledine R, Borges K, Bowie D, Traynelis SF (1999) The glutamate receptor ion channels. *Pharmacol Rev* 51:7-61.
- Dominguez-Gerpe L, Rey-Mendez M (2003) Evolution of the thymus size in response to physiological and random events throughout life. *Microsc Res Tech* 62:464-476.
- Ebisui O, Fukata J, Murakami N, Kobayashi H, Segawa H, Muro S, Hanaoka I, Naito Y, Masui Y, Ohmoto Y, et al. (1994) Effect of IL-1 receptor antagonist and antiserum to TNF-alpha on LPS-induced plasma ACTH and corticosterone rise in rats. *Am J Physiol* 266:E986-992.
- Engeland CG, Nielsen DV, Kavaliers M, Ossenkopp KP (2001) Locomotor activity changes following lipopolysaccharide treatment in mice: a multivariate assessment of behavioral tolerance. *Physiol Behav* 72:481-491.
- Ericsson A, Liu C, Hart RP, Sawchenko PE (1995) Type 1 Interleukin-1 Receptor in the rat brain: Distribution, regulation, and relationship to sites of IL-1-Induced cellular activation. *J Comp Neurol* 361:681-698.
- Fagoaga OR, Yellon SM, Nehlsen-Cannarella SL (2000) Maturation of lymphocyte immunophenotypes and memory T helper cell differentiation during development in mice. *Dev Immunol* 8:47-60.
- Fang M, Yin Y, Chen H, Hu Z, Davies H, Ling S (2013) Contribution of Rag1 to spatial memory ability in rats. *Behav Brain Res* 236:200-209.
- Figueiredo HF, Bodie BL, Tauchi M, Dolgas CM, Herman JP (2003) Stress integration after acute and chronic predator stress: differential activation of central stress circuitry and sensitization of the hypothalamo-pituitary-adrenocortical axis. *Endocrinology* 144:5249-5258.

- Flajnik MF, Kasahara M (2001) Comparative genomics of the MHC: glimpses into the evolution of the adaptive immune system. *Immunity* 15:351-362.
- Foster JA, Quan N, Stern EL, Kristensson K, Herkenham M (2002) Induced neuronal expression of class I major histocompatibility complex mRNA in acute and chronic inflammation models. *J Neuroimmunol* 131:83-91.
- Fourgeaud L, Davenport CM, Tyler CM, Cheng TT, Spencer MB, Boulanger LM (2010) MHC class I modulates NMDA receptor function and AMPA receptor trafficking. *Proc Natl Acad Sci U S A* 107:22278-22283.
- Frick LR, Williams K, Pittenger C (2013) Microglial dysregulation in psychiatric disease. *Clin Dev Immunol* 2013:608654.
- Fuller RW (1994) Uptake inhibitors increase extracellular serotonin concentration measured by brain microdialysis. *Life Sci* 55:163-167.
- Gaillard RC, Daneva T, Hadid R, Muller K, Spinedi E (1998) The hypothalamo-pituitary-adrenal axis of athymic Swiss nude mice. The implications of T lymphocytes in the ACTH release from immune cells. *Ann N Y Acad Sci* 840:480-490.
- Garay PA, McAllister AK (2010) Novel roles for immune molecules in neural development: implications for neurodevelopmental disorders. *Front Synaptic Neurosci* 2:136.
- Gatch MB, Wallis CJ, Lal H (1999) Effects of NMDA antagonists on ethanol-withdrawal induced "anxiety" in the elevated plus maze. *Alcohol* 19:207-211.
- Gentsch C, Lichtsteiner M, Feer H (1987) Open field and elevated plus-maze: a behavioural comparison between spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) rats and the effects of chlordiazepoxide. *Behav Brain Res* 25:101-107.
- Glover DA, Steele AC, Stuber ML (2005) Preliminary evidence for lymphocyte distribution differences at rest and after acute psychological stress in PTSD-symptomatic women. *Brain Behav Immun* 19:243-251.
- Goddard CA, Butts DA, Shatz CJ (2007) Regulation of CNS synapses by neuronal MHC class I. *Proc Natl Acad Sci U S A* 104:6828-6833.
- Goel N, Bale TL (2010) Sex differences in the serotonergic influence on the hypothalamic-pituitary-adrenal stress axis. *Neuroendocrinology* 151:1784-1794.
- Golub Y, Kaltwasser SF, Mauch CP, Herrmann L, Schmidt U, Holsboer F, Czisch M, Wotjak CT (2011) Reduced hippocampus volume in the mouse model of Posttraumatic Stress Disorder. *J Psychiatr Res* 45:650-659.
- Goujon E, Parnet P, Laye S, Combe C, Dantzer R (1996) Adrenalectomy enhances pro-inflammatory cytokines gene expression, in the spleen, pituitary and brain of mice in response to lipopolysaccharide. *Brain Res Mol Brain Res* 36:53-62.
- Gray JA (1971) Sex differences in emotional behaviour in mammals including man: endocrine bases. *Acta Psychol (Amst)* 35:29-46.

- Gray TS, Carney ME, Magnuson DJ (1989) Direct projections from the central amygdaloid nucleus to the hypothalamic paraventricular nucleus: possible role in stress-induced adrenocorticotropin release. *Neuroendocrinology* 50:433-446.
- Greetfeld M, Schmidt MV, Ganea K, Sterlemann V, Liebl C, Muller MB (2009) A single episode of restraint stress regulates central corticotrophin-releasing hormone receptor expression and binding in specific areas of the mouse brain. *J Neuroendocrinol* 21:473-480.
- Gresack JE, Schafe GE, Orr PT, Frick KM (2009) Sex differences in contextual fear conditioning are associated with differential ventral hippocampal extracellular signal-regulated kinase activation. *Neuroscience* 159:451-467.
- Gross C, Zhuang X, Stark K, Ramboz S, Oosting R, Kirby L, Santarelli L, Beck S, Hen R (2002) Serotonin_{1A} receptor acts during development to establish normal anxiety-like behaviour in the adult. *Nature* 416:396-400.
- Grova N, Schroeder H, Farinelle S, Prodhomme E, Valley A, Muller CP (2008) Sub-acute administration of benzo[a]pyrene (B[a]P) reduces anxiety-related behaviour in adult mice and modulates regional expression of N-methyl-D-aspartate (NMDA) receptors genes in relevant brain regions. *Chemosphere* 73:S295-302.
- Grupe DW, Oathes DJ, Nitschke JB (2013) Dissecting the anticipation of aversion reveals dissociable neural networks. *Cereb Cortex* 23:1874-1883.
- Haas DA, George SR (1988) Gonadal regulation of corticotropin-releasing factor immunoreactivity in hypothalamus. *Brain Research Bulletin* 20:361-367.
- Handley SL, Mithani S (1984) Effects of alpha-adrenoceptor agonists and antagonists in a maze-exploration model of 'fear'-motivated behavior. *Naunyn-Schmiedeberg's Archives of Pharmacology* 327.
- Harris AP, Holmes MC, de Kloet ER, Chapman KE, Seckl JR (2013) Mineralocorticoid and glucocorticoid receptor balance in control of HPA axis and behaviour. *Psychoneuroendocrinology* 38:648-658.
- Hart BL (1988) Biological basis of the behavior of sick animals. *Neurosci Biobehav Rev* 12:123-137.
- Hefner K, Holmes A (2007) Ontogeny of fear-, anxiety- and depression-related behavior across adolescence in C57BL/6J mice. *Behav Brain Res* 176:210-215.
- Heijtz RD, Wang S, Anuar F, Qian Y, Bjorkholm B, Samuelsson A, Hibberd ML, Forssberg H, Pettersson S Normal gut microbiota modulates brain development and behavior. *Proc Natl Acad Sci U S A* 108:3047-3052.
- Heisler LK, Zhou L, Bajwa P, Hsu J, Tecott LH (2007) Serotonin 5-HT_{2C} receptors regulate anxiety-like behavior. *Genes Brain Behav* 6:491-496.
- Hek K, Direk N, Newson RS, Hofman A, Hoogendijk WJ, Mulder CL, Tiemeier H (2013) Anxiety disorders and salivary cortisol levels in older adults: a population-based study. *Psychoneuroendocrinology* 38:300-305.

- Hendrick V, Altshuler LL, Gitlin MJ, Delrahim S, Hammen C (2000) Gender and bipolar illness. *J Clin Psych* 61:393-396; quiz 397.
- Hensler JG (2006) Serotonergic modulation of the limbic system. *Neurosci Biobehav Rev* 30:203-214.
- Herkenham M, Lee HY, Baker RA (1998) Temporal and spatial patterns of c-fos mRNA induced by intravenous interleukin-1: a cascade of non-neuronal cellular activation at the blood-brain barrier. *J Comp Neurol* 400:175-196.
- Holmes A (2001) Targeted gene mutation approaches to the study of anxiety-like behavior in mice. *Neurosci Biobehav Rev* 25:261-273.
- Holstege G (1992) The emotional motor system. *Eur J Morphol* 30:67-79.
- Huang G-J, Smith AL, Gray DGD, Cosgrove C, Singer BH, Edwards A, Sim S, Parent JM, Johnson A, Mott R, Mathis D, Klennerman P, Benoist C, Flint J (2010) A genetic and functional relationship between T cells and cellular proliferation in the adult hippocampus. *PLoS Biology* 8: 1-10.
- Huh GS, Boulanger LM, Du H, Riquelme PA, Brotz TM, Shatz CJ (2000) Functional requirement for class I MHC in CNS development and plasticity. *Science* 290:2155-2159.
- Ingi T, Krumins AM, Chidiac P, Brothers GM, Chung S, Snow BE, Barnes CA, Lanahan AA, Siderovski DP, Ross EM, Gilman AG, Worley PF (1998) Dynamic regulation of RGS2 suggests a novel mechanism in G-protein signaling and neuronal plasticity. *J Neurosci* 18:7178-7188.
- Jacobson L, Sapolsky R (1991) The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. *Endocrine Reviews* 12:118-134.
- Jakovcecki M, Schachner M, Morellini F (2011) Susceptibility to the long-term anxiogenic effects of an acute stressor is mediated by the activation of the glucocorticoid receptors. *Neuropharmacology* 61:1297-1305.
- Janeway CA, Travers P, Walport M, Shlomchik MJ (2005) *Immunobiology*, 6th Edition. New York, NY: Garland Science Publishing.
- Jankord R, Herman JP (2008) Limbic regulation of hypothalamo-pituitary-adrenocortical function during acute and chronic stress. *Ann N Y Acad Sci* 1148:64-73.
- Jiang X, Xing G, Yang C, Verma A, Zhang L, Li H (2009) Stress impairs 5-HT_{2A} receptor-mediated serotonergic facilitation of GABA release in juvenile rat basolateral amygdala. *Neuropsychopharmacology* 34:410-423.
- Jirillo E, Maffione AB, Greco B, Cannuscio B, Calvella R, Covelli V (1993) Triazolobenzodiazepines exert immunopotentiating activities on normal human peripheral blood lymphocytes. *Immunopharmacol Immunotoxicol* 15:307-319.
- Johnston AL, File SE (1991) Sex differences in animal tests of anxiety. *Physiol Behav* 49:245-250.
- Juszczak GR, Blaszczyk J, Sadowski B, Sliwa AT, Wolak P, Tymosiak-Zielinska A, Lisowski P, Swiergiel AH (2008) Lipopolysaccharide does not affect acoustic startle reflex in mice. *Brain Behav Immun* 22:74-79.

- Kammermeier PJ, Ikeda SR (1999) Expression of RGS2 alters the coupling of metabotropic glutamate receptor 1a to M-type K⁺ and N-type Ca²⁺ channels. *Neuron* 22:819-829.
- Kawa I, Carter JD, Joyce PR, Doughty CJ, Frampton CM, Wells JE, Walsh AE, Olds RJ (2005) Gender differences in bipolar disorder: age of onset, course, comorbidity, and symptom presentation. *Bipolar disorders* 7:119-125.
- Kawate T, Abo T, Hinuma S, Kumagai K (1981) Studies of the bioperiodicity of the immune response. II. Co-variations of murine T and B cells and a role of corticosteroid. *J Immunol* 126:1364-1367.
- Kellendonk C, Gass P, Kretz O, Schutz G, Tronche F (2002) Corticosteroid receptors in the brain: gene targeting studies. *Brain Res Bull* 57:73-83.
- Kenna GA, Roder-Hanna N, Leggio L, Zywiak WH, Clifford J, Edwards S, Kenna JA, Shoaff J, Swift RM (2012) Association of the 5-HTT gene-linked promoter region (5-HTTLPR) polymorphism with psychiatric disorders: review of psychopathology and pharmacotherapy. *Pharmgenomics Pers Med* 5:19-35.
- Kessler RC, Amminger GP, Aguilar-Gaxiola S, Alonso J, Lee S, Ustun TB (2007) Age of onset of mental disorders: a review of recent literature. *Curr Opin Psychiatry* 20:359-364.
- Kim SK, Wang KC, Hong SJ, Chung CK, Lim SY, Kim YY, Chi JG, Kim CJ, Chung YN, Kim HJ, Cho BK (2003) Gene expression profile analyses of cortical dysplasia by cDNA arrays. *Epilepsy Res* 56:175-183.
- Kipnis J, Cohen H, Cardon M, Ziv Y, Schwartz M (2004) T cell deficiency leads to cognitive dysfunction: implications for therapeutic vaccination for schizophrenia and other psychiatric conditions. *Proc Natl Acad Sci U S A* 101:8180-8185.
- Kirsch S, Thijssen S, Alarcon Salvador S, Heine GH, van Bentum K, Fliser D, Sester M, Sester U (2012) T-cell numbers and antigen-specific T-cell function follow different circadian rhythms. *J Clin Immunol* 32:1381-1389.
- Kirschbaum C, Kudielka BM, Gaab J, Schommer NC, Hellhammer DH (1999) Impact of gender, menstrual cycle phase, and oral contraceptives on the activity of the hypothalamus-pituitary-adrenal axis. *Psychosom Med* 61:154-162.
- Kishimoto T, Radulovic J, Radulovic M, Lin CR, Schrick C, Hooshmand F, Hermanson O, Rosenfeld MG, Spiess J (2000) Deletion of *crhr2* reveals an anxiolytic role for corticotropin-releasing hormone receptor-2. *Nat Genet* 24:415-419.
- Kitamura D, Roes J, Kuhn R, Rajewsky K (1991) A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature* 350:423-426.
- Kiyama Y, Manabe T, Sakimura K, Kawakami F, Mori H, Mishina M (1998) Increased thresholds for long-term potentiation and contextual learning in

- mice lacking the NMDA-type glutamate receptor {epsilon}1 subunit. *J Neurosci* 18:6704-6712.
- Klein LC, Corwin EJ (2002) Seeing the unexpected: how sex differences in stress responses may provide a new perspective on the manifestation of psychiatric disorders. *Curr Psych Rep* 4:441-448.
- Klein SL (2000) The effects of hormones on sex differences in infection: from genes to behavior. *Neurosci Biobehav Rev* 24:627-638.
- Klemenhagen KC, Gordon JA, David DJ, Hen R, Gross CT (2006) Increased fear response to contextual cues in mice lacking the 5-HT1A receptor. *Neuropsychopharmacology* 31:101-111.
- Kluger MJ (1986) Is fever beneficial? *Yale J Biol Med* 59:89-95.
- Koen N, Stein DJ (2011) Pharmacotherapy of anxiety disorders: a critical review. *Dialogues Clin Neurosci* 13:423-437.
- Kolozsi E, Mackenzie RN, Rouillet FI, Decatzenaro D, Foster JA (2009) Prenatal exposure to valproic acid leads to reduced expression of synaptic adhesion molecule neuroligin 3 in mice. *Neuroscience* 163:1201-1210.
- Koo JW, Duman RS (2009) Interleukin-1 receptor null mutant mice show decreased anxiety-like behavior and enhanced fear memory. *Neurosci Lett* 456:39-43.
- Kornstein SG, Schatzberg AF, Yonkers KA, Thase ME, Keitner GI, Ryan CE, Schlager D (1995) Gender differences in presentation of chronic major depression. *Psychopharmacology bulletin* 31:711-718.
- Kotlinska J, Biala G (1999) Effects of the NMDA/glycine receptor antagonist, L-701,324, on morphine- and cocaine-induced place preference. *Pol J Pharmacol* 51:323-330.
- Kusnecov AW, Liang R, Shurin G (1999) T-lymphocyte activation increases hypothalamic and amygdaloid expression of CRH mRNA and emotional reactivity to novelty. *J Neurosci* 19:4533-4543.
- Landfield PW, Eldridge JC (1994) The glucocorticoid hypothesis of age-related hippocampal neurodegeneration: role of dysregulated intraneuronal calcium. *Ann N Y Acad Sci* 746:308-321; discussion 321-306.
- Landgraf R, Wigger A (2002) High vs low anxiety-related behavior rats: an animal model of extremes in trait anxiety. *Behav Genet* 32:301-314.
- Lange T, Dimitrov S, Born J (2010) Effects of sleep and circadian rhythm on the human immune system. *Ann N Y Acad Sci* 1193:48-59.
- Lapiz-Bluhm MD, Bondi CO, Doyen J, Rodriguez GA, Bedard-Arana T, Morilak DA (2008) Behavioural assays to model cognitive and affective dimensions of depression and anxiety in rats. *J Neuroendocrinol* 20:1115-1137.
- Laurie DJ, Seeburg PH (1994) Regional and developmental heterogeneity in splicing of the rat brain NMDAR1 mRNA. *J Neurosci* 14:3180-3194.
- LeDoux JE (2000) Emotion circuits in the brain. *Annu Rev Neurosci* 23:155-184.
- Leonardo ED, Hen R (2008) Anxiety as a developmental disorder. *Neuropsychopharmacology* 33:134-140.

- Lepicard EM, Joubert C, Hagneau I, Perez-Diaz F, Chapouthier G (2000) Differences in anxiety-related behavior and response to diazepam in BALB/cByJ and C57BL/6J strains of mice. *Pharmacol Biochem Behav* 67:739-748.
- Lewinsohn PM, Gotlib IH, Lewinsohn M, Seeley JR, Allen NB (1998) Gender differences in anxiety disorders and anxiety symptoms in adolescents. *J Abnorm Psychol* 107:109-117.
- Li Q, Luo T, Jiang X, Wang J (2012) Anxiolytic effects of 5-HT(1)A receptors and anxiogenic effects of 5-HT(2)C receptors in the amygdala of mice. *Neuropharmacology* 62:474-484.
- Liebsch G, Landgraf R, Engelmann M, Lorsch P, Holsboer F (1999) Differential behavioural effects of chronic infusion of CRH 1 and CRH 2 receptor antisense oligonucleotides into the rat brain. *J Psychiatr Res* 33:153-163.
- Linda H, Hammarberg H, Piehl F, Khademi M, Olsson T (1999) Expression of MHC class I heavy chain and beta2-microglobulin in rat brainstem motoneurons and nigral dopaminergic neurons. *J Neuroimmunol* 101:76-86.
- Ljunggren Hg, Van Kaer L, Ashton-Rickardt PG, Tonegawa S, Ploegh HL (1995) Differential reactivity of residual CD8+ T lymphocytes in TAP1 and beta 2-microglobulin mutant mice. *Eur J Immunol* 25:174-178.
- Ljunggren HG, Stam NJ, Ohlen C, Neefjes JJ, Hogland P, Heemels MT, Bastin J, Schumacher TN, Townsend A, Karre K, al. e (1990) Empty MHC class I molecules come out in the cold. *Nature* 346:476-480.
- Lovallo WR, Farag NH, Vincent AS (2010) Use of a resting control day in measuring the cortisol response to mental stress: diurnal patterns, time of day, and gender effects. *Psychoneuroendocrinology* 35:1253-1258.
- Lowry CA, Johnson PL, Hay-Schmidt A, Mikkelsen J, Shekhar A (2005) Modulation of anxiety circuits by serotonergic systems. *Stress* 8:233-246.
- Macpherson AJ, Uhr T (2004) Compartmentalization of the mucosal immune responses to commensal intestinal bacteria. *Ann N Y Acad Sci* 1029:36-43.
- Macpherson AJ, Gatto D, Sainsbury E, Harriman GR, Hengartner H, Zinkernagel RM (2000) A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science* 288:2222-2226.
- Makino S, Smith MA, Gold PW (1995) Increased expression of corticotropin-releasing hormone and vasopressin messenger ribonucleic acid (mRNA) in the hypothalamic paraventricular nucleus during repeated stress: association with reduction in glucocorticoid receptor mRNA levels. *Endocrinology* 136:3299-3309.
- Matta SG, Linner KM, Sharp BM (1993) Interleukin-1 alpha and interleukin-1 beta stimulate adrenocorticotropin secretion in the rat through a similar hypothalamic receptor(s): effects of interleukin-1 receptor antagonist protein. *Neuroendocrinology* 57:14-22.

- Maxeiner H-G, Rojewski MT, Schmitt A, Tumani H, Bechter K, Schmitt M (2009) Flow cytometric analysis of T cell subsets in paired samples of cerebrospinal fluid and peripheral blood from patients with neurological and psychiatric disorders. *Brain Behav Immun* 23:134-142.
- McVey Neufeld K-A (2012) Impact of commensal intestinal microbiota on nervous system development and function. Open Access Dissertations and Theses: Paper 6728.
- Melik Parsadaniantz S, Levin N, Lenoir V, Roberts JL, Kerdelhue B (1994) Human interleukin 1 beta: corticotropin releasing factor and ACTH release and gene expression in the male rat: in vivo and in vitro studies. *J Neurosci Res* 37:675-682.
- Merali Z, Du L, Hrdina P, Palkovits M, Faludi G, Poulter MO, Anisman H (2004) Dysregulation in the suicide brain: mRNA expression of corticotropin-releasing hormone receptors and GABA(A) receptor subunits in frontal cortical brain region. *J Neurosci* 24:1478-1485.
- Miesfeld R, Okret S, Wikstrom AC, Wrange O, Gustafsson JA, Yamamoto KR (1984) Characterization of a steroid hormone receptor gene and mRNA in wild-type and mutant cells. *Nature* 312:779-781.
- Millan MJ (2003) The neurobiology and control of anxious states. *Prog Neurobiol* 70:83-244.
- Miller BH, Schultz LE, Gulati A, Su AI, Pletcher MT (2010) Phenotypic characterization of a genetically diverse panel of mice for behavioral despair and anxiety. *PLoS One* 5:e14458.
- Milner LC, Crabbe JC (2008) Three murine anxiety models: results from multiple inbred strain comparisons. *Genes Brain Behav* 7:496-505.
- Miyamoto A, Wake H, Moorhouse AJ, Nabekura J (2013) Microglia and synapse interactions: fine tuning neural circuits and candidate molecules. *Front Cell Neurosci* 7:70.
- Miyamoto Y, Yamada K, Noda Y, Mori H, Mishina M, Nabeshima T (2001) Hyperfunction of dopaminergic and serotonergic neuronal systems in mice lacking the NMDA receptor {epsilon}1 subunit. *J Neurosci* 21:750-757.
- Mizukawa K, Tseng IM, Otsuka N (1989) Quantitative electron microscopic analysis of postnatal development of zinc-positive nerve endings in the rat amygdala using Timm's sulphide silver technique. *Brain Res Dev Brain Res* 50:197-203.
- Mo R, Chen J, Grolleau-Julius A, Murphy HS, Richardson BC, Yung RL (2005) Estrogen regulates CCR gene expression and function in T lymphocytes. *J Immunol* 174:6023-6029.
- Mombaerts P, Mizoguchi E, Ljunggren HG, Iacomini J, Ishikawa H, Wang L, Grusby MJ, Glimcher LH, Winn HJ, Bhan AK, et al. (1994) Peripheral lymphoid development and function in TCR mutant mice. *Int Immunol* 6:1061-1070.
- Montgomery KC (1955a) The relation between fear induced by novel stimulation and exploratory behavior. *J Comp Physiol Psychol* 48:254-260.

- Montgomery KC (1955b) The relation between fear induced by novel stimulation and exploratory behaviour. *Journal of Comparative and Physiological Psychology* 48:7.
- Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 12:529-540.
- Moore EM, Linsenbardt DN, Melon LC, Boehm SL, 2nd (2011) Ontogenetic differences in adolescent and adult C57BL/6J and DBA/2J mice: anxiety-like, locomotor, and consummatory behaviors. *Dev Psychobiol* 53:141-156.
- Moriya T, Kouzu Y, Shibata S, Kadotani H, Fukunaga K, Miyamoto E, Yoshioka T (2000) Close linkage between calcium/calmodulin kinase II alpha/beta and NMDA-2A receptors in the lateral amygdala and significance for retrieval of auditory fear conditioning. *Eur J Neurosci* 12:3307-3314.
- Morris RGM (1981) Spatial localization does not require the presence of local cues. *Learning and Motivation* 12:239-260.
- Muller MB, Zimmermann S, Sillaber I, Hagemeyer TP, Deussing JM, Timpl P, Kormann MS, Droste SK, Kuhn R, Reul JM, Holsboer F, Wurst W (2003) Limbic corticotropin-releasing hormone receptor 1 mediates anxiety-related behavior and hormonal adaptation to stress. *Nat Neurosci* 6:1100-1107.
- Myers DA, Gibson M, Schulkin J, Greenwood Van-Meerveld B (2005) Corticosterone implants to the amygdala and type 1 CRH receptor regulation: effects on behavior and colonic sensitivity. *Behav Brain Res* 161:39-44.
- Nakazawa T, Komai S, Watabe AM, Kiyama Y, Fukaya M, Arima-Yoshida F, Horai R, Sudo K, Ebine K, Delawary M, Goto J, Umemori H, Tezuka T, Iwakura Y, Watanabe M, Yamamoto T, Manabe T (2006) NR2B tyrosine phosphorylation modulates fear learning as well as amygdaloid synaptic plasticity. *Embo J* 25:2867-2877.
- Neufeld KA, Kang N, Bienenstock J, Foster JA (2011a) Effects of intestinal microbiota on anxiety-like behavior. *Commun Integr Biol* 4:492-494.
- Neufeld KM, Kang N, Bienenstock J, Foster JA (2011b) Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterol Motil* 23:255-264, e119.
- Newson MJ, Pope GR, Roberts EM, Lolait SJ, O'Carroll AM (2013) Stress-dependent and gender-specific neuroregulatory roles of the apelin receptor in the hypothalamic-pituitary-adrenal axis response to acute stress. *J Endocrinol* 216:99-109.
- Nichols DE, Nichols CD (2008) Serotonin receptors. *Chem Rev* 108:1614-1641.
- Niewoehner B, Single FN, Hvalby O, Jensen V, Meyer zum Alten Borgloh S, Seeburg PH, Rawlins JN, Sprengel R, Bannerman DM (2007) Impaired spatial working memory but spared spatial reference memory following

- functional loss of NMDA receptors in the dentate gyrus. *Eur J Neurosci* 25:837-846.
- Nishi M, Kawata M (2006) Brain corticosteroid receptor dynamics and trafficking: Implications from live cell imaging. *Neuroscientist* 12:119-133.
- Nolen-Hoeksema S (2012) Emotion regulation and psychopathology: the role of gender. *Annu Rev Clin Psychol* 8:161-187.
- Oldehinkel AJ, Bouma EM (2011) Sensitivity to the depressogenic effect of stress and HPA-axis reactivity in adolescence: a review of gender differences. *Neurosci Biobehav Rev* 35:1757-1770.
- Oliveira AL, Thams S, Lidman O, Hokfelt PF, Karre K, Linda H, Cullheim S (2004) A role for the MHC class I molecules in synaptic plasticity and regeneration of neurons after axotomy. *Proc Natl Acad Sci U S A* 101.
- Oliveira-Dos-Santos AJ, Matsumoto G, Snow BE, Bai D, Houston FP, Wishaw IQ, Mariathasan S, Sasaki T, Wakeham A, Ohashi PS, Roder JC, Barnes CA, Siderovski DP, Penninger JM (2000) Regulation of T cell activation, anxiety, and male aggression by RGS2. *Proc Natl Acad Sci U S A* 97:12272-12277.
- Ortega JE, Gonzalez-Lira V, Horrillo I, Herrera-Marschitz M, Callado LF, Meana JJ (2013) Additive effect of rimonabant and citalopram on extracellular serotonin levels monitored with in vivo microdialysis in rat brain. *Eur J Pharmacol* 709:13-19.
- Otte C, Hart S, Neylan TC, Marmar CR, Yaffe K, Mohr DC (2005) A meta-analysis of cortisol response to challenge in human aging: importance of gender. *Psychoneuroendocrinology* 30:80-91.
- Oyola MG, Portillo W, Reyna A, Foradori CD, Kudwa A, Hinds L, Handa RJ, Mani SK (2012) Anxiolytic effects and neuroanatomical targets of estrogen receptor-beta (ERbeta) activation by a selective ERbeta agonist in female mice. *Endocrinology* 153:837-846.
- Palanza P (2001) Animal models of anxiety and depression: how are females different? *Neurosci Biobehav Rev* 25:219-233.
- Paolicelli RC, Bolasco G, Pagani F, Maggi L, Scianni M, Panzanelli P, Giustetto M, Ferreira TA, Guiducci E, Dumas L, Ragozzino D, Gross CT (2011) Synaptic pruning by microglia is necessary for normal brain development. *Science* 333:1456-1458.
- Park JE, Kim SW, Park Q, Jeong DU, Yu BH (2005) Lymphocyte subsets and mood states in panic disorder patients. *J Korean Med Sci* 20:215-219.
- Paxinos G FK (2001) The mouse brain in stereotaxic coordinates. New York: Academic Press.
- Paylor R, Tracy R, Wehner J, Rudy JW (1994) DBA/2 and C57BL/6 mice differ in contextual fear but not auditory fear conditioning. *Behav Neurosci* 108:810-817.
- Pellow S, File SE (1986) Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat. *Pharm Biochem Behav* 24:525-529.

- Pellow S, Chopin P, File SE, Briley M (1985) Validation of open : closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Meth* 14:149-167.
- Penit C, Vasseur F (1989) Cell proliferation and differentiation in the fetal and early postnatal mouse thymus. *J Immunol* 142:3369-3377.
- Perretti M, Duncan GS, Flower RJ, Peers SH (1993) Serum corticosterone, interleukin-1 and tumour necrosis factor in rat experimental endotoxaemia: comparison between Lewis and Wistar strains. *Br J Pharmacol* 110:868-874.
- Pitychoutis PM, Nakamura K, Tsonis PA, Papadopoulou-Daifoti Z (2009) Neurochemical and behavioral alterations in an inflammatory model of depression: sex differences exposed. *Neuroscience* 159:1216-1232.
- Pohlack ST, Nees F, Liebscher C, Cacciaglia R, Diener SJ, Ridder S, Woermann FG, Flor H (2012) Hippocampal but not amygdalar volume affects contextual fear conditioning in humans. *Hum Brain Mapp* 33:478-488.
- Ponder CA, Kliethermes CL, Drew MR, Muller J, Das K, Risbrough VB, Crabbe JC, Gilliam TC, Palmer AA (2007) Selection for contextual fear conditioning affects anxiety-like behaviors and gene expression. *Genes Brain Behav* 6:736-749.
- Preil J, Muller MB, Gesing A, Reul JM, Sillaber I, van Gaalen MM, Landgrebe J, Holsboer F, Stenzel-Poore M, Wurst W (2001) Regulation of the hypothalamic-pituitary-adrenocortical system in mice deficient for CRH receptors 1 and 2. *Endocrinology* 142:4946-4955.
- Propes MJ, Johnson RW (1997) Role of corticosterone in the behavioral effects of central interleukin-1 beta. *Physiol Behav* 61:7-13.
- Qiu L, Zhu CL, Wang XY, Xu FL (2007) Changes of cell proliferation and differentiation in the developing brain of mouse. *Neurosci Bull* 23:46-52.
- Quan N, Stern EL, Whiteside MB, Herkenham M (1999) Induction of pro-inflammatory cytokine mRNAs in the brain after peripheral injection of subseptic doses of lipopolysaccharide in the rat. *J Neuroimmunol* 93:72-80.
- Ramboz S, Oosting R, Amara DA, Kung HF, Blier P, Mendelsohn M, Mann JJ, Brunner D, Hen R (1998) Serotonin receptor 1A knockout: an animal model of anxiety-related disorder. *Proc Natl Acad Sci U S A* 95:14476-14481.
- Ramos A, Berton O, Mormede P, Chaouloff F (1997) A multiple-test study of anxiety-related behaviours in six inbred rat strains. *Behav Brain Res* 85:57-69.
- Ramseier H, Lichtensteiger W, Schlumpf M (1993) In vitro inhibition of cellular immune responses by benzodiazepines and PK 11195. Effects on mitogen- and alloantigen-driven lymphocyte proliferation and on IL-1, IL-2 synthesis and IL-2 receptor expression. *Immunopharmacol Immunotoxicol* 15:557-582.

- Raone A, Cassanelli A, Scheggi S, Rauggi R, Danielli B, De Montis MG (2007) Hypothalamus-pituitary-adrenal modifications consequent to chronic stress exposure in an experimental model of depression in rats. *Neuroscience* 146:1734-1742.
- Rhodes ME, Balestreire EM, Czambel RK, Rubin RT (2002) Estrous cycle influences on sexual diergism of HPA axis responses to cholinergic stimulation in rats. *Brain Res Bull* 59:217-225.
- Rilett KC, Ellegood J, Friedel M, MacKenzie RN, Lai JKY, Lerch JP, Foster JA (2013) Functional loss of T cells results in sex differences in anxiety phenotype and neuroanatomy. In: *The Canadian College of Neuropsychopharmacology Meeting*. Toronto, ON.
- Robison RJ, Reimherr FW, Marchant BK, Faraone SV, Adler LA, West SA (2008) Gender differences in 2 clinical trials of adults with attention-deficit/hyperactivity disorder: a retrospective data analysis. *J Clin Psych* 69:213-221.
- Rodgers RJ, Cole JC (1993) Influence of social isolation, gender, strain, and prior novelty on plus-maze behaviour in mice. *Physiol Behav* 54:729-736.
- Rodgers RJ, Johnson NJ (1995) Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety. *Pharmacol Biochem Behav* 52:297-303.
- Rodgers RJ, Dalvi A (1997) Anxiety, defence and the elevated plus-maze. *Neurosci Biobehav Rev* 21:801-810.
- Roy BN, Reid RL, Van Vugt DA (1999) The effects of estrogen and progesterone on corticotropin-releasing hormone and arginine vasopressin messenger ribonucleic acid levels in the paraventricular nucleus and supraoptic nucleus of the rhesus monkey. *Endocrinology* 140:2191-2198.
- Russig H, Durrer A, Yee BK, Murphy CA, Feldon J (2003) The acquisition, retention and reversal of spatial learning in the morris water maze task following withdrawal from an escalating dosage schedule of amphetamine in wistar rats. *Neuroscience* 119:167-179.
- Ruttle PL, Shirtcliff EA, Serbin LA, Fisher DB, Stack DM, Schwartzman AE (2011) Disentangling psychobiological mechanisms underlying internalizing and externalizing behaviors in youth: longitudinal and concurrent associations with cortisol. *Horm Behav* 59:123-132.
- Salazar A, Gonzalez-Rivera BL, Redus L, Parrott JM, O'Connor JC (2012) Indoleamine 2,3-dioxygenase mediates anhedonia and anxiety-like behaviors caused by peripheral lipopolysaccharide immune challenge. *Horm Behav* 62:202-209.
- Sankar A, MacKenzie RN, Foster JA (2012) Loss of class I MHC function alters behaviour and stress reactivity. *Journal of Neuroimmunology*.
- Sapolsky R, Rivier C, Yamamoto G, Plotsky P, Vale W (1987) Interleukin-1 stimulates the secretion of hypothalamic corticotropin-releasing factor. *Science* 238:522-524.

- Sarabdjitsingh RA, Meijer OC, Schaaf MJ, de Kloet ER (2009) Subregion-specific differences in translocation patterns of mineralocorticoid and glucocorticoid receptors in rat hippocampus. *Brain Res* 1249:43-53.
- Scheetz AJ, Constantine-Paton M (1994) Modulation of NMDA receptor function: implications for vertebrate neural development. *Faseb J* 8:745-752.
- Scheibe S, Preuschhof C, Cristi C, Bagby RM (2003) Are there gender differences in major depression and its response to antidepressants? *Journal of affective disorders* 75:223-235.
- Scollay R, Smith J, Stauffer V (1986) Dynamics of early T cells: prothymocyte migration and proliferation in the adult mouse thymus. *Immunol Rev* 91:129-157.
- Seale JV, Wood SA, Atkinson HC, Bate E, Lightman SL, Ingram CD, Jessop DS, Harbuz MS (2004) Gonadectomy reverses the sexually divergent patterns of circadian and stress-induced hypothalamic-pituitary-adrenal axis activity in male and female rats. *J Neuroendocrinol* 16:516-524.
- Shear MK, Cloitre M, Pine D, Ross J (2005) Clinical importance of sex differences. In: *Business Briefing: Women's Healthcare*.
- Shepard JD, Barron KW, Myers DA (2000) Corticosterone delivery to the amygdala increases corticotropin-releasing factor mRNA in the central amygdaloid nucleus and anxiety-like behavior. *Brain Res* 861:288-295.
- Shepard JD, Barron KW, Myers DA (2003) Stereotaxic localization of corticosterone to the amygdala enhances hypothalamo-pituitary-adrenal responses to behavioral stress. *Brain Res* 963:203-213.
- Sidor MM (2009) Behavioural and molecular outcomes of early life immune challenge in mice. In: *Psychiatry and Behavioural Neuroscience*, pp xvi, 188. Hamilton, Ontario: McMaster University.
- Sidor MM, Rilett KC, Foster JA (2010a) Validation of an automated system for measuring anxiety-related behaviours in the elevated plus maze. *Journal of Neurosci Meth* 188:7-13.
- Sidor MM, Amath A, MacQueen G, Foster JA (2010b) A developmental characterization of mesolimbocortical serotonergic gene expression changes following early immune challenge. *J Neurosci Meth* 171:734-746.
- Sidor MM, Dzirasa K, Parekh P, Tye KM, Warden MR, Deisseroth K, McClung CA (2013) Chronic optogenetic stimulation of ventral tegmental dopamine neurons drives distinct and separable features of manic-like behaviour. In: *The Canadian College of Neuropsychopharmacology Meeting*. Toronto, ON.
- Somerville LH, Whalen PJ, Kelley WM (2010) Human bed nucleus of the stria terminalis indexes hypervigilant threat monitoring. *Biol Psych* 68:416-424.
- Spruijt BM, van Hooff JA, Gispen WH (1992) Ethology and neurobiology of grooming behavior. *Physiol Rev* 72:825-852.
- Stein MB, Simmons AN, Feinstein JS, Paulus MP (2007) Increased amygdala and insula activation during emotion processing in anxiety-prone subjects. *Am J Psych* 164:318-327.

- Stiller AL, Drugan RC, Hazi A, Kent SP (2011a) Stress resilience and vulnerability: The association with rearing conditions, endocrine function, immunology, and anxious behavior. *Psychoneuroendocrinology* 36:1383-1395.
- Stiller AL, Drugan RC, Hazi A, Kent SP (2011b) Stress resilience and vulnerability: The association with rearing conditions, endocrine function, immunology, and anxious behavior. *Psychoneuroendocrinology*.
- Swiergiel AH, Dunn AJ (2007) Effects of interleukin-1beta and lipopolysaccharide on behavior of mice in the elevated plus-maze and open field tests. *Pharmacol Biochem Behav* 86:651-659.
- Szymanski S, Lieberman JA, Alvir JM, Mayerhoff D, Loebel A, Geisler S, Chakos M, Koreen A, Jody D, Kane J (1995) Gender differences in onset of illness, treatment response, course, and biologic indexes in first-episode schizophrenic patients. *Am J Psych* 152:698-703.
- Taniguchi S, Nakazawa T, Tanimura A, Kiyama Y, Tezuka T, Watabe AM, Katayama N, Yokoyama K, Inoue T, Izumi-Nakaseko H, Kakuta S, Sudo K, Iwakura Y, Umemori H, Inoue T, Murphy NP, Hashimoto K, Kano M, Manabe T, Yamamoto T (2009) Involvement of NMDAR2A tyrosine phosphorylation in depression-related behaviour. *Embo J* 28:3717-3729.
- Tarr AJ, Chen Q, Wang Y, Sheridan JF, Quan N (2012) Neural and behavioral responses to low-grade inflammation. *Behav Brain Res* 235:334-341.
- Tracey KJ, Lowry SF, Fahey TJ, 3rd, Albert JD, Fong Y, Hesse D, Beutler B, Manogue KR, Calvano S, Wei H, et al. (1987) Cachectin/tumor necrosis factor induces lethal shock and stress hormone responses in the dog. *Surg Gynecol Obstet* 164:415-422.
- Trullas R, Skolnick P (1993) Differences in fear motivated behaviors among inbred mouse strains. *Psychopharmacology (Berl)* 111:323-331.
- Turnbull AV, Rivier CL (1999) Regulation of the Hypothalamic-Pituitary-Adrenal Axis by Cytokines: Actions and Mechanisms of Action. *Physiological Reviews* 79:1-79.
- van Eijk LT, Dorresteyn MJ, Smits P, van der Hoeven JG, Netea MG, Pickkers P (2007) Gender differences in the innate immune response and vascular reactivity following the administration of endotoxin to human volunteers. *Crit Care Med* 35:1464-1469.
- Van Kaer L, Ashton-Rickardt PG, Ploegh HL, Tonegawa S (1992) TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and CD4-8+ T cells. *Cell* 71:1205-1214.
- Vesga-Lopez O, Schneier FR, Wang S, Heimberg RG, Liu SM, Hasin DS, Blanco C (2008) Gender differences in generalized anxiety disorder: results from the National Epidemiologic Survey on Alcohol and Related Conditions (NESARC). *Journal Clin Psych* 69:1606-1616.
- Viau V, Bingham B, Davis J, Lee P, Wong M (2005) Gender and puberty interact on the stress-induced activation of parvocellular neurosecretory neurons

- and corticotropin-releasing hormone messenger ribonucleic acid expression in the rat. *Endocrinology* 146:137-146.
- Vicente A, Varas A, Sacedon R, Jimenez E, Munoz JJ, Zapata AG (1998) Appearance and maturation of T-cell subsets during rat thymus ontogeny. *Develop Immunol* 5:319.
- Voikar V, Koks S, Vasar E, Rauvala H (2001) Strain and gender differences in the behavior of mouse lines commonly used in transgenic studies. *Physiology & Behavior* 72:271-281.
- von Engelhardt J, Doganci B, Jensen V, Hvalby O, Gongrich C, Taylor A, Barkus C, Sanderson DJ, Rawlins JN, Seeburg PH, Bannerman DM, Monyer H (2008) Contribution of hippocampal and extra-hippocampal NR2B-containing NMDA receptors to performance on spatial learning tasks. *Neuron* 60:846-860.
- Walf AA, Koonce C, Manley K, Frye CA (2009) Proestrous compared to diestrous wildtype, but not estrogen receptor beta knockout, mice have better performance in the spontaneous alternation and object recognition tasks and reduced anxiety-like behavior in the elevated plus and mirror maze. *Behav Brain Res* 196:254-260.
- Wang J, Dunn AJ (1999) The role of interleukin-6 in the activation of the hypothalamo-pituitary-adrenocortical axis and brain indoleamines by endotoxin and interleukin-1 beta. *Brain Res* 815:337-348.
- Weinstein Y, Ran S, Segal S (1984) Sex-associated differences in the regulation of immune responses controlled by the MHC of the mouse. *J Immunol* 132:656-661.
- Weinstock M, Razin M, Schorer-Apelbaum D, Men D, McCarty R (1998) Gender differences in sympathoadrenal activity in rats at rest and in response to footshock stress. *Int J Dev Neurosci* 16:289-295.
- Weiser MJ, Foradori CD, Handa RJ (2010) Estrogen receptor beta activation prevents glucocorticoid receptor-dependent effects of the central nucleus of the amygdala on behavior and neuroendocrine function. *Brain Res* 1336:78-88.
- Weiser MJ, Goel N, Sandau US, Bale TL, Handa RJ (2008) Androgen regulation of corticotropin-releasing hormone receptor 2 (CRHR2) mRNA expression receptor binding in the rat brain. *Exp Neurol* 214:62-58.
- Weissman MM, Olfson M (1995) Depression in women: implications for health care research. *Science* 269:799-801.
- Weisstaub NV, Zhou M, Lira A, Lambe E, Gonzalez-Maeso J, Hornung JP, Sibille E, Underwood M, Itohara S, Dauer WT, Ansorge MS, Morelli E, Mann JJ, Toth M, Aghajanian G, Sealton SC, Hen R, Gingrich JA (2006) Cortical 5-HT_{2A} receptor signaling modulates anxiety-like behaviors in mice. *Science* 313:536-540.
- Whitacre CC (2001) Sex differences in autoimmune disease. *Nat Immunol* 2:777-780.

- Wilhelm I, Born J, Kudielka BM, Schlotz W, Wust S (2007) Is the cortisol awakening rise a response to awakening? *Psychoneuroendocrinology* 32:358-366.
- Wilkinson PO, Goodyer IM (2011) Childhood adversity and allostatic overload of the hypothalamic-pituitary-adrenal axis: a vulnerability model for depressive disorders. *Dev Psychopathol* 23:1017-1037.
- Wolfram M, Bellingrath S, Kudielka BM (2011) The cortisol awakening response (CAR) across the female menstrual cycle. *Psychoneuroendocrinology* 36:905-912.
- Yaguchi T, Nagata T, Yang D, Nishizaki T Interleukin-18 regulates motor activity, anxiety and spatial learning without affecting synaptic plasticity. *Behav Brain Res* 206:47-51.
- Ziv Y, Ron N, Butovsky O, Landa G, Sudai E, Greenberg N, Cohen H, Kipnis J, Schwartz M (2006) Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood. *Nat Neurosci* 9:268-275.
- Zoccola PM, Dickerson SS (2012) Assessing the relationship between rumination and cortisol: a review. *J Psychosom Res* 73:1-9.
- Zohar I, Weinstock M (2011) Differential effect of prenatal stress on the expression of corticotrophin-releasing hormone and its receptors in the hypothalamus and amygdala in male and female rats. *J Neuroendocrinol* 23:320-328.