EARLY LIFE IMMUNE CHALLENGE IN MICE

BEHAVIOURAL AND MOLECULAR OUTCOMES OF EARLY LIFE IMMUNE CHALLENGE IN MICE

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ABSTRACT

Although historically treated as separate systems, there is considerable interaction between the immune system and brain. It has become increasingly clear that immune-brain communication is important to both health and disease. An immunogenic challenge given during the first postnatal week in rodents impacts the developing central nervous system (CNS) leading to long-term behavioural and molecular alterations reflective of enhanced stress-reactivity. Anxiety and depression are stress-related pathologies with a proposed neurodevelopmental origin suggesting that perturbation to neonatal immune-brain signalling may contribute to psychopathology. The current body of work examined the long-term impact of an early immune challenge on behavioural and molecular phenotypes associated with anxiety and depression. Mice were administered lipopolysaccharide (LPS) on postnatal days three and five. The emergence of anxiety-related behaviour was characterized along the developmental trajectory of LPS-mice concurrent with changes to serotonergic neurocircuitry. Adult depressive-related behaviour was assessed in the forced swim test (FST) along with hippocampal neurogenesis as revealed by immunoreactivity for bromodeoxyuridine (BrdU) and doublecortin (DCX). The results demonstrated a sex-specific alteration in both the temporal emergence and phenotypic variant of anxiety-related behaviours displayed by LPS-mice. This was accompanied by changes to CNS serotonergic-related gene expression that coincided with a critical developmental time window essential to the establishment of emotionality. Adult LPS-mice exhibited hyperactivity during the FST

that was accompanied by increased doublecortin immunoreactivity in the dorsal and ventral hippocampus, reflecting enhanced immature neuronal differentiation. The current results demonstrate that an early immune challenge impacts the developing CNS leading to enhanced emotional-reactivity. Altered serotonergic neurocircuitry and adult hippocampal neurogenesis may underlie behavioural abnormalities. The current body of work demonstrates a preeminent role for early-life immune disturbance in psychopathology and advances understanding of how immune-brain signalling impacts the developing CNS and confers risk for later disease.

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ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
5HTT	Serotonin transporter
ACTH	Adrenocorticotropic hormone
BrdU	Bromodeoxyuridine
CNS	Central nervous system
CORT	Corticosterone
COX	Cyclo-oxygenase
CRH	Corticotrophin releasing hormone
DCX	Doublecortin
DR	Dorsal raphe
EPM	Elevated plus maze
FST	Forced swim test
GABA	Gamma-aminobutyric acid
HPA	Hypothalamic-pituitary-adrenal axis
iNOS	Inducible nitric oxide synthase
IL	Interleukin
i.p.	Intraperitoneal
LPS	Lipopolysaccharide
MDD	Major Depressive Disorder
mRNA	Messenger ribonucleic acid
NSF	Novelty suppressed feeding test
P	Postnatal day
PVN	Paraventricular nucleus
OCD	Obsessive-Compulsive Disorder
SAL	Saline
SSRI	Selective serotonin reuptake inhibitor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TPH	Tryptophan hydroxylase

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STATEMENT OF CONTRIBUTIONS

The author would like to acknowledge the following individuals for their experimental contributions: Aysah Amath for her help with *in situ* hybridization image capture and data collection; Patrick Martin who was the second observer for immunohistochemical cell counting; and Kelly Rilett who acted as a second observer, provided intellectual contribution to logistics of data collection, and helped in the preparation of the schematic figures for validation of the elevated plus maze. Author's contributions associated with each study are presented sequentially: Paper 1. Experimental design, data collection (animal breeding and general care, behavioural testing), data analysis and interpretation, preparation and writing of manuscript. Paper 2. Experimental design, primer design and generation of probes, *in situ* hybridization, collection of *in situ* images, data analysis and interpretation, preparation and writing of manuscript. Paper 3. Data collection (behavioural testing, immunohistochemical cell counting), data analysis and interpretation, preparation and writing of manuscript. Paper 4. (appendix) Study design and organization, data collection, data analysis and interpretation, preparation and writing of manuscript.

CHAPTER 1. INTRODUCTION

1.1. Immune-brain communication and vulnerability to disease

Immune-brain communication is both continual and bidirectional. This reciprocal communication is essential to healthy brain function and is especially important during the early postnatal period which represents a time of enhanced vulnerability for the developing central nervous system (CNS). Aberrant neuroimmune interactions have been implicated in a variety of neurological diseases (Patrick and Lindstrom, 1973; Solimena et al., 1990; Rogers et al., 1994; Darnell and Posner, 2003; McGeer and McGeer, 2003) including psychiatric disorders such as anxiety and depression (Anisman, 2009). The proposed developmental origin of these latter disorders (Ansorge et al., 2007; Leonardo and Hen, 2008) presents the intriguing possibility that altered neonatal immune-brain signalling can influence the developmental trajectory of the CNS and confer risk for later psychopathology. The current body of work is aimed at delineating the long-term consequences of an early-life immunogenic challenge on behavioural and molecular phenotypes associated with anxiety and depression. This is crucial in understanding the contribution of early immune disturbance to the pathogenesis of these disorders and has broad implications for understanding how early immune-brain signalling modifies susceptibility and vulnerability to disease.

1.2. Preclinical models of early-life adversity

Early environmental stressors can have long-term consequences on the developing CNS. Clinical and preclinical evidence demonstrates that exposure to perinatal stress or trauma increases the risk for the later development of anxiety and depression (Heim and Nemeroff, 2001), suggesting a developmental origin and programming of psychopathology (Ansorge et al., 2007; Leonardo and Hen, 2008). Examples of established preclinical rodent models of early adversity include postnatal maternal separation and maternal deprivation. Work in these animal models has examined the long-term consequences of early-life stress on the modulation of behavioural and molecular phenotypes associated with anxiety, depression (collectively termed emotionality), and stress-reactivity (Levine, 1957; Denenberg and Smith, 1963; Plotsky and Meaney, 1993; Shanks et al., 1995; Liu et al., 1997; Kikusui et al., 2004). Maternal separation experiments involve removal of the dam from the pups for three hours daily throughout much of the pre-weaning period. This results in behavioural deficits indicative of enhanced emotionality and stress-reactivity (Plotsky and Meaney, 1993; Lee et al., 2007; Lambas-Senas et al., 2009; Romeo et al., 2003; Parfitt et al., 2004) that are accompanied by altered CNS expression levels or function of glucocorticoid receptors (Meaney and Aitken, 1985), GABA receptors (Hsu et al., 2003), serotonin (Gardner et al., 2009), and brain-derived neurotrophic factor (BDNF) (Lippmann et al., 2007). Maternal deprivation due to early weaning leads to increased aggressive and anxiety-related behaviours in adult rodents (Kikusui et al., 2004; Kanari et al., 2005). This behavioural

phenotype is accompanied by a molecular signature representative of enhanced emotionality, such as increased basal and stress-induced plasma corticosterone (CORT) and hippocampal pathology including decreased BDNF protein levels, reduced serotonin receptor mRNA expression, and altered adult neurogenesis (Kikusui et al., 2006; Nakamura et al., 2008; Kikusui et al., 2009).

Exogenous factors such as the timing and duration of early-life insult, and endogenous factors such as sex of the animal, can modify the long-term outcome of early adversity (Lehmann and Feldon, 2000; Millstein and Holmes, 2007). For instance, in contrast to the effects of prolonged maternal separation, shorter bouts (approximately 15 min daily) result in a reduction of corticotrophin releasing hormone (CRH) mRNA, adrenocorticotropic hormone (ACTH) and CORT in response to stress (Plotsky and Meaney, 1993; Parfitt et al., 2004) and attenuate anxiety-like behaviours in mice genetically modified to exhibit enhanced emotionality (Zanettini et al., 2009). Sexual dichotomy is present in maternally deprived rodents, with male and female rodents displaying differential adult behavioural and molecular phenotypes in response to identical early-life adversity (Wigger and Neumann, 1999; Romeo et al., 2003; Ono et al., 2008; Kikusui et al., 2009). While the majority of these models were originally characterized in rats, accumulating studies have utilized mice which permits the exploration of potential genetic influences on early adversity and contribution to later pathology (Muller and Keck, 2002; Millstein and Holmes, 2007). Collectively, these studies demonstrate the importance of early environmental manipulation on neurodevelopment and its impact on stress-related behavioural and molecular phenotypes.

1.3. The Early Immune Challenge Model

1.3.1. Acute effects of peripheral lipopolysaccharide administration

Early immune challenge with the bacterial endotoxin lipopolysaccharide (LPS) has emerged as an additional rodent model to study the long-term impact of early-life stressors on CNS development. LPS is a component of the cell wall of gram-negative bacteria and is a major pathogen-associated molecular pattern recognized by the innate immune system through toll-like receptor (TLR) signalling. TLRs are components of the innate immune system that participate in pathogen recognition (Ozinsky et al., 2000). LPS interacts with TLR4 (Poltorak et al., 1998), found on circulating monocytes, tissue macrophages, and constitutively expressed in cells located on the circumventricular organs of the CNS (Laflamme and Rivest, 2001) and on brain endothelial cells. LPS-TLR4-CD14 binding leads to transduction of a proinflammatory signal that activates genes encoding cytokines (IL-1β, Il-6, and TNF-α), chemokines, and enzymes such as COX-2 and iNOS. These soluble mediators can act centrally to orchestrate a physiological cascade of events that include induction of fever (Kozak et al., 1994), a pattern of behavioural alterations collectively termed sickness behaviour (Hart, 1988), and robust activation of the hypothalamic-pituitary-adrenal (HPA) axis (Smith et al., 1994; Whiteside et al., 1999; Vakharia and Hinson, 2005) which leads to increased plasma ACTH and CORT (Givalois et al., 1994).

The neonatal physiological response to an immunogenic challenge is similar to that experienced in adult rodents. A single injection with sub-septic doses of LPS during the first postnatal week leads to a transient increase in peripheral pro-inflammatory cytokines (Walker et al., 2009b) and activation of the HPA-axis as revealed by increased *c-fos* and CRH mRNA expression in the hypothalamic PVN (Dent et al., 1999), reduced median eminence CRH levels (Shanks and Meaney, 1994), and increased plasma ACTH and CORT (Shanks and Meaney, 1994; Walker et al., 2004b). This indicates that early endotoxin challenge can alter centrally mediated processes in a manner that is similar to the innate immune response in adults. Work aimed at understanding the impact of early immune challenge on CNS development is outlined below.

1.3.2. Long-term impact of peripheral lipopolysaccharide administration

Rodents exposed to an early immune challenge with LPS display an array of long-term behavioural and physiological alterations. These include cognitive abnormalities (Bilbo et al., 2005; Bilbo et al., 2006), altered neuroimmune functioning (Boisse et al., 2004; Spencer et al., 2006b), modified risk for disease (Shanks et al., 2000; Hodgson et al., 2001; Breivik et al., 2002; Spencer et al., 2007; Galic et al., 2008; Ellestad et al., 2009), and enhanced stress-reactivity and altered emotionality in adulthood (Shanks et al., 1995; Granger et al., 1996; Shanks et al., 2000; Breivik et al., 2002; Walker et al., 2004a; Bilbo et al., 2007; Walker et al., 2008; Walker et al., 2009a). Similar to other early-life adversity models, the long-term consequences of LPS challenge are highly dependant on

factors such as the timing of exposure during ontogeny (Spencer et al., 2005; Spencer et al., 2006a; Harre et al., 2008) and sex which can differentially impact disease susceptibility (Shanks et al., 1995; Tenk et al., 2007; Tenk et al., 2008; Walker et al., 2009a). Specifically, LPS challenge on postnatal day (P)3 and 5 (relatively comparable to third trimester human pregnancy) leads to a unique profile of sex-specific long-term behavioural and molecular alterations that converge on modification of stress-reactivity and emotionality; an effect not observed with later exposure or exposures of shorter duration (see Table 1). At the molecular level this is revealed by increased basal CRH mRNA expression in the PVN and increased basal or stress-induced plasma CORT in P3, P5 challenged rodents (Shanks et al., 1995; Shanks et al., 2000). Decreased levels of glucocorticoid receptor density in the hypothalamus, hippocampus, and frontal cortex indicate a reduced capacity for negative feedback control of the stress-axis (Shanks et al., 1995). This is accompanied by enhanced emotional-reactivity and increased anxiety-related behaviours in adulthood (Breivik et al., 2002; Walker et al., 2004a; Walker et al., 2008; Walker et al., 2009a). Importantly, an endotoxin exposure of shorter duration during the first postnatal week (single injection on P4 or P7) or a later exposure on P14 does not increase stress-reactivity or emotionality (see Table 1). In fact, one study reported a blunted CORT response to a stressor experienced in adulthood in P4 challenged rodents (Bilbo et al., 2007). This indicates that the timing of exposure during early development is critical in establishing the long-term consequences of an early immune challenge.

Table 1. Adult stress-related behavioural and molecular phenotypes in the early immune challenge model

Model	Rat Strain	LPS (i.p.)	Sex	Adult Phenotype	Reference
P3, P5	Long Evans	0.05mg/kg	Female *Male	Increased plasma ACTH and CORT response to restraint stress; increased CRH mRNA in PVN*; increased CRH* and AVP content in ME; decreased GR density in hypothalamus, hippocampus, and frontal cortex	Shanks et al., 1995
	Sprague-Dawley	0.05mg/kg	Male	Increased basal and noise-stress induced plasma CORT; prolonged hyperactivity in response to noise-stress	Shanks et al., 2000
	Lewis	0.05mg/kg	Male	Increased anxiety-like behaviour in EPM, open field, and social interaction tests	Breivik et al., 2002
	Fischer 344	0.05mg/kg	Male	Increased anxiety-like behaviour in EPM	Walker et al., 2004
	Long Evans	0.05mg/kg	Male	Exaggerated acoustic startle response following restraint stress; Increased stress-induced plasma CORT	Walker et al., 2008
	Wistar	0.05mg/kg	Female *Male	Exaggerated acoustic startle response; increased basal anxiety-like behaviour in hide box/open field, EPM*; stress-induced behavioural inhibition (hypoactivity)*; increased stress-induced CORT	Walker et al., 2009
P4	Sprague-Dawley	live *E. Coli*	Male	No difference in basal or stress-induced plasma CORT response; no difference in activity levels in open field	Bilbo et al., 2006
	Sprague-Dawley	live *E. Coli*	Male	Blunted CORT response to tail shock; antidepressant response in SPT; no difference in basolateral amygdala 5-HT protein	Bilbo et al., 2007
P7	Sprague-Dawley	0.1mg/kg	Male	No difference in anxiety-like behaviour in EPM	Spencer et al., 2006
P14	Sprague-Dawley	0.1mg/kg	Male	No anxiety-like behaviour in EPM and open-field (exploratory and locomotor indices included); no depressive-like behaviour in FST; reduced novel object exploration	Spencer et al., 2005
	Sprague-Dawley	0.1mg/kg	Male	No difference in anxiety-like behaviour in EPM	Spencer et al., 2006

5-HT: serotonin; ACTH: adrenocorticotropic hormone; AVP: arginine vasopressin; CRH: corticotrophin releasing hormone; CORT: corticosterone; EPM: elevated plus maze; FST: forced swim test; GR: glucocorticoid receptor; i.p. intraperitoneal; LPS: lipopolysaccharide; ME: median eminence; P: postnatal day; PVN: paraventricular nucleus; SPT: sucrose preference test

1.3.3. Early immune challenge: modelling an etiological factor in affective disorders

The convergence of P3, P5 LPS challenge on adult stress-related phenotypes suggests that this particular model offers a unique translational approach to understanding stress-related pathologies. Notably, similarities in the profile of physiological and behavioural alterations between P3, P5 LPS-challenged rodents and mood disorder patients (see Table 2) establish this model as a viable tool by which to examine the role of the early immune environment in the etiology of stress-related disorders such as anxiety and depression. This is especially relevant given the evidence linking anxiety and depression to immune system disturbance (Anisman, 2009). Patients with anxiety and/or major depression disorder (MDD) exhibit increased peripheral blood inflammatory biomarkers consistent with a pro-inflammatory phenotype, suggesting a link between immune system activation and mood abnormalities (for review see: Goldstein et al., 2009; Miller et al., 2009). This includes a general imbalance in the T-helper type (Th)1/Th2 cytokine profile in MDD (Kim et al., 2007; Sutcigil et al., 2007) with specific increases in peripheral TNF-α, IL-6, and IL-1β levels reported in MDD (Maes et al., 1995; Mikova et al., 2001; Alesci et al., 2005) and anxiety (Maes et al., 1999; Gill et al., 2008; Hoge et al., 2009). The increased prevalence of comorbid illnesses with a prominent immune basis, such as asthma (Katon et al., 2007), inflammatory bowel disorders (Kurina et al., 2001), diabetes (Engum, 2007), and cardiovascular illness (Kawachi et al., 1994; Ford et al., 1998), in patients with anxiety and depression points to underlying immune system abnormalities or a shared pathogenic basis. Indeed, early immune challenge in rodents

Table 2. Early immune challenge as a model for affective disorders

Mood Disorder Patients	References	P3, P5 Immune Challenge Model	References
Primary anxiety disorder or high comorbidity of anxiety	Simon et al., 2009	Increased adult anxiety-related behaviours	Breivik et al., 2002; Walker et al., 2004, 2009b
Altered HPA function	Plotsky et al., 1998	Increased behavioural and physiological stress response (CORT, ACTH); decreased GR binding in hypothalamus, hippocampus, frontal cortex; blunted response to dexamethasone suppression	Shanks et al., 1995, 2000; Nilsson et al., 2002; Walker et al., 2008
Increased prevalence of substance abuse	Bolten et al., 2009	Increased behavioural sensitization to drugs of reward	Tenk et al., 2007
Altered circadian rhythms	Germain and Kupfer, 2008	Altered behavioural and endocrine diurnal rhythmicity	Shanks et al., 2000; Breivik et al., 2002
Altered peripheral immune markers	Raison et al., 2006	Impaired natural killer-cell activity, increased plasma IL-6, decreased plasma IFN-gamma; altered immunoglobulin production; altered predisposition to adult inflammatory disease	Hodgson et al., 2001; Breivik et al., 2002; Shanks et al., 2000; Walker et al., 2009a
Increased incidence of metabolic disturbances	McIntyre et al., 2009	Altered insulin sensitivity, glucose homeostasis, neuroendocrine activity, and adipose deposition	Nilsson et al., 2002; Knox et al., 2009; Li et al., 2007; Walker et al., 2006

leads to long-term immune abnormalities that may drive or contribute to disease etiology (Breivik et al., 2002; Galic et al., 2008; Hodgson et al., 2002). Therefore, a careful exploration of anxiety and depressive-related behaviours and associated pathophysiology in early LPS challenged rodents is essential in further elucidating the role of early immune perturbation in the etiology of stress-related psychopathology. Outlined below is a discussion of the behavioural tools available for the preclinical assessment of anxiety and depressive-related behaviours.

1.4. Preclinical assessment of anxiety and depression

A repertoire of ethologically relevant and valid behavioural tools are available that model and test aspects of rodent emotionality. Validity is based on resemblance to human psychiatric disease in terms of symptoms, behavioural signs, underlying biological mechanisms, and sensitivity to mood modifying treatments (McKinney and Bunney, 1969). While no one test can fully recapitulate the complex heterogeneity of human anxiety or depression, these tests do capture discrete clinical features or endophenotypes (Gottesman and Gould, 2003; see Tables 3, 4). In the aggregate, these tests become powerful tools by which to profile a range of anxiety and depressive-related behaviours and represent an important step in the identification of underlying neuropathology.

Table 3. Preclinical tests of anxiety-like behaviour

Clinical symptom*	Preclinical measure	Anxiety Test
Avoidance of anxiety-inducing areas	Avoidance of exposed brightly lit areas	Open field, light-dark, elevated plus maze, novelty suppressed feeding test
Anxiety induced during social engagements	Decreased social interaction with unfamiliar conspecific	Social interaction test
Anxiety-inducing obsessions and anxiety-reducing compulsions	Increased marble-burying and excessive grooming	Marble-burying test; grooming
Heightened startle response	Increased acoustic startle response	Acoustic startle
Separation anxiety	Increased pup ultrasonic vocalizations upon separation from Mom	Ultrasonic vocalization
Increased arousal and avoidance to stimuli associated with previous trauma	Increased freezing response to fear conditioned cue or context	Pavlovian fear conditioning

* based on symptoms provided in the *Diagnostic and Statistical Manual-IV* for diagnosis of anxiety disorder; N.B. not an inclusive list.

Table modified from: Cryan and Holmes (2005) Nat. Rev. Drug Discovery, 4, 775-790.

Table 4. Preclinical tests of depressive-like behaviours

Clinical symptom*	Preclinical measure	Test/Model
Psychomotor impairment	Immobility (inability to sustain effort)	Forced swim test, tail suspension test, learned helplessness
Loss of interest in pleasurable activities	Reduced intake of positive reward	Sucrose preference test
Changes in appetite or weight	Decreased body mass in response to chronic stress	Restraint stress, chronic mild stress
Difficulty performing everyday tasks (e.g. personal hygiene)	Poor coat condition	Chronic mild stress
Altered sleep pattern, circadian rhythms	Altered diurnal/nocturnal activity patterns	Home cage activity; wheel running

* based on symptoms provided in the *Diagnostic and Statistical Manual-IV* for diagnosis of anxiety disorder; N.B. Not an inclusive list.

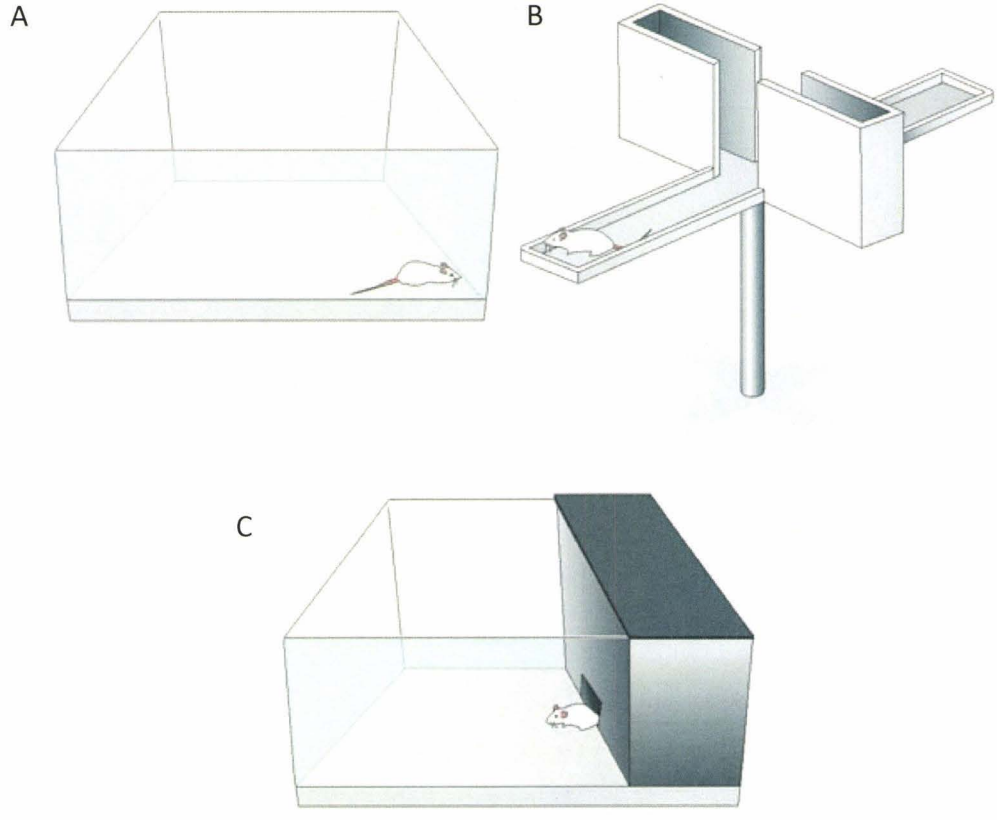
Table modified from: Cryan and Holmes (2005) Nat. Rev. Drug Discovery, 4, 775-790.

1.4.1. Anxiety-like behaviour: Exploratory approach-avoidance paradigm

Exploratory tests of anxiety are based on ethological tasks that exploit the natural behaviour of a rodent (Cryan and Holmes, 2005). The innate tendency for rodents to approach and explore novel environments and the opposing natural aversion to exposed brightly lit areas form the conceptual basis of these tests. Anxiety is assessed by measuring the degree to which an animal engages in the respective approach versus avoidance behaviours. The exploratory based tests are currently the most widely used tests for assaying anxiety-like behaviour (Holmes, 2001).

The open field (Hall, 1936), light-dark test (Crawley and Goodwin, 1980), and elevated plus maze (EPM; (Handley and Mithani, 1984)) are examples of well established and widely used exploratory based tests of anxiety (see Figure 1). Anxiety is measured in these tests by avoidance of aversive zones that are represented by the centre of the open field, illuminated area of the light-dark box, and open arms of the EPM. Indeed, anxiety modifying interventions alter the extent to which a rodent explores these aversive zones. Increased exploration, measured in units of time and/or distance travelled, of the centre of the open field, light arena of the light-dark box, and open arms of the EPM in response to treatment with anxiolytic compounds (e.g. benzodiazepines) is interpreted as reduced anxiety-like behaviour (Crawley and Goodwin, 1980; Crawley, 1981; Handley and Mithani, 1984; Pellow et al., 1985; Belzung et al., 1987; Lister, 1987; Prut and Belzung, 2003). The corresponding decrease in exploration of these aversive zones in response to

Exploratory based tests of anxiety



Modified from: Cryan & Holmes (2005) Nat. Rev., 4, 775-790.

Figure 1. Examples of exploratory based tests for assessment of rodent anxiety-related behaviour. These tests are considered ethologically relevant as they exploit the innate approach-avoidance behaviour that rodents display when confronted with novel environments such as the open field (**A**), elevated plus maze (**B**), and light/dark box (**C**). Anxiety is assessed by measuring avoidance of the aversive centre of the open field, open arms of the elevated plus maze, and light compartment of the light/dark box.

anxiogenic compounds is interpreted as increased anxiety-like behaviour. Behaviours such as directed exploration (e.g. rearing) and general locomotion can be assayed in addition to the aforementioned standard measures of anxiety-like behaviour (Crawley et al., 1984; Lister, 1987; Rodgers and Johnson, 1995). In fact, dissociating locomotion from emotionality during exploratory based tests of anxiety is important in ruling out any potential confounding effects of altered mobility on the capacity of a rodent to explore and move about the testing environment (Weiss et al., 1998). There has been some debate and concern as to which measures across different tests constitute a single particular behaviour. To this end, statistical methods such as factor analysis have been helpful in identifying measures that are related (factor together) across tests (Ramos et al., 1997; Milner and Crabbe, 2008; Brigman et al., 2009), albeit results have not always been consistent (Ramos et al., 1998; Holmes et al., 2003). This inconsistency, in part, has contributed to the growing consensus that anxiety is multidimensional with each test representing partially overlapping constructs (Cryan and Holmes, 2005) and thereby measuring different facets of anxiety-like behaviour (Belzung and Le Pape, 1994; Ramos and Mormede, 1998; van Gaalen and Steckler, 2000; Turri et al., 2001; Holmes et al., 2003). This latter point argues for the use of a battery of tests to capture and measure the full repertoire of anxiety-related behaviours (van Gaalen and Steckler, 2000). The reproducible modification of anxiety-like behaviour across a battery of tests would, therefore, provide a strong argument for an anxiety-like phenotype.

1.4.2. Depressive-like behaviour: Behavioural despair paradigm

Stress is considered a major predisposing factor to MDD as it often precipitates and proceeds illness onset and that of subsequent mood episodes in vulnerable individuals (Kendler et al., 1995; Kendler et al., 1999). Accordingly, animal tests to assess depressive-related behaviours involve exposure to stressful situations. A variety of different tests have been developed, each measuring a different facet of depressive symptomatology (see Table 4). For instance, both the tail suspension test and forced swim test (FST) involve the induction and measurement of behavioural despair; whereas the sucrose preference test measures anhedonia, a loss of interest in pleasurable activities. The most widely used of these tests is the FST, originally developed by Porsolt and colleagues (Porsolt et al., 1977a; Porsolt et al., 1977b). Rodents exposed to an inescapable cylinder filled with tepid water will initially engage in active escape behaviours such as swimming and climbing. As the test progresses, mice will naturally adopt a passive coping strategy characterized by floating or immobility. This pattern of behaviour forms the conceptual basis of the FST with measures such as duration of immobility and latency to float as indicators of depressive behaviour (Porsolt et al., 1977a; Porsolt et al., 1977b; Castagne et al., 2009). These behaviours are sensitive to a range of pharmacological and non-pharmacological antidepressant treatments (REM sleep deprivation, electroconvulsive shock therapy, environmental enrichment, and voluntary exercise; (Porsolt et al., 1978; Bjornebekk et al., 2005)) that increase active escape-

directed behaviours, which is interpreted as an antidepressant-like response. Conversely, factors known to play an etiological role in inducing or increasing susceptibility to clinical depression, such as early-life stress (Papaioannou et al., 2002; Lee et al., 2007), chronic stress (Solberg et al., 1999; Tannenbaum et al., 2002; Dunn and Swiergiel, 2008; Kompagne et al., 2008; Veena et al., 2009), or a genetic vulnerability to stress-related pathology (El Yacoubi et al., 2003; Cryan and Mombereau, 2004; Bjornebekk et al., 2005) increase immobility in the FST as evidence of increased depressive-like behaviour. Finally, individual differences in immobility within rodent strains can be mapped to particular quantitative trait loci implicated in MDD (Yoshikawa et al., 2002). Together this demonstrates the suitability of the FST as a tool for assessing depressive-related phenotypes in rodents.

1.4.3. The automated advantage

A variety of automated systems have been developed and validated for obtaining behavioural measures during tests of anxiety and depressive-related behaviours (Young and Johnson, 1991; Hedou et al., 2001; Noldus et al., 2001; Torres and Escarabajal, 2002; Kurtuncu et al., 2005). These systems rely on video tracking or use a series of apparatus embedded photobeams that translate rodent movement into objective spatiotemporal measures. The advent of automated testing has addressed several inherent limitations such as rapidity of obtaining data and inter-rater subjective biases. Indeed even slight differences in observer definitions of a given behaviour can drastically alter the measured outcomes contributing to both intra- and inter-laboratory inconsistency. Therefore,

individual laboratories should validate automated equipment to ascertain reliability of the system against observer-derived measurements. In this regard, the forced swim test apparatus used in the present study was previously validated (Kurtuncu et al., 2005) while the elevated plus maze was validated in our laboratory (validation paper is provided in appendix).

1.5. Cellular basis of anxiety- and depressive-related behaviours

The long-term behavioural and molecular consequences of early immune challenge are highly dependant on when endotoxin exposure occurs in relation to the distinct developmental time course of differing brain systems and processes. Immune challenge during these critical periods has the potential to 'program' or alter the developmental trajectory of systems relevant to emotionality. Previous work in the early immune challenge literature has focused mainly on LPS programming of the neonatal stress-axis (Shanks et al., 1995; Shanks et al., 2000) or cytokine-HPA axis imbalance (Bilbo and Schwarz, 2009) in relation to adult behavioural dysfunction. Although the exact neural substrates that underlie anxiety and depression have yet to be fully elucidated, two theories have emerged which have received little attention in the early immune challenge literature: altered serotonergic signalling during postnatal development and altered hippocampal neurogenesis. An investigation as to the contribution of these factors to altered emotionality in LPS-rodents can yield insight into underlying

neuropathology with the ultimate goal of advancing our understanding of disease pathophysiology.

1.5.1. Serotonin in the pathogenesis of affective disorders

Serotonin or 5-hydroxytryptamine (5-HT) is a major modulatory neurotransmitter of the CNS. There is a long-standing association between 5-HT and affective disorders (Woolley, 1963) with a substantial body of clinical literature to support a role for aberrant 5-HT function in anxiety and depression. Key findings in mood disorder patients include reduced cerebrospinal fluid levels of the serotonin metabolite, 5-H1AA (Asberg et al., 1976), increased depressive symptoms in response to rapid depletion of tryptophan, the amino acid precursor for 5-HT synthesis (Delgado et al., 1990; Smith et al., 1997), and lower CNS binding of the 5-HT transporter and 5-HT receptors (Parsey et al., 2006; Boldrini et al., 2008). Genetic studies have revealed that an allelic polymorphism in the 5-HT transporter gene that acts to decrease transporter activity, density, and gene transcription (Lesch et al., 1996), interacts with early life adversity to modify risk for the later development of depression (Caspi et al., 2003). This evidence, together with the efficacy of serotonin reuptake inhibitors (SRIs) and partial serotonin agonists/antagonists for the treatment of anxiety and/or depression (Davidson et al., 1999; Baldwin and Lopes, 2009; Goodwin et al., 2009) highlight the preeminent role for serotonin in the pathogenesis of mood disorders.

1.5.2. Serotonergic neurocircuitry of emotionality

A large proportion of 5-HT neurons can be found in discrete clusters residing in the dorsal raphe (DR) nucleus of the brainstem. A subset of these neurons found within the mid-rostrocaudal and caudal aspect of the DR send efferent projections to key corticolimbic structures implicated in regulating stress and anxiety (Lowry, 2002). These regions include the amygdala, hippocampus, hypothalamus and frontal cortex. Together with the DR they form the mesolimbocortical serotonergic system - a neurocircuit implicated in regulating emotionality (Lowry et al., 2005). Aberrant 5-HT signalling within this circuit is proposed to mediate altered anxiety-related behaviour (Lowry, 2002). There are currently fourteen identified 5-HT receptor sub-types that transduce 5-HT signalling (Barnes and Sharp, 1999). The most studied with regards to emotionality are the post-synaptic receptors 5HT1A, 2A and 2C, the 5-HT transporter (5HTT) and the somatodendritic 5HT1A receptor (Holmes, 2008). An altered spatial and temporal profile of 5-HT receptor gene expression within this circuit would ultimately act to alter 5-HT signalling and anxiety-related behaviours.

1.5.3. Serotonergic signalling during early postnatal development

In addition to its role as a neurotransmitter in the adult CNS, 5-HT is involved in the regulation and refinement of synaptic connections during brain development. Recent preclinical evidence has emerged demonstrating that altered 5-HT signalling during development, and not adulthood, is essential to the establishment of anxiety (Gross et al.,

2002; Ansorge et al., 2004). This has shifted the focus of research to the early postnatal period and has contributed to the growing recognition that anxiety is a neurodevelopmental disorder (Gross and Hen, 2004; Leonardo and Hen, 2008).

The period between the first and third postnatal week in rodents is a time of intense synaptic refinement and appears critical to the development of the neurocircuitry mediating emotionality. For instance, transient pharmacological inhibition of the 5-HT transporter during P4-P21 results in long-term alterations in emotional-reactivity (Ansorge et al., 2004). Expression of the 5HT1A receptor between P14-P21 is essential for the normal establishment of adult anxiety-like behaviour (Gross et al., 2002). Conditional knockout of this receptor between P14-P21 leads to permanent modification of anxiety-related behaviour in adult rodents (Gross et al., 2002) with indicators of anxiety emerging as early as the third postnatal week (Gross and Hen, 2004; Leonardo and Hen, 2008). This suggests that early development is the appropriate time window for examining anxiety-related behavioural and molecular phenotypes. To date, work in the early immune challenge model has focused solely on outcomes measured in adulthood as an experimental endpoint. Given that LPS injection during adulthood is known to alter 5-HT signalling (Hollis et al., 2006), it is intriguing to suggest that such a mechanism might also operate during development. Even a subtle and transient disturbance of serotonin signalling during ontogeny is enough to confer increased emotionality. For instance, a transient increase in 5HT1A receptor expression during the second postnatal week accompanied a permanent increase in anxiety-related behaviour associated with early-life

stress (Goodfellow et al., 2009). LPS challenge administered during early postnatal development may alter 5-HT neurocircuitry early in life with implications for the normal development of emotionality. *An early postnatal examination of 5-HT gene expression concurrent with a temporal characterization of the emergence of anxiety-related behaviours will yield insight into the role of serotonin neurocircuitry in the development of anxiety.*

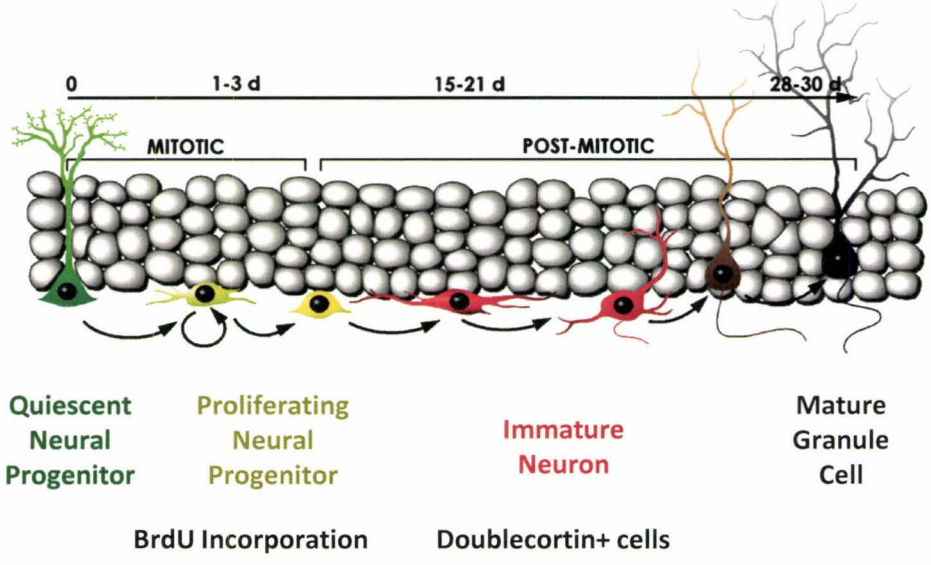
1.5.4. Hippocampal neurogenesis

The discovery of adult neurogenesis, the ongoing production of new neurons in the adult brain, marked a profound paradigm shift in our view of the dynamic nature of the adult brain and lead to dissolution of the dogma that the brain was static beyond development. This phenomenon has been described in the CNS of birds (Paton and Nottebohm, 1984), rodents (Altman and Das, 1965; Cameron et al., 1993), non-human primates (Gould et al., 1998; Gould et al., 1999; Kornack and Rakic, 1999), and humans (Eriksson et al., 1998; Sanai et al., 2004; Curtis et al., 2007). Adult neurogenesis is confined to the olfactory bulbs and the dentate gyrus of the hippocampus, although there is accumulating evidence to support the possibility that neurogenesis may extend beyond these two sites (for review see: Gould, 2007).

1.5.5. The neurogenic cascade: from progenitors to mature neurons

Neurogenesis can be considered a process characterized by contiguous stages rather than a singular event (see Figure 2). In the rodent hippocampus, this process takes approximately 4 weeks (Cameron et al., 1993). Progenitor cells reside in the subgranular zone of the hippocampal dentate gyrus and begin to proliferate in response to a variety of intrinsic and extrinsic regulating factors. These proliferating cells migrate up through the granule cell layers and begin to differentiate into immature neurons. The final step in this cascade is functional integration and survival of mature granule cell neurons (van Praag et al., 2002; Toni et al., 2008). Experimental identification of each stage can be useful in teasing apart the differential impact a given treatment has on the neurogenic process (i.e. proliferation, differentiation, mature neuronal survival). This is accomplished through detection of stage-specific endogenous or exogenously administered cell markers that identify sub-populations of cells (Figure 2). For instance, proliferating cells can be identified through in vivo labelling with the thymidine analog, 5-bromo-2'-deoxyuridine (BrdU), which is selectively incorporated into dividing cells and can be visualized using immunohistochemistry (Miller and Nowakowski, 1988; Bauer and Patterson, 2005). Other stages can be identified through immunoreactivity to constitutively expressed cell markers, such as the immature neuronal marker doublecortin (DCX; (Brown et al., 2003b)). Using a combination of these markers, one can readily quantify cell numbers to assess the impact a given treatment has on different stages of the neurogenic cascade.

Neurogenic cascade in the dentate gyrus of the hippocampus



Modified from: Encinas et al., (2006) PNAS, 103, 8233-8238.

Figure 2. Neurogenesis is a process characterized by contiguous stages that range from proliferation of progenitor cells through to cellular differentiation, maturation, and survival of functionally integrated mature granule neurons. Each step of this cascade can be experimentally identified by stage-specific cell markers. For instance, bromodeoxyuridine (BrdU) is taken up by dividing cells upon systemic administration and can be used as a marker for proliferating neural progenitors. Doublecortin is a protein expressed in young post-mitotic neurons and is used to identify the immature neuronal population.

1.5.6. Hippocampal neurogenesis in the pathogenesis of affective disorders

Aside from the well established role of the hippocampus in mediating spatial learning and memory (Winocur et al., 2006; Saxe et al., 2007; Jessberger et al., 2009), there is increasing evidence to support a role for hippocampal neurogenesis in affective disorders. A variety of treatments with known antidepressant efficacy increase cell proliferation and neurogenesis in the hippocampus of rodents including environmental enrichment (Kempermann et al., 1997), voluntary exercise (van Praag et al., 1999), acute sleep deprivation (Grassi Zucconi et al., 2006), electroconvulsive shock treatment (Scott et al., 2000), and several different classes of pharmacological antidepressants (Malberg et al., 2000). This coupled with the evidence that exposure to stress or chronic corticosterone treatment (a precipitating factor for depression) reduces hippocampal neurogenesis and/or cell proliferation (Gould and Cameron, 1996; Gould et al., 1997; Gould et al., 1998; Malberg and Duman, 2003; Murray et al., 2008), forged the way for the neurogenic theory of depression (Kempermann and Kronenberg, 2003).

There have been relatively few studies conducted to directly measure neurogenesis in psychiatric patients, largely owing to a lack of non-invasive tools (although see: Pereira et al., 2007). Of those conducted, one reported a decrease of the neural progenitor population in non-treated MDD patients compared with controls (Boldrini et al., 2009). Another study, however, failed to find an association with MDD but did report decreased neurogenesis in schizophrenia (Reif et al., 2007). There is, however, supporting circumstantial evidence for an etiological role of neurogenesis in

depression such as reduced performance on neurogenesis-dependant cognitive tasks (Becker et al., 2009) and reduced hippocampal volume (Sheline et al., 1999) in patients with MDD, which may reflect a reduced capacity for neurogenesis. There is also recent evidence demonstrating that antidepressants increase hippocampal neurogenesis in patients with MDD (Boldrini et al., 2009). Patients treated with antidepressants (SSRIs and tricyclics) exhibited an increase in their neuronal progenitor cell population compared with drug-naive MDD patients and healthy controls (Boldrini et al., 2009). Although this may reflect a non-functional by-product of antidepressant treatment, seminal studies using rodent models of anxiety/depression have demonstrated the requirement of hippocampal neurogenesis for some of the behavioural effects of antidepressants (Santarelli et al., 2003; David et al., 2009). Subsequent work, however, has demonstrated the dependence of this phenomenon on mouse strain (Holick et al., 2008), type of antidepressant administered (Meshi et al., 2006; Surget et al., 2008), and the behavioural paradigm used to assess emotionality (David et al., 2009). This evidence does not discount neurogenesis in antidepressant mechanisms of action, but rather cautions for a balanced view of the etiological role of neurogenesis in the context of other contributing cellular and biochemical factors (i.e. altered serotonin (Jacobs et al., 2000) and neurotrophic factor signalling (D'Sa and Duman, 2002)). Collectively, there is convincing evidence provided by preclinical and clinical studies to support a role of neurogenesis in mood disorders.

1.5.7. Early-life events alter adult hippocampal neurogenesis

The adult rodent hippocampal granular layer is comprised of cells whose majority are born during the first week of life (Muramatsu et al., 2007). This raises the possibility that early experience can modify granule cell production (Tanapat et al., 1998). Retention of neonatally-born cells through to adulthood suggests that any perturbation coinciding with granule cell development may lead to long-term functional alterations and possibly impact hippocampal function, including neurogenesis. Early-life stress is known to have an acute (Tanapat et al., 1998) and chronic impact (Mirescu et al., 2004; Kikusui et al., 2009) on precursor cell proliferation or immature neuron production in the rodent. A recent microarray study identified key genes that are altered in response to an acute LPS challenge in the P7 immature rat brain (Eklind et al., 2006). Some of these genes are known to underlie processes associated with neurogenesis, suggesting that LPS challenge may alter neurogenic processes during development, with long-term consequences to its continuing function and to associated behaviours. Indeed, the hippocampus is implicated in a variety of functions that are altered by early LPS challenge. For instance, the hippocampus is a major site of negative feedback of the HPA axis (Sapolsky et al., 1989), is important in modulating anxiety-like behaviours (Deacon et al., 2002; Bannerman et al., 2003), and has a well established role in spatial learning and memory (Winocur et al., 2006; Saxe et al., 2007; Jessberger et al., 2009). As LPS rodents show alterations in all of these processes, it is likely that altered hippocampal development may underlie some of

these disturbances. *Despite this evidence, no studies to date have directly examined hippocampal neurogenesis concurrent with depressive-related behaviours in adult rodents challenged with LPS during the first neonatal week.*

1.6. Current diagnostic view of anxiety and depression

Although historically treated as separate entities, there is considerable overlap in terms of comorbidity and clinical features between anxiety and depression. This is reflected in the modern psychiatric view of these two disorders as constituting differing parts on a mood continuum. Furthermore, it is likely that a shared neurobiological basis exists as the presence of an anxiety disorder in early life often predicts the development of depression in adulthood (Bittner et al., 2007). Indeed, even on a molecular level there exists a mechanistic convergence between serotonin signalling and neurogenesis both during adulthood (Gould, 1999; Jacobs et al., 2000; Radley and Jacobs, 2002; Banasr et al., 2004) and early development (Yan et al., 1997). Therefore any associated pathogenic mechanisms explored should not be considered exclusive to one disorder or another. On this note, the following studies examine the behavioural and molecular changes associated with an early immune challenge focusing on early-life anxiety and adult depressive-related phenotypes.

CHAPTER 2. HYPOTHESIS AND AIMS

Central hypothesis: Based on the clinical and pre-clinical evidence presented, it is proposed that early endotoxin exposure impacts CNS development leading to behavioural and molecular phenotypes associated with altered stress-reactivity. This hypothesis is addressed by the following three studies:

Hypotheses: i) early immune challenge leads to an early emergence and persistence of anxiety-related behaviours in LPS rodents; ii) this is accompanied by alterations to developing serotonin neurocircuitry during a developmental time window important for the establishment of anxiety-related behaviours; iii) early immune challenge incurs a depressive-like behavioural phenotype characterized molecularly by altered hippocampal neurogenesis.

Aims:

1. Determine the developmental trajectory of anxiety-related behaviours in male and female LPS-mice.
2. Examine the spatial and temporal expression of serotonin-related genes during postnatal development following an early immune challenge.
3. Assess adult depressive-like behaviour concurrent with hippocampal cell proliferation and immature neuronal differentiation.

CHAPTER 3.

Sex impacts the temporal emergence and phenotype of anxiety-related behaviours following an early immune challenge in mice

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Abstract

Sexual dichotomy exists in the development, presentation, and course of many neuropsychiatric disorders, including anxiety. Anxiety disorders are one of the earliest psychiatric illnesses to manifest, suggesting that the early environment interacts with sex to shape CNS development. A role for immune system programming of the developing CNS has emerged in relation to anxiety. Adult rodents neonatally exposed to an immune challenge exhibit increased anxiety-related behaviours. No studies to date, however, have determined how sex interacts with early immune challenge to shape the developmental trajectory of anxiety-related behaviours. Mice were administered lipopolysaccharide (LPS; 0.05mg/kg, i.p.) or saline on postnatal days (P) three and five. Anxiety-related behaviour was assessed during early development on P14, P21, and P28, and re-assessed in adulthood at 10 and 12 weeks. Results reveal sex-specificity in the temporal emergence and phenotypic profile of anxiety-related behaviours displayed by LPS-mice. Male LPS-mice exhibited behavioural inhibition in early development (P21) that persisted into adulthood. Female LPS-mice exhibited increased avoidance behaviours in adulthood only. In both sexes, neonatal LPS challenge interacted with early indices of increased emotionality to influence adult anxiety-related behaviours. This suggests that although overt behavioural differences were not always present in juvenile LPS-mice, groups exhibiting high early-life emotionality were more susceptible to the impact of LPS challenge. These results demonstrate complex interactions between sex, individual differences, and the immune system in shaping the developmental trajectory of anxiety-

related behaviours. Understanding the sex-specificity of outcomes related to affective disorders has important implications for disease detection and treatment.

3.1. Introduction

Early-life adversity is an established risk factor for the later development of psychiatric disorders. Both clinical and preclinical studies have demonstrated that early stressful life events can markedly alter adult stress-reactivity and predispose to anxiety (Stein et al., 1996; Heim and Nemeroff, 2001). Indeed, rodent models of early-life manipulation, such as maternal separation, result in a modification of adult anxiety-related behaviour and associated neurobiological substrates (Plotsky and Meaney, 1993; Romeo et al., 2003; Kikusui et al., 2004) with prolonged periods of maternal separation leading to enhanced fear and anxiety in adult mice (Romeo et al., 2003) and dysregulation of the HPA axis in adult rats (Plotsky and Meaney, 1993). Many of the long-term behavioural and molecular consequences of early-life adversity exhibit a sexual dichotomy (McIntosh et al., 1999; Papaioannou et al., 2002; Romeo et al., 2003; Eklund and Arborelius, 2006; Kikusui et al., 2009; Oreland et al., 2009), demonstrating the importance of sex in influencing the development of adult stress-related psychopathology. There are reported sex differences in the clinical onset, presentation, course, and/or treatment response of many psychiatric disorders with a proposed neurodevelopmental origin including schizophrenia, major depression, and anxiety (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).

There has been a growing body of literature linking the immune system to anxiety modification (Silverman et al., 2007; Swiergiel and Dunn, 2007; Nautiyal et al., 2008). Indeed, immune abnormalities are proposed to play an etiological role in affective disorders (Anisman, 2009). Early immune challenge has emerged as a model of early-life adversity (Shanks and Meaney, 1994) with a distinct focus on the impact of immune activation during CNS development on stress-related phenotypes. Postnatal challenge with lipopolysaccharide (LPS), a cell wall constituent of gram-negative bacteria, during the first week of life (postnatal day (P)3 and 5) enhances the neuroendocrine and behavioural response to stress in adult rats (Shanks et al., 2000; Hodgson et al., 2001; Walker et al., 2008; Walker et al., 2009) and increases basal or stress-induced anxiety-related behaviours (Breivik et al., 2002; Walker et al., 2004; Walker et al., 2009). These effects are dependent on the timing of postnatal challenge as no anxiety-related differences are observed if LPS challenge occurs after the first postnatal week (Spencer et al., 2005; Spencer et al., 2006). The consequences of early-life stress are also sex-dependant (Weinstock et al., 1992; Romeo et al., 2003; Eklund and Arborelius, 2006; Slotten et al., 2006). Male and female rodents have different susceptibility to the acute (Shanks et al., 1994; Shanks and Meaney, 1994) and long-term consequences of LPS challenge (Shanks et al., 1995; Tenk et al., 2007; Tenk et al., 2008) with a recent study demonstrating clear differences in anxiety-related behaviours of adult male and female LPS-challenged rats (Walker et al., 2009). This sexual dichotomy suggests that the early immune challenge model can be used to investigate how sex interacts with the immune system during development to impact anxiety-related behaviours.

Whereas the majority of the early immune challenge literature has focused on alterations detected during adulthood, the possibility that anxiety-like behaviour emerges earlier in development has not been fully explored. This has clinical relevance, given that anxiety disorders are often the first psychiatric illness to manifest (Rogers et al., 1999; Kessler et al., 2005). In mice, the time window between the first and third postnatal week is essential to the establishment of anxiety. Conditional knockout of the serotonin (5-HT)-1A receptor (Gross et al., 2002) and transient inhibition of the 5HT transporter (Ansorge et al., 2004) during this time modulates anxiety-related behaviours with differences in emotional-reactivity emerging as early as the third week of life (P21; (Leonardo and Hen, 2008)). The initial aim of the study, therefore, was to determine the impact of sex on the temporal emergence and behavioural phenotypes of emotionality from early development through to adulthood in mice neonatally exposed to LPS.

Finally, as childhood indicators of anxiety often predict later psychiatric illness (Schwartz et al., 1999; Prior et al., 2000; Gladstone et al., 2005), we wanted to explore whether early-life differences in emotional-reactivity were associated with adult behavioural abnormalities. Specifically, early-life stress is known to differentially impact adult anxiety in high versus low anxiety rodents (Neumann et al., 2005; Bosch et al., 2006). Given that we employed repeated measures over time, this allowed us to examine whether LPS challenge differentially impacts adult anxiety-related behaviours in groups of female and male mice displaying high versus low emotional-reactivity in early development.

3.2. Methods

3.2.1. Animals. CD-1 mice were bred in-house from male and female mice (8-10 w) obtained from Charles River. Postnatal day (P) 0 denotes day of birth. A total of 10 litters were used; litter size ranged from 8-13 with litters culled to a maximum of 12 on P1. Mice were weaned on P21, ear punched to identify individuals, and separated based on sex into a maximum of 5 pups/cage.

3.2.2. Injections. On P3 and P5, whole litters were given an i.p. injection of either 0.05 mg/kg lipopolysaccharide (E. coli LPS; Sigma) in 50 μl/g or an equal volume of saline (SAL) between 07:00-9:00 h. Dams were removed from the homecage and returned once all pups had received an injection. Maternal separation did not exceed 5 m.

3.2.3. Growth and development. Mice were weighed daily from P1-P7 and weekly thereafter. Eye opening was assessed beginning P10 as a physical screen to monitor postnatal development. A score of 0 = no eyes open; 1 = one eye open; 2 = both eyes open; average score/treatment group is presented.

3.2.4. Behavioural testing. All behavioural testing was performed during the animal's active cycle between 20:00-03:00 h. At least one week separated each behavioural test. Given that the same cohort of animals were to be followed throughout development, we developed an anxiety-test battery. This was conducted in order to avoid confounding issues with repeated test exposure (Rodgers and Shepherd, 1993; Holmes et al., 2001).

3.2.4.1. Open field. P14 pups were separated from the mom and brought into a dimly lit testing room in their homecage. All pups had achieved full eye opening at the time of testing. Pups were individually placed into plexiglass chambers (Kinderscientific Smart Cage Rack System; field dimensions: 9.5" wide x 18.0" long) consisting of infrared beams used to detect horizontal and vertical activity. This system was interfaced to a PC computer running MotorMonitor software. Pups were allowed to explore the chamber for a period of 15 m and data was collected in the form of photobeam breaks as an indication of activity. After all pups had undergone testing, they were returned to the mom as a group; maternal separation did not exceed 1 h. At 10 wks, mice were allowed to habituate in a dark outer room for 45 m before being individually placed into a brightly lit arena (Kinderscientific's Smart Cage System). Distance travelled and time spent in the centre versus periphery was recorded by MonitorMonitor software for 30 m.

3.2.4.2. Light-dark. Anxiety-like behaviour was assessed using a 10 m light-dark test. Mice were individually tested using the Kinderscientific Smart Cage Rack system and behaviour scored by MotorMonitor software. Each chamber was equipped with a black plexiglass box on one side of the chamber; a small doorway allowed free exploration of dark and light sides of the chambers. Mice were allowed to habituate in their homecage for 15 m in a dimly lit outer room, individually brought into the testing room and placed into the light side of the chamber.

3.2.4.3. Novelty suppressed feeding (NSF). On P28, mice were tested for anxiety-like behaviour using the NSF paradigm. Mice were food deprived 24 h prior to testing with

water provided *ad libitum*. Food deprivation was time staggered to ensure mice were deprived for exactly 24 h. On test day, mice were placed into a holding cage and allowed to habituate to the testing room for 10 m. Mice were individually placed into a brightly lit novel arena (plexiglass with clear sides; 24" X 24") consisting of a single piece of white filter paper with one small pellet (~2g) placed atop in the centre of the arena. Mice were placed into the box facing the corner and the latency to begin feeding was recorded manually. In order for feeding to be scored, the animal had to pick up the pellet while standing on its hunches and begin eating. Picking up, pulling, pushing or nibbling at the pellet was not considered feeding. The test was scored up to a max. of 5 m; if feeding did not occur within this time, a score of 5 m was given. Immediately following, mice were placed into their homecage and the amount of food consumed was recorded for 5 m.

3.2.4.4. Elevated plus maze (EPM). At 12 weeks of age, anxiety-like behaviour was assessed using a 5 m EPM test. This device is a '+' shaped black plexiglas maze, consisting of two opposing closed arms (flanked by 15cm opaque black plexiglass walls), and two open arms (no walls). Animals were allowed to habituate in a dimly lit outer room for 10 m and then transported individually into a similarly lit testing room. Mice were placed in the center of the plus maze facing a closed arm. Behaviour was scored using Kinderscientific's MotorMonitor software.

3.2.5. Statistics and data analysis. Data were analyzed using a two-way analysis of variance (ANOVA) with sex (female/male) and treatment (SAL/LPS) as main factors. followed by the Student's t-test to analyze within sex differences for each behavioural

test. Within subject data for growth and development over time were analyzed using repeated measures ANOVA. Males were ranked into high and low emotional-reactivity according to rearing scores obtained during the light/dark test at P21. Scores above the group median were considered low reactivity, whereas those below the median were assigned as high reactivity (i.e. low rearing = high emotional reactivity). Female ranking for high and low reactivity levels was based on the % distance travelled in the light chamber of the light/dark test at P21 (above/below median considered low/high-reactivity). Adult behaviour for high and low groups was compared using the Student's t-test. Statistics were performed using the Prism 4.0a statistical software for Macintosh. A two-tailed p-value less than 0.05 was considered statistically significant. Significance is denoted as the following: $*p<0.05$. All values are expressed as mean ± SEM.

3.3. Results

3.3.1. Postnatal growth and development

There was a significant effect of time ($F(7,98)=508.3$, $p<0.0001$) but no main effect of treatment on pre-weaning body mass (Fig. 1A). Physical development was assessed through monitoring of eye opening. No difference was detected between groups, with all mice achieving full eye opening by the end of the second postnatal week (Fig. 1B).

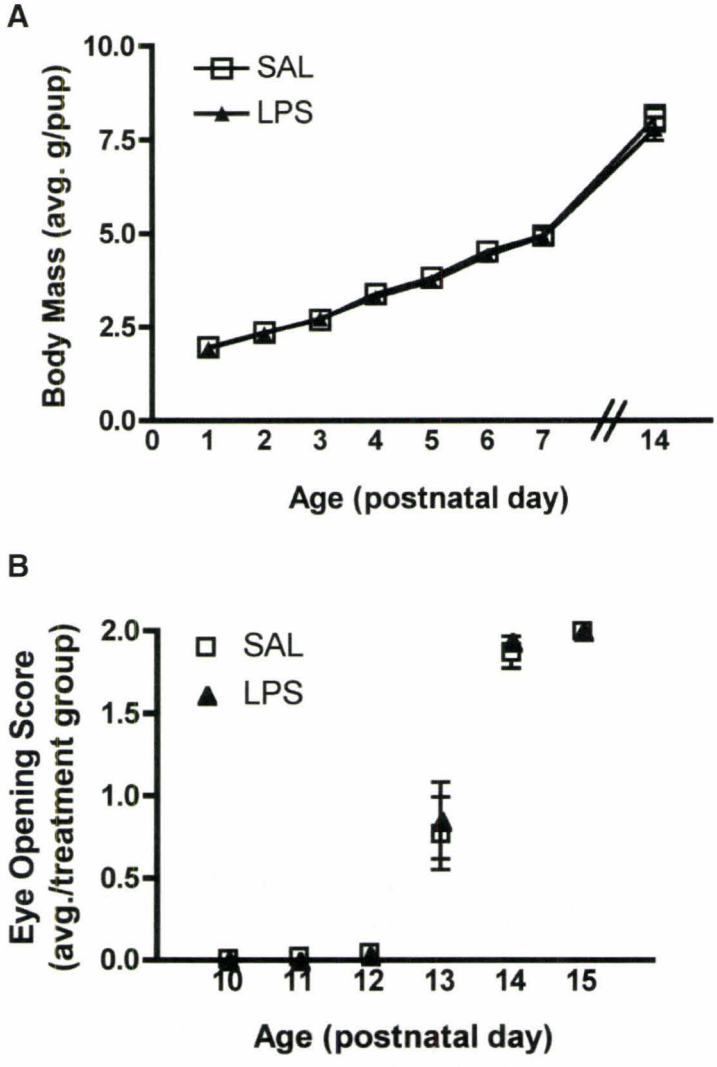

Figure 1. Physical growth was monitored during early development in mice treated with lipopolysaccharide (LPS) or saline (SAL) on postnatal days (P)3 and 5. **(A)** There was no significant impact of treatment on growth as indicated by body mass over the first two weeks of life. **(B)** Eye opening was assessed as an indicator of physical development, with no differences emerging between treatment groups. Eye opening was scored as follows: 0=no eyes open, 1=one eye open, 2=both eyes open. Data are plotted as mean ± SEM for 51 SAL and 51 LPS mice.

3.3.2. Locomotor activity

Given that quadrupedal locomotion in rodents emerges during the second postnatal week (Altman and Sudarshan, 1975; Clarke and Still, 2001; Glynn et al., 2007), locomotor activity was assessed beginning at P14 in the open field, and subsequently assessed in the light/dark apparatus at P21, the open field at 10 w (adult), and the EPM at 12 w. Total distance travelled was used as a standard measure of activity. Figure 2A depicts locomotor behaviour observed during early development in LPS-mice. There was a significant impact of treatment on the total distance P14 pups travelled ($F(1,97)=10.66$, $p=0.0016$; Fig. 2A). Female and male LPS-pups travelled less total distance during the 15 m open-field test (female: $t(45)=2.45$, $p=0.018$; male: $t(52)=2.21$, $p=0.032$; Fig. 2A). A significant main effect of treatment was also found at P21 during light-dark testing ($F(1,93)=4.04$, $p=0.047$; Fig. 2A). Although female LPS-mice showed no difference in locomotion ($t(50)=0.61$, $p=0.54$), male LPS-mice continued to show hypo-locomotion compared with male controls ($t(43)=2.29$, $p=0.027$; Fig. 2A). Locomotor behaviour was re-assessed in adulthood in the EPM and open field (Fig. 2B). There was a significant impact of treatment on total distance travelled during the EPM ($F(1,99)=6.35$, $p=0.013$; Fig. 2B). Posthoc analysis revealed that LPS-males travelled significantly less distance in the EPM than male saline controls ($t(49)=3.53$, $p=0.009$). Collectively, the data suggests that male LPS-mice exhibit a hypo-locomotor phenotype that develops early in life and persists through to adulthood. This male phenotype is specific to tests of shorter duration as no significant treatment differences were detected during the 30 min open field test in adulthood ($F(1,98)=1.17$, $p=0.28$; Fig. 2B).

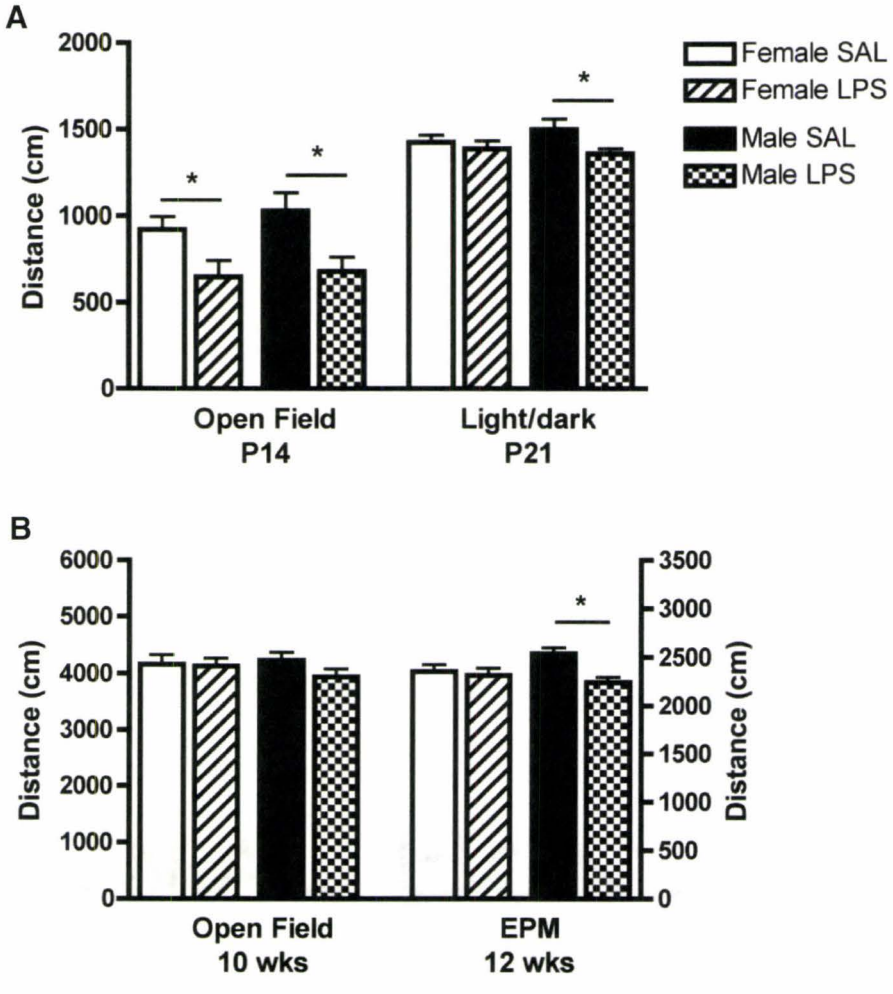

Figure 2. Locomotor behaviour as indicated by overall distance travelled during tests of anxiety-like behaviour across the development of female and male mice treated with LPS or SAL during the first postnatal week. Locomotion was assessed early in development on **(A)** postnatal days (P)14 and 21 and re-assessed within the same cohort later in **(B)** adulthood at 10 and 12 weeks of age. EPM=elevated plus maze. Data are plotted as mean ± SEM. P14: SAL F/M, n=24/26, LPS F/M, n=23/28; P21: SAL F/M, n=29/17, LPS F/M, n=23/28; 10w,12w : SAL F/M, n=29/26, LPS F/M, n=23/25. *$p<0.05$.

3.3.3. Anxiety-related behaviour

Figure 3A depicts anxiety-like behaviour measured in female and male LPS/SAL mice using the open field at P14 and the light/dark test at P21. To control for differences in locomotion detected throughout development (Fig. 2), the distance travelled in the centre aversive zone was calculated as a ratio of total distance travelled and used as an index of anxiety (% distance travelled). There was no significant treatment effect on % distance travelled in either the centre of the open field at P14 ($F(1,98)=1.58$, $p=0.21$; Fig.3A) or light chamber of the light/dark box at P21 ($F(1,93)=0.02$, $p=0.90$; Fig. 3A).

Anxiety-like behaviour was assessed at P28 using the NSF (Fig. 3B). No significant treatment effect was detected in the latency to begin feeding in the novel environment ($F(1,90)=0.50$, $p=0.48$; Fig. 3B). There were no differences in appetitive motivation between groups as measured by amount of food consumed during a five-minute period following novel environment testing ($F(1,90)=0.17$, $p=0.69$; data not shown).

Anxiety-like behaviour was re-assessed in adulthood using the open field at 10 w and the elevated plus maze at 12 w. To assess anxiety-like behaviour specifically, behaviour during the first 5 m of the open field was considered. This measure captures the initial reactivity to novelty, which may differ from behavioural performance after longer exposures to the open field. There was a significant sex effect on % distance travelled in the centre of the open field during the first 5 m ($F(1,98)=18.47$, $p<0.0001$; Fig. 3C), with females of both treatment groups travelling less centre distance than males.

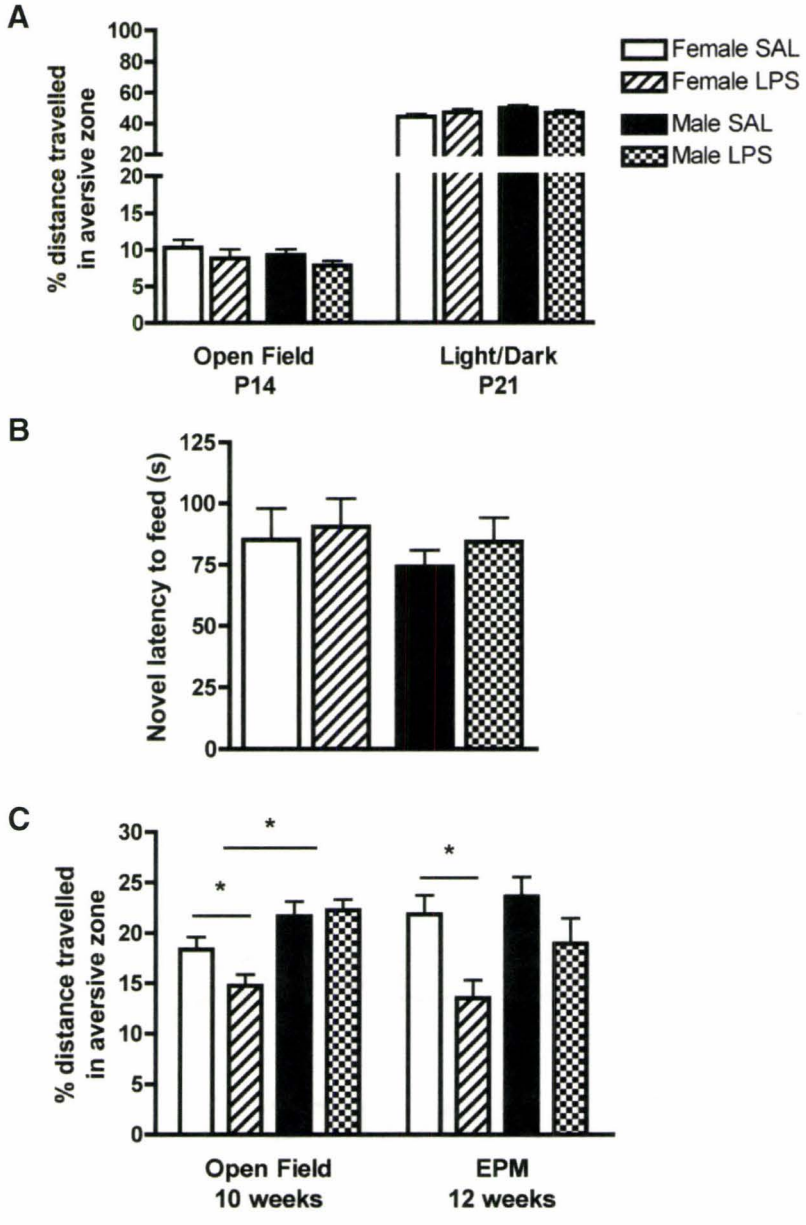

Figure 3. Measures of anxiety-related behaviour across development. (**A, B**) No differences emerged during early postnatal testing (B=P28). (**C**) At both 10 and 12 wks, female LPS-mice exhibited a significant reduction in % distance travelled in the respective aversive zones of each test (i.e. open field=centre; EPM=open arms) compared with female saline controls. Data are plotted as mean ± SEM. P14: SAL F/M, n=24/26, LPS F/M, n=23/28, P21: SAL F/M, n=29/17, LPS F/M, n=23/28; P28: SAL F/M, n=27/21, LPS F/M, n=21/25; 10 w: SAL F/M, n=29/26, LPS F/M, n=22/25; 12 w: SAL F/M, n=29/26, LPS F/M, n=23/25. *$p<0.05$.

Females LPS-mice also travelled less distance relative to female saline controls during this time ($t(49)=2.07$, $p=0.044$; Fig. 3C). There was no significant difference on this parameter when the full 30 m was considered (data not shown). There was a main effect of treatment on % distance travelled in the open arms of the elevated plus maze at 12 w ($F(1,99)=9.97$, $p=0.002$; Fig. 3C). Posthoc analysis revealed that female LPS-mice travelled less distance in the open arms compared with SAL-females ($t(50)=3.16$, $p=0.003$; Fig. 3C). Taken together, this demonstrates increased anxiety-like behaviour in adult female LPS-mice.

3.3.4. Exploratory behaviour

Indicators of exploratory behaviour included rearing in both the light/dark and open field tests and closed arm entries in the elevated plus maze (Crawley et al., 1984; Holmes et al., 2003). There was a significant treatment effect on rearing behaviour during the open field test at P14 ($F(1,98)=7.99$, $p=0.006$; Fig. 4A) and light/dark test at P21 ($F(1,93)=4.37$, $p=0.039$; Fig. 4A) that was identified posthoc as a reduced frequency of rearing in male LPS-mice compared to male controls (P14: $t(52)=2.41$, $p=0.019$; P21: $t(43)=2.29$, $p=0.027$).

Exploratory behaviour was re-assessed during open-field testing at 10 w and EPM testing at 12 w of age. There was a significant main effect of sex ($F(1,98)=4.18$, $p=0.044$) on number of rears in the open field, with LPS-males rearing less compared to male controls over the 30 min of the open field test ($t(49)=2.204$, $p=0.032$; Fig. 4B). No difference in rearing emerged between LPS and SAL females. A significant main effect

of treatment was found for EPM closed arm entries ($F(1,99)=7.94$, $p=0.006$). Posthoc analysis revealed this effect was specific to males, with LPS-males entering the closed arms less than SAL-males ($t(49)=3.245$, $p=0.002$; Fig. 4B). As deficits in exploration during exploratory-based tests of anxiety-like behaviour contain an emotionality component to them (Gray and N., 2000; Holmes, 2001), the behaviour of LPS-males can be interpreted as an early emergence of decreased exploration and therefore an increase in emotional-reactivity.

3.3.5. Early emotional-reactivity influences adult anxiety-related behaviour

To determine whether early-life differences in emotional-reactivity influenced the development of adult anxiety, we classified mice into high or low emotional-reactivity based on P21 behaviour and examined the corresponding adult behaviour in those groups (see methods). Behaviour on P21 was chosen given that rodent emotional-reactivity to a novel environment emerges at P20 (Leonardo and Hen, 2008). This implies that behavioural differences detected at this time are likely a result of differences in emotionality. Female mice were separated into high and low emotional-reactivity based on P21 avoidance behaviour to the aversive light chamber during light/dark testing. As LPS-males manifested emotional-reactivity through exploratory deficits, male mice were separated into high and low groups based on P21 rearing behaviour.

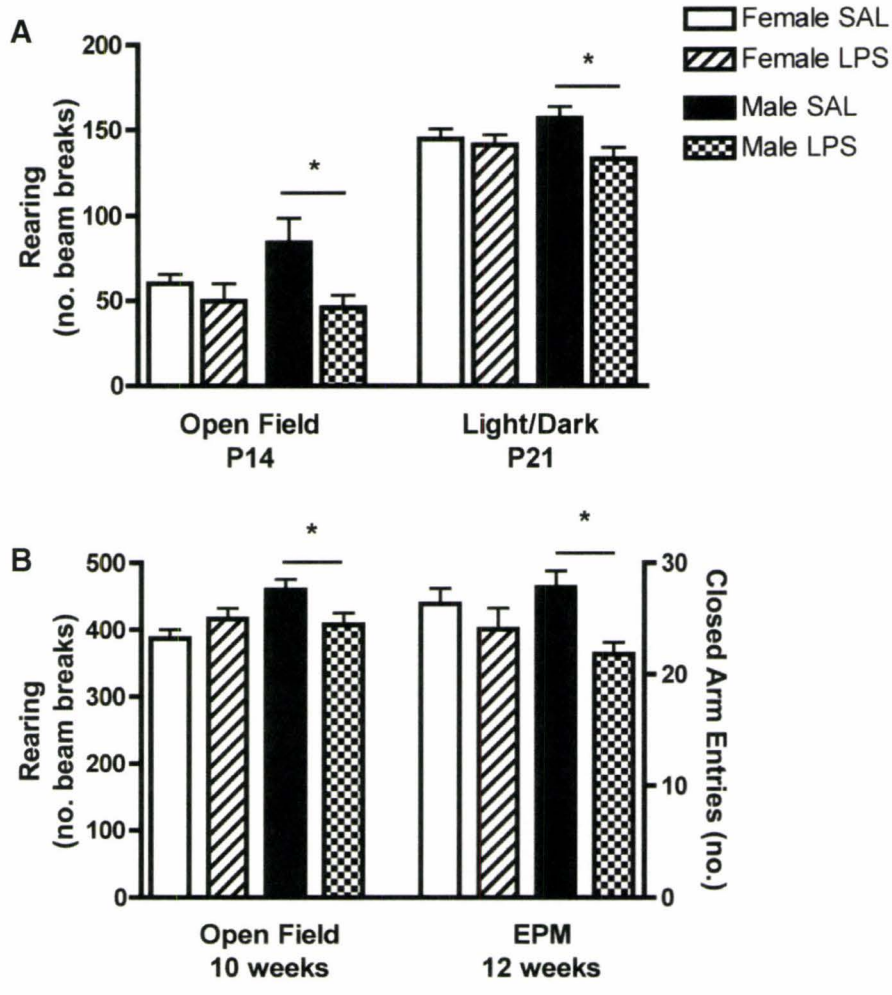

Figure 4. Cohorts of mice were assessed for exploratory behaviour during (A) development (P14-P21) and (B) adulthood (10 and 12 wks). **(A)** Rearing was used as a measure of exploratory behaviour in the open field and light/dark test. Male LPS-mice exhibited a significant reduction in frequency of rearing compared to male saline (SAL) controls at P14 and P21. **(B)** Exploratory behaviour was re-assessed in adulthood. Frequency of closed arm entries was used as an indicator of exploration in the EPM. Male LPS-mice exhibited reduced rearing during the open field test. Similarly, the frequency of closed arm entries in the EPM was reduced in LPS-males relative to male SAL controls. Data are plotted as mean ± SEM. P14: SAL F/M, n=24/26, LPS F/M, n=23/28, P21: SAL F/M, n=29/17, LPS F/M, n=23/28; P28: SAL F/M, n=27/21, LPS F/M, n=21/25; 10 w: SAL F/M, n=29/26, LPS F/M, n=22/25; 12 w: SAL F/M, n=29/26, LPS F/M, n=23/25. *$p<0.05$.

Figure 5A depicts adult anxiety-like behaviour in the open field and elevated plus maze in female mice defined as having high or low emotional-reactivity early in life as indicated by the % distance travelled in the light chamber of the light/dark box. In adulthood, high-reactive LPS-females exhibited a decrease in the % distance travelled in the centre of the open field ($t(22)=1.85$, $p=0.077$) and open arms of the elevated plus maze ($t(22)=2.75$, $p=0.012$) compared with high-reactive SAL-females. No treatment differences were detected between low-reactive groups (open field: $t(22)=1.03$, $p=0.31$; EPM: $t(23)=1.60$, $p=0.12$).

There was a significant difference in adult exploratory behaviour in high versus low reactive LPS-males in the open field ($t(23)=2.19$, $p=0.039$, Fig. 5B) and elevated plus maze ($t(23)=2.04$, $p=0.05$, Fig. 5B). There was no difference in adult exploration between high- and low-reactive SAL-males. LPS-males with high-reactivity early in life exhibited increased adult emotional-reactivity as reflected by a significant decrease in adult open field rearing ($t(16)=3.18$, $p=0.006$) and plus-maze closed arm entries ($t(16)=3.62$, $p=0.002$) compared with high-reactive SAL-males, with no treatment differences detected between low-reactive groups (open field: $t(20)=0.40$, $p=0.70$; EPM: $t(20)=1.41$, $p=0.17$). Collectively, this suggests that early immune challenge increases vulnerability to adult anxiety-related behaviours in groups displaying high emotional-reactivity in early-life.

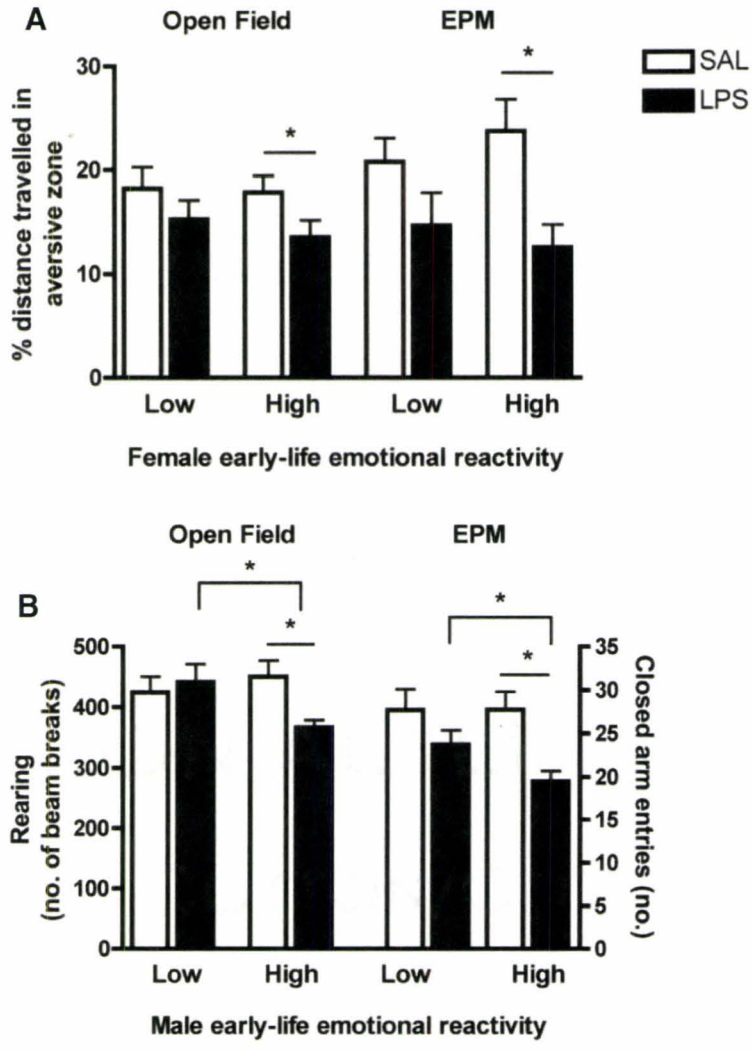

Figure 5. Mice were separated into high or low groups based on early-life emotional-reactivity. Adult anxiety-related behaviour in the open field and elevated plus maze (EPM) were then compared in female (A) and male (B) mice exhibiting high versus low early-life emotional-reactivity. **(A)** LPS-challenge potentiated an increase in adult anxiety-related behaviours in the open field and EPM in female LPS-mice displaying high early life emotional-reactivity. **(B)** Male LPS-mice exhibiting high early life emotional-reactivity continued to show increased emotionality in adulthood as indicated by reduced exploratory behaviour in the open field and EPM in high-reactive groups. Data are plotted as mean ± SEM. Low: SAL F/M, n=14/9, LPS F/M, n= 10/13; High: SAL F/M, n=14/7, LPS F/M, n=10/11. *$p<0.05$.

3.4. Discussion

The current study determined the impact of sex on the behavioural profile of mice exposed to a neonatal endotoxin challenge, focusing on anxiety-related behaviours through development. The results provide important evidence to demonstrate a sexual dichotomy in the emergence and presentation of anxiety-related behaviours. Overall, LPS-mice displayed increased emotional-reactivity with sex-specific behavioural phenotypes emerging in early-life males and in adult females. Within the neonatal immune challenge group, we found that early-life differences in emotional reactivity were associated with adult anxiety-related behaviours.

The current findings are consistent with the growing body of literature supporting sex differences in the acute and long-term effect of neonatal LPS exposure on postnatal days three and five (P3, P5). For instance, acute administration of LPS leads to greater adrenocorticotropic hormone and corticosterone (CORT) plasma levels in female rat pups compared to males (Shanks et al., 1994). Female-specific reports of long-term alterations include increased locomotor sensitization to the dopamine agonist, quinpirole (Tenk et al., 2007) and increased CORT response to chronic stress (Hodgson and Knott, 2002). The majority of reports on sex differences in the long-term effects of postnatal LPS, however, indicate an increased susceptibility of disease in males. This includes decreased locomotion in response to a subsequent adult LPS challenge (Tenk et al., 2008), increased anxiety-like behaviour in response to restraint stress (Walker et al., 2009), increased basal CRH mRNA in the PVN (Shanks et al., 1995), impaired natural killer cell activity, and

decreased resistance to tumor colonization (Hodgson and Knott, 2002). The present findings add to this growing literature by demonstrating sex-specific alterations in emotional-reactivity in early immune challenged rodents. This finding has clinical relevance given the reported sex differences in the incidence, age of onset, and clinical presentation of many psychiatric disorders (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). The existence of sex-differences in any disorder necessitates the examination and consideration of the influence of sex in basic neuroscience research (Cahill, 2006). The early immune challenge model provides a novel tool for studying sex-specific behavioural characteristics that are relevant to the clinical course and presentation of anxiety.

The current findings extend previous reports of increased emotional-reactivity in adult LPS P3, P5 rodents (Shanks et al., 2000; Breivik et al., 2002; Walker et al., 2004; Walker et al., 2008; Walker et al., 2009). In the current study, female LPS-mice demonstrated anxiety-related behaviour characterized by avoidance behaviour to aversive environments (centre of open field and open arm of plus-maze). Male LPS-mice exhibited novelty-induced behavioural inhibition as evidenced by decreased exploratory locomotion across a range of novel environments. Rodents with high levels of anxiety-like behaviour often exhibit behavioural inhibition and reduced exploratory locomotion (Gray and N., 2000; Holmes, 2001). Therefore, it is possible that the current observed male behaviour also represents a form of enhanced emotional-reactivity. The distinct

behavioural profile of emotional-reactivity displayed between female and male LPS-mice recapitulates the different clinical features that men and women present with across a range of anxiety disorders (Castle et al., 1995; Turgeon et al., 1998; Weinstock, 1999; Steiner et al., 2005; Simon et al., 2006; Labad et al., 2008; Vesga-Lopez et al., 2008). Together this highlights the importance of using a battery of behavioural tests and examining multiple measures to fully capture the repertoire of anxiety-related behaviours in male and female rodents.

Behavioural characterization through development revealed differential timing for the onset of anxiety-related behaviours in female and male LPS-mice. Female LPS-mice presented with an adult-onset phenotype, whereas male LPS-mice experienced an early and persistent behavioural phenotype. This is interesting in light of the increased incidence of psychiatric diagnoses in women after puberty (Earls, 1987) and an increased incidence of psychiatric illness in boys prior to puberty (Seeman, 1997). Although it is possible that the presence of early-life anxiety in females was not detected by the behavioural tests used, the present finding of adult-onset anxiety is consistent with previous findings in early immune challenged rodents (Walker et al., 2004). In the study by Walker et al (2004), anxiety-like behaviour was measured in cohorts of adolescent and adult LPS rats using the elevated plus maze, with increased anxiety reported in adulthood specifically. Therefore it is plausible that anxiety emerges in adulthood and that the negative finding early in life is independent of the behavioural test used. However, future studies to re-assess anxiety-like behaviour using different tests would confirm the

temporal component of the emergence of female anxiety-like behaviour.

Repeated measures over time allowed for the examination as to whether early differences in emotional-reactivity were associated with adult anxiety-related behaviours. This is a clinically relevant question given that early indicators of childhood behavioural inhibition and anxiety are often predictive for the later development of anxiety disorders (Schwartz et al., 1999; Prior et al., 2000; Gladstone et al., 2005). In the current study, neonatal exposure to LPS was associated with an increase in adult anxiety-related behaviours in female and male mice exhibiting high emotional-reactivity in early life. In this regard, one study has reported differential stress responses to an LPS challenge in rodents with high versus low anxiety such that high anxiety adult rats experienced a greater rise in plasma CORT two hours after an immune challenge with LPS compared to low anxiety rats (Salome et al., 2008). This suggests that early immune challenge may interact with innate differences in early life to shape the development of anxiety-related behaviours and is consistent with a stress-diathesis model whereby early-life stressors interact with underlying vulnerability to increase risk of later disease (McEwen and Stellar, 1993).

The exact mechanisms underlying the current reported sex-differences in anxiety-related behaviour remain speculative. There are known sex differences in the HPA and immune response to stressors in both rodents and humans (Shanks et al., 1994; Da Silva, 1999; Silva et al., 2002). A sex-specific response to an early immune challenge may differentially impact the developing brain, resulting in the distinct developmental

trajectory of anxiety-related behaviours currently observed between female and male mice. Further study is required to determine the contribution of both sex hormone dependant and independent effects on the observed results. The current findings support a growing body of literature linking the immune system to anxiety (Silverman et al., 2007; Swiergiel and Dunn, 2007; Nautiyal et al., 2008) and extend these results to demonstrate that sex is integral in shaping anxiety-related behavioural outcomes following an immune challenge during early development.

3.5. References

Altman J, Sudarshan K (1975) Postnatal development of locomotion in the laboratory rat. Animal Behaviour 23:896-920.

Andia AM, Zisook S, Heaton RK, Hesselink J, Jernigan T, Kuck J, Morganville J, Braff DL (1995) Gender differences in schizophrenia. The Journal of nervous and mental disease 183:522-528.

Anisman H (2009) Cascading effects of stressors and inflammatory immune system activation: implications for major depressive disorder. J Psychiatry Neurosci 34:4-20.

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 (New York, NY) 306:879-881.

Bosch OJ, Kromer SA, Neumann ID (2006) Prenatal stress: opposite effects on anxiety and hypothalamic expression of vasopressin and corticotropin-releasing hormone in rats selectively bred for high and low anxiety. Eur J Neurosci 23:541-551.

Breivik T, Stephan M, Brabant GE, Straub RH, Pabst R, von Horsten S (2002) Postnatal lipopolysaccharide-induced illness predisposes to periodontal disease in adulthood. Brain, behavior, and immunity 16:421-438.

Cahill L (2006) Why sex matters for neuroscience. Nature reviewsNeuroscience 7:477-484.

Castle DJ, Deale A, Marks IM (1995) Gender differences in obsessive compulsive disorder. The Australian and New Zealand Journal of Psychiatry 29:114-117.

Clarke KA, Still J (2001) Development and consistency of gait in the mouse. Physiology & behavior 73:159-164.

Crawley JN, Skolnick P, Paul SM (1984) Absence of intrinsic antagonist actions of benzodiazepine antagonists on an exploratory model of anxiety in the mouse. Neuropharmacology 23:531-537.

Da Silva JA (1999) Sex hormones and glucocorticoids: interactions with the immune system. Ann N Y Acad Sci 876:102-117; discussion 117-108.

Earls F (1987) Sex differences in psychiatric disorders: origins and developmental influences. Psychiatric developments 5:1-23.

Eklund MB, Arborelius L (2006) Twice daily long maternal separations in Wistar rats decreases anxiety-like behaviour in females but does not affect males. Behav Brain Res 172:278-285.

Gladstone GL, Parker GB, Mitchell PB, Wilhelm KA, Malhi GS (2005) Relationship between self-reported childhood behavioral inhibition and lifetime anxiety disorders in a clinical sample. Depression and anxiety 22:103-113.

Glynn D, Sizemore RJ, Morton AJ (2007) Early motor development is abnormal in complexin 1 knockout mice. Neurobiology of disease 25:483-495.

Gray J, N. M, eds (2000) The neuropsychology of anxiety: Oxford University press.

Gross C, Zhuang X, Stark K, Ramboz S, Oosting R, Kirby L, Santarelli L, Beck S, Hen R (2002) Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. Nature 416:396-400.

Heim C, Nemeroff CB (2001) The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. Biol Psychiatry 49:1023-1039.

Hendrick V, Altshuler LL, Gitlin MJ, Delrahim S, Hammen C (2000) Gender and bipolar illness. The Journal of clinical psychiatry 61:393-396; quiz 397.

Hodgson DM, Knott B (2002) Potentiation of tumor metastasis in adulthood by neonatal endotoxin exposure: sex differences. Psychoneuroendocrinology 27:791-804.

Hodgson DM, Knott B, Walker FR (2001) Neonatal endotoxin exposure influences HPA responsivity and impairs tumor immunity in Fischer 344 rats in adulthood. Pediatric research 50:750-755.

Holmes A (2001) Targeted gene mutation approaches to the study of anxiety-like behavior in mice. Neuroscience and biobehavioral reviews 25:261-273.

Holmes A, Iles JP, Mayell SJ, Rodgers RJ (2001) Prior test experience compromises the anxiolytic efficacy of chlordiazepoxide in the mouse light/dark exploration test. Behavioural brain research 122:159-167.

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.

Kessler RC, Berglund P, Demler O, Jin R, Merikangas KR, Walters EE (2005) Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. Archives of General Psychiatry 62:593-602.

Kikusui T, Takeuchi Y, Mori Y (2004) Early weaning induces anxiety and aggression in adult mice. Physiol Behav 81:37-42.

Kikusui T, Ichikawa S, Mori Y (2009) Maternal deprivation by early weaning increases corticosterone and decreases hippocampal BDNF and neurogenesis in mice. Psychoneuroendocrinology 34:762-772.

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. Current psychiatry reports 4:441-448.

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.

Labad J, Menchon JM, Alonso P, Segalas C, Jimenez S, Jaurrieta N, Leckman JF, Vallejo J (2008) Gender differences in obsessive-compulsive symptom dimensions. Depression and anxiety 25:832-838.

Leonardo ED, Hen R (2008) Anxiety as a developmental disorder. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 33:134-140.

McEwen BS, Stellar E (1993) Stress and the individual. Mechanisms leading to disease. Archives of Internal Medicine 153:2093-2101.

McIntosh J, Anisman H, Merali Z (1999) Short- and long-periods of neonatal maternal separation differentially affect anxiety and feeding in adult rats: gender-dependent effects. Brain Res Dev Brain Res 113:97-106.

Nautiyal KM, Ribeiro AC, Pfaff DW, Silver R (2008) Brain mast cells link the immune system to anxiety-like behavior. Proceedings of the National Academy of Sciences of the United States of America 105:18053-18057.

Neumann ID, Wigger A, Kromer S, Frank E, Landgraf R, Bosch OJ (2005) Differential effects of periodic maternal separation on adult stress coping in a rat model of extremes in trait anxiety. Neuroscience 132:867-877.

Oreland S, Pickering C, Gokturk C, Oreland L, Arborelius L, Nylander I (2009) Two repeated maternal separation procedures differentially affect brain 5-hydroxytryptamine transporter and receptors in young and adult male and female rats. Brain Res.

Papaioannou A, Gerozissis K, Prokopiou A, Bolaris S, Stylianopoulou F (2002) Sex differences in the effects of neonatal handling on the animal's response to stress and the vulnerability for depressive behaviour. Behav Brain Res 129:131-139.

Plotsky PM, Meaney MJ (1993) Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. Brain Res Mol Brain Res 18:195-200.

Prior M, Smart D, Sanson A, Oberklaid F (2000) Does shy-inhibited temperament in childhood lead to anxiety problems in adolescence? Journal of the American Academy of Child and Adolescent Psychiatry 39:461-468.

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. The Journal of clinical psychiatry 69:213-221.

Rodgers RJ, Shepherd JK (1993) Influence of prior maze experience on behaviour and response to diazepam in the elevated plus-maze and light/dark tests of anxiety in mice. Psychopharmacology 113:237-242.

Rogers MP, Warshaw MG, Goisman RM, Goldenberg I, Rodriguez-Villa F, Mallya G, Freeman SA, Keller MB (1999) Comparing primary and secondary generalized anxiety disorder in a long-term naturalistic study of anxiety disorders. Depression and anxiety 10:1-7.

Romeo RD, Mueller A, Sisti HM, Ogawa S, McEwen BS, Brake WG (2003) Anxiety and fear behaviors in adult male and female C57BL/6 mice are modulated by maternal separation. Horm Behav 43:561-567.

Salome N, Tasiemski A, Dutriez I, Wigger A, Landgraf R, Viltart O (2008) Immune challenge induces differential corticosterone and interleukin-6 responsiveness in rats bred for extremes in anxiety-related behavior. Neuroscience 151:1112-1118.

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.

Schwartz CE, Snidman N, Kagan J (1999) Adolescent social anxiety as an outcome of inhibited temperament in childhood. Journal of the American Academy of Child and Adolescent Psychiatry 38:1008-1015.

Seeman MV (1997) Psychopathology in women and men: focus on female hormones. The American Journal of Psychiatry 154:1641-1647.

Shanks N, Meaney MJ (1994) Hypothalamic-pituitary-adrenal activation following endotoxin administration in the developing rat: a CRH-mediated effect. Journal of neuroendocrinology 6:375-383.

Shanks N, McCormick CM, Meaney MJ (1994) Sex differences in hypothalamic-pituitary-adrenal responding to endotoxin challenge in the neonate: reversal by gonadectomy. Brain researchDevelopmental brain research 79:260-266.

Shanks N, Larocque S, Meaney MJ (1995) Neonatal endotoxin exposure alters the development of the hypothalamic-pituitary-adrenal axis: early illness and later responsivity to stress. The Journal of neuroscience : the official journal of the Society for Neuroscience 15:376-384.

Shanks N, Windle RJ, Perks PA, Harbuz MS, Jessop DS, Ingram CD, Lightman SL (2000) Early-life exposure to endotoxin alters hypothalamic-pituitary-adrenal function and predisposition to inflammation. Proceedings of the National Academy of Sciences of the United States of America 97:5645-5650.

Silva C, Ines LS, Nour D, Straub RH, da Silva JA (2002) Differential male and female adrenal cortical steroid hormone and cortisol responses to interleukin-6 in humans. Ann N Y Acad Sci 966:68-72.

Silverman MN, Macdougall MG, Hu F, Pace TW, Raison CL, Miller AH (2007) Endogenous glucocorticoids protect against TNF-alpha-induced increases in anxiety-like behavior in virally infected mice. Mol Psychiatry 12:408-417.

Simon NM, Zalta AK, Worthington JJ, 3rd, Hoge EA, Christian KM, Stevens JC, Pollack MH (2006) Preliminary support for gender differences in response to fluoxetine for generalized anxiety disorder. Depression and anxiety 23:373-376.

Slotten HA, Kalinichev M, Hagan JJ, Marsden CA, Fone KC (2006) Long-lasting changes in behavioural and neuroendocrine indices in the rat following neonatal maternal separation: gender-dependent effects. Brain Res 1097:123-132.

Spencer SJ, Heida JG, Pittman QJ (2005) Early life immune challenge--effects on behavioural indices of adult rat fear and anxiety. Behavioural brain research 164:231-238.

Spencer SJ, Martin S, Mouihate A, Pittman QJ (2006) Early-life immune challenge: defining a critical window for effects on adult responses to immune challenge. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 31:1910-1918.

Stein MB, Walker JR, Anderson G, Hazen AL, Ross CA, Eldridge G, Forde DR (1996) Childhood physical and sexual abuse in patients with anxiety disorders and in a community sample. The American Journal of Psychiatry 153:275-277.

Steiner M, Allgulander C, Ravindran A, Kosar H, Burt T, Austin C (2005) Gender differences in clinical presentation and response to sertraline treatment of generalized anxiety disorder. Human psychopharmacology 20:3-13.

Swiergiel AH, Dunn AJ (2007) Effects of interleukin-1beta and lipopolysaccharide on behavior of mice in the elevated plus-maze and open field tests. Pharmacology, biochemistry, and behavior 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. The American Journal of Psychiatry 152:698-703.

Tenk CM, Kavaliers M, Ossenkopp KP (2008) Sexually dimorphic effects of neonatal immune system activation with lipopolysaccharide on the behavioural response to a homotypic adult immune challenge. International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience 26:331-338.

Tenk CM, Foley KA, Kavaliers M, Ossenkopp KP (2007) Neonatal immune system activation with lipopolysaccharide enhances behavioural sensitization to the dopamine agonist, quinpirole, in adult female but not male rats. Brain, behavior, and immunity 21:935-945.

Turgeon L, Marchand A, Dupuis G (1998) Clinical features in panic disorder with agoraphobia: a comparison of men and women. Journal of anxiety disorders 12:539-553.

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). The Journal of clinical psychiatry 69:1606-1616.

Walker AK, Nakamura T, Byrne RJ, Naicker S, Tynan RJ, Hunter M, Hodgson DM (2009) Neonatal lipopolysaccharide and adult stress exposure predisposes rats to anxiety-like behaviour and blunted corticosterone responses: Implications for the double-hit hypothesis. Psychoneuroendocrinology 34:1515-1525.

Walker FR, March J, Hodgson DM (2004) Endotoxin exposure in early life alters the development of anxiety-like behaviour in the Fischer 344 rat. Behavioural brain research 154:63-69.

Walker FR, Knott B, Hodgson DM (2008) Neonatal endotoxin exposure modifies the acoustic startle response and circulating levels of corticosterone in the adult rat but only following acute stress. Journal of psychiatric research 42:1094-1103.

Weinstock LS (1999) Gender differences in the presentation and management of social anxiety disorder. The Journal of clinical psychiatry 60 Suppl 9:9-13.

Weinstock M, Matlina E, Maor GI, Rosen H, McEwen BS (1992) Prenatal stress selectively alters the reactivity of the hypothalamic-pituitary adrenal system in the female rat. Brain Res 595:195-200.

CHAPTER 4.

An ontogenetic characterization of mesolimbocortical serotonergic gene expression changes following early immune challenge

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Abstract

An immunogenic challenge during early postnatal development leads to long-term changes in behavioural and physiological measures reflecting enhanced emotionality and anxiety. Altered CNS serotonin (5-HT) signalling during the third postnatal week is thought to modify the developing neurocircuitry governing anxiety-like behaviour. Changes in 5-HT signalling during this time window may underlie increased emotionality reported in early immune challenge rodents. Here we examine both the spatial and temporal profile of 5-HT related gene expression, including 5HT1A, 2A, 2C receptors, the 5-HT transporter (5HTT), and tryptophan hydroxylase 2 (TPH2) during early development (postnatal days [P] 14-28) in mice challenged with lipopolysaccharide (LPS) during the first postnatal week. Expression levels were measured using *in situ* hybridization in regions associated with mediating emotive behaviours: the dorsal raphe (DR), amygdala, hippocampus and prefrontal cortex (PFC). Increased TPH2 and 5HTT expression in the ventral DR of LPS-mice accompanied decreased expression of ventral DR 5HT1A and dorsomedial DR 5HTT. In the forebrain, increased hippocampal 5HT1A and amygdala 5HT2C expression along with decreased hippocampal and PFC 5HT2C expression were detected in LPS-mice. These changes were restricted to P14-21. Transient changes in 5-HT expression coincide with the critical time window in which 5-HT disturbance leads to permanent modification of anxiety-related behaviours. This suggests that alterations in CNS 5-HT during development may underlie the enhanced emotionality associated with an early immune challenge.

4.1. Introduction

Peripheral administration of lipopolysaccharide (LPS), a component of gram-negative bacteria, during early postnatal development leads to long-term changes in rodent physiology and behaviour (Shanks et al., 1995; Shanks et al., 2000; Hodgson et al., 2001; Breivik et al., 2002; Nilsson et al., 2002; Bilbo et al., 2005; Boisse et al., 2005; Spencer et al., 2005; Bilbo et al., 2007; Tenk et al., 2007; Galic et al., 2008; Tenk et al., 2008). A well replicated finding is that LPS exposure on postnatal day (P) 3 and 5 leads to enhanced emotional-reactivity and anxiety-like behaviour in adulthood (Shanks et al., 1995; Breivik et al., 2002; Walker et al., 2004a; Walker et al., 2008; Walker et al., 2009). Although these behaviours have been well characterized, the underlying pathophysiological determinants have yet to be fully elucidated. In particular, the molecular changes that are occurring early in development have not been considered.

LPS-challenge acts on the neonatal stress axis to increase both plasma adrenocorticotropic hormone and corticosterone following administration (Shanks and Meaney, 1994; Dent et al., 1999; Walker et al., 2004b). As LPS is a robust activator of the hypothalamic-pituitary-axis, much focus has been given to the long-term consequences of neonatal stress-axis programming by early LPS challenge (Shanks and Meaney, 1994; Shanks et al., 2000). Immune challenge also acts on brain neuromodulatory circuits, such as the serotonergic (5-HT) system. For instance, LPS administration activates 5-HT neurons in the dorsal raphe (Hollis et al., 2006), increases

extracellular concentrations of 5-HT in the hippocampus (Linthorst et al., 1995; Linthorst and Reul, 1998) and alters 5-HT activity in various limbic forebrain regions implicated in regulating emotionality (Pitychoutis et al., 2009). This suggests that LPS associated alterations in 5-HT signalling may contribute to the long-term alterations in emotional-reactivity and anxiety-related behaviours in early immune challenged rodents. No studies to date, however, have explored the impact of early immune challenge on the developing 5-HT system.

Experiments using genetic modification of the serotonergic system in mice provide evidence that anxiety can be viewed as a neurodevelopmental disorder. The 5-HT1A receptor knockout mouse displays increased anxiety-related behaviours in adulthood (Parks et al., 1998). However, conditional forebrain expression of the 5HT1A receptor between the ages of P14-P21 restores normal anxiety-related behaviours in these mice (Gross et al., 2002). Conversely, pharmacological blockade of the 5HT1A receptor during this time reproduces the adult knockout phenotype of increased anxiety-related behaviours (Lo Iacono and Gross, 2008). A similar phenotype of enhanced emotionality is observed following functional blockade of the 5-HT transporter (5HTT) with fluoxetine during early postnatal development (P4-P21) but not later (Ansorge et al., 2004). Collectively, these studies suggests that anxiety-related behavioural deficits are a consequence of altered 5-HT during early development and not during adulthood. Specifically, the time window between P14 and P21 appears critical for the later establishment of rodent anxiety-like behaviour (Leonardo and Hen, 2008).

In this study, we examined the spatial and temporal expression of serotonin-related genes during postnatal development (P14-P28) following early immune challenge. As disruption of 5-HT receptor expression during this time can have long-term consequences on a variety of neuronal circuits with relevance to anxiety (Patel and Zhou, 2005), we examined the receptors 5HT1A, 2A and 2C, along with the 5-HTT and the rate-limiting enzyme for 5-HT production, tryptophan hydroxylase (TPH2). Serotonin gene expression was measured in brain regions implicated in the pathophysiology of affective disorders such as the dorsal raphe (DR), prefrontal cortex (PFC), basolateral amygdala (BLA), and the hippocampus (Lowry et al., 2005).

4.2. Methods

4.2.1. Animals. Female and male CD-1 mice (8-10 w; Charles River) were bred in house and the resulting offspring were used in these experiments (n=8 litters). Postnatal day (P) 0 denotes day of birth. Litters were culled to a maximum of 12 on P1. Weighing occurred at the same time daily from P1-P7 and weekly thereafter. Mice were weaned on P21 and housed in standard cages (max. 5/cage) on a 12h:12h light:dark cycle (lights on at 07:00) with food and water provided *ad libitum*. All procedures were approved by the McMaster University Animal Research Ethics Board and carried out in accordance with the guidelines described in the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care 1993).

4.2.2. Experimental procedure. On P3 and P5, pups were given an i.p. injection of either 0.05 mg/kg lipopolysaccharide (E. coli LPS; Sigma) in 50 ul/g or an equal volume of saline (SAL; 0.9%) between 7:00-9:00 h. Pups from one litter received either LPS or SAL (n=24 LPS, n=24 SAL). Dams were removed from the homecage and returned once all pups had received an injection. Maternal separation did not exceed 10 m.

At P14, P17, P21 and P28, brains were rapidly removed following decapitation, frozen in -60°C isopentane, and stored at -70°C until cryostat sectioning. A series of 12μm coronal sections were collected through the prefrontal cortex (bregma 1.70-1.54), basolateral amygdala (bregma 2.46-2.54) dorsal hippocampus (bregma 1.46-1.94), ventral hippocampus (bregma 2.30-2.54), and the mid-rostrocaudal dorsal raphe (bregma 4.48-4.60), according to the stereotaxic atlas of Paxinos and Franklin, 2003. The mid-rostrocaudal level was chosen for analysis of serotonin gene expression given that serotonergic neurons found here project to anatomical regions implicated in mediating anxiety states (Lowry, 2002). Sections were mounted onto gelatin-coated glass slides, dried on a slide warmer (30°C) for 10 min and stored at -35°C until processing. A total of 48 male and female mice were used for mRNA analysis (6 per treatment per time point: P14, P17, P21 and P28).

4.2.3. Riboprobes. The 5HT1A receptor riboprobe was generously provided by Dr. Pat Levitt, Vanderbilt University, Nashville, TN. Riboprobes for 5HT2A, 5HT2C, 5HTT and TPH2 were generated in our laboratory (see Table 1 for primer sequences and the product size obtained). Complimentary DNA was generated through polymerase chain reaction

and ligated into the pGEM-T easy expression vector (Promega). Target sequences were confirmed by DNA sequencing (Mobix Lab, McMaster University).

4.2.4. In situ hybridization. *In situ* hybridization procedures were carried out as previously described (Foster et al., 2002). Both sense and antisense riboprobes were transcribed from linearized plasmid with ^{35} S-UTP (specific activity >1,000 Ci/mmol; Perkin Elmer, Boston, MA) using T3, T7 or Sp6 polymerase. Slide-mounted sections were fixed in 4% formaldehyde in PBS, acetylated with fresh 0.25% acetic anhydride in 0.1 M triethanolamine–HCl (pH 8.0), dehydrated in increasing concentrations of ethanol, and delipidated with chloroform. Tissue sections were then hybridized (approximately 500,000 CPM / section) overnight (18 h) at 55°C in a humidified chamber with radiolabeled riboprobe diluted in hybridization buffer (0.6 M NaCl, 10 mM Tris pH 8.0, 1 mM ethylenediaminetetraacetic acid pH 8.0, 10% Dextran sulfate, 0.01% sheared salmon sperm DNA, 0.5% total yeast RNA, type XI, 0.01% yeast transfer RNA, 1X Denhardt's solution). Slides were washed in 20 µg/ml ribonuclease solution for 30 m to reduce non-specific binding followed by 1 h each in 2XSSC at 50°C, 0.2XSSC at 55°C and 0.2XSSC at 60°C. Slides were dehydrated through a graded serious of ethanol and air dried for autoradiography. Specificity of the antisense probe was determined by a lack of sense signal on tissue sections containing corresponding regions of interest.

Table 1. Primers used to synthesize cDNA for generation of 5-HT riboprobes.

Riboprobe	Primer Position	Primer Sequence (5'-3')	Product size (bp)
5HT2A	Forward	CCAGGAGGGGCTTATTCTTT	309
	Reverse	TGTGGAATCCCCTCTCTTTG	
5HT2C	Forward	AGACCAGAATGATGCACATGAC	834
	Reverse	GAGAGCTTGGAAGGATCTGAAA	
5HTT	Forward	CTGGGTTTGGATAGCACGTT	560
	Reverse	ATTTCCGTTGGTGTTTCAGG	
TPH2	Forward	CTGGGGATTTGATGCCTAGA	329
	Reverse	GGAATTGTGCGAGGAATGTT	

4.2.5. Autoradiography. Slides and ^{14}C plastic standards containing known amounts of radioactivity (American Radiochemicals) were placed in X-ray cassettes and apposed to film (Biomax MR, Kodak) for a specified length of time based on the probe used and region of interest. Images were captured using a Qiacam digital camera (Quorum Technologies) using a constant illumination light source (Northern Light Precision Illuminator, Imaging Research). NIH image analysis software (http://rsb.info.nih.gov.libaccess.lib.mcmaster.ca/nih-image) running on a Macintosh computer-based image analysis system was used for quantification of mRNA expression. For regions where mRNA was homogenous, light transmittance (OD) was converted to radioactivity levels (disintegrations per minute [DPM]) using the Rodbard curve applied to the ^{14}C standards. In regions where mRNA signal was not homogenous, density slice, which measures both light transmittance and area of the mRNA signal, was used. Light

transmittance was converted to DPM and multiplied by signal area to yield an integrated density. Rostrocaudal levels of the DR were confirmed by comparing the *in situ* image with atlases of immunostaining or mRNA expression for TPH2, 5HTT and 5HT1A (Abrams et al., 2004; Clark et al., 2006). The mean DPM or integrated density was calculated for each brain region per animal as an average of two sections.

4.2.6. Statistics. Data were analyzed using a two-way ANOVA with treatment (SAL/LPS) and time as main factors. The Student's t-test was used when analyzing treatment effects at specific time points during development. The P17 time point for 5HTT expression in the dorsomedial was disregarded due to compromised tissue. Statistics was performed using the Prism 4.0a statistical software for Macintosh. A two-tailed p-value less than 0.05 was considered statistically significant. Significance is denoted as the following: *$p<0.05$. All values are expressed as mean ± SEM.

4.3. Results

4.3.1. Dorsal raphe: TPH2, 5HTT, 5HT1A

In situ hybridization signal for TPH2, 5HTT and 5HT1A were measured in the mid-rostrocaudal DR of SAL and LPS mice from P14-28. Given the functional and topographic organization of the DR (Abrams et al., 2004; Lowry et al., 2008), sub-regions of the DR were analyzed separately: dorsomedial, ventromedial, lateral wings, and median raphe (see Fig.1A, 2A, 3A). The spatial expression profile for TPH2, 5HTT and 5HT1A within the mid-rostrocaudal sub-regions of the DR was consistent with known

expression patterns of these genes during adulthood (Clark et al., 2006). Expression for TPH2 was highest in the ventromedial DR, followed by the dorsomedial DR, median raphe and lateral wings. There was a significant effect of time ($F_{3,33}$=6.17, p=0.002) on TPH2 mRNA expression in the dorsomedial aspect of the DR with expression increasing between the second postnatal week and P28 in both treatment groups (Fig. 1B). There was an increase in TPH2 signal in LPS-mice at P17 in the dorsomedial DR relative to SAL controls (P17: t_9= 2.181, p=0.057; Fig. 1B), although this did not quite reach statistical significance. TPH2 mRNA expression in the lateral wings of LPS-mice was significantly increased at P14 (t_8= 3.997, p=0.006; Fig. 1C). No significant differences in mRNA expression were detected in the ventromedial DR or median raphe (Fig 1D, Fig. 1E).

There was a significant impact of treatment on 5HTT expression in the dorsomedial DR ($F_{1,15}$=5.35, p=0.03; Fig 2B). This reflected a decreased expression in LPS-mice compared with saline controls at both P14 and P21, which reached significance at P14 (t_7=4.61, p=0.002). There was a significant increase in 5HTT expression in the lateral wings of LPS-mice at P17 (t_5=2.88, p=0.035; Fig. 2C). No expression differences were detected in the ventromedial DR or median raphe (Fig. 2D, E).

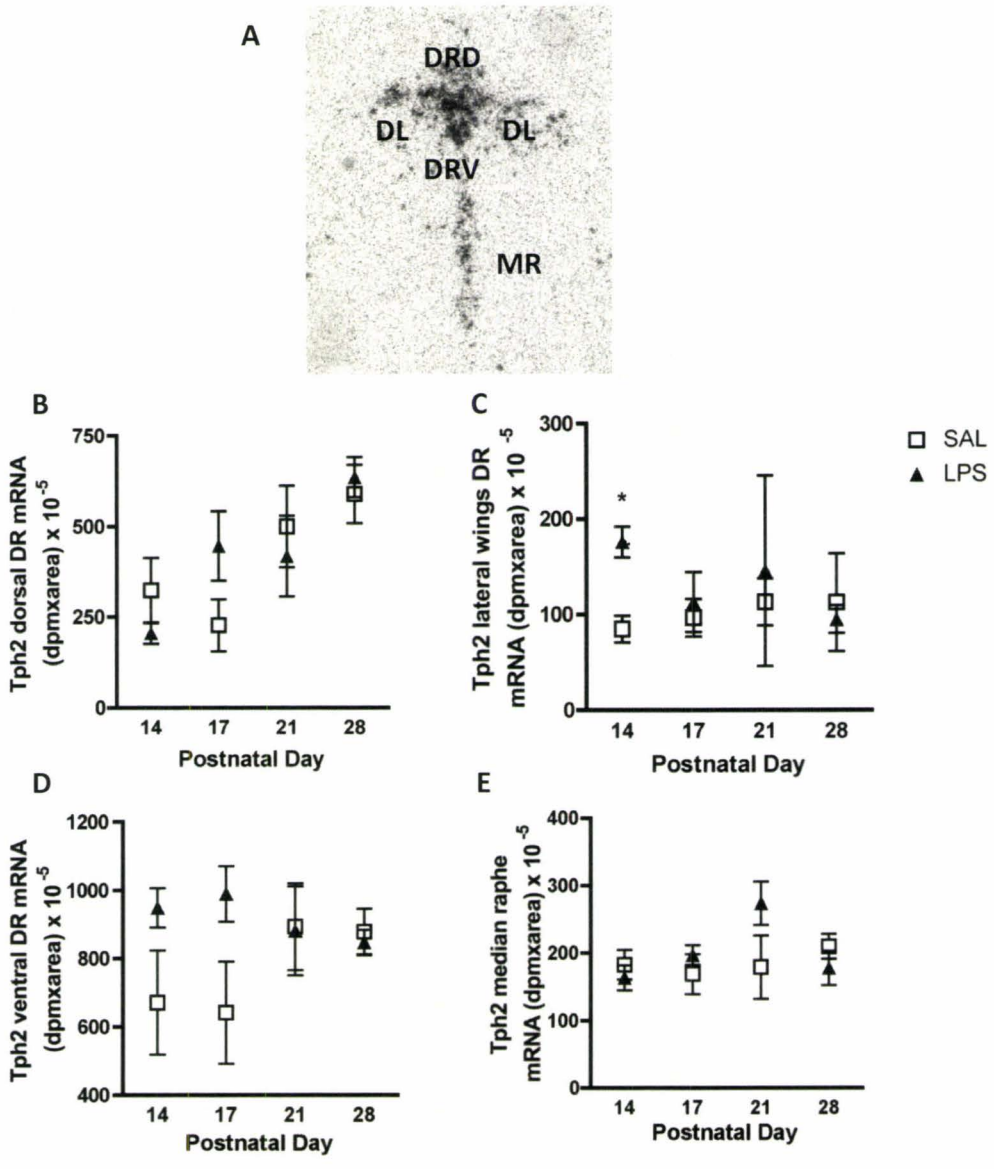

Figure 1. Representative *in situ* hybridization autoradiographic coronal section of tryptophan hydroxylase (TPH2) mRNA expression in the mouse mid rostrocaudal dorsal raphe (~bregma 4.48). Sub-regions for quantification of TPH2 expression are labelled in **A**. Developmental expression of TPH2 mRNA in the dorsomedial (**B**) dorsal lateral (**C**) ventral (**D**) and median (**E**) raphe nucleus of mice exposed to a postnatal injection of lipopolysaccharide (LPS) or saline (SAL). Values represent mean integrated density, ± SEM based on the average of two sections per animal (SAL/LPS, n=6/time point). DR=dorsal raphe; DRD=dorsomedial DR; DRV=ventral DR; DL=lateral wings; MR=median raphe. *$p<0.05$.

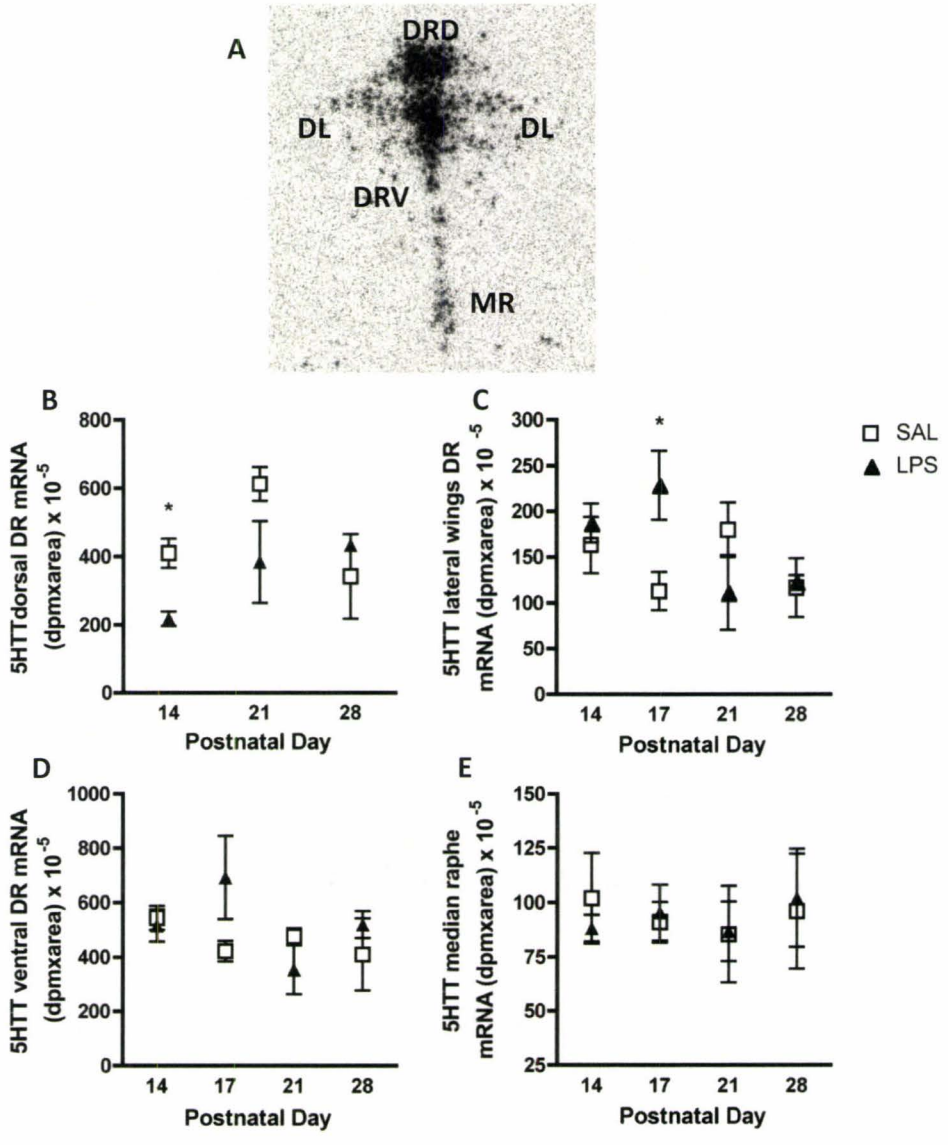

Figure 2. Representative *in situ* hybridization image of a coronal section showing 5HTT mRNA expression (**A**). Sub-regions for quantification of 5HTT expression are labelled in **A**. (**B**) Note that values for P17 are missing due to compromised tissue and lack of sufficient N for graph or analysis purposes. Values represent mean integrated density, ± SEM based on the average of two sections per animal (SAL/LPS, n=6/time point). DR=dorsal raphe; DRD=dorsomedial DR; DRV=ventral DR; DL=lateral wings; MR=median raphe. *p<0.05.

5HT1A expression was strongest in the dorsomedial and ventromedial DR and lowest in the lateral wings and median raphe (Fig. 3A). There was a steady increase in dorsomedial 5HT1A expression in both treatment groups between P14-P28 (Fig. 3B). There was a treatment effect in the ventromedial DR ($F_{1,36}$=3.88, p=0.05; Fig. 3D) such that 5HT1A expression was significantly decreased in LPS-mice relative to SAL controls at P17 ($p<0.05$). No significant differences were detected in the lateral wings or median raphe (Fig. 3C, E).

4.3.2. Prefrontal cortex: 5HT2C, 5HT2A

The expression of 5HT2C and 5HT2A mRNA was measured in the prelimbic and infralimbic cortices of SAL and LPS mice during the first 4 weeks of postnatal development (Fig. 4). 5HT2C expression was strongest in cortical layer V of the prefrontal cortex at all time points examined (Fig. 4A). There was a significant effect of time on 5HT2C mRNA expression in layer V of the prefrontal cortex ($F_{3,37}$=31.95, $p<0.0001$; Fig. 4B) reflecting strong expression at P14 that steadily declined in both treatment groups from P14 through to P28. Posthoc analyses revealed a significant decrease in 5HT2C mRNA expression in LPS-mice compared with SAL on P14 ($p<0.05$).

5HT2A expression was strongest in layer II/III of the prefrontal cortex in both treatment groups (Fig. 4C). No time or treatment effects were found for 5HT2A mRNA expression (Fig. 4D).

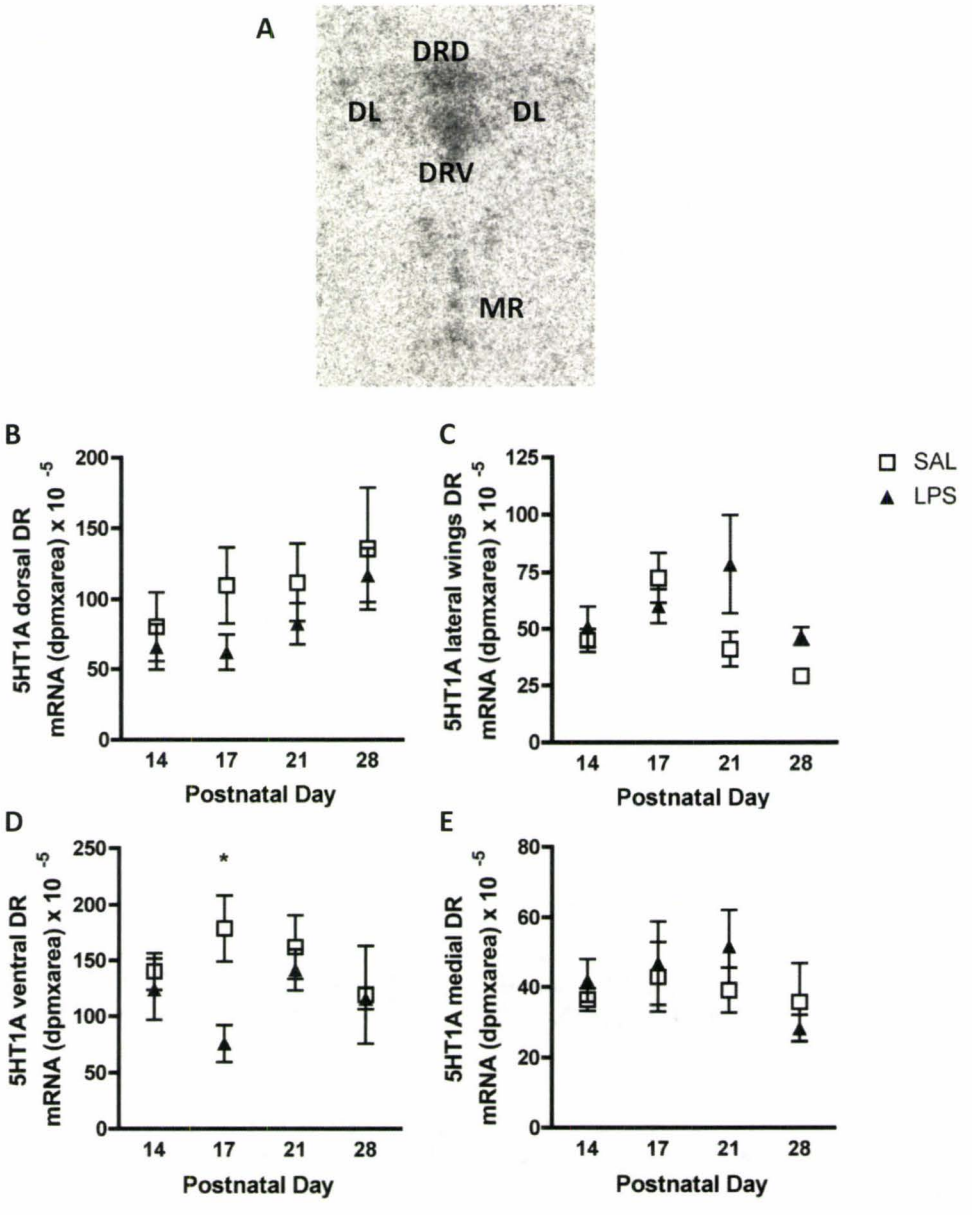

Figure 3. Representative *in situ* hybridization image of a coronal section showing 5HT1A receptor mRNA expression (**A**). Sub-regions for quantification of 5HT1A expression are labelled in **A**. Values represent mean integrated density, ± SEM based on the average of two sections per animal (SAL/LPS, n=6/time point). DR=dorsal raphe; DRD=dorsomedial DR; DRV=ventral DR; DL=lateral wings; MR=median raphe. *p<0.05.

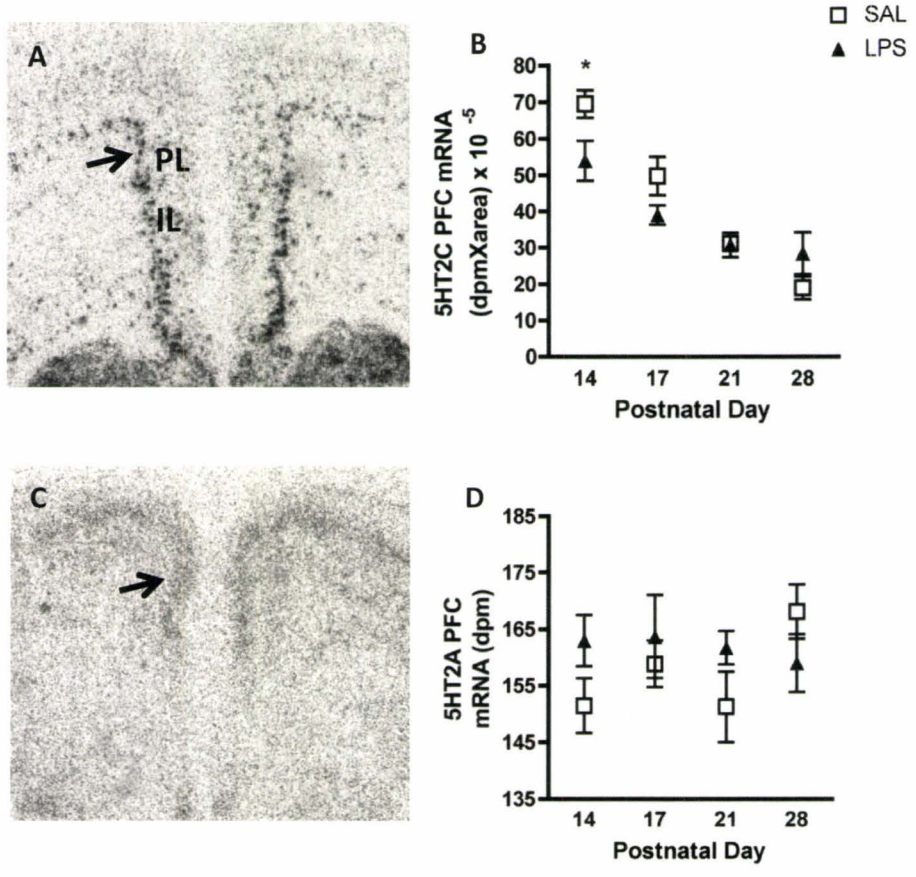

Figure 4. Representative *in situ* hybridization autoradiographic coronal section of 5HT2C receptor (**A**) and 5HT2A receptor (**C**) mRNA expression in the mouse prefrontal cortex. Arrow points to layer V of the prefrontal cortex where 5HT2C expression was quantified in the prelimbic (PL) and infralimbic (IL) regions and layer II/III for 5HT2A. Developmental expression of 5HT2C (**B**) and 2A (**D**) receptor mRNA in SAL and LPS mice. Values represent mean integrated density for 2C and mean disintegrations per minute (dpm) for 2A, ± SEM based on the average of two sections per animal (SAL/LPS, n=6/time point). *p<0.05.

4.3.3. Amygdaloid complex: 5HT2C

5HT2C mRNA expression was detected in the basolateral, basolateral posterior, basomedial, and lateral dorsal regions of the amygdala (Fig. 5A). In all regions, 5HT2C expression was highest at P14 and declined over time, with lowest expression detected at P28. This temporal expression is consistent with the known expression pattern of 5HT2 receptors during early postnatal development (Morilak and Ciaranello, 1993). Specifically, there was a significant impact of time on 5HT2C mRNA expression in the lateral amygdala ($F_{3,30}$ = 3.01, p=0.046; Fig. 5B) with SAL controls exhibiting a greater decline in mRNA expression between P14-P21 than LPS-mice. There was also a significant impact of time on 5HT2C mRNA expression in the basolateral amygdala ($F_{3,30}$ = 5.28, p=0.005; Fig. 5C), with mRNA expression decreasing significantly between P14 and P21 in both treatment groups (SAL: $p<0.05$; LPS: $p<0.05$). No significant differences were found for 5HT2C mRNA expression in the basolateral posterior (Fig. 5D) or basomedial amygdala (Fig. 5E).

4.3.4. Hippocampal formation: 5HT1A, 5HT2C

5HT1A mRNA expression was analyzed in the dentate gyrus (DG) and CA1 sub-regions of the dorsal hippocampus (Fig. 6A). A weak expression signal was detected in the CA3 region, but was too low to reliably quantify. There was a significant effect of time ($F_{1,37}$=3.05, p=0.04; Fig. 6B) on 5HT1A mRNA expression in the DG, reflecting an

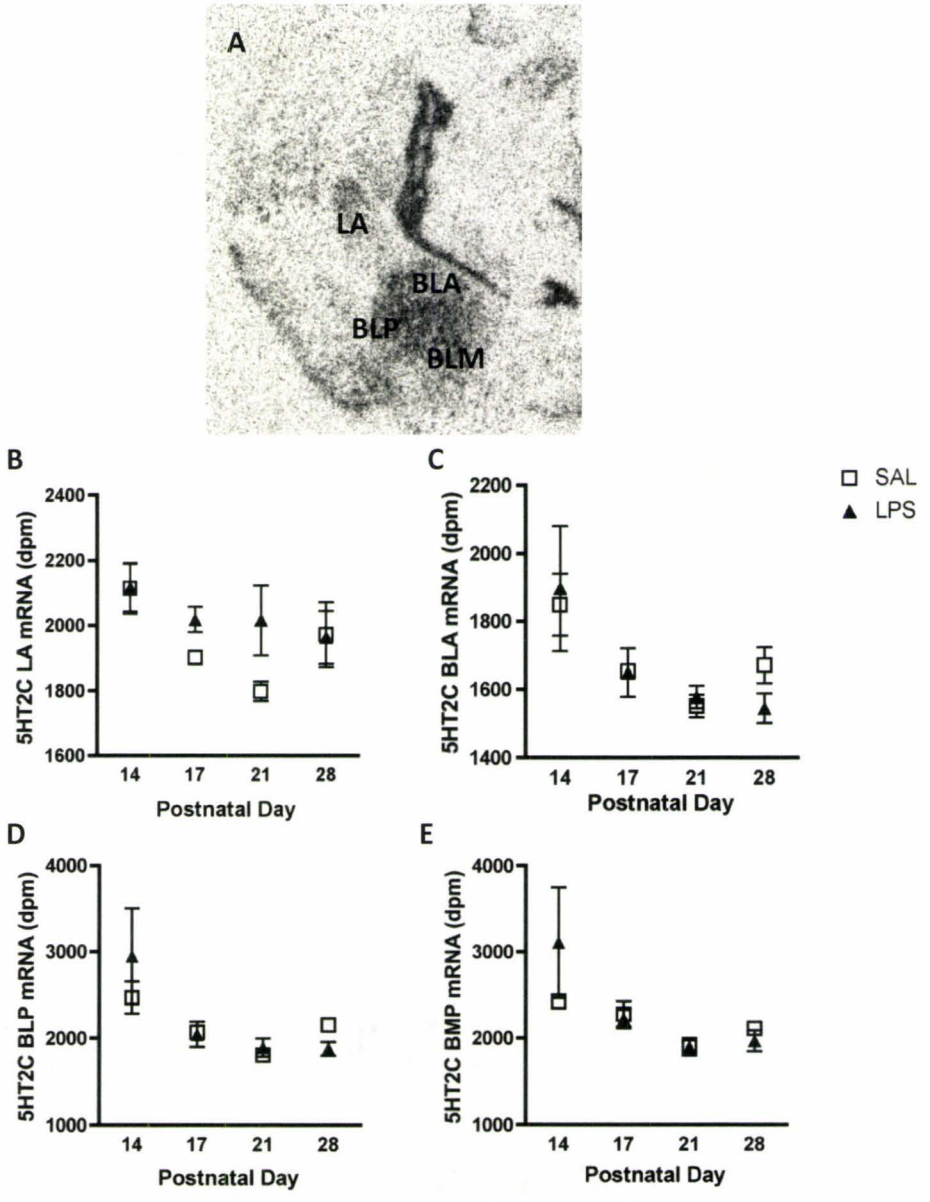

Figure 5. The expression pattern of 5HT2C receptor mRNA was quantified over the first 4 postnatal weeks in SAL and LPS mice (B-E). (**A**) Representative autoradiographic coronal section of 5HT2C receptor mRNA expression in the amygdala (~ bregma 2.54). Sub-regions analyzed are labelled as LA=lateral amygdala, BLA=basolateral amygdala, BLP=basolateral posterior, BLM=basomedial amygdala. Values represent mean disintegrations per minute (dpm), ± SEM based on average of two sections/animal (SAL/LPS, n=6/time point).

increase in expression in LPS mice between P14-P17 ($p<0.05$) and in SAL mice between P17-P21 ($p<0.001$). There was also a significant impact of treatment ($F_{1,37}=4.34$, $p=0.04$; Fig. 6B) on 5HT1A expression in the DG, with an increase in mRNA expression at P17 in LPS-mice relative to SAL controls ($p<0.01$). There was a significant effect of time in the CA1 region ($F_{1.37}=23.04$, $p=0.0001$; Fig. 6C) reflecting a steady increase in expression in both LPS and SAL mice between P14-P28 (SAL, LPS, $p<0.001$).

5HT2C receptor mRNA expression was detected in the CA3 region of the ventral hippocampus (Fig. 7A); no expression was detected in the dorsal hippocampus. There was an effect of treatment on 5HT2C receptor mRNA expression ($F_{1,35}=4.06$, $p=0.05$; Fig. 7B), with a significant decrease in expression observed in LPS-mice compared with SAL controls on P14 ($p<0.05$).

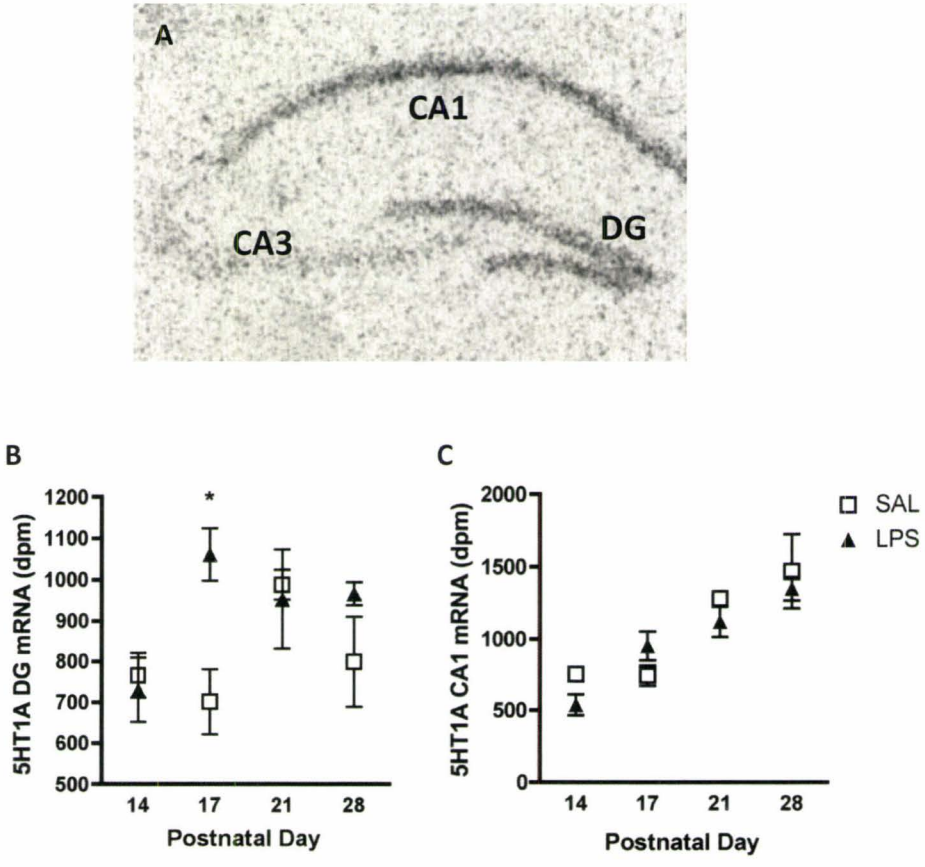

Figure 6. Representative autoradiographic coronal section of 5HT1A receptor expression in the dorsal hippocampal level used for analysis (Bregma ~ 1.46). Sub-regions of the dorsal hippocampus used in analysis are labelled (**A**). Expression pattern of 5HT1A mRNA in the dentate gyrus (**B**) and CA1 (**C**) of SAL and LPS mice. (**B**) There was a significant increase in expression measured at P17 in the DG of LPS-mice. (**C**) There was no treatment effect on 5HT1A mRNA expression in the CA1 region, although both groups exhibited a general increase in expression between P14-P28. Values represent mean disintegrations per minute (dpm), ± SEM based on the average of two sections per animal (SAL/LPS, n=6/time point). DG=dentate gyrus. *$p<0.05$.

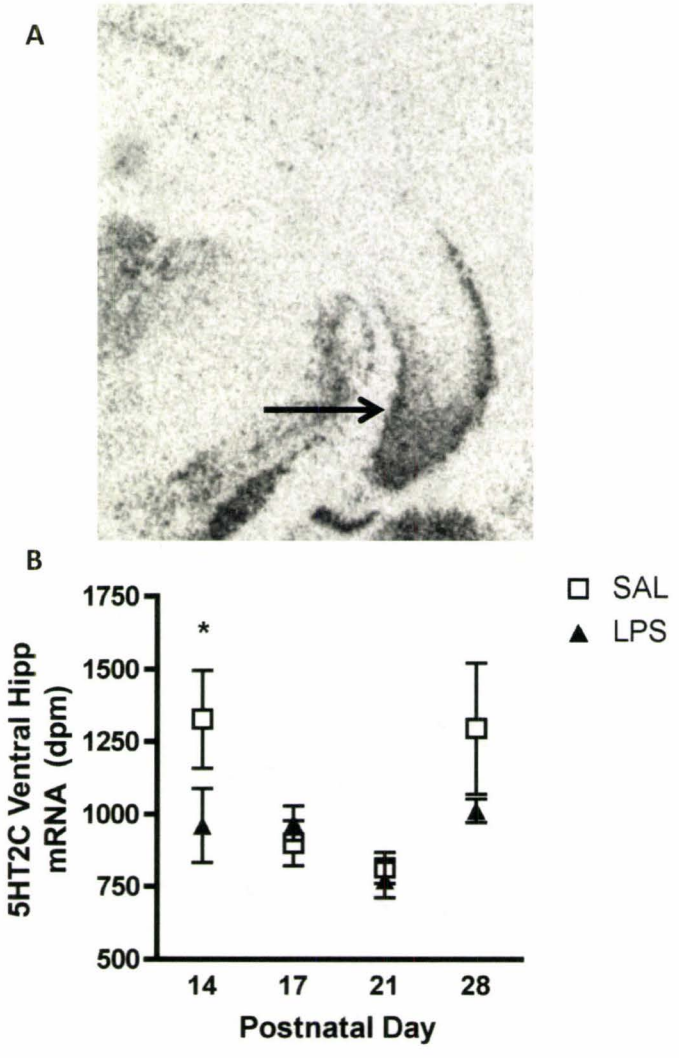

Figure 7. The effects of early postnatal treatment with lipopolysaccharide (LPS) or saline on 5HT2C receptor mRNA expression in the ventral hippocampus during early development. (**A**) Representative autoradiographic coronal section of 5HT2C expression in the ventral hippocampus (~bregma=2.48) with arrow pointing to the hippocampal region that was considered ventral for this analysis. (**B**) There was a significant decrease in 5HT2C receptor mRNA expression in LPS-mice on P14. Values represent mean disintegrations per minute (dpm), ± SEM based on the average of two sections per animal (SAL/LPS, n=6/time point). *$p<0.05$.

4.4. Discussion

The current study characterized the spatiotemporal pattern of serotonin (5-HT)-related gene expression during early postnatal development (P14-28) following an early immune challenge. Results demonstrate that exposure to an immune challenge during the first postnatal week (P3, P5) alters the expression level of 5-HT related genes both at forebrain post-synaptic sites and in the brain stem DR during early development. These changes were transient, occurring during the third postnatal week (P14-P21), with differences no longer detected by P28. Given that 5-HT receptor expression reaches adult levels between P21-P28 (Roth et al., 1991; Morilak and Ciaranello, 1993; Patel and Zhou, 2005), the results suggest that early immune challenge specifically targets developing 5-HT neurocircuitry.

Early LPS-challenge alters brain stem 5-HT neurocircuitry

Early immune challenge led to altered 5-HT receptor mRNA expression that was most evident in the ventral portion of the DR (lateral wings and ventromedial DR). The ventral portion of the DR contains a functionally distinct sub-population of 5-HT neurons that are believed to be involved in the regulation of emotionality or passive emotional coping responses (Gardner et al., 2005), in part through projections to areas such as the basolateral amygdala (Peyron et al., 1998). Specifically, we found an early immune challenge associated increase in ventral DR TPH2 mRNA expression and a decrease in the inhibitory 5HT1A autoreceptor which would be expected to increase 5-HT

neurotransmission. A similar phenotype of increased TPH2 and 5-HT neurons in the DR is observed in post-mortem tissue from patients with major depression (Bach-Mizrachi et al., 2006; Bach-Mizrachi et al., 2008). This finding may appear paradoxical in the context of the widely held hypothesis of reduced 5-HT in depression, although it is possible that this reflects a compensatory mechanism for reduced forebrain 5-HT. Alternatively, the recent finding of elevated 5-HT turnover in unmedicated patients with depression (Barton et al., 2008) and anxiety (Esler et al., 2007) that normalizes with antidepressant treatment indicates increased 5-HT production and would be consistent with an increase in TPH2 mRNA expression and/or reduced autoreceptor 5HT1A expression. When interpreting these results it is important to caution that extrapolation from knowledge of receptor function in adulthood can be misleading as 5-HT receptors have expanded and distinct roles during development (Beique et al., 2004; Patel and Zhou, 2005). Therefore, although the exact functional consequences of altered 5-HT gene expression in the DR are uncertain, our results combined with a recent report of increased 5-HT mRNA expression in the ventral portion of the DR in rats exposed to early-life adversity (Gardner et al., 2009a; Gardner et al., 2009b) suggest that the DR may be a common target of early-life stressors. Indeed, acute endotoxin administration is known to activate DR 5-HT neurons within the mesolimbocortical circuit (Hollis et al., 2006; Lowry et al., 2007). This alteration in 5-HT transmission in the DR during early development may set the stage for downstream alterations in forebrain 5-HT receptors.

Early LPS challenge alters forebrain 5-HT neurocircuitry

We found increased 5HT1A receptor expression in the hippocampal dentate gyrus of LPS-mice during the third postnatal week. Elevated 5HT1A receptor expression in the CA1 region of the hippocampus during early postnatal development is a robust finding in early-life adversity models (Vazquez et al., 2000; Vazquez et al., 2002; Goodfellow et al., 2009) and is proposed to represent a compensatory mechanism in a compromised neuronal environment (Goodfellow et al., 2009). The regional specificity of our finding (i.e. dentate gyrus) has important implications given that the 5HT1A receptor is essential in providing neurotrophic support to developing hippocampal granule cells during the first few weeks of postnatal development (Yan et al., 1997). LPS-mice also exhibited a decrease in 5HT2C expression in the ventral hippocampus, a region associated with governing emotionality (Bannerman et al., 2003; Snyder et al., 2008) and a target of antidepressant treatment (Banasr et al., 2006). Together with altered 5HT1A mRNA expression, this demonstrates a general disturbance in 5-HT signalling in the hippocampus of LPS-mice during early postnatal development. The hippocampus governs functions that are altered in early immune challenge rodents, such as stress-axis regulation (Shanks et al., 1995; Shanks et al., 2000), emotionality (Breivik et al., 2002; Walker et al., 2004a; Bilbo et al., 2007; Walker et al., 2008), and learning and memory (Bilbo et al., 2005; Bilbo et al., 2006; Kohman et al., 2008). Disturbed 5-HT signalling during ontogeny may impact the developing hippocampus with long-term ramifications to hippocampal-dependant functions in LPS-mice.

5HT2C receptor expression is believed to govern anxiety at the level of the amygdala presumably by enhancing excitatory communication to projecting regions such as the paraventricular nucleus, controlling HPA activation (Holmes, 2008). The lateral amygdala, specifically, is implicated in evaluating the affective significance of sensory events and therefore plays a role in mediating aspects of emotionality. The elevated expression of 5HT2C receptors in the lateral amygdala of LPS-mice would ostensibly act to enhance 5-HT signalling and may be associated with increased anxiety-related behaviours reported in the model. 5HT2C receptors are also important in controlling excitation/inhibition of the prefrontal cortex (PFC) both directly and indirectly through amygdala-PFC innervation. This is important in the context that there also exists a prominent PFC-DR afferent projection (Peyron et al., 1998) that modifies DR activation in relation to the perceived controllability of a stressor (Amat et al., 2005). The current observation of altered 5HT2C receptor expression in the forebrains of LPS-mice would modify the nature of the amygdala-PFC-DR signal and possibly impact subsequent stress-induced DR activation.

Implications of transient alteration of 5-HT gene expression during development

The transient nature of gene expression perturbation in LPS-mice suggests that the long-term consequences of a neonatal immunogenic challenge are not regulated at the level of gene transcription. Indeed, a recent study demonstrated transient changes in 5HT1A receptor gene expression during the second postnatal week in animals exposed to

early-life stress. Although the behavioural outcomes of early-life stress were enduring, differences in receptor expression were not observed beyond the second postnatal week (Goodfellow et al., 2009). This suggests that the long-term functional alterations in 5-HT signalling are possibly mediated downstream of the receptor. The 5-HT receptors considered in the current study are exclusively G-protein coupled. As stated by Goodfellow et al. (2009) in interpreting their findings, previous work has demonstrated that receptor function in response to stress is reduced via an uncoupling of 5-HT receptors from their G-protein effectors (Okuhara and Beck, 1998). This uncoupling posits an alternative method by which 5-HT signalling may be altered in adulthood independent of transcriptional regulation. Interestingly, altered G-protein coupling may be related to RNA editing and the production of distinct receptor isoforms that differ in activity and activation potential (Herrick-Davis et al., 1999; Niswender et al., 1999; Wang et al., 2000). Altered 5-HT receptor editing has been associated with enhanced emotionality as stress-reactive strains of mice exhibit differences in basal rates of 5-HT receptor editing in the PFC and amygdala (Hackler et al., 2006). Significantly increased levels of 5-HT receptor editing were also found in the PFC of patients with a history of depression (Gurevich et al., 2002). With relevance to the current study, the 5HT2C receptor undergoes pre-RNA editing in response to early maternal separation (Bhansali et al., 2007). Therefore, similarities in overall 5-HT receptor expression in adult LPS-mice does not discount the possibility that the relative expression levels of different receptor isoforms may be present, thereby acting to modify 5-HT signalling in adulthood.

Conclusion

The early postnatal period is a time of intensive synaptic refinement (Sur and Leamey, 2001). Serotonin is believed to play a pivotal role in modulating synaptic activity in developing neural circuits and is responsible for refinement of synaptic connectivity during this time. The relative alterations in 5-HT gene expression in LPS-mice suggest a differential developmental trajectory of 5-HT governed neurocircuitry that has the potential to exert long-lasting effects. The accumulating evidence demonstrating that genetic (Gross et al., 2002) or pharmacological (Ansorge et al., 2004; Tsetsenis et al., 2007; Lo Iacono and Gross, 2008) modification of 5-HT during the second-third postnatal week is sufficient to induce anxiety-related behaviour in adulthood, suggests that transient changes in 5-HT signalling during development may underlie reported altered emotionality in adult LPS-rodents (Breivik et al., 2002; Walker et al., 2004a; Bilbo et al., 2007; Walker et al., 2008). The current study demonstrates changes to 5-HT-related gene expression both at forebrain post-synaptic sites and in the brain stem DR. This suggests that a neonatal immunogenic challenge is a powerful modifier of 5-HT signalling during early postnatal development.

4.5 References

Abrams JK, Johnson PL, Hollis JH, Lowry CA (2004) Anatomic and functional topography of the dorsal raphe nucleus. Ann N Y Acad Sci 1018:46-57.

Amat J, Baratta MV, Paul E, Bland ST, Watkins LR, Maier SF (2005) Medial prefrontal cortex determines how stressor controllability affects behavior and dorsal raphe nucleus. Nat Neurosci 8:365-371.

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 (New York, NY) 306:879-881.

Bach-Mizrachi H, Underwood MD, Tin A, Ellis SP, Mann JJ, Arango V (2008) Elevated expression of tryptophan hydroxylase-2 mRNA at the neuronal level in the dorsal and median raphe nuclei of depressed suicides. Mol Psychiatry 13:507-513, 465.

Bach-Mizrachi H, Underwood MD, Kassir SA, Bakalian MJ, Sibille E, Tamir H, Mann JJ, Arango V (2006) Neuronal tryptophan hydroxylase mRNA expression in the human dorsal and median raphe nuclei: major depression and suicide. Neuropsychopharmacology 31:814-824.

Banasr M, Soumier A, Hery M, Mocaer E, Daszuta A (2006) Agomelatine, a new antidepressant, induces regional changes in hippocampal neurogenesis. Biological psychiatry 59:1087-1096.

Bannerman DM, Grubb M, Deacon RM, Yee BK, Feldon J, Rawlins JN (2003) Ventral hippocampal lesions affect anxiety but not spatial learning. Behav Brain Res 139:197-213.

Barton DA, Esler MD, Dawood T, Lambert EA, Haikerwal D, Brenchley C, Socratous F, Hastings J, Guo L, Wiesner G, Kaye DM, Bayles R, Schlaich MP, Lambert GW (2008) Elevated brain serotonin turnover in patients with depression: effect of genotype and therapy. Arch Gen Psychiatry 65:38-46.

Beique JC, Chapin-Penick EM, Mladenovic L, Andrade R (2004) Serotonergic facilitation of synaptic activity in the developing rat prefrontal cortex. J Physiol 556:739-754.

Bhansali P, Dunning J, Singer SE, David L, Schmauss C (2007) Early life stress alters adult serotonin 2C receptor pre-mRNA editing and expression of the alpha subunit of the heterotrimeric G-protein G q. J Neurosci 27:1467-1473.

Bilbo SD, Rudy JW, Watkins LR, Maier SF (2006) A behavioural characterization of neonatal infection-facilitated memory impairment in adult rats. Behavioural brain research 169:39-47.

Bilbo SD, Levkoff LH, Mahoney JH, Watkins LR, Rudy JW, Maier SF (2005) Neonatal infection induces memory impairments following an immune challenge in adulthood. Behavioral neuroscience 119:293-301.

Bilbo SD, Yirmiya R, Amat J, Paul ED, Watkins LR, Maier SF (2007) Bacterial infection early in life protects against stressor-induced depressive-like symptoms in adult rats. Psychoneuroendocrinology 33:261-269.

Boisse L, Spencer SJ, Mouihate A, Vergnolle N, Pittman QJ (2005) Neonatal immune challenge alters nociception in the adult rat. Pain 119:133-141.

Breivik T, Stephan M, Brabant GE, Straub RH, Pabst R, von Horsten S (2002) Postnatal lipopolysaccharide-induced illness predisposes to periodontal disease in adulthood. Brain, behavior, and immunity 16:421-438.

Clark MS, McDevitt RA, Neumaier JF (2006) Quantitative mapping of tryptophan hydroxylase-2, 5-HT1A, 5-HT1B, and serotonin transporter expression across the anteroposterior axis of the rat dorsal and median raphe nuclei. J Comp Neurol 498:611-623.

Dent GW, Smith MA, Levine S (1999) The ontogeny of the neuroendocrine response to endotoxin. Brain researchDevelopmental brain research 117:21-29.

Esler M, Lambert E, Alvarenga M, Socratous F, Richards J, Barton D, Pier C, Brenchley C, Dawood T, Hastings J, Guo L, Haikerwal D, Kaye D, Jennings G, Kalff V, Kelly M, Wiesner G, Lambert G (2007) Increased brain serotonin turnover in panic disorder patients in the absence of a panic attack: reduction by a selective serotonin reuptake inhibitor. Stress 10:295-304.

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. Journal of neuroimmunology 131:83-91.

Galic MA, Riazi K, Heida JG, Mouihate A, Fournier NM, Spencer SJ, Kalynchuk LE, Teskey GC, Pittman QJ (2008) Postnatal inflammation increases seizure susceptibility in adult rats. The Journal of neuroscience : the official journal of the Society for Neuroscience 28:6904-6913.

Gardner KL, Thrivikraman KV, Lightman SL, Plotsky PM, Lowry CA (2005) Early life experience alters behavior during social defeat: focus on serotonergic systems. Neuroscience 136:181-191.

Gardner KL, Hale MW, Lightman SL, Plotsky PM, Lowry CA (2009a) Adverse early life experience and social stress during adulthood interact to increase serotonin transporter mRNA expression. Brain Res 1305:47-63.

Gardner KL, Hale MW, Oldfield S, Lightman SL, Plotsky PM, Lowry CA (2009b) Adverse experience during early life and adulthood interact to elevate tph2 mRNA expression in serotonergic neurons within the dorsal raphe nucleus. Neuroscience 163:991-1001.

Goodfellow NM, Benekareddy M, Vaidya VA, Lambe EK (2009) Layer II/III of the prefrontal cortex: Inhibition by the serotonin 5-HT1A receptor in development and stress. J Neurosci 29:10094-10103.

Gross C, Zhuang X, Stark K, Ramboz S, Oosting R, Kirby L, Santarelli L, Beck S, Hen R (2002) Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. Nature 416:396-400.

Gurevich I, Tamir H, Arango V, Dwork AJ, Mann JJ, Schmauss C (2002) Altered editing of serotonin 2C receptor pre-mRNA in the prefrontal cortex of depressed suicide victims. Neuron 34:349-356.

Hackler EA, Airey DC, Shannon CC, Sodhi MS, Sanders-Bush E (2006) 5-HT(2C) receptor RNA editing in the amygdala of C57BL/6J, DBA/2J, and BALB/cJ mice. Neurosci Res 55:96-104.

Herrick-Davis K, Grinde E, Niswender CM (1999) Serotonin 5-HT2C receptor RNA editing alters receptor basal activity: implications for serotonergic signal transduction. J Neurochem 73:1711-1717.

Hodgson DM, Knott B, Walker FR (2001) Neonatal endotoxin exposure influences HPA responsivity and impairs tumor immunity in Fischer 344 rats in adulthood. Pediatric research 50:750-755.

Hollis JH, Evans AK, Bruce KP, Lightman SL, Lowry CA (2006) Lipopolysaccharide has indomethacin-sensitive actions on Fos expression in topographically organized subpopulations of serotonergic neurons. Brain, behavior, and immunity 20:569-577.

Holmes A (2008) Genetic variation in cortico-amygdala serotonin function and risk for stress-related disease. Neuroscience and biobehavioral reviews 32:1293-1314.

Kohman RA, Tarr AJ, Sparkman NL, Bogale TM, Boehm GW (2008) Neonatal endotoxin exposure impairs avoidance learning and attenuates endotoxin-induced sickness behavior and central IL-1beta gene transcription in adulthood. Behavioural brain research 194:25-31.

Leonardo ED, Hen R (2008) Anxiety as a developmental disorder. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 33:134-140.

Linthorst AC, Reul JM (1998) Brain neurotransmission during peripheral inflammation. Ann N Y Acad Sci 840:139-152.

Linthorst AC, Flachskamm C, Muller-Preuss P, Holsboer F, Reul JM (1995) Effect of bacterial endotoxin and interleukin-1 beta on hippocampal serotonergic neurotransmission, behavioral activity, and free corticosterone levels: an in vivo microdialysis study. J Neurosci 15:2920-2934.

Lo Iacono L, Gross C (2008) Alpha-Ca2+/calmodulin-dependent protein kinase II contributes to the developmental programming of anxiety in serotonin receptor 1A knock-out mice. J Neurosci 28:6250-6257.

Lowry CA (2002) Functional subsets of serotonergic neurones: implications for control of the hypothalamic-pituitary-adrenal axis. J Neuroendocrinol 14:911-923.

Lowry CA, Johnson PL, Hay-Schmidt A, Mikkelsen J, Shekhar A (2005) Modulation of anxiety circuits by serotonergic systems. Stress 8:233-246.

Lowry CA, Evans AK, Gasser PJ, Hale MW, Staub DR, Shekhar A (2008) Topographical organization and chemoarchitecture of the dorsal raphe nucleus and the median raphe nucleus. In: Serotonin and sleep: molecular, functional and clinical aspects (Monti JM, Pandi-Perumal BL, Jacobs BL, Nutt DL, eds), pp 25–68. Birkhauser, Basel, Switzerland.

Lowry CA, Hollis JH, de Vries A, Pan B, Brunet LR, Hunt JR, Paton JF, van Kampen E, Knight DM, Evans AK, Rook GA, Lightman SL (2007) Identification of an immune-responsive mesolimbocortical serotonergic system: potential role in regulation of emotional behavior. Neuroscience 146:756-772.

Morilak DA, Ciaranello RD (1993) Ontogeny of 5-hydroxytryptamine2 receptor immunoreactivity in the developing rat brain. Neuroscience 55:869-880.

Nilsson C, Jennische E, Ho HP, Eriksson E, Bjorntorp P, Holmang A (2002) Postnatal endotoxin exposure results in increased insulin sensitivity and altered activity of neuroendocrine axes in adult female rats. European journal of endocrinology / European Federation of Endocrine Societies 146:251-260.

Niswender CM, Copeland SC, Herrick-Davis K, Emeson RB, Sanders-Bush E (1999) RNA editing of the human serotonin 5-hydroxytryptamine 2C receptor silences constitutive activity. J Biol Chem 274:9472-9478.

Okuhara DY, Beck SG (1998) Corticosteroids alter 5-hydroxytryptamine1A receptor-effector pathway in hippocampal subfield CA3 pyramidal cells. J Pharmacol Exp Ther 284:1227-1233.

Parks CL, Robinson PS, Sibille E, Shenk T, Toth M (1998) Increased anxiety of mice lacking the serotonin1A receptor. Proceedings of the National Academy of Sciences of the United States of America 95:10734-10739.

Patel TD, Zhou FC (2005) Ontogeny of 5-HT1A receptor expression in the developing hippocampus. Brain Res Dev Brain Res 157:42-57.

Peyron C, Petit JM, Rampon C, Jouvet M, Luppi PH (1998) Forebrain afferents to the rat dorsal raphe nucleus demonstrated by retrograde and anterograde tracing methods. Neuroscience 82:443-468.

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.

Roth BL, Hamblin MW, Ciaranello RD (1991) Developmental regulation of 5-HT2 and 5-HT1c mRNA and receptor levels. Brain Res Dev Brain Res 58:51-58.

Shanks N, Meaney MJ (1994) Hypothalamic-pituitary-adrenal activation following endotoxin administration in the developing rat: a CRH-mediated effect. Journal of neuroendocrinology 6:375-383.

Shanks N, Larocque S, Meaney MJ (1995) Neonatal endotoxin exposure alters the development of the hypothalamic-pituitary-adrenal axis: early illness and later responsivity to stress. The Journal of neuroscience : the official journal of the Society for Neuroscience 15:376-384.

Shanks N, Windle RJ, Perks PA, Harbuz MS, Jessop DS, Ingram CD, Lightman SL (2000) Early-life exposure to endotoxin alters hypothalamic-pituitary-adrenal function

and predisposition to inflammation. Proceedings of the National Academy of Sciences of the United States of America 97:5645-5650.

Snyder JS, Radik R, Wojtowicz JM, Cameron HA (2008) Anatomical gradients of adult neurogenesis and activity: Young neurons in the ventral dentate gyrus are activated by water maze training. Hippocampus 19:360-370.

Spencer SJ, Heida JG, Pittman QJ (2005) Early life immune challenge--effects on behavioural indices of adult rat fear and anxiety. Behavioural brain research 164:231-238.

Tenk CM, Kavaliers M, Ossenkopp KP (2008) Sexually dimorphic effects of neonatal immune system activation with lipopolysaccharide on the behavioural response to a homotypic adult immune challenge. International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience 26:331-338.

Tenk CM, Foley KA, Kavaliers M, Ossenkopp KP (2007) Neonatal immune system activation with lipopolysaccharide enhances behavioural sensitization to the dopamine agonist, quinpirole, in adult female but not male rats. Brain, behavior, and immunity 21:935-945.

Tsetsenis T, Ma XH, Lo Iacono L, Beck SG, Gross C (2007) Suppression of conditioning to ambiguous cues by pharmacogenetic inhibition of the dentate gyrus. Nat Neurosci 10:896-902.

Vazquez DM, Lopez JF, Van Hoers H, Watson SJ, Levine S (2000) Maternal deprivation regulates serotonin 1A and 2A receptors in the infant rat. Brain research 855:76-82.

Vazquez DM, Eskandari R, Zimmer CA, Levine S, Lopez JF (2002) Brain 5-HT receptor system in the stressed infant rat: implications for vulnerability to substance abuse. Psychoneuroendocrinology 27:245-272.

Walker AK, Nakamura T, Byrne RJ, Naicker S, Tynan RJ, Hunter M, Hodgson DM (2009) Neonatal lipopolysaccharide and adult stress exposure predisposes rats to anxiety-like behaviour and blunted corticosterone responses: Implications for the double-hit hypothesis. Psychoneuroendocrinology 34:1515-1525.

Walker FR, March J, Hodgson DM (2004a) Endotoxin exposure in early life alters the development of anxiety-like behaviour in the Fischer 344 rat. Behavioural brain research 154:63-69.

Walker FR, Knott B, Hodgson DM (2008) Neonatal endotoxin exposure modifies the acoustic startle response and circulating levels of corticosterone in the adult rat but only following acute stress. Journal of psychiatric research 42:1094-1103.

Walker FR, Brogan A, Smith R, Hodgson DM (2004b) A profile of the immediate endocrine, metabolic and behavioural responses following a dual exposure to endotoxin in early life. Physiology & behavior 83:495-504.

Wang Q, O'Brien PJ, Chen CX, Cho DS, Murray JM, Nishikura K (2000) Altered G protein-coupling functions of RNA editing isoform and splicing variant serotonin2C receptors. J Neurochem 74:1290-1300.

Yan W, Wilson CC, Haring JH (1997) 5-HT1a receptors mediate the neurotrophic effect of serotonin on developing dentate granule cells. Brain Res Dev Brain Res 98:185-190.

CHAPTER 5.

Altered adult hippocampal neurogenesis following early immune challenge in mice

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Abstract

Neonatal exposure to an immune challenge leads to long-term behavioural and molecular changes in stress-reactivity. Enhanced stress-reactivity is related to both depressive-like behaviour and deficits in hippocampal neurogenesis. Given this, we sought to measure depressive-like behaviour in the forced swim test (FST) concurrent with hippocampal cell proliferation and neurogenesis in adult mice neonatally exposed to lipopolysaccharide (LPS; 0.05mg/kg, i.p.) on postnatal days 3 and 5. Neurogenesis was measured separately in the ventral and dorsal hippocampus to account for the functional dissociation that exists along the dorsal-ventral hippocampal axis. Hippocampal cell proliferation was measured using the immunohistochemical marker BrdU and immature neuronal differentiation was assessed using doublecortin (DCX). Our findings reveal that both female and male LPS-mice took longer to engage in immobility, spent less time immobile, and exhibited more active behaviour during the FST, suggesting altered reactivity to swim stress. This was accompanied by an increase in DCX-positive cells in both the dorsal and ventral hippocampus that was specific to female LPS-mice. These findings demonstrate that exposure to an immune challenge during critical developmental time periods leads to long-term alterations in stress-reactivity that are accompanied by changes to adult hippocampal neurogenesis.

5.1. Introduction

Immunological challenge during specific developmental time periods leads to long-term stress-related behavioural and molecular changes. Neonatal exposure to lipopolysaccharide (LPS) results in alterations to stress-reactivity and behaviour in adult rodents (Bilbo et al., 2005a; Bilbo et al., 2005b; Bilbo et al., 2006; Boisse et al., 2004; Ellis et al., 2005; Nilsson et al., 2002; Shanks et al., 1995; Shanks et al., 2000; Spencer et al., 2005a; Spencer et al., 2005b; Spencer et al., 2006; Walker et al., 2004). Reactivity to restraint stress (Shanks et al., 1995), noise stress (Shanks et al., 2000) or the stress of a novel environment (Nilsson et al., 2002) is increased in adult rats exposed to LPS on postnatal days 3 and 5 (P3 and P5) as measured by the magnitude of the corticosterone and adrenocorticotropic hormone response. Reports of increased expression of corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) mRNAs in the median eminence (Shanks et al., 1995), increased expression of glucocorticoid receptor (GR) protein in the hypothalamus (Nilsson et al., 2002), and reduced GR receptor binding levels in the hypothalamus, hippocampus, and frontal cortex (Shanks et al., 1995), provide evidence that early immune challenge leads to long-term molecular changes in CNS stress circuitry. Behavioural changes have been reported, including reduced social interaction (Breivik et al., 2002), increased anxiety-like behaviour (Walker et al., 2004) and an increased locomotor response to both noise stress (Shanks et al., 2000) and novelty (Breivik et al., 2002; Nilsson et al., 2002). In experiments where LPS was delayed to P7, P14, or P21, basal differences in anxiety-like behaviour were no longer detected (Spencer

et al., 2005b; Spencer et al., 2006), suggesting that the timing of immune challenge is important to the impact on CNS development.

Increased stress-reactivity in adult rodents is related to increased depressive-like behaviour in the forced swim test (FST; (Rygula et al., 2005) and decreased cell proliferation in the subgranular zone of the dentate gyrus (Bjornebekk et al., 2005). As mice challenged early in life with LPS show basal differences in stress-reactivity and behaviour, we hypothesized that mice with neonatal LPS challenge would show increased depressive-like behaviour in the FST and decreased hippocampal neurogenesis. Neurogenesis was considered separately in the dorsal and ventral hippocampus given the functional dissociation that exists along the dorsal-ventral poles of the hippocampus (Moser and Moser, 1998; Sahay and Hen, 2007). Lesion studies, for instance, have demonstrated a preferential role of the dorsal hippocampus in spatial learning and memory (Moser et al., 1993), whereas the predominate role of the ventral sub-region appears to be regulation of emotionality (Bannerman et al., 2003; Kjelstrup et al., 2002). Indeed, the ventral aspect sends projections to limbic regions sub-serving emotionality and stress-related behaviours such as the amygdala, prefrontal cortex, and regions associated with the hypothalamic-pituitary-adrenal axis (Herman et al., 1995; Ishikawa and Nakamura, 2006; Verwer et al., 1997). Elucidating region-specific alterations to hippocampal neurogenesis in LPS-mice will offer insight into the differing susceptibility of these regions to early immune challenge and their possible contribution to behavioural dysfunction. Using an established model of early immune challenge, the experiments

described below examine the impact of early LPS challenge on depressive-like behaviour using the FST as well as on hippocampal neurogenesis in adult mice.

5.2. Methods

5.2.1. Animals. Forty mice (22 males, 18 females) of a heterogeneous strain (breeding colony mix of C57Bl/6, Swiss Webster, and DBA-2 strains) were used. These mice were derived from five litters bred in-house. Day of birth is denoted as postnatal day (P)0 with litters culled to 10 pups on P1. Mice were weaned at 21 days of age and separated based on sex into cages of 3-5. Animals were left undisturbed except for weekly weighing. Mice were maintained on a reverse 10 h:14 h light:dark cycle (lights on at 20:00 h) and housed with unlimited access to food and water. All procedures were approved by the McMaster University Animal Research Ethics Board and carried out in accordance with the guidelines described in the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care 1993).

5.2.2. LPS challenge. On P3 and P5, half of each litter received an intraperitoneal (i.p.) injection of either 0.05mg/kg LPS (E. coli; Sigma) in 50 µl/g or an equal volume of saline (SAL, 0.9%) between 07:00 and 08:00 h. Prepared solutions were aliquoted into working stock vials of 500µl and frozen at -20°C. Solutions were thawed and sterile filtered immediately prior to use. Dams were removed from the home cage prior to pup injections. Pups were weighed and injected individually, placed back into the home cage,

and the mother returned once all pups had received an injection. Maternal separation was no longer than 5 m.

5.2.3. Forced swim test. Depressive-like behaviour was assessed at 12 wk with a modified version of Porsolt's forced swim test using the validated Kinder Scientific automated FST system. This system has been validated for use with mice (Kurtuncu et al., 2005) and was further validated in our laboratory through significant correlation of the automated behavioural data with that obtained by videotape and analyzed by blinded trained observers. Testing was performed over two trials; a 10 m trial on day 1 followed by a 5 m trial 24 h later (day 2). Behavioural testing occurred during the animal's active period, between 08:00 and 11:00 h, under dim-lighting conditions. Mice were placed in a clear plexiglass cylinder (diameter = 24cm; height = 45cm) filled to a depth of 30 cm with water (temp 24-25^{o}C). The FST apparatus was interfaced to a PC laptop computer that provided automated scoring of both passive (immobility) and active (horizontal and vertical movements) behaviours using MotorMonitor software (KinderScientific). Time to float was defined as the first instance to an immobile posture such that no photobeams were broken within a three second time period. Automated data obtained using this definition correlated highest with observer-derived measurements of immobility during validation of the FST system in our laboratory. Behaviour was analyzed during the first 6 minutes on day 1 and for the full 5 minutes on day 2.

5.2.4. BrdU preparation and injection. Bromodeoxyuridine (BrdU) was used to label dividing neurons in the adult mouse CNS (Miller and Nowakowski, 1988). A sub-set of

mice was chosen (n=24) for immunohistochemical analysis and BrdU was administered four days after completion of day 2 of the FST (~12 wk). BrdU (50μg/g body weight, Sigma) was dissolved in 0.9% NaCl to a final concentration of 10mg/ml and sterile filtered prior to use. Given that the adult mouse s-phase of the cell cycle has been calculated to be ~6h (Burns and Kuan 2005), three pulse i.p. injections of BrdU were administered, separated by two hour intervals (6 h total). Mice were deeply anaesthetized with sodium pentobarbital two hours after the final BrdU injection. Animals were transcardially perfused with 1X phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PF) in PBS. Brains were collected and post-fixed in 4% PF at room temperature for 4 hours then transferred to fresh 1XPBS for immunohistochemical staining. To control for the possible impact of behavioural testing on neurogenesis, a randomly chosen sub-set of mice that received BrdU did not undergo the FST (n=6). These mice had no significant difference in cell proliferation or neurogenesis and so were included in the final immunohistochemical dataset.

5.2.5. Immunohistochemistry. Immunohistochemical staining for both BrdU and doublecortin (DCX) was performed by Neuroscience Associates (www.NSAlabs.com). Brains were sectioned sagittally at 40μm and 1 in 6 adjacent sections were processed for BrdU or DCX according to NSA's standard protocols. Briefly, free-floating sections were incubated overnight at room temperature with either sheep anti-BrdU (1:10,000; Novus Biologicals) or goat anti-DCX (1:30,000; Santa Cruz Biomedical). Following rinses in PBS, the appropriate biotinylated secondary was applied: Vecta Elite anti-sheep

or anti-goat IgG, respectively (Vector Labs). Sections were washed and treated with an avidin-biotin complex (Vectastain, ABC kit, Vector Labs). For visualization, DCX sections were treated with diaminobenzidine tetrahydrochloride (DAB) and BrdU sections with DAB-nickel. Sections were mounted on gelatinized glass slides and coverslipped.

5.2.6. Cell quantification. BrdU+ and DCX+ cell counts were estimated independently by two blinded observers by sampling every sixth section in a series along the entire extent of the lateromedial axis of the hippocampus. BrdU+ cells were optically counted in the subgranular zone (SGZ), granular cell layer (GCL) and hilus (HL) of the dentate gyrus (DG) at 40X on a light microscope (Zeiss Axiophot 2). The SGZ was defined as one cell width from the bottom demarcation of the GCL. DCX+ cells were counted in the GCL of the DG on captured 25X images. Labelled DCX and BrdU cells occurring in the upper most focal plane were not included in the counts. Total BrdU+ and DCX+ cell count estimates were obtained by multiplying the total number of positive cells counted in a series by the inverse of the sectioning sample fraction (i.e. 1/6), to yield "cells per dentate gyrus". Labelled cells in the dorsal and ventral DG were counted separately to determine potential regional differences in proliferative rate and immature neuronal formation. As sections were taken parallel to the dorsal-ventral axis along the sagittal plane, the dorsal and ventral regions were easily discernable and regional cell counts began after the natural separation of the dorsal and ventral DG. On average, 18-20 dorsal hippocampal sections were counted per animal, of these, 3-5 sections included the ventral hippocampus.

5.2.7. Statistics and data analysis. Body weight data were analyzed using a two-way repeated measures analysis of variance (ANOVA) with time and treatment as main factors. Forced swim test data were analyzed using repeated measures ANOVA with sex (female/male) and treatment (LPS/SAL) as between subjects variables and time (Day) as a within subjects factor. Two-way repeated ANOVAs were performed where appropriate to further specify group differences. Analyses of the neurogenesis data were performed using a two-way ANOVA with sex and treatment as main factors. The Bonferroni test was used for post-hoc analyses. Statistics were performed using SPSS and GraphPad Prism version 4.0a software for Macintosh. A two-tailed p-value less than 0.05 was considered statistically significant. For the FST data, three LPS and one SAL treated animal had to be excluded from analysis due to hardware malfunction. One SAL mouse was removed from ventral BrdU immunohistochemical analysis due to sub-optimal staining. The ventral hippocampus was torn in DCX sections for five SAL and two LPS mice and could not be accurately counted. Data are presented as mean +/- SEM.

5.3. Results

5.3.1. No impact of LPS on postnatal growth and development

Animals were weighed during the pre- and post-weaning period to assess the impact of treatment on growth and development. There was no significant effect of treatment on body mass during the pre- ($F_{1,8}$=0.24, p=0.639; Fig. 1A) or post-weaning period ($F_{1,38}$=0.005, p=0.944; Fig. 1B).

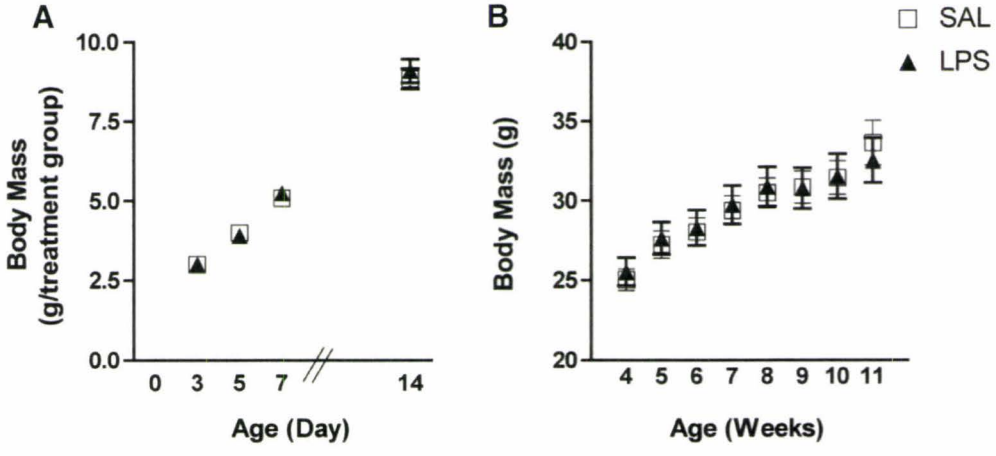
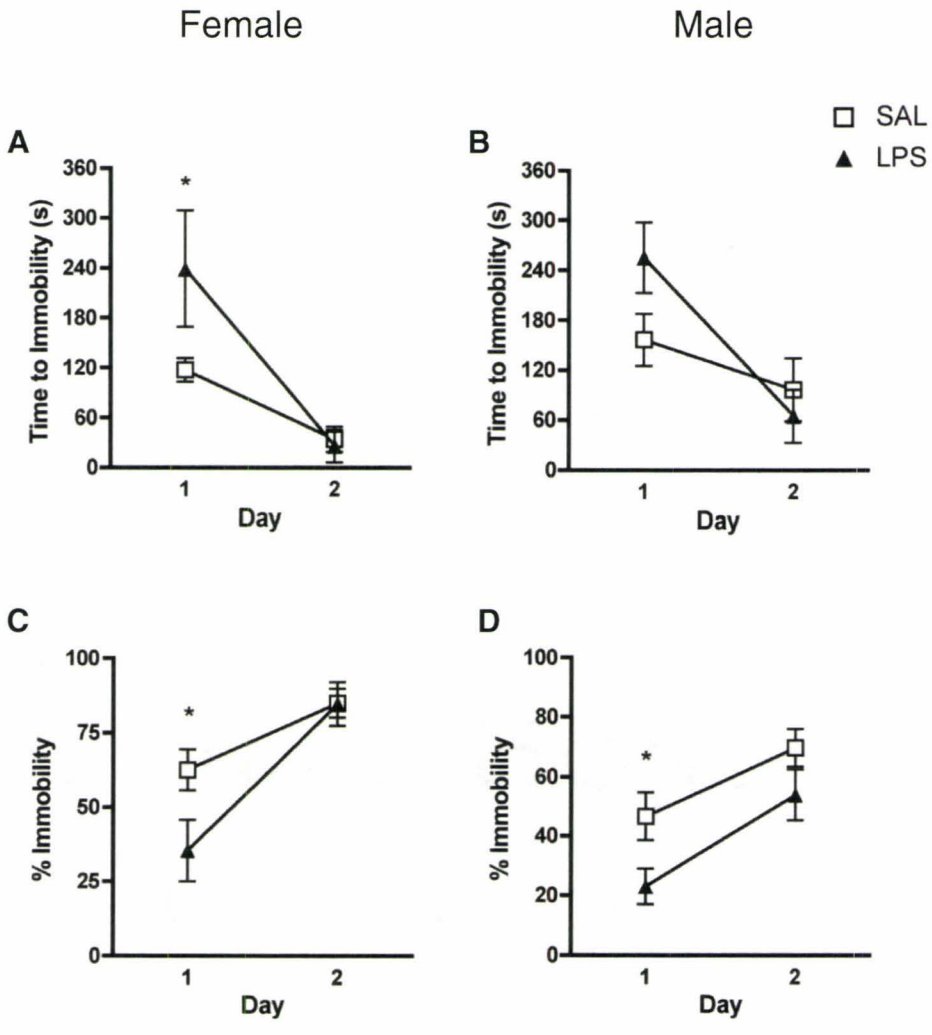


Figure 1. LPS injections on postnatal day 3 and 5 had no effect on body mass during either the (**A**) pre- or (**B**) post- weaning period. Pre-weaning weights are expressed as mean body mass per treatment group (5 litters/treatment). Data are plotted as mean +/- SEM. Post-weaning: n=20 pups/treatment.

5.3.2. Early LPS challenge alters forced swim test performance

Postnatal exposure to LPS had a significant long-term impact on passive (immobility) and active (horizontal and vertical movements) behaviours in adult LPS-mice relative to SAL controls. There was a significant effect of day ($F_{1,26}$=22.68, p<0.001) and a treatment x day interaction ($F_{1,26}$=5.14, p=0.03; Fig. 2A) on time to first immobility, with further analysis indicating that female LPS-mice took longer to engage in immobile behaviour than SAL controls on day 1 of the test (p<0.05). On day 2, female LPS-mice took less time to engage in immobility relative to their day 1 levels (p<0.001; Fig. 2A), such that no difference was detected between LPS and SAL females on day 2 (p>0.05). Male LPS-mice exhibited a similar pattern of behaviour on day 1 and 2 of the FST, although differences between groups of male animals were not statistically significant (Fig. 2B).

There was a significant effect of day ($F_{1,26}$=21.54, p<0.001), treatment ($F_{1,26}$=7.38, p<0.012), and a significant day x treatment interaction ($F_{1,26}$=4.48, p=0.044) on total time spent immobile. Male and female LPS-mice spent less time immobile on day 1 compared with SAL controls (p<0.05). With repeated exposure to the FST, both female and male LPS-mice showed an increase in the percentage of time spent immobile relative to day 1 (p<0.05; Fig. 2C,D). As a result, LPS and SAL-mice exhibited similar levels of immobility on day 2 of the FST. There was also a significant effect of sex ($F_{1,26}$=7.84, p=0.01) such that female mice, regardless of treatment condition, spent more time


Female
Male
SAL
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A
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C
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Time to Immobility (s)
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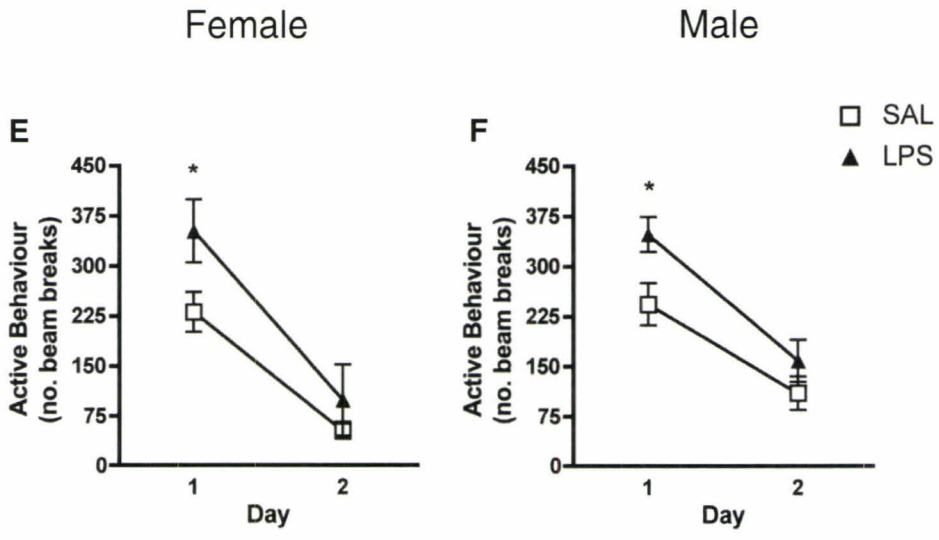

Figure 2. SAL and LPS-mice were exposed to the forced swim test (FST) at 12 weeks of age. Day 1 of the FST consisted of a 10 min session followed by a 5 min session 24 h later on day 2. Time to first immobility (A,B), % time spent immobile (C,D), and horizontal and vertical movements (E,F) were measured on day 1 and 2 in female and male mice. (**A**) Female LPS-mice took significantly longer to become immobile on day 1 of the FST compared with controls. On day 2, female LPS-mice took significantly less time to become immobile relative to day 1, diminishing the difference between treatment groups on day 2 of testing. (**B**) Male LPS-mice displayed a similar behavioural profile to female LPS-mice on day 1 and 2 of the FST. (**C**) Both female and (**D**) male LPS-mice spent significantly less time immobile compared with controls on day 1 of the FST; there was no difference in immobility time between groups on day 2. (**E**) Female and (**F**) male LPS-mice showed a greater amount of activity during day 1 of the FST. There was no difference in active behaviours between treatment groups on day 2 of testing. % time spent immobile and horizontal and vertical movements are depicted for the first 6 min on day 1 and for 5 min on day 2. Data are presented as mean +/- SEM. SAL n=17; LPS, n=13. *$p<0.05$.

immobile during the FST. The greater time to first immobility and less time spent immobile throughout the duration of the test on day 1 suggest that LPS-mice exhibited less passive behaviour in response to acute swim stress.

There was a significant impact of treatment ($F_{1,26}$=12.77, p=0.001) on active behaviour reflecting an increase in overall total horizontal and vertical movements in both female and male LPS-mice compared with SAL controls (Fig. 2E, F).

5.3.3. Early LPS challenge does not alter adult hippocampal cell proliferation

To obtain an estimate of hippocampal progenitor cell proliferation, BrdU-labelled cells were quantified 2 hrs after 3 pulse injections of BrdU were administered. The majority of BrdU-labelled cells were found in clusters of 3-5 within the SGZ of animals from both groups (Fig. 3A). Absolute numbers of BrdU-positive cells were determined along the extent of the dorsal and ventral DG separately and within the subgranular zone, granular cell layer, and hilar sub-regions of each. Analyses revealed no effect on the number of BrdU-positive cells in any region of the dorsal or ventral DG (p's >0.05; Fig. 3B, C).

5.3.4. Early LPS challenge alters adult hippocampal neurogenesis

Doublecortin (DCX)-labelled cells were quantified as a measure of immature neuronal differentiation. DCX-positive cells were predominately found with their soma situated within the SGZ with dendrites extending up through the granule layer towards or

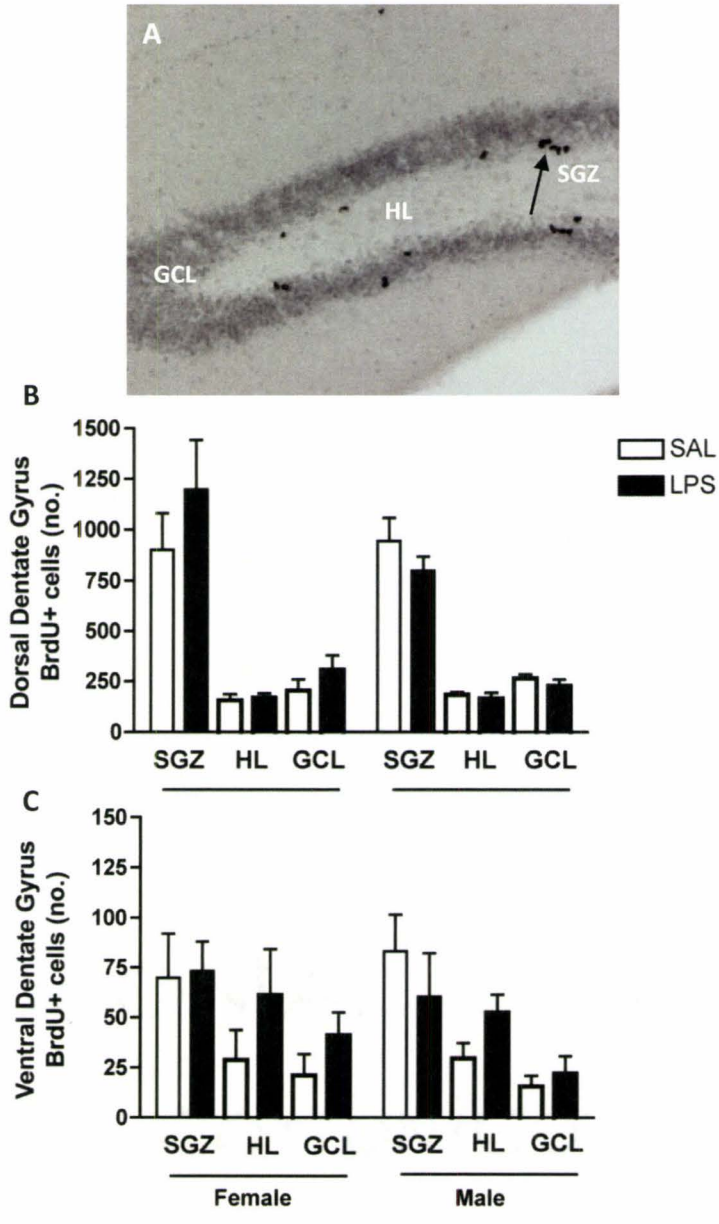

Figure 3. Adult hippocampal cell proliferation in mice exposed neonatally to SAL or LPS. (**A**) Representative image of BrdU immunohistochemistry in the dentate gyrus (DG). Arrow points to representative cluster of BrdU+ cells stained with nickel-DAB. Total BrdU+ cells per DG were counted in the dorsal (B) and ventral (C) hippocampus of male and female mice. (**B,C**) There was no significant difference between treatment groups in the number of BrdU+ cells in any region of the dorsal or ventral DG. SGZ=subgranular zone; HL=hilus; GCL=granule cell layer. Data are plotted as mean +/- SEM. Dorsal DG: SAL n=12; LPS n=12. Ventral DG: SAL n=11; LPS n=12.

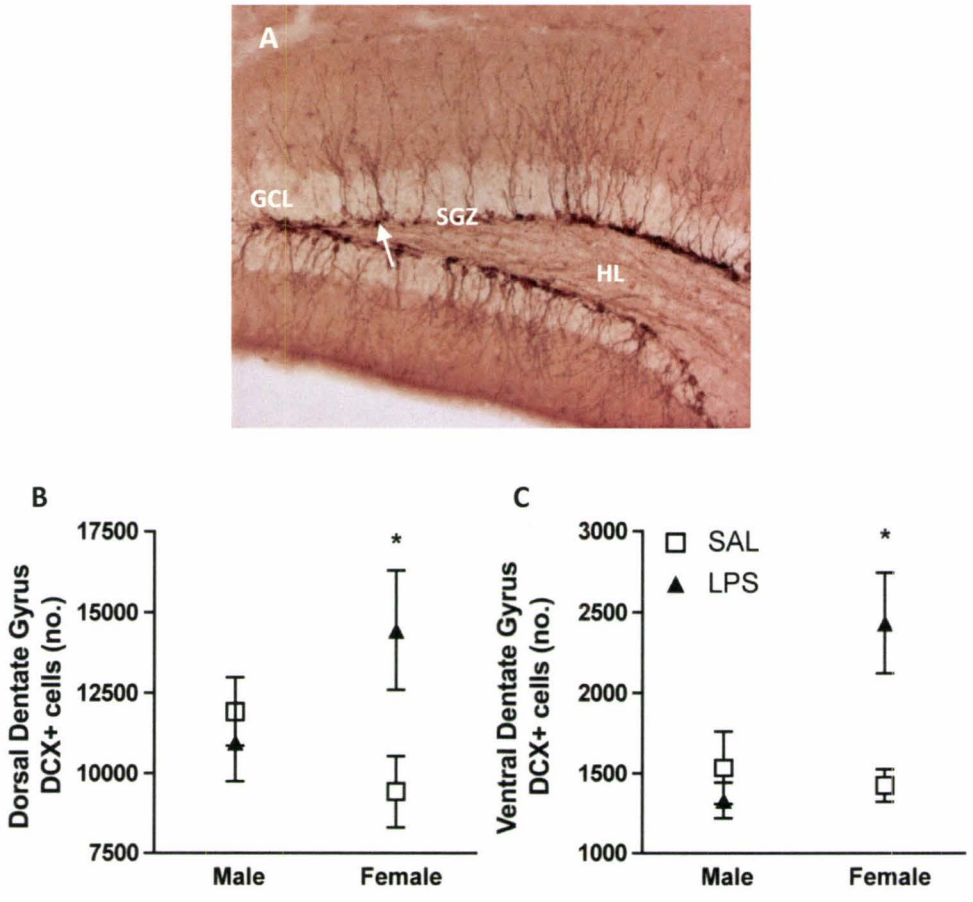

Figure 4. Hippocampal sections of adult SAL and LPS mice were stained with antibodies to DCX for assessment of neurogenesis. DCX+ cells were counted in the dorsal and ventral dentate gyrus (DG) and are expressed as total cells/DG. (**A**) Representative image of DCX immunoreactivity in the DG. Arrow points to representative DCX+ cell. (**B**) Female LPS-mice had a significantly greater number of DCX+ cells in the dorsal DG compared with female controls. (**C**) In the ventral dentate gyrus, female-LPS mice had a significantly greater number of DCX+ cells compared with both female controls and LPS-males. SGZ=subgranular zone; HL=hilus; GCL=granule cell layer. Data are plotted as mean +/- SEM. Dorsal DG: SAL n=12 (6/sex); LPS n=12 (7 male, 5 female). Ventral DG: SAL n=7 (3 male, 4 female); LPS n=10 (5/sex). *$p<0.05$.

terminating within the molecular layer in animals from both groups (Fig. 4A). There was a significant sex x treatment interaction in the number of DCX-positive cells measured in the dorsal DG ($F_{1,20}$=5.25, p=0.033; Fig. 4B). Female LPS-mice had a greater number of DCX-positive cells in the dorsal DG compared to female SAL controls ($p<0.05$). There was also a significant sex x treatment interaction ($F_{1,20}$=7.39, p=0.018; Fig. 4C) and a significant sex effect ($F_{1,20}$=4.94, p=0.045) on the number of DCX-labelled cells in the ventral DG; female LPS-mice had an increased number of DCX-positive cells in the ventral DG relative to both female controls ($p<0.05$) and LPS-males ($p<0.01$).

5.4. Discussion

The present study examined the long-term impact of an early immune challenge on adult depressive-like behaviour, as measured using the FST, concurrent with alterations to hippocampal cell proliferation and new neuron formation. Early exposure to LPS had a long-term impact on FST performance that was associated with sex-specific alterations to hippocampal neurogenesis along the dorsal-ventral axis.

Mice exposed to LPS early in life exhibited more active behaviour in contrast to control animals after acute exposure to the FST – a phenotype similar to that observed when animals are pre-treated with antidepressant medication. This behaviour is consistent with a recent report in rats that were exposed to *E. Coli* early in life and protected against the depressive symptoms induced by inescapable tail shock when administered in adulthood (Bilbo et al., 2008). Although it is intriguing to suggest that

exposure to an immune stimulus early in life confers resilience to stressful conditions later in adulthood, the behaviour observed here in the FST might also reflect other behavioural phenotypes. For instance, mice with altered serotonergic signalling, such as the 5HT1A receptor knockout mouse, display antidepressive-like behaviour in the FST but an increased anxiety and stress response (Parks et al., 1998; Toth, 2003). Thus, LPS mice might have enhanced reactivity to swim stress rather than a true antidepressant response. Such an interpretation is supported by the finding that injections of LPS given during the same critical time windows as employed in this study (single injection on P3 and on P5) lead to behavioural hyperactivity in response to noise stress (Shanks et al., 2000) and to the stress of a novel environment (Breivik et al., 2002; Nilsson et al., 2002). As stress-induced hyperactivity can be a confounding factor in rodent models of depression (Strekalova et al., 2005), the FST results in the current study may be indicative of altered reactivity to an acute stressor, with a tendency for animals to increase activity when presented with a novel environment. Notably, similar behavioural patterns in the FST have been observed in both BDNF conditional knockout mice and TrkB knockout mice (Chan et al., 2006; Zorner et al., 2003). Taken together, these results extend previous findings that demonstrate long-term alterations to stress-reactivity in early endotoxin exposed rodents.

LPS-females had an increase in dorsal and ventral hippocampal neurogenesis as measured using the immature neuronal marker, doublecortin (DCX). Increased neurogenesis does account for the mood elevating effects of some (Airan et al., 2007; Santarelli et al., 2003), but not all (Holick et al., 2008; Meshi et al., 2006), antidepressant

treatments. Neurogenesis, however, is also associated with pathological states such as temporal lobe epilepsy and megencephaly (Almgren et al., 2007; Parent and Lowenstein, 1997), underscoring the notion that more is not always better (Scharfman and Hen, 2007). Similarly, less is not always bad, as performance in select hippocampal-dependant learning tasks, such as in the radial maze, may improve after interventions that suppress hippocampal neurogenesis (Saxe et al., 2007). These studies illustrate the complex relationship between neurogenesis and hippocampal function.

The functional dissociation that exists along the ventral-dorsal poles of the hippocampus suggests that alterations to neurogenesis within these two regions may confer different behavioural consequences. Altered ventral hippocampal neurogenesis in female LPS-mice may be associated with the reputed increase in stress-reactivity observed during the FST. Lesion studies have demonstrated a direct association between ventral hippocampal function and emotionality as lesions of the ventral hippocampus decrease anxiety-like behaviour in a variety of behavioural tests, including the elevated plus maze (Bannerman et al., 2004; Degroot and Treit, 2004). This evidence combined with the recent finding that newborn neurons in the ventral dentate gyrus may contribute to the expression of altered emotionality (Snyder et al., 2008), suggests that newborn neurons in the ventral hippocampus of female LPS-mice may be associated with enhanced stress-reactivity. The caveat here is that both female and male LPS-mice exhibited a similar behavioural profile in response to swim stress, but increased neurogenesis was specific to female LPS-mice. If neurogenesis were indeed linked to the

FST behavioural profile, one might expect altered neurogenesis in both sexes. The finding that only female-LPS mice exhibit increased neurogenesis could suggest that altered neurogenesis does not drive the current behavioural phenotype. Alternatively, it could suggest that different etiopathological mechanisms underlie a similar behavioural readout between the sexes. In this regard, the FST behaviour of LPS-males may be independent of changes to neurogenesis. Regardless, a future avenue of research should explore sex-specific behavioural differences that may be relevant to the current reported sex differences in hippocampal neurogenesis. These findings highlight the importance of examining behavioural and molecular phenomena independently in female and male rodents and add to the accumulating evidence of sex-specific behavioural and molecular alterations in rodents exposed to an early immune challenge (Hodgson and Knott, 2002; Shanks et al., 1994; Tenk et al., 2007; Tenk et al., 2008).

Increased neurogenesis in female LPS-mice occurred independent of increases to cell proliferation in the subgranular zone as measured using BrdU. The neurogenic cascade proceeds from the proliferation of precursor cells in the subgranular zone through to differentiation and cell survival. Neurogenesis is tightly regulated by a homeostatic balance between the production of new neurons and cell death – a process that is important for maintaining proper size and function of the hippocampus. To have an increased number of immature neurons without the preceding increase in proliferating neuronal precursors suggests alterations to one or more steps in the neurogenic cascade. For instance, this could reflect either enhanced cell survival of proliferating cells or

inadequate apoptosis. Future studies will assess subsequent steps in the cascade to determine possible mechanisms underlying the observed increase in the immature neuronal population. This will also include assessing long-term survival of young neurons.

Our finding of enhanced stress-reactivity extends previous findings with the early immune challenge model. This study is novel in that it is the first to examine the long-term impact of early immune challenge on adult hippocampal neurogenesis and to report enhanced neurogenesis in adult female LPS-mice. Our findings are in agreement with the growing body of literature demonstrating the different susceptibility of the sexes to early environmental challenge. The observed sex difference in this study, with respect to new neuron formation, suggests that further studies using the early immune challenge model include and account for potential sex differences. The present study highlights the importance of the early immune environment to the developing CNS and demonstrates that early immune perturbation has long-term consequences to behavioural stress-reactivity and hippocampal neurogenesis.

5.5. References

Airan, R.D., Meltzer, L.A., Roy, M., Gong, Y., Chen, H., Deisseroth, K., 2007. High-speed imaging reveals neurophysiological links to behavior in an animal model of depression. Science 317, 819-823.

Almgren, M., Persson, A.S., Fenghua, C., Witgen, B.M., Schalling, M., Nyengaard, J.R., Lavebratt, C., 2007. Lack of potassium channel induces proliferation and survival causing increased neurogenesis and two-fold hippocampus enlargement. Hippocampus 17, 292-304.

Bannerman DM, Grubb M, Deacon RM, Yee BK, Feldon J, Rawlins JN (2003) Ventral hippocampal lesions affect anxiety but not spatial learning. Behav Brain Res 139:197-213.

Bannerman, D.M., Matthews, P., Deacon, R.M., Rawlins, J.N., 2004. Medial septal lesions mimic effects of both selective dorsal and ventral hippocampal lesions. Behav Neurosci 118, 1033-1041.

Bilbo, S.D., Biedenkapp, J.C., Der-Avakian, A., Watkins, L.R., Rudy, J.W., Maier, S.F., 2005a. Neonatal infection-induced memory impairment after lipopolysaccharide in adulthood is prevented via caspase-1 inhibition. J Neurosci 25, 8000-8009.

Bilbo, S.D., Levkoff, L.H., Mahoney, J.H., Watkins, L.R., Rudy, J.W., Maier, S.F., 2005b. Neonatal infection induces memory impairments following an immune challenge in adulthood. Behav Neurosci 119, 293-301.

Bilbo, S.D., Rudy, J.W., Watkins, L.R., Maier, S.F., 2006. A behavioural characterization of neonatal infection-facilitated memory impairment in adult rats. Behav Brain Res 169, 39-47.

Bilbo, S.D., Yirmiya, R., Amat, J., Paul, E.D., Watkins, L.R., Maier, S.F., 2008. Bacterial infection early in life protects against stressor-induced depressive-like symptoms in adult rats. Psychoneuroendocrinology 33, 261-269.

Bjornebekk, A., Mathe, A.A., Brene, S., 2005. The antidepressant effect of running is associated with increased hippocampal cell proliferation. Int J Neuropsychopharmacol 8, 357-368.

Boisse, L., Mouihate, A., Ellis, S., Pittman, Q.J., 2004. Long-term alterations in neuroimmune responses after neonatal exposure to lipopolysaccharide. J Neurosci 24, 4928-4934.

Bondolfi, L., Ermini, F., Long, J.M., Ingram, D.K., Jucker, M., 2004. Impact of age and caloric restriction on neurogenesis in the dentate gyrus of C57BL/6 mice. Neurobiol Aging 25, 333-340.

Breivik, T., Stephan, M., Brabant, G.E., Straub, R.H., Pabst, R., von Horsten, S., 2002. Postnatal lipopolysaccharide-induced illness predisposes to periodontal disease in adulthood. Brain Behav Immun 16, 421-438.

Chan, J.P., Unger, T.J., Byrnes, J., Rios, M., 2006. Examination of behavioral deficits triggered by targeting Bdnf in fetal or postnatal brains of mice. Neuroscience 142, 49-58.

Degroot, A., Treit, D., 2004. Anxiety is functionally segregated within the septo-hippocampal system. Brain Res 1001, 60-71.

Ellis, S., Mouihate, A., Pittman, Q.J., 2005. Early life immune challenge alters innate immune responses to lipopolysaccharide: implications for host defense as adults. Faseb J 19, 1519-1521.

Herman, J.P., Cullinan, W.E., Morano, M.I., Akil, H., Watson, S.J., 1995. Contribution of the ventral subiculum to inhibitory regulation of the hypothalamo-pituitary-adrenocortical axis. J Neuroendocrinol 7, 475-482.

Hodgson, D.M., Knott, B., 2002. Potentiation of tumor metastasis in adulthood by neonatal endotoxin exposure: sex differences. Psychoneuroendocrinology 27, 791-804.

Holick, K.A., Lee, D.C., Hen, R., Dulawa, S.C., 2008. Behavioral effects of chronic fluoxetine in BALB/cJ mice do not require adult hippocampal neurogenesis or the serotonin 1A receptor. Neuropsychopharmacology 33, 406-417.

Ishikawa, A., Nakamura, S., 2006. Ventral hippocampal neurons project axons simultaneously to the medial prefrontal cortex and amygdala in the rat. J Neurophysiol 96, 2134-2138.

Kjelstrup KG, Tuvnes FA, Steffenach HA, Murison R, Moser EI, Moser MB (2002) Reduced fear expression after lesions of the ventral hippocampus. Proceedings of the National Academy of Sciences of the United States of America 99:10825-10830.

Kurtuncu, M., Luka, L.J., Dimitrijevic, N., Uz, T., Manev, H., 2005. Reliability assessment of an automated forced swim test device using two mouse strains. J Neurosci Methods 149, 26-30.

Meshi, D., Drew, M.R., Saxe, M., Ansorge, M.S., David, D., Santarelli, L., Malapani, C., Moore, H., Hen, R., 2006. Hippocampal neurogenesis is not required for behavioral effects of environmental enrichment. Nat Neurosci 9, 729-731.

Miller, M.W., Nowakowski, R.S., 1988. Use of bromodeoxyuridine-immunohistochemistry to examine the proliferation, migration and time of origin of cells in the central nervous system. Brain Res 457, 44-52.

Moser, M.B., Moser, E.I., 1998. Functional differentiation in the hippocampus. Hippocampus 8, 608-619.

Moser E, Moser MB, Andersen P (1993) Spatial learning impairment parallels the magnitude of dorsal hippocampal lesions, but is hardly present following ventral lesions. The Journal of neuroscience : the official journal of the Society for Neuroscience 13:3916-3925.

Nilsson, C., Jennische, E., Ho, H.P., Eriksson, E., Bjorntorp, P., Holmang, A., 2002. Postnatal endotoxin exposure results in increased insulin sensitivity and altered activity of neuroendocrine axes in adult female rats. Eur J Endocrinol 146, 251-260.

Parent, J.M., Lowenstein, D.H., 1997. Mossy fiber reorganization in the epileptic hippocampus. Curr Opin Neurol 10, 103-109.

Parks, C.L., Robinson, P.S., Sibille, E., Shenk, T., Toth, M., 1998. Increased anxiety of mice lacking the serotonin1A receptor. Proc Natl Acad Sci U S A 95, 10734-10739.

Petersen, A., Wortwein, G., Gruber, S.H., Mathe, A.A., 2008. Escitalopram reduces increased hippocampal cytogenesis in a genetic rat depression model. Neurosci Lett 436, 305-308.

Rygula, R., Abumaria, N., Flugge, G., Fuchs, E., Ruther, E., Havemann-Reinecke, U., 2005. Anhedonia and motivational deficits in rats: impact of chronic social stress. Behav Brain Res 162, 127-134.

Sahay, A., Hen, R., 2007. Adult hippocampal neurogenesis in depression. Nat Neurosci 10, 1110-1115.

Santarelli, L., Saxe, M., Gross, C., Surget, A., Battaglia, F., Dulawa, S., Weisstaub, N., Lee, J., Duman, R., Arancio, O., Belzung, C., Hen, R., 2003. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science 301, 805-809.

Saxe, M.D., Malleret, G., Vronskaya, S., Mendez, I., Garcia, A.D., Sofroniew, M.V., Kandel, E.R., Hen, R., 2007. Paradoxical influence of hippocampal neurogenesis on working memory. Proc Natl Acad Sci U S A 104, 4642-4646.

Scharfman, H.E., Hen, R., 2007. Neuroscience. Is more neurogenesis always better? Science 315, 336-338.

Seri, B., Garcia-Verdugo, J.M., McEwen, B.S., Alvarez-Buylla, A., 2001. Astrocytes give rise to new neurons in the adult mammalian hippocampus. J Neurosci 21, 7153-7160.

Shanks, N., Larocque, S., Meaney, M.J., 1995. Neonatal endotoxin exposure alters the development of the hypothalamic-pituitary-adrenal axis: early illness and later responsivity to stress. J Neurosci 15, 376-384.

Shanks, N., McCormick, C.M., Meaney, M.J., 1994. Sex differences in hypothalamic-pituitary-adrenal responding to endotoxin challenge in the neonate: reversal by gonadectomy. Brain Res Dev Brain Res 79, 260-266.

Shanks, N., Windle, R.J., Perks, P.A., Harbuz, M.S., Jessop, D.S., Ingram, C.D., Lightman, S.L., 2000. Early-life exposure to endotoxin alters hypothalamic-pituitary-adrenal function and predisposition to inflammation. Proc Natl Acad Sci USA 97, 5645-5650.

Snyder, J.S., Radik, R., Wojtowicz, J.M., Cameron, H.A., 2008. Anatomical gradients of adult neurogenesis and activity: Young neurons in the ventral dentate gyrus are activated by water maze training. Hippocampus 19, 360-370.

Spencer, S.J., Auer, R.N., Pittman, Q.J., 2005a. Rat neonatal immune challenge alters adult responses to cerebral ischaemia. J Cereb Blood Flow Metab 26, 456-467.

Spencer, S.J., Heida, J.G., Pittman, Q.J., 2005b. Early life immune challenge--effects on behavioural indices of adult rat fear and anxiety. Behav Brain Res 164, 231-238.

Spencer, S.J., Martin, S., Mouihate, A., Pittman, Q.J., 2006. Early-Life Immune Challenge: Defining a Critical Window for Effects on Adult Responses to Immune Challenge. Neuropsychopharmacology 31, 1910-1918.

Steiner, B., Kronenberg, G., Jessberger, S., Brandt, M.D., Reuter, K., Kempermann, G., 2004. Differential regulation of gliogenesis in the context of adult hippocampal neurogenesis in mice. Glia 46, 41-52.

Strekalova, T., Spanagel, R., Dolgov, O., Bartsch, D., 2005. Stress-induced hyperlocomotion as a confounding factor in anxiety and depression models in mice. Behav Pharmacol 16, 171-180.

Tenk, C.M., Foley, K.A., Kavaliers, M., Ossenkopp, K.P., 2007. Neonatal immune system activation with lipopolysaccharide enhances behavioural sensitization to the dopamine agonist, quinpirole, in adult female but not male rats. Brain Behavior and Immunity 21, 935-945.

Tenk, C.M., Kavaliers, M., Ossenkopp, K.P., 2008. Sexually dimorphic effects of neonatal immune system activation with lipopolysaccharide on the behavioural response to a homotypic adult immune challenge. International Journal of Developmental Neuroscience 26, 331-338.

Toth, M., 2003. 5-HT1A receptor knockout mouse as a genetic model of anxiety. Eur J Pharmacol 463, 177-184.

Verwer, R.W., Meijer, R.J., Van Uum, H.F., Witter, M.P., 1997. Collateral projections from the rat hippocampal formation to the lateral and medial prefrontal cortex. Hippocampus 7, 397-402.

Walker, F.R., March, J., Hodgson, D.M., 2004. Endotoxin exposure in early life alters the development of anxiety-like behaviour in the Fischer 344 rat. Behav Brain Res 154, 63-69.

Zorner, B., Wolfer, D.P., Brandis, D., Kretz, O., Zacher, C., Madani, R., Grunwald, I., Lipp, H.P., Klein, R., Henn, F.A., Gass, P., 2003. Forebrain-specific trkB-receptor knockout mice: behaviorally more hyperactive than "depressive". Biol Psychiatry 54, 972-982.

CHAPTER 6. DISCUSSION

6.1. Summary of findings

The present body of work examined the long-term impact of a neonatal immunogenic challenge on stress-related behavioural and molecular phenotypes during both early postnatal development and adulthood. Alterations to serotonin (5-HT) neurocircuitry during postnatal development were assessed in parallel with the emergence of anxiety-related behaviours in mice exposed to an early LPS challenge. In adulthood, mice were assessed for anxiety- and depressive-related behaviours concurrent with alterations to adult hippocampal neurogenesis.

Results demonstrate that a neonatal LPS challenge alters emotionality in a sex- and temporal-specific manner. Male LPS-mice exhibited an early and persistent behavioural phenotype consistent with enhanced anxiety-related behaviour, whereas female LPS-mice exhibited late-onset emotionality. The resultant antidepressant phenotype observed in adulthood was similar between the sexes. Transient changes to 5-HT signalling during early postnatal development and altered adult hippocampal neurogenesis may provide a functional link to some of these behavioural abnormalities.

The current results extend accumulating clinical and preclinical evidence demonstrating that exposure to early-life adversity is associated with the development of altered emotional-reactivity (Levine, 1957; Denenberg and Smith, 1963; Plotsky and Meaney, 1993; Shanks et al., 1995; Liu et al., 1997; Heim and Nemeroff, 2001; Kikusui

et al., 2004) and highlight the prominent role that immune-brain signalling has on influencing the development of anxiety- and depressive-related phenotypes. Both altered 5-HT signalling and adult hippocampus have been suggested to play an etiological role in clinical and preclinical anxiety and depression, suggesting that these two mechanisms may underlie the current finding of altered emotionality in LPS-mice.

6.2. Reconciliation of an anxiety- and antidepressant-like behavioural phenotype

Early LPS-challenge evoked a behavioural phenotype consistent with both enhanced anxiety-like behaviour and an antidepressant response. As mentioned previously, without further study it is difficult to accurately interpret the results obtained in the forced swim test in terms of whether the behavioural profile reflects enhanced stress-reactivity or a true antidepressant response (study 3; chapter 5). As the argument for a stress-reactive phenotype has already been explored (see pg. 111-12), the focus here is on an antidepressant response. In this case, the argument surrounds the reconciliation of two apparent paradoxical behaviours co-existing within the same animal model.

6.2.1. Potential strain differences in stress-related phenotypes

One potentially confounding factor needs to be addressed, however, relating to the potential for a train-dependant effect on the observed behavioural phenotypes. The current studies employed two different strains of mice: the CD-1 strain for assessment of anxiety-related behaviours and a mixed strain (C57Bl/6, Swiss Webster, and DBA-2) for

assessment of depressive-like behaviour. Not only are there known strain differences in endogenous levels of emotional-reactivity (Trullas and Skolnick, 1993; Ramos et al., 1997; Griebel et al., 2000; Holmes et al., 2002; Ducottet and Belzung, 2005; Ibarguen-Vargas et al., 2008; Milner and Crabbe, 2008; Brigman et al., 2009) and in the long-term behavioural and molecular outcomes associated with early-life adversity (Millstein et al., 2006; Millstein and Holmes, 2007), potential strain-dependant effects have been proposed to account for discrepant findings within the early immune challenge model itself. With that said, however, we were successful in replicating the FST antidepressant phenotype, originally present in the mixed strain, in CD-1 mice which exhibited enhanced anxiety-related behaviours (see Figure 1). The presence of both increased anxiety-like behaviour and an antidepressant response within the CD-1 strain argues against a strain-dependant FST effect. The reproducibility of this behaviour in a different strain also demonstrates the robustness of the early immune challenge model to induce similar stress-related behavioural phenotypes across independent strains of mice.

6.2.2. Role for serotonin in mediating a complex behavioural phenotype

The presence of enhanced anxiety-related behaviour and an antidepressant response is not unique to the early immune challenge model. This is best exemplified by the pleiotropic actions of a genetically driven loss of 5HT1A receptor expression and function. The 5HT1A receptor knockout mouse displays a behavioural phenotype consistent with both enhanced anxiety-related behaviour and an antidepressant

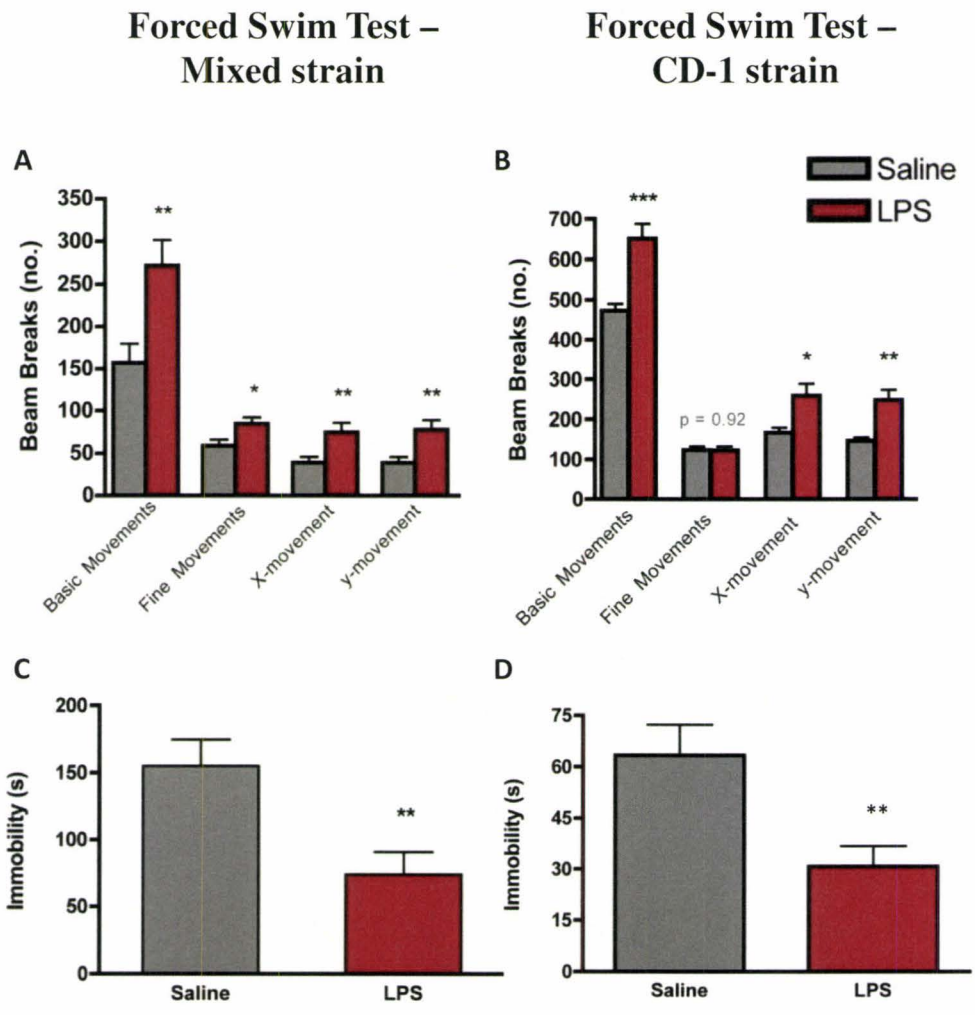

Figure 1. Comparison of forced swim test (FST) behaviour in two mouse strains that were used in the current studies. Both mixed (C57Bl/6, Swiss Webster, DBA-2) and CD-1 strains of early LPS challenged mice exhibited a similar antidepressant profile of behaviour during the FST in adulthood as reflected by increased active behaviours: horizontal (x), vertical (y) and total (basic) movements; and decreased immobility relative to saline controls. This suggests that the antidepressant phenotype of early LPS challenge rodents is a robust finding that is independent of strain. Mixed (~12w): Saline, n=15; LPS, n=13; CD-1 (~13w) : Saline, n=17; LPS, n=20. *p<0.05, **p<0.01.

behavioural phenotype (Parks et al., 1998). These paradoxical findings have been attributed to the complex and opposing actions of 5-HT receptors throughout the brain (Holmes, 2008). Indeed, there are a variety of 5-HT receptors dispersed throughout the CNS, each with distinct properties that are partially determined by location (Barnes and Sharp, 1999). As any one receptor can act at multiple nodes within the DR-corticolimbic circuit, it is not uncommon for a given receptor subtype to exert opposing actions as a result of this spatial distribution. The current finding of altered 5-HT-related gene expression across different regions of the CNS in LPS-rodents could, therefore, account for the complex behavioural phenotype reported both here and previously (Granger et al., 1996; Breivik et al., 2002; Walker et al., 2004a; Spencer et al., 2005; Bilbo et al., 2007; Harre et al., 2008; Walker et al., 2008; Walker et al., 2009a). Further support for the involvement of altered 5-HT signalling in mediating enhanced emotionality in LPS-rodents lies in the transient nature of the observed expression changes. LPS-rodents displayed altered 5-HT related gene expression restricted to the third postnatal week. Both genetic knockout (Gross et al., 2002) and pharmacological blockade (Ansorge et al., 2004; Lo Iacono and Gross, 2008; Vinkers et al., 2009) of 5-HT receptors restricted to the second-third postnatal week is sufficient to produce long-lasting changes in emotionality. Together, the current work strongly supports a role for altered 5-HT circuitry in mediating the complex behavioural phenotype of LPS-rodents. The similarity between the behavioural profile of LPS-rodents and the heterogeneous symptom profile of mood disorder patients suggests that the early LPS model offers unique insight into the

phenotypic variability displayed by patients and establishes it as a valuable tool for future study of associated pathophysiology.

6.2.3. Exploring the role for additional neurotransmitter systems

The possibility exists that alterations to additional neurotransmitter systems contribute to aberrant behaviour in LPS-rodents. Gamma-aminobutyric acid (GABA) neurotransmission has an established role in mediating (Liu et al., 2007; Depino et al., 2008) and treating anxiety-related disorders (Rudolph et al., 1999). In addition, GABA is critical to the regulation of hippocampal excitability and the promotion of neuronal differentiation from proliferating hippocampal progenitor cells (Tozuka et al., 2005). Based on this known ascribed function, a compelling speculation is that altered GABA signalling may account for both altered hippocampal neurogenesis and anxiety-related behaviours in LPS-mice. The evidence that rats exposed to early-life adversity exhibit alterations in GABAnergic receptors accompanied by a behavioural profile strikingly similar to that reported here (Caldji et al., 2000; Hsu et al., 2003) suggests a possible etiopathogenic role for altered GABAnergic transmission in LPS-rodents. This is further supported by the combined evidence that early postnatal 5-HT receptor function is essential to the normal development of GABA receptor function (Vinkers et al., 2009) and that transient disruption of GABA receptors during early postnatal development is sufficient to permanently affect the developmental programming of anxiety-related behaviours (Depino et al., 2008). Collectively, this suggests that the GABAnergic system represents a promising alternative target for future research.

6.3. Adult hippocampal neurogenesis and emotionality

A surprising finding was that of increased hippocampal neurogenesis in adult LPS-mice. An acute injection of endotoxin is known to have a negative impact on hippocampal neurogenesis both during adulthood (Ekdahl et al., 2003; Monje et al., 2003) and early postnatal development (Bland et al., 2009). This immediate effect on neurogenesis may be mediated, in part, by the known regulatory actions of proinflammatory cytokines on hippocampal neurogenesis (Vallieres et al., 2002; Ekdahl et al., 2003; Monje et al., 2003; Koo and Duman, 2008). Young hippocampal granule cells developing in an LPS-induced proinflammatory milieu may compensate by altering their intrinsic properties (Jakubs et al., 2008) leading to a steady-state increase in neuronal production. Although the immediate impact of LPS injection on hippocampal neurogenesis was not assessed in the current study, chronic elevations in proinflammatory cytokines such as TNF-alpha (Galic et al., 2008) and IL-6 (Breivik et al., 2002) have been reported in adult LPS-rodents and may exert a long-term influence on neurogenesis. Interestingly, TNF-alpha can facilitate excitatory actions on hippocampal neurons through up-regulation of AMPA/kainite receptors *in vitro* (Ogoshi et al., 2005). Given that neurogenesis is regulated via an activity-dependant mechanism (Deisseroth et al., 2004), elevated TNF-alpha levels may contribute to its enhancement in LPS-mice. Additionally, the close relationship between 5-HT and neurogenesis (Gould, 1999) indicates that the long-term alterations to 5-HT signalling suggested by the current work may be a contributing factor. Further study is required to fully characterize the exact impact early

immune challenge has on different stages of the neurogenic cascade (see Figure on pg. 24). Given that LPS injections were given at a time of peak granule cell development (Muramatsu et al., 2007), the migration, differentiation, long-term survival and/or electrophysiological properties of these developing cells may differ which would have implications for their long-term functioning.

6.3.1. Functional implications of altered hippocampal neurogenesis

The functional implications of altered neurogenesis should not be based on the false presumption that a positive correlative relationship always exists between neurogenesis and associated behavioural effects (Scharfman and Hen, 2007). Rather any deviation from control levels, regardless of directionality, should be viewed as pathogenic especially when accompanied by aberrant behaviour. The most compelling evidence for this comes from animal models of epilepsy where an increase in neurogenesis accompanies epileptic seizures (Parent et al., 1998; Madsen et al., 2000; Scott et al., 2000). Therefore, an increase is not always beneficial nor is a decrease necessarily detrimental.

Early immune challenge increased the immature neuronal population in LPS-mice independent of an increase in proliferating cells as identified by BrdU immunoreactivity. This dissociation suggests that nascent neurons are particularly susceptible to the effects of an early immune challenge. Indeed, this specific population of neurons is targeted by other forms of early-life adversity such as maternal deprivation (Mirescu et al., 2004),

suggesting that the behavioural alterations currently observed, and within the LPS-model in general, may be attributed to an alteration in immature neurons. The distinct properties of immature neurons make them uniquely positioned to exert a greater relative influence on hippocampal function (Doetsch and Hen, 2005).

Although the observed increase in hippocampal neurogenesis would appear consistent with the antidepressant behaviour of LPS-mice, there has been a recent surge of evidence demonstrating that immature neurons regulate anxiety (Santarelli et al., 2003; Ageta et al., 2008; Bergami et al., 2008; Eadie et al., 2009) and not depressive-related behaviours, *per se* (Revest et al., 2009). This evidence is consistent with a number of studies which have failed to demonstrate an association between inhibition of neurogenesis and precipitation of depressive-related behaviours (Vollmayr et al., 2003; Pollak et al., 2008; Surget et al., 2008; David et al., 2009). The emerging association to anxiety does not necessarily negate a role for neurogenesis in depressive-related behaviours but suggests a wider role in regulating different aspects of emotionality. Indeed, the requirement of hippocampal neurogenesis for the positive behavioural effects of some antidepressants is a well established finding (Santarelli et al., 2003; Li et al., 2008; Pollak et al., 2008; Surget et al., 2008; David et al., 2009).

The recent causal link between neurogenesis and anxiety-related behaviour (Revest et al., 2009) is exciting within the context of the current findings which demonstrate both enhanced anxiety-related behaviours and increased immature neuronal differentiation in LPS-mice. Given the functional dissociation that exists along the

hippocampal dorso-ventral axis (Bannerman et al., 2004), with the ventral hippocampus implicated in mediating emotionality (Bannerman et al., 2003), the observed alteration in ventral hippocampal neurogenesis specifically, may underlie enhanced emotionality in early LPS challenge rodents. Young neurons found in the ventral region, for instance, are activated in response to stress (Snyder et al., 2008) and mice with altered anxiety-related behaviours exhibit abnormal ventral hippocampal neurogenesis (Eadie et al., 2009). It should be noted that as neurogenic differences were not currently observed in male LPS-mice, altered neurogenesis may play a preferential role in mediating the behavioural phenotype of females. The presence of early onset anxiety-related behaviour in male LPS-mice (study 1; Chapter 3) suggests a different phenotypic variant of anxiety that may be associated with a distinct pathogenic mechanism.

6.4. A mechanistic convergence

The current finding of altered 5-HT and hippocampal neurogenesis should not be treated as separate and distinct pathological entities. Indeed, there exists a convergence and synergy between serotonin signalling and neurogenesis both during adulthood (Gould, 1999; Jacobs et al., 2000; Radley and Jacobs, 2002; Banasr et al., 2004) and granule cell development (Yan et al., 1997). For instance, 5-HT is known to have a regulatory influence on hippocampal neurogenesis, as blockade of the 5HT1A receptor reduces the number of proliferating cells (Radley and Jacobs, 2002) while 5-HT enhancement increases the basal rate of neurogenesis (Jacobs et al., 2000). Furthermore,

the current findings do not preclude the contributing role of alterations to HPA function which is an established finding in LPS-rodents (Shanks et al., 1995; Shanks et al., 2000). The more likely scenario is that a mechanistic convergence exists between these factors (see Figure 2). Briefly, in addition to its prominent role in mediating emotionality, 5-HT can impact HPA activity via actions at all brain regions examined in the current study. Altered 5-HT signalling during development could possibly disrupt the developing HPA-axis which would then further exacerbate modifications to developing neurocircuitry in LPS-mice via stress hormone actions on 5-HT. Additionally, the hippocampus is a major neuroanatomical regulator of the stress-axis (Sapolsky et al., 1989). Altered hippocampal neurogenesis and function would have an impact on regulation of the HPA-axis which would, in turn, have a profound influence on hippocampal neurogenesis given the established role of stress hormones on both the adult (Gould and Cameron, 1996; Gould et al., 1997; Gould et al., 1998) and developing hippocampus (Tanapat et al., 1998). Ultimately, the molecular changes associated with an early immune challenge might represent a compensatory homeostatic mechanism in an environment characterized by chronic exposure to stress hormones, altered 5-HT signalling and hippocampal neurogenesis, elevated proinflammatory cytokines, and a host of downstream deleterious factors.

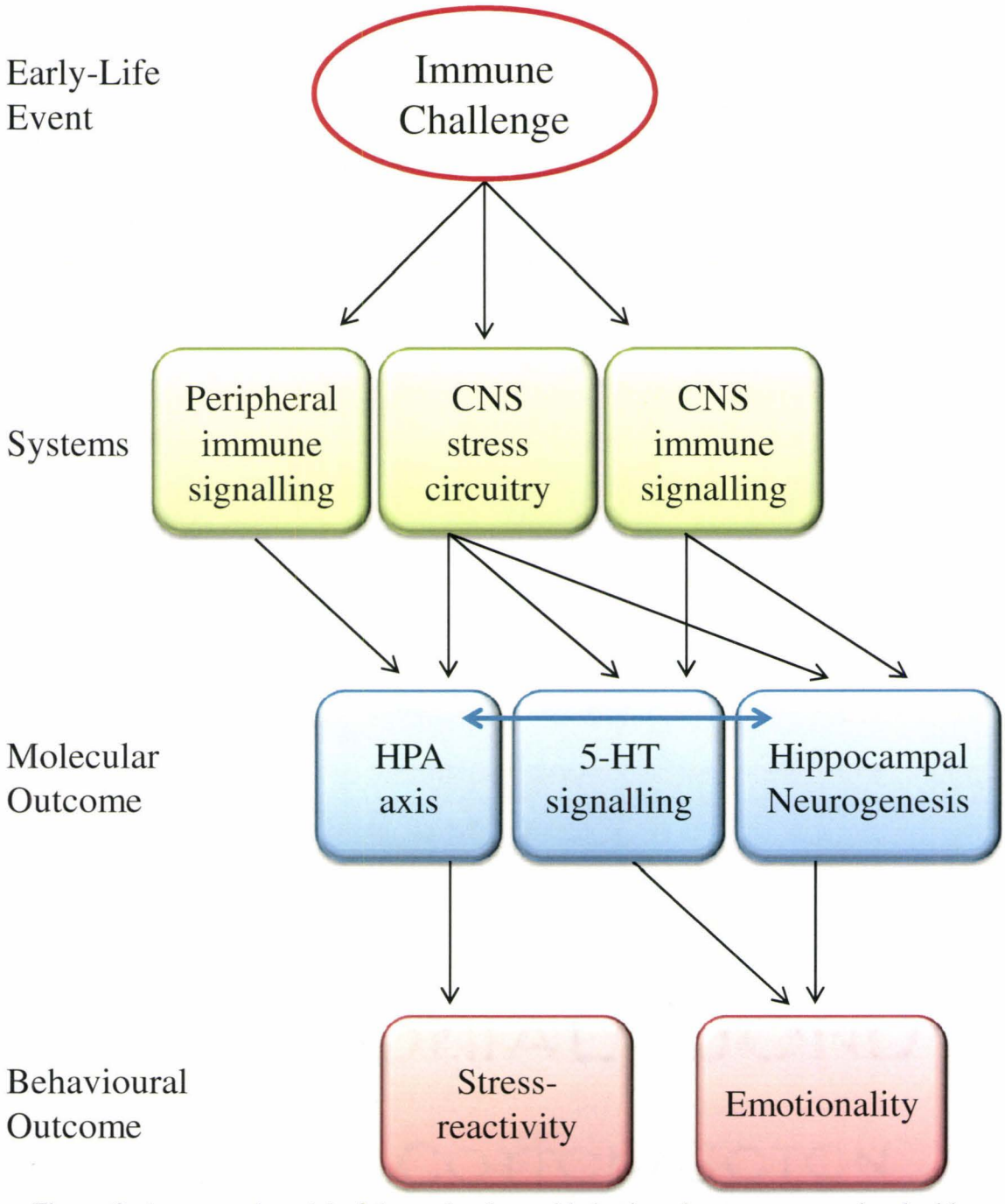

Figure 2. A proposed model of the molecular and behavioural outcomes associated with an early immune challenge, integrating findings from the current body of work. An immunogenic challenge during development activates a variety of systems that can impact CNS function with implications for long-term alterations. At the molecular level, there exists a mechanistic convergence (represented by blue arrow) whereby altered functioning of one factor further drives alterations to other pathogenic factors. Together, this has the potential to impact stress-reactivity and emotionality.

6.5. Importance of sex differences in neuroscience research

The sexual dichotomy observed within the current behavioural and molecular findings is consistent with the clinical picture of mood disorders. For instance, sex differences in the clinical presentation of social and generalized anxiety disorder have been reported (Weinstock, 1999; Steiner et al., 2005; Simon et al., 2006; Vesga-Lopez et al., 2008) with women experiencing greater severity of agoraphobic avoidance than men (Turgeon et al., 1998). There are differences in the types of obsession and compulsions displayed in OCD, with men experiencing an earlier age of onset and chronicity of symptoms (Castle et al., 1995; Labad et al., 2008). This sexual dichotomy speaks to a larger issue in neuroscience research which has historical relied on male rodents for modelling psychopathology despite the burgeoning data demonstrating sex differences in clinical and preclinical brain structure and function (Cahill, 2006). The biological basis of this dichotomy is beyond the scope of the current work, but may involve complex immune-neuroendocrine actions acting during early development. It would be misleading however, to attribute these differences entirely to variations in sex hormones as this would ignore the profound influence of genetics on inducing sex differences in the brain, independent of hormonal actions (Kolbinger et al., 1991; Beyer et al., 1992). It is likely that the resultant behavioural phenotype of LPS-mice involves a complex interaction between the combined effects of sex, early adversity, and genetic vulnerability on the developing brain. In the aggregate, these results argue for the use of both sexes when modelling psychiatric disease.

6.6. Clinical Implications

The presence of heightened emotionality in early life often increases vulnerability to the later development of subsequent mood disorders, such as anxiety or major depression (Bittner et al., 2007). Consistent with this, a subtle finding of the current work is that high emotional-reactivity during development interacts with early-life stress (LPS challenge) to enhance susceptibility to increased emotionality in adulthood (study 1; Chapter 3). This is a clinically relevant finding given that a major goal of psychiatric research is to identify sub-populations of at-risk individuals who are most likely to develop a disorder. The current finding is of particular interest for future research using the early immune challenge model as it allows for the early identification of animals susceptible to the development of adult behavioural dysfunction and for the exploration of distinct pathogenic mechanisms at work within this sub-population. This is useful for determining whether pharmacological or environmental intervention in an at-risk population can reverse or mitigate the development of behavioural abnormalities.

6.6.1. Identification of critical developmental time windows: implications for intervention

Anxiety disorders are often the earliest psychiatric disorder to emerge and are highly comorbid with a range of other psychiatric illnesses (Kessler et al., 2005). This early manifestation underscores the necessity for the early detection and treatment of anxiety with the potential to reduce vulnerability to subsequent disorders (i.e. major

depression). The current study identified developmental time windows in which intervention may attenuate or prevent future pathology. The observed disruption of postnatal 5-HT-related gene expression in LPS-mice suggests that an early developmental time window may be an appropriate target.

The majority of pharmaceuticals indicated for the treatment of anxiety and depression act on serotonin or GABA receptors. This presents a complication however, given the distinct and often opposing roles that neurotransmitters and their receptors appear to have during early development relative to adulthood (Roth et al., 1991; Patel and Zhou, 2005). For instance, whereas stress increases hippocampal 5HT1A receptor expression in the developing brain (Vazquez et al., 2000; Andrews et al., 2004) it decreases receptor expression in adulthood (Neumaier et al., 2000). Such differential properties are not restricted to 5-HT receptors, as the major inhibitory neurotransmitter in the adult CNS, GABA, functions as an excitatory transmitter during early CNS development (Wang and Kriegstein, 2009). Collectively, this suggests that pharmacological intervention targeted at specific receptors may not exert the intended beneficial effect. This is demonstrated by a recent study in which juvenile mice treated with the SSRI, fluoxetine, exhibited increased anxiety-related behaviour rather than the expected decrease (Oh et al., 2009). In this case, non-pharmacological intervention in the form of environmental enrichment may prove beneficial. For instance, the enriching effect of postnatal handling between P1-P14 was able to reduce social anxiety and attenuate stress-induced CORT release in adult 5HT1A knockout mice (Zanettini et al.,

2009). Voluntary exercise during early postnatal development may also prove beneficial as this form of environmental enrichment has been shown preclinically or clinically to increase neurotrophic factors (Russo-Neustadt et al., 1999; Trejo et al., 2008), hippocampal neurogenesis (van Praag et al., 1999; Brown et al., 2003a; Bjornebekk et al., 2005; Pereira et al., 2007), alter 5-HT receptor levels (Greenwood et al., 2005), reduce inflammation (Cotman et al., 2007; Nichol et al., 2008; Parachikova et al., 2008), attenuate the behavioural and physiological stress response (Fox et al., 2008), and is associated with affect enhancement (Binder et al., 2004; Bjornebekk et al., 2005; Duman et al., 2008; Greenwood et al., 2008). Therefore, exercise during postnatal development or adulthood may be able to compensate for any deleterious effects of early LPS-challenge. The extent to which pharmacological or environmental intervention can reverse behavioural and molecular alterations in LPS-rodents would strengthen the validity of the model as a tool to study psychopathology and represents a promising avenue for future research.

6.7. Conclusion

Immune-brain communication is essential to health and disease. Immune dysregulation has been implicated in a range of neurological (Patrick and Lindstrom, 1973; Solimena et al., 1990; Rogers et al., 1994; Darnell and Posner, 2003; McGeer and McGeer, 2003) and psychiatric disorders (Dale et al., 2005; Vargas et al., 2005; Arion et al., 2007; Anisman, 2009). The present body of work demonstrates that immunological

challenge during critical time points in development has long-term consequences to stress-related behavioural and molecular phenotypes, highlighting the prominent role of the immune system during brain development. It is likely that the long-term behavioural and molecular consequences of an early immune challenge are the result of a culmination of pathogenic mechanisms and factors acting throughout development. The present study demonstrates that altered 5-HT signalling and hippocampal neurogenesis are potential contributing factors to this pathogenic cascade and may provide a functional link between early immune perturbation and altered anxiety- and depressive-related behaviours. Together with altered HPA functioning, these factors may act as a synergistic driving force promoting or contributing to disease emergence and progression. This work provides a framework for future study on the role of early immune disturbance in psychopathology and advances understanding of how early immune-brain signalling influences the developing CNS and risk for subsequent disease.

References

Ageta H, Murayama A, Migishima R, Kida S, Tsuchida K, Yokoyama M, Inokuchi K (2008) Activin in the brain modulates anxiety-related behavior and adult neurogenesis. PLoS ONE 3:e1869.

Alesci S, Martinez PE, Kelkar S, Ilias I, Ronsaville DS, Listwak SJ, Ayala AR, Licinio J, Gold HK, Kling MA, Chrousos GP, Gold PW (2005) Major depression is associated with significant diurnal elevations in plasma interleukin-6 levels, a shift of its circadian rhythm, and loss of physiological complexity in its secretion: clinical implications. J Clin Endocrinol Metab 90:2522-2530.

Altman J, Das GD (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 124:319-335.

Andrews MH, Kostaki A, Setiawan E, McCabe L, Matthews SG (2004) Developmental regulation of 5-HT1A receptor mRNA in the fetal limbic system: response to antenatal glucocorticoid. Brain Res Dev Brain Res 149:39-44.

Anisman H (2009) Cascading effects of stressors and inflammatory immune system activation: implications for major depressive disorder. J Psychiatry Neurosci 34:4-20.

Ansorge MS, Hen R, Gingrich JA (2007) Neurodevelopmental origins of depressive disorders. Curr Opin Pharmacol 7:8-17.

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 (New York, NY) 306:879-881.

Arion D, Unger T, Lewis DA, Levitt P, Mirnics K (2007) Molecular evidence for increased expression of genes related to immune and chaperone function in the prefrontal cortex in schizophrenia. Biol Psychiatry 62:711-721.

Asberg M, Thoren P, Traskman L, Bertilsson L, Ringberger V (1976) "Serotonin depression"--a biochemical subgroup within the affective disorders? Science 191:478-480.

Baldwin DS, Lopes AT (2009) Agomelatine improves symptoms of generalised anxiety disorder. Evid Based Ment Health 12:54.

Banasr M, Hery M, Printemps R, Daszuta A (2004) Serotonin-induced increases in adult cell proliferation and neurogenesis are mediated through different and common 5-HT

receptor subtypes in the dentate gyrus and the subventricular zone. Neuropsychopharmacology 29:450-460.

Bannerman DM, Grubb M, Deacon RM, Yee BK, Feldon J, Rawlins JN (2003) Ventral hippocampal lesions affect anxiety but not spatial learning. Behav Brain Res 139:197-213.

Bannerman DM, Rawlins JN, McHugh SB, Deacon RM, Yee BK, Bast T, Zhang WN, Pothuizen HH, Feldon J (2004) Regional dissociations within the hippocampus--memory and anxiety. Neuroscience and biobehavioral reviews 28:273-283.

Barnes NM, Sharp T (1999) A review of central 5-HT receptors and their function. Neuropharmacology 38:1083-1152.

Bauer S, Patterson PH (2005) The cell cycle-apoptosis connection revisited in the adult brain. J Cell Biol 171:641-650.

Becker S, Macqueen G, Wojtowicz JM (2009) Computational modeling and empirical studies of hippocampal neurogenesis-dependent memory: Effects of interference, stress and depression. Brain Res 1299:45-54.

Belzung C, Le Pape G (1994) Comparison of different behavioral test situations used in psychopharmacology for measurement of anxiety. Physiology & behavior 56:623-628.

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.

Bergami M, Rimondini R, Santi S, Blum R, Gotz M, Canossa M (2008) Deletion of TrkB in adult progenitors alters newborn neuron integration into hippocampal circuits and increases anxiety-like behavior. Proceedings of the National Academy of Sciences of the United States of America 105:15570-15575.

Beyer C, Eusterschulte B, Pilgrim C, Reisert I (1992) Sex steroids do not alter sex differences in tyrosine hydroxylase activity of dopaminergic neurons in vitro. Cell Tissue Res 270:547-552.

Bilbo SD, Schwarz JM (2009) Early-life programming of later-life brain and behavior: a critical role for the immune system. Front Behav Neurosci 3:14.

Bilbo SD, Rudy JW, Watkins LR, Maier SF (2006) A behavioural characterization of neonatal infection-facilitated memory impairment in adult rats. Behavioural brain research 169:39-47.

Bilbo SD, Levkoff LH, Mahoney JH, Watkins LR, Rudy JW, Maier SF (2005) Neonatal infection induces memory impairments following an immune challenge in adulthood. Behavioral neuroscience 119:293-301.

Bilbo SD, Yirmiya R, Amat J, Paul ED, Watkins LR, Maier SF (2007) Bacterial infection early in life protects against stressor-induced depressive-like symptoms in adult rats. Psychoneuroendocrinology 33:261-269.

Binder E, Droste SK, Ohl F, Reul JM (2004) Regular voluntary exercise reduces anxiety-related behaviour and impulsiveness in mice. Behavioural brain research 155:197-206.

Bittner A, Egger HL, Erkanli A, Jane Costello E, Foley DL, Angold A (2007) What do childhood anxiety disorders predict? Journal of child psychology and psychiatry, and allied disciplines 48:1174-1183.

Bjornebekk A, Mathe AA, Brene S (2005) The antidepressant effect of running is associated with increased hippocampal cell proliferation. The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP) 8:357-368.

Bland ST, Beckley JT, Young S, Tsang V, Watkins LR, Maier SF, Bilbo SD (2009) Enduring consequences of early-life infection on glial and neural cell genesis within cognitive regions of the brain. Brain Behav Immun (Sept 25; ahead of print).

Boisse L, Mouihate A, Ellis S, Pittman QJ (2004) Long-term alterations in neuroimmune responses after neonatal exposure to lipopolysaccharide. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:4928-4934.

Boldrini M, Underwood MD, Mann JJ, Arango V (2008) Serotonin-1A autoreceptor binding in the dorsal raphe nucleus of depressed suicides. J Psychiatr Res 42:433-442.

Boldrini M, Underwood MD, Hen R, Rosoklija GB, Dwork AJ, John Mann J, Arango V (2009) Antidepressants increase neural progenitor cells in the human hippocampus. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 34:2376-2389.

Breivik T, Stephan M, Brabant GE, Straub RH, Pabst R, von Horsten S (2002) Postnatal lipopolysaccharide-induced illness predisposes to periodontal disease in adulthood. Brain, behavior, and immunity 16:421-438.

Brigman JL, Mathur P, Lu L, Williams RW, Holmes A (2009) Genetic relationship between anxiety-related and fear-related behaviors in BXD recombinant inbred mice. Behav Pharmacol 20:204-209.

Brown J, Cooper-Kuhn CM, Kempermann G, Van Praag H, Winkler J, Gage FH, Kuhn HG (2003a) Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis. The European journal of neuroscience 17:2042-2046.

Brown JP, Couillard-Despres S, Cooper-Kuhn CM, Winkler J, Aigner L, Kuhn HG (2003b) Transient expression of doublecortin during adult neurogenesis. J Comp Neurol 467:1-10.

Cahill L (2006) Why sex matters for neuroscience. Nature reviewsNeuroscience 7:477-484.

Caldji C, Francis D, Sharma S, Plotsky PM, Meaney MJ (2000) The effects of early rearing environment on the development of GABAA and central benzodiazepine receptor levels and novelty-induced fearfulness in the rat. Neuropsychopharmacology 22:219-229.

Cameron HA, Woolley CS, McEwen BS, Gould E (1993) Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. Neuroscience 56:337-344.

Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H, McClay J, Mill J, Martin J, Braithwaite A, Poulton R (2003) Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. Science 301:386-389.

Castagne V, Porsolt RD, Moser P (2009) Use of latency to immobility improves detection of antidepressant-like activity in the behavioral despair test in the mouse. Eur J Pharmacol 616:128-133.

Castle DJ, Deale A, Marks IM (1995) Gender differences in obsessive compulsive disorder. The Australian and New Zealand Journal of Psychiatry 29:114-117.

Cotman CW, Berchtold NC, Christie LA (2007) Exercise builds brain health: key roles of growth factor cascades and inflammation. Trends in neurosciences 30:464-472.

Crawley J, Goodwin FK (1980) Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. Pharmacol Biochem Behav 13:167-170.

Crawley JN (1981) Neuropharmacologic specificity of a simple animal model for the behavioral actions of benzodiazepines. Pharmacol Biochem Behav 15:695-699.

Crawley JN, Skolnick P, Paul SM (1984) Absence of intrinsic antagonist actions of benzodiazepine antagonists on an exploratory model of anxiety in the mouse. Neuropharmacology 23:531-537.

Cryan JF, Mombereau C (2004) In search of a depressed mouse: utility of models for studying depression-related behavior in genetically modified mice. Mol Psychiatry 9:326-357.

Cryan JF, Holmes A (2005) The ascent of mouse: advances in modelling human depression and anxiety. Nat Rev Drug Discov 4:775-790.

Curtis MA, Kam M, Nannmark U, Anderson MF, Axell MZ, Wikkelso C, Holtas S, van Roon-Mom WM, Bjork-Eriksson T, Nordborg C, Frisen J, Dragunow M, Faull RL, Eriksson PS (2007) Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. Science 315:1243-1249.

D'Sa C, Duman RS (2002) Antidepressants and neuroplasticity. Bipolar Disord 4:183-194.

Dale RC, Heyman I, Giovannoni G, Church AW (2005) Incidence of anti-brain antibodies in children with obsessive-compulsive disorder. Br J Psychiatry 187:314-319.

Darnell RB, Posner JB (2003) Paraneoplastic syndromes involving the nervous system. N Engl J Med 349:1543-1554.

David DJ, Samuels BA, Rainer Q, Wang JW, Marsteller D, Mendez I, Drew M, Craig DA, Guiard BP, Guilloux JP, Artymyshyn RP, Gardier AM, Gerald C, Antonijevic IA, Leonardo ED, Hen R (2009) Neurogenesis-dependent and -independent effects of fluoxetine in an animal model of anxiety/depression. Neuron 62:479-493.

Davidson JR, DuPont RL, Hedges D, Haskins JT (1999) Efficacy, safety, and tolerability of venlafaxine extended release and buspirone in outpatients with generalized anxiety disorder. J Clin Psychiatry 60:528-535.

Deacon RM, Bannerman DM, Rawlins JN (2002) Anxiolytic effects of cytotoxic hippocampal lesions in rats. Behav Neurosci 116:494-497.

Deisseroth K, Singla S, Toda H, Monje M, Palmer TD, Malenka RC (2004) Excitation-neurogenesis coupling in adult neural stem/progenitor cells. Neuron 42:535-552.

Delgado PL, Charney DS, Price LH, Aghajanian GK, Landis H, Heninger GR (1990) Serotonin function and the mechanism of antidepressant action. Reversal of

antidepressant-induced remission by rapid depletion of plasma tryptophan. Arch Gen Psychiatry 47:411-418.

Denenberg VH, Smith SA (1963) Effects of infantile stimulation and age upon behavior. J Comp Physiol Psychol 56:307-312.

Dent GW, Smith MA, Levine S (1999) The ontogeny of the neuroendocrine response to endotoxin. Brain researchDevelopmental brain research 117:21-29.

Depino AM, Tsetsenis T, Gross C (2008) GABA homeostasis contributes to the developmental programming of anxiety-related behavior. Brain Res 1210:189-199.

Doetsch F, Hen R (2005) Young and excitable: the function of new neurons in the adult mammalian brain. Curr Opin Neurobiol 15:121-128.

Ducottet C, Belzung C (2005) Correlations between behaviours in the elevated plus-maze and sensitivity to unpredictable subchronic mild stress: evidence from inbred strains of mice. Behav Brain Res 156:153-162.

Duman CH, Schlesinger L, Russell DS, Duman RS (2008) Voluntary exercise produces antidepressant and anxiolytic behavioral effects in mice. Brain research 1199:148-158.

Dunn AJ, Swiergiel AH (2008) Effects of acute and chronic stressors and CRF in rat and mouse tests for depression. Ann N Y Acad Sci 1148:118-126.

Eadie BD, Zhang WN, Boehme F, Gil-Mohapel J, Kainer L, Simpson JM, Christie BR (2009) Fmr1 knockout mice show reduced anxiety and alterations in neurogenesis that are specific to the ventral dentate gyrus. Neurobiol Dis 36:361-373.

Ekdahl CT, Claasen JH, Bonde S, Kokaia Z, Lindvall O (2003) Inflammation is detrimental for neurogenesis in adult brain. Proc Natl Acad Sci U S A 100:13632-13637.

Eklind S, Hagberg H, Wang X, Savman K, Leverin AL, Hedtjarn M, Mallard C (2006) Effect of lipopolysaccharide on global gene expression in the immature rat brain. Pediatric research 60:161-168.

El Yacoubi M, Bouali S, Popa D, Naudon L, Leroux-Nicollet I, Hamon M, Costentin J, Adrien J, Vaugeois JM (2003) Behavioral, neurochemical, and electrophysiological characterization of a genetic mouse model of depression. Proc Natl Acad Sci U S A 100:6227-6232.

Ellestad KK, Tsutsui S, Noorbakhsh F, Warren KG, Yong VW, Pittman QJ, Power C (2009) Early life exposure to lipopolysaccharide suppresses experimental autoimmune

encephalomyelitis by promoting tolerogenic dendritic cells and regulatory T cells. Journal of immunology (Baltimore, Md: 1950) 183:298-309.

Engum A (2007) The role of depression and anxiety in onset of diabetes in a large population-based study. J Psychosom Res 62:31-38.

Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (1998) Neurogenesis in the adult human hippocampus. Nat Med 4:1313-1317.

Ford DE, Mead LA, Chang PP, Cooper-Patrick L, Wang NY, Klag MJ (1998) Depression is a risk factor for coronary artery disease in men: the precursors study. Arch Intern Med 158:1422-1426.

Fox JH, Hammack SE, Falls WA (2008) Exercise is associated with reduction in the anxiogenic effect of mCPP on acoustic startle. Behavioral neuroscience 122:943-948.

Galic MA, Riazi K, Heida JG, Mouihate A, Fournier NM, Spencer SJ, Kalynchuk LE, Teskey GC, Pittman QJ (2008) Postnatal inflammation increases seizure susceptibility in adult rats. The Journal of neuroscience : the official journal of the Society for Neuroscience 28:6904-6913.

Gardner KL, Hale MW, Oldfield S, Lightman SL, Plotsky PM, Lowry CA (2009) Adverse experience during early life and adulthood interact to elevate tph2 mRNA expression in serotonergic neurons within the dorsal raphe nucleus. Neuroscience 10:991-1001.

Gill J, Vythilingam M, Page GG (2008) Low cortisol, high DHEA, and high levels of stimulated TNF-alpha, and IL-6 in women with PTSD. J Trauma Stress 21:530-539.

Givalois L, Dornand J, Mekaouche M, Solier MD, Bristow AF, Ixart G, Siaud P, Assenmacher I, Barbanel G (1994) Temporal cascade of plasma level surges in ACTH, corticosterone, and cytokines in endotoxin-challenged rats. Am J Physiol 267:R164-170.

Goldstein BI, Kemp DE, Soczynska JK, McIntyre RS (2009) Inflammation and the phenomenology, pathophysiology, comorbidity, and treatment of bipolar disorder: a systematic review of the literature. J Clin Psychiatry 70:1078-1090.

Goodfellow NM, Benekareddy M, Vaidya VA, Lambe EK (2009) Layer II/III of the prefrontal cortex: Inhibition by the serotonin 5-HT1A receptor in development and stress. J Neurosci 29:10094-10103.

Goodwin GM, Emsley R, Rembry S, Rouillon F (2009) Agomelatine prevents relapse in patients with major depressive disorder without evidence of a discontinuation syndrome: a 24-week randomized, double-blind, placebo-controlled trial. J Clin Psychiatry 70:1128-1137.

Gottesman, II, Gould TD (2003) The endophenotype concept in psychiatry: etymology and strategic intentions. Am J Psychiatry 160:636-645.

Gould E (1999) Serotonin and hippocampal neurogenesis. Neuropsychopharmacology 21:46S-51S.

Gould E (2007) How widespread is adult neurogenesis in mammals? Nat Rev Neurosci 8:481-488.

Gould E, Cameron HA (1996) Regulation of neuronal birth, migration and death in the rat dentate gyrus. Dev Neurosci 18:22-35.

Gould E, McEwen BS, Tanapat P, Galea LA, Fuchs E (1997) Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. J Neurosci 17:2492-2498.

Gould E, Tanapat P, McEwen BS, Flugge G, Fuchs E (1998) Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. Proc Natl Acad Sci U S A 95:3168-3171.

Gould E, Reeves AJ, Fallah M, Tanapat P, Gross CG, Fuchs E (1999) Hippocampal neurogenesis in adult Old World primates. Proc Natl Acad Sci U S A 96:5263-5267.

Granger DA, Hood KE, Ikeda SC, Reed CL, Block ML (1996) Neonatal endotoxin exposure alters the development of social behavior and the hypothalamic-pituitary-adrenal axis in selectively bred mice. Brain, behavior, and immunity 10:249-259.

Grassi Zucconi G, Cipriani S, Balgkouranidou I, Scattoni R (2006) 'One night' sleep deprivation stimulates hippocampal neurogenesis. Brain Res Bull 69:375-381.

Greenwood BN, Strong PV, Brooks L, Fleshner M (2008) Anxiety-like behaviors produced by acute fluoxetine administration in male Fischer 344 rats are prevented by prior exercise. Psychopharmacology 199:209-222.

Greenwood BN, Foley TE, Day HE, Burhans D, Brooks L, Campeau S, Fleshner M (2005) Wheel running alters serotonin (5-HT) transporter, 5-HT1A, 5-HT1B, and alpha 1b-adrenergic receptor mRNA in the rat raphe nuclei. Biological psychiatry 57:559-568.

Griebel G, Belzung C, Perrault G, Sanger DJ (2000) Differences in anxiety-related behaviours and in sensitivity to diazepam in inbred and outbred strains of mice. Psychopharmacology (Berl) 148:164-170.

Gross C, Hen R (2004) The developmental origins of anxiety. Nat Rev Neurosci 5:545-552.

Gross C, Zhuang X, Stark K, Ramboz S, Oosting R, Kirby L, Santarelli L, Beck S, Hen R (2002) Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. Nature 416:396-400.

Hall CS (1936) Emotional behavior in the rat. III. The relationship between emotionality and ambulatory activity. Journal of Comparative Psychology 22:345-352.

Handley SL, Mithani S (1984) Effects of alpha-adrenoceptor agonists and antagonists in a maze-exploration model of 'fear'-motivated behaviour. Naunyn-Schmiedeberg's archives of pharmacology 327:1-5.

Harre EM, Galic MA, Mouihate A, Noorbakhsh F, Pittman QJ (2008) Neonatal inflammation produces selective behavioural deficits and alters N-methyl-D-aspartate receptor subunit mRNA in the adult rat brain. The European journal of neuroscience 27:644-653.

Hart BL (1988) Biological basis of the behavior of sick animals. Neurosci Biobehav Rev 12:123-137.

Hedou G, Pryce C, Di Iorio L, Heidbreder CA, Feldon J (2001) An automated analysis of rat behavior in the forced swim test. Pharmacol Biochem Behav 70:65-76.

Heim C, Nemeroff CB (2001) The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. Biol Psychiatry 49:1023-1039.

Hodgson DM, Knott B, Walker FR (2001) Neonatal endotoxin exposure influences HPA responsivity and impairs tumor immunity in Fischer 344 rats in adulthood. Pediatric research 50:750-755.

Hoge EA, Brandstetter K, Moshier S, Pollack MH, Wong KK, Simon NM (2009) Broad spectrum of cytokine abnormalities in panic disorder and posttraumatic stress disorder. Depress Anxiety 26:447-455.

Holick KA, Lee DC, Hen R, Dulawa SC (2008) Behavioral effects of chronic fluoxetine in BALB/cJ mice do not require adult hippocampal neurogenesis or the serotonin 1A

receptor. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 33:406-417.

Hollis JH, Evans AK, Bruce KP, Lightman SL, Lowry CA (2006) Lipopolysaccharide has indomethacin-sensitive actions on Fos expression in topographically organized subpopulations of serotonergic neurons. Brain, behavior, and immunity 20:569-577.

Holmes A (2001) Targeted gene mutation approaches to the study of anxiety-like behavior in mice. Neuroscience and biobehavioral reviews 25:261-273.

Holmes A (2008) Genetic variation in cortico-amygdala serotonin function and risk for stress-related disease. Neuroscience and biobehavioral reviews 32:1293-1314.

Holmes A, Wrenn CC, Harris AP, Thayer KE, Crawley JN (2002) Behavioral profiles of inbred strains on novel olfactory, spatial and emotional tests for reference memory in mice. Genes Brain Behav 1:55-69.

Holmes A, Kinney JW, Wrenn CC, Li Q, Yang RJ, Ma L, Vishwanath J, Saavedra MC, Innerfield CE, Jacoby AS, Shine J, Iismaa TP, Crawley JN (2003) Galanin GAL-R1 receptor null mutant mice display increased anxiety-like behavior specific to the elevated plus-maze. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 28:1031-1044.

Hsu FC, Zhang GJ, Raol YS, Valentino RJ, Coulter DA, Brooks-Kayal AR (2003) Repeated neonatal handling with maternal separation permanently alters hippocampal GABAA receptors and behavioral stress responses. Proceedings of the National Academy of Sciences of the United States of America 100:12213-12218.

Ibarguen-Vargas Y, Surget A, Touma C, Palme R, Belzung C (2008) Multifaceted strain-specific effects in a mouse model of depression and of antidepressant reversal. Psychoneuroendocrinology 33:1357-1368.

Jacobs BL, Praag H, Gage FH (2000) Adult brain neurogenesis and psychiatry: a novel theory of depression. Mol Psychiatry 5:262-269.

Jakubs K, Bonde S, Iosif RE, Ekdahl CT, Kokaia Z, Kokaia M, Lindvall O (2008) Inflammation regulates functional integration of neurons born in adult brain. J Neurosci 28:12477-12488.

Jessberger S, Clark RE, Broadbent NJ, Clemenson GD, Jr., Consiglio A, Lie DC, Squire LR, Gage FH (2009) Dentate gyrus-specific knockdown of adult neurogenesis impairs spatial and object recognition memory in adult rats. Learning & memory (Cold Spring Harbor, NY) 16:147-154.

Kanari K, Kikusui T, Takeuchi Y, Mori Y (2005) Multidimensional structure of anxiety-related behavior in early-weaned rats. Behav Brain Res 156:45-52.

Katon W, Lozano P, Russo J, McCauley E, Richardson L, Bush T (2007) The prevalence of DSM-IV anxiety and depressive disorders in youth with asthma compared with controls. J Adolesc Health 41:455-463.

Kawachi I, Sparrow D, Vokonas PS, Weiss ST (1994) Symptoms of anxiety and risk of coronary heart disease. The Normative Aging Study. Circulation 90:2225-2229.

Kempermann G, Kronenberg G (2003) Depressed new neurons--adult hippocampal neurogenesis and a cellular plasticity hypothesis of major depression. Biol Psychiatry 54:499-503.

Kempermann G, Kuhn HG, Gage FH (1997) More hippocampal neurons in adult mice living in an enriched environment. Nature 386:493-495.

Kendler KS, Karkowski LM, Prescott CA (1999) Causal relationship between stressful life events and the onset of major depression. Am J Psychiatry 156:837-841.

Kendler KS, Kessler RC, Walters EE, MacLean C, Neale MC, Heath AC, Eaves LJ (1995) Stressful life events, genetic liability, and onset of an episode of major depression in women. Am J Psychiatry 152:833-842.

Kessler RC, Berglund P, Demler O, Jin R, Merikangas KR, Walters EE (2005) Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. Archives of General Psychiatry 62:593-602.

Kikusui T, Takeuchi Y, Mori Y (2004) Early weaning induces anxiety and aggression in adult mice. Physiol Behav 81:37-42.

Kikusui T, Ichikawa S, Mori Y (2009) Maternal deprivation by early weaning increases corticosterone and decreases hippocampal BDNF and neurogenesis in mice. Psychoneuroendocrinology 34:762-772.

Kikusui T, Nakamura K, Kakuma Y, Mori Y (2006) Early weaning augments neuroendocrine stress responses in mice. Behav Brain Res 175:96-103.

Kim YK, Na KS, Shin KH, Jung HY, Choi SH, Kim JB (2007) Cytokine imbalance in the pathophysiology of major depressive disorder. Prog Neuropsychopharmacol Biol Psychiatry 31:1044-1053.

Kolbinger W, Trepel M, Beyer C, Pilgrim C, Reisert I (1991) The influence of genetic sex on sexual differentiation of diencephalic dopaminergic neurons in vitro and in vivo. Brain Res 544:349-352.

Kompagne H, Bardos G, Szenasi G, Gacsalyi I, Harsing LG, Levay G (2008) Chronic mild stress generates clear depressive but ambiguous anxiety-like behaviour in rats. Behav Brain Res 193:311-314.

Koo JW, Duman RS (2008) IL-1beta is an essential mediator of the antineurogenic and anhedonic effects of stress. Proc Natl Acad Sci U S A 105:751-756.

Kornack DR, Rakic P (1999) Continuation of neurogenesis in the hippocampus of the adult macaque monkey. Proc Natl Acad Sci U S A 96:5768-5773.

Kozak W, Conn CA, Kluger MJ (1994) Lipopolysaccharide induces fever and depresses locomotor activity in unrestrained mice. Am J Physiol 266:R125-135.

Kurina LM, Goldacre MJ, Yeates D, Gill LE (2001) Depression and anxiety in people with inflammatory bowel disease. J Epidemiol Community Health 55:716-720.

Kurtuncu M, Luka LJ, Dimitrijevic N, Uz T, Manev H (2005) Reliability assessment of an automated forced swim test device using two mouse strains. Journal of neuroscience methods 149:26-30.

Labad J, Menchon JM, Alonso P, Segalas C, Jimenez S, Jaurrieta N, Leckman JF, Vallejo J (2008) Gender differences in obsessive-compulsive symptom dimensions. Depression and anxiety 25:832-838.

Laflamme N, Rivest S (2001) Toll-like receptor 4: the missing link of the cerebral innate immune response triggered by circulating gram-negative bacterial cell wall components. FASEB J 15:155-163.

Lambas-Senas L, Mnie-Filali O, Certin V, Faure C, Lemoine L, Zimmer L, Haddjeri N (2009) Functional correlates for 5-HT(1A) receptors in maternally deprived rats displaying anxiety and depression-like behaviors. Prog Neuropsychopharmacol Biol Psychiatry 33:262-268.

Lee JH, Kim HJ, Kim JG, Ryu V, Kim BT, Kang DW, Jahng JW (2007) Depressive behaviors and decreased expression of serotonin reuptake transporter in rats that experienced neonatal maternal separation. Neurosci Res 58:32-39.

Lehmann J, Feldon J (2000) Long-term biobehavioral effects of maternal separation in the rat: consistent or confusing? Rev Neurosci 11:383-408.

Leonardo ED, Hen R (2008) Anxiety as a developmental disorder. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 33:134-140.

Lesch KP, Bengel D, Heils A, Sabol SZ, Greenberg BD, Petri S, Benjamin J, Muller CR, Hamer DH, Murphy DL (1996) Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. Science 274:1527-1531.

Levine S (1957) Infantile experience and resistance to physiological stress. Science 126:405.

Li Y, Luikart BW, Birnbaum S, Chen J, Kwon CH, Kernie SG, Bassel-Duby R, Parada LF (2008) TrkB regulates hippocampal neurogenesis and governs sensitivity to antidepressive treatment. Neuron 59:399-412.

Lippmann M, Bress A, Nemeroff CB, Plotsky PM, Monteggia LM (2007) Long-term behavioural and molecular alterations associated with maternal separation in rats. Eur J Neurosci 25:3091-3098.

Lister RG (1987) The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology 92:180-185.

Liu D, Diorio J, Tannenbaum B, Caldji C, Francis D, Freedman A, Sharma S, Pearson D, Plotsky PM, Meaney MJ (1997) Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. Science 277:1659-1662.

Liu GX, Cai GQ, Cai YQ, Sheng ZJ, Jiang J, Mei Z, Wang ZG, Guo L, Fei J (2007) Reduced anxiety and depression-like behaviors in mice lacking GABA transporter subtype 1. Neuropsychopharmacology 32:1531-1539.

Lo Iacono L, Gross C (2008) Alpha-Ca2+/calmodulin-dependent protein kinase II contributes to the developmental programming of anxiety in serotonin receptor 1A knock-out mice. J Neurosci 28:6250-6257.

Lowry CA (2002) Functional subsets of serotonergic neurones: implications for control of the hypothalamic-pituitary-adrenal axis. J Neuroendocrinol 14:911-923.

Lowry CA, Johnson PL, Hay-Schmidt A, Mikkelsen J, Shekhar A (2005) Modulation of anxiety circuits by serotonergic systems. Stress 8:233-246.

Madsen TM, Treschow A, Bengzon J, Bolwig TG, Lindvall O, Tingstrom A (2000) Increased neurogenesis in a model of electroconvulsive therapy. Biol Psychiatry 47:1043-1049.

Maes M, Meltzer HY, Bosmans E, Bergmans R, Vandoolaeghe E, Ranjan R, Desnyder R (1995) Increased plasma concentrations of interleukin-6, soluble interleukin-6, soluble interleukin-2 and transferrin receptor in major depression. J Affect Disord 34:301-309.

Maes M, Lin AH, Delmeire L, Van Gastel A, Kenis G, De Jongh R, Bosmans E (1999) Elevated serum interleukin-6 (IL-6) and IL-6 receptor concentrations in posttraumatic stress disorder following accidental man-made traumatic events. Biol Psychiatry 45:833-839.

Malberg JE, Duman RS (2003) Cell proliferation in adult hippocampus is decreased by inescapable stress: reversal by fluoxetine treatment. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 28:1562-1571.

Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. The Journal of neuroscience : the official journal of the Society for Neuroscience 20:9104-9110.

McGeer EG, McGeer PL (2003) Inflammatory processes in Alzheimer's disease. Prog Neuropsychopharmacol Biol Psychiatry 27:741-749.

McKinney WT, Jr., Bunney WE, Jr. (1969) Animal model of depression. I. Review of evidence: implications for research. Arch Gen Psychiatry 21:240-248.

Meaney MJ, Aitken DH (1985) The effects of early postnatal handling on hippocampal glucocorticoid receptor concentrations: temporal parameters. Brain Res 354:301-304.

Meshi D, Drew MR, Saxe M, Ansorge MS, David D, Santarelli L, Malapani C, Moore H, Hen R (2006) Hippocampal neurogenesis is not required for behavioral effects of environmental enrichment. Nature neuroscience 9:729-731.

Mikova O, Yakimova R, Bosmans E, Kenis G, Maes M (2001) Increased serum tumor necrosis factor alpha concentrations in major depression and multiple sclerosis. Eur Neuropsychopharmacol 11:203-208.

Miller AH, Maletic V, Raison CL (2009) Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. Biol Psychiatry 65:732-741.

Miller MW, Nowakowski RS (1988) Use of bromodeoxyuridine-immunohistochemistry to examine the proliferation, migration and time of origin of cells in the central nervous system. Brain research 457:44-52.

Millstein RA, Holmes A (2007) Effects of repeated maternal separation on anxiety- and depression-related phenotypes in different mouse strains. Neurosci Biobehav Rev 31:3-17.

Millstein RA, Ralph RJ, Yang RJ, Holmes A (2006) Effects of repeated maternal separation on prepulse inhibition of startle across inbred mouse strains. Genes Brain Behav 5:346-354.

Milner LC, Crabbe JC (2008) Three murine anxiety models: results from multiple inbred strain comparisons. Genes Brain Behav 7:496-505.

Mirescu C, Peters JD, Gould E (2004) Early life experience alters response of adult neurogenesis to stress. Nat Neurosci 7:841-846.

Monje ML, Toda H, Palmer TD (2003) Inflammatory blockade restores adult hippocampal neurogenesis. Science 302:1760-1765.

Muller MB, Keck ME (2002) Genetically engineered mice for studies of stress-related clinical conditions. J Psychiatr Res 36:53-76.

Muramatsu R, Ikegaya Y, Matsuki N, Koyama R (2007) Neonatally born granule cells numerically dominate adult mice dentate gyrus. Neuroscience 148:593-598.

Murray F, Smith DW, Hutson PH (2008) Chronic low dose corticosterone exposure decreased hippocampal cell proliferation, volume and induced anxiety and depression like behaviours in mice. Eur J Pharmacol 583:115-127.

Nakamura K, Kikusui T, Takeuchi Y, Mori Y (2008) Changes in social instigation- and food restriction-induced aggressive behaviors and hippocampal 5HT1B mRNA receptor expression in male mice from early weaning. Behav Brain Res 187:442-448.

Neumaier JF, Sexton TJ, Hamblin MW, Beck SG (2000) Corticosteroids regulate 5-HT(1A) but not 5-HT(1B) receptor mRNA in rat hippocampus. Brain Res Mol Brain Res 82:65-73.

Nichol KE, Poon WW, Parachikova AI, Cribbs DH, Glabe CG, Cotman CW (2008) Exercise alters the immune profile in Tg2576 Alzheimer mice toward a response coincident with improved cognitive performance and decreased amyloid. Journal of neuroinflammation 5:13.

Noldus LP, Spink AJ, Tegelenbosch RA (2001) EthoVision: a versatile video tracking system for automation of behavioral experiments. Behavior research methods, instruments, & computers : a journal of the Psychonomic Society, Inc 33:398-414.

Ogoshi F, Yin HZ, Kuppumbatti Y, Song B, Amindari S, Weiss JH (2005) Tumor necrosis-factor-alpha (TNF-alpha) induces rapid insertion of Ca2+-permeable alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA)/kainate (Ca-A/K) channels in a subset of hippocampal pyramidal neurons. Exp Neurol 193:384-393.

Oh JE, Zupan B, Gross S, Toth M (2009) Paradoxical Anxiogenic Response of Juvenile Mice to Fluoxetine. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 34:2197-2207.

Ono M, Kikusui T, Sasaki N, Ichikawa M, Mori Y, Murakami-Murofushi K (2008) Early weaning induces anxiety and precocious myelination in the anterior part of the basolateral amygdala of male Balb/c mice. Neuroscience 156:1103-1110.

Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, Schroeder L, Aderem A (2000) The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc Natl Acad Sci U S A 97:13766-13771.

Papaioannou A, Gerozissis K, Prokopiou A, Bolaris S, Stylianopoulou F (2002) Sex differences in the effects of neonatal handling on the animal's response to stress and the vulnerability for depressive behaviour. Behav Brain Res 129:131-139.

Parachikova A, Nichol KE, Cotman CW (2008) Short-term exercise in aged Tg2576 mice alters neuroinflammation and improves cognition. Neurobiology of disease 30:121-129.

Parent JM, Janumpalli S, McNamara JO, Lowenstein DH (1998) Increased dentate granule cell neurogenesis following amygdala kindling in the adult rat. Neuroscience letters 247:9-12.

Parfitt DB, Levin JK, Saltstein KP, Klayman AS, Greer LM, Helmreich DL (2004) Differential early rearing environments can accentuate or attenuate the responses to stress in male C57BL/6 mice. Brain Res 1016:111-118.

Parks CL, Robinson PS, Sibille E, Shenk T, Toth M (1998) Increased anxiety of mice lacking the serotonin1A receptor. Proceedings of the National Academy of Sciences of the United States of America 95:10734-10739.

Parsey RV, Hastings RS, Oquendo MA, Huang YY, Simpson N, Arcement J, Huang Y, Ogden RT, Van Heertum RL, Arango V, Mann JJ (2006) Lower serotonin transporter binding potential in the human brain during major depressive episodes. Am J Psychiatry 163:52-58.

Patel TD, Zhou FC (2005) Ontogeny of 5-HT1A receptor expression in the developing hippocampus. Brain Res Dev Brain Res 157:42-57.

Paton JA, Nottebohm FN (1984) Neurons generated in the adult brain are recruited into functional circuits. Science 225:1046-1048.

Patrick J, Lindstrom J (1973) Autoimmune response to acetylcholine receptor. Science 180:871-872.

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. Journal of neuroscience methods 14:149-167.

Pereira AC, Huddleston DE, Brickman AM, Sosunov AA, Hen R, McKhann GM, Sloan R, Gage FH, Brown TR, Small SA (2007) An in vivo correlate of exercise-induced neurogenesis in the adult dentate gyrus. Proceedings of the National Academy of Sciences of the United States of America 104:5638-5643.

Plotsky PM, Meaney MJ (1993) Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. Brain Res Mol Brain Res 18:195-200.

Pollak DD, Monje FJ, Zuckerman L, Denny CA, Drew MR, Kandel ER (2008) An animal model of a behavioral intervention for depression. Neuron 60:149-161.

Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282:2085-2088.

Porsolt RD, Bertin A, Jalfre M (1977a) Behavioral despair in mice: a primary screening test for antidepressants. Archives Internationales de Pharmacodynamie et de Therapie 229:327-336.

Porsolt RD, Le Pichon M, Jalfre M (1977b) Depression: a new animal model sensitive to antidepressant treatments. Nature 266:730-732.

Porsolt RD, Anton G, Blavet N, Jalfre M (1978) Behavioural despair in rats: a new model sensitive to antidepressant treatments. Eur J Pharmacol 47:379-391.

Prut L, Belzung C (2003) The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur J Pharmacol 463:3-33.

Radley JJ, Jacobs BL (2002) 5-HT1A receptor antagonist administration decreases cell proliferation in the dentate gyrus. Brain Res 955:264-267.

Ramos A, Mormede P (1998) Stress and emotionality: a multidimensional and genetic approach. Neuroscience and biobehavioral reviews 22:33-57.

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.

Ramos A, Mellerin Y, Mormede P, Chaouloff F (1998) A genetic and multifactorial analysis of anxiety-related behaviours in Lewis and SHR intercrosses. Behav Brain Res 96:195-205.

Reif A, Schmitt A, Fritzen S, Lesch KP (2007) Neurogenesis and schizophrenia: dividing neurons in a divided mind? Eur Arch Psychiatry Clin Neurosci 257:290-299.

Revest JM, Dupret D, Koehl M, Funk-Reiter C, Grosjean N, Piazza PV, Abrous DN (2009) Adult hippocampal neurogenesis is involved in anxiety-related behaviors. Mol Psychiatry 14:959-967.

Rodgers RJ, Johnson NJ (1995) Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety. Pharmacology, biochemistry, and behavior 52:297-303.

Rogers SW, Andrews PI, Gahring LC, Whisenand T, Cauley K, Crain B, Hughes TE, Heinemann SF, McNamara JO (1994) Autoantibodies to glutamate receptor GluR3 in Rasmussen's encephalitis. Science 265:648-651.

Romeo RD, Mueller A, Sisti HM, Ogawa S, McEwen BS, Brake WG (2003) Anxiety and fear behaviors in adult male and female C57BL/6 mice are modulated by maternal separation. Horm Behav 43:561-567.

Roth BL, Hamblin MW, Ciaranello RD (1991) Developmental regulation of 5-HT2 and 5-HT1c mRNA and receptor levels. Brain Res Dev Brain Res 58:51-58.

Rudolph U, Crestani F, Benke D, Brunig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H, Mohler H (1999) Benzodiazepine actions mediated by specific gamma-aminobutyric acid(A) receptor subtypes. Nature 401:796-800.

Russo-Neustadt A, Beard RC, Cotman CW (1999) Exercise, antidepressant medications, and enhanced brain derived neurotrophic factor expression. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 21:679-682.

Sanai N, Tramontin AD, Quinones-Hinojosa A, Barbaro NM, Gupta N, Kunwar S, Lawton MT, McDermott MW, Parsa AT, Manuel-Garcia Verdugo J, Berger MS, Alvarez-Buylla A (2004) Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. Nature 427:740-744.

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O, Belzung C, Hen R (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science (New York, NY) 301:805-809.

Sapolsky RM, Armanini MP, Sutton SW, Plotsky PM (1989) Elevation of hypophysial portal concentrations of adrenocorticotropin secretagogues after fornix transection. Endocrinology 125:2881-2887.

Saxe MD, Malleret G, Vronskaya S, Mendez I, Garcia AD, Sofroniew MV, Kandel ER, Hen R (2007) Paradoxical influence of hippocampal neurogenesis on working memory. Proceedings of the National Academy of Sciences of the United States of America 104:4642-4646.

Scharfman HE, Hen R (2007) Neuroscience. Is more neurogenesis always better? Science (New York, NY) 315:336-338.

Scott BW, Wojtowicz JM, Burnham WM (2000) Neurogenesis in the dentate gyrus of the rat following electroconvulsive shock seizures. Exp Neurol 165:231-236.

Shanks N, Meaney MJ (1994) Hypothalamic-pituitary-adrenal activation following endotoxin administration in the developing rat: a CRH-mediated effect. Journal of neuroendocrinology 6:375-383.

Shanks N, Larocque S, Meaney MJ (1995) Neonatal endotoxin exposure alters the development of the hypothalamic-pituitary-adrenal axis: early illness and later responsivity to stress. The Journal of neuroscience : the official journal of the Society for Neuroscience 15:376-384.

Shanks N, Windle RJ, Perks PA, Harbuz MS, Jessop DS, Ingram CD, Lightman SL (2000) Early-life exposure to endotoxin alters hypothalamic-pituitary-adrenal function and predisposition to inflammation. Proceedings of the National Academy of Sciences of the United States of America 97:5645-5650.

Sheline YI, Sanghavi M, Mintun MA, Gado MH (1999) Depression duration but not age predicts hippocampal volume loss in medically healthy women with recurrent major depression. J Neurosci 19:5034-5043.

Simon NM, Zalta AK, Worthington JJ, 3rd, Hoge EA, Christian KM, Stevens JC, Pollack MH (2006) Preliminary support for gender differences in response to fluoxetine for generalized anxiety disorder. Depression and anxiety 23:373-376.

Smith KA, Fairburn CG, Cowen PJ (1997) Relapse of depression after rapid depletion of tryptophan. Lancet 349:915-919.

Smith T, Hewson AK, Quarrie L, Leonard JP, Cuzner ML (1994) Hypothalamic PGE2 and cAMP production and adrenocortical activation following intraperitoneal endotoxin injection: in vivo microdialysis studies in Lewis and Fischer rats. Neuroendocrinology 59:396-405.

Snyder JS, Radik R, Wojtowicz JM, Cameron HA (2008) Anatomical gradients of adult neurogenesis and activity: Young neurons in the ventral dentate gyrus are activated by water maze training. Hippocampus 19:360-370.

Solberg LC, Horton TH, Turek FW (1999) Circadian rhythms and depression: effects of exercise in an animal model. Am J Physiol 276:R152-161.

Solimena M, Folli F, Aparisi R, Pozza G, De Camilli P (1990) Autoantibodies to GABA-ergic neurons and pancreatic beta cells in stiff-man syndrome. N Engl J Med 322:1555-1560.

Spencer SJ, Heida JG, Pittman QJ (2005) Early life immune challenge--effects on behavioural indices of adult rat fear and anxiety. Behavioural brain research 164:231-238.

Spencer SJ, Martin S, Mouihate A, Pittman QJ (2006a) Early-life immune challenge: defining a critical window for effects on adult responses to immune challenge. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 31:1910-1918.

Spencer SJ, Boisse L, Mouihate A, Pittman QJ (2006b) Long term alterations in neuroimmune responses of female rats after neonatal exposure to lipopolysaccharide. Brain, behavior, and immunity 20:325-330.

Spencer SJ, Hyland NP, Sharkey KA, Pittman QJ (2007) Neonatal immune challenge exacerbates experimental colitis in adult rats: potential role for TNF-alpha. American journal of physiologyRegulatory, integrative and comparative physiology 292:R308-315.

Steiner M, Allgulander C, Ravindran A, Kosar H, Burt T, Austin C (2005) Gender differences in clinical presentation and response to sertraline treatment of generalized anxiety disorder. Human psychopharmacology 20:3-13.

Surget A, Saxe M, Leman S, Ibarguen-Vargas Y, Chalon S, Griebel G, Hen R, Belzung C (2008) Drug-dependent requirement of hippocampal neurogenesis in a model of depression and of antidepressant reversal. Biol Psychiatry 64:293-301.

Sutcigil L, Oktenli C, Musabak U, Bozkurt A, Cansever A, Uzun O, Sanisoglu SY, Yesilova Z, Ozmenler N, Ozsahin A, Sengul A (2007) Pro- and anti-inflammatory cytokine balance in major depression: effect of sertraline therapy. Clin Dev Immunol 2007:76396.

Tanapat P, Galea LA, Gould E (1998) Stress inhibits the proliferation of granule cell precursors in the developing dentate gyrus. Int J Dev Neurosci 16:235-239.

Tannenbaum B, Tannenbaum GS, Sudom K, Anisman H (2002) Neurochemical and behavioral alterations elicited by a chronic intermittent stressor regimen: implications for allostatic load. Brain Res 953:82-92.

Tenk CM, Kavaliers M, Ossenkopp KP (2008) Sexually dimorphic effects of neonatal immune system activation with lipopolysaccharide on the behavioural response to a homotypic adult immune challenge. International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience 26:331-338.

Tenk CM, Foley KA, Kavaliers M, Ossenkopp KP (2007) Neonatal immune system activation with lipopolysaccharide enhances behavioural sensitization to the dopamine agonist, quinpirole, in adult female but not male rats. Brain, behavior, and immunity 21:935-945.

Toni N, Laplagne DA, Zhao C, Lombardi G, Ribak CE, Gage FH, Schinder AF (2008) Neurons born in the adult dentate gyrus form functional synapses with target cells. Nature neuroscience 91: 901-907.

Torres C, Escarabajal MD (2002) Validation of a behavioral recording automated system in the elevated plus-maze test. Life Sciences 70:1751-1762.

Tozuka Y, Fukuda S, Namba T, Seki T, Hisatsune T (2005) GABAergic excitation promotes neuronal differentiation in adult hippocampal progenitor cells. Neuron 47:803-815.

Trejo JL, Llorens-Martin MV, Torres-Aleman I (2008) The effects of exercise on spatial learning and anxiety-like behavior are mediated by an IGF-I-dependent mechanism related to hippocampal neurogenesis. Molecular and cellular neurosciences 37:402-411.

Trullas R, Skolnick P (1993) Differences in fear motivated behaviors among inbred mouse strains. Psychopharmacology (Berl) 111:323-331.

Turgeon L, Marchand A, Dupuis G (1998) Clinical features in panic disorder with agoraphobia: a comparison of men and women. Journal of anxiety disorders 12:539-553.

Turri MG, Datta SR, DeFries J, Henderson ND, Flint J (2001) QTL analysis identifies multiple behavioral dimensions in ethological tests of anxiety in laboratory mice. Curr Biol 11:725-734.

Vakharia K, Hinson JP (2005) Lipopolysaccharide directly stimulates cortisol secretion by human adrenal cells by a cyclooxygenase-dependent mechanism. Endocrinology 146:1398-1402.

Vallieres L, Campbell IL, Gage FH, Sawchenko PE (2002) Reduced hippocampal neurogenesis in adult transgenic mice with chronic astrocytic production of interleukin-6. J Neurosci 22:486-492.

van Gaalen MM, Steckler T (2000) Behavioural analysis of four mouse strains in an anxiety test battery. Behavioural brain research 115:95-106.

van Praag H, Kempermann G, Gage FH (1999) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nature neuroscience 2:266-270.

van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH (2002) Functional neurogenesis in the adult hippocampus. Nature 415:1030-1034.

Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo CA (2005) Neuroglial activation and neuroinflammation in the brain of patients with autism. Ann Neurol 57:67-81.

Vazquez DM, Lopez JF, Van Hoers H, Watson SJ, Levine S (2000) Maternal deprivation regulates serotonin 1A and 2A receptors in the infant rat. Brain research 855:76-82.

Veena J, Srikumar BN, Raju TR, Shankaranarayana Rao BS (2009) Exposure to enriched environment restores the survival and differentiation of new born cells in the hippocampus and ameliorates depressive symptoms in chronically stressed rats. Neurosci Lett 455:178-182.

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). The Journal of clinical psychiatry 69:1606-1616.

Vinkers CH, Oosting RS, van Bogaert MJ, Olivier B, Groenink L (2009) Early-Life Blockade of 5-HT(1A) Receptors Alters Adult Anxiety Behavior and Benzodiazepine Sensitivity. Biol Psychiatry.

Vollmayr B, Simonis C, Weber S, Gass P, Henn F (2003) Reduced cell proliferation in the dentate gyrus is not correlated with the development of learned helplessness. Biol Psychiatry 54:1035-1040.

Walker AK, Nakamura T, Byrne RJ, Naicker S, Tynan RJ, Hunter M, Hodgson DM (2009a) Neonatal lipopolysaccharide and adult stress exposure predisposes rats to anxiety-like behaviour and blunted corticosterone responses: Implications for the double-hit hypothesis. Psychoneuroendocrinology 34:1515-1525.

Walker FR, March J, Hodgson DM (2004a) Endotoxin exposure in early life alters the development of anxiety-like behaviour in the Fischer 344 rat. Behavioural brain research 154:63-69.

Walker FR, Knott B, Hodgson DM (2008) Neonatal endotoxin exposure modifies the acoustic startle response and circulating levels of corticosterone in the adult rat but only following acute stress. Journal of psychiatric research 42:1094-1103.

Walker FR, Hodyl NA, Hodgson DM (2009b) Neonatal bacterial endotoxin challenge interacts with stress in the adult male rat to modify KLH specific antibody production but not KLH stimulated ex vivo cytokine release. J Neuroimmunol 207:57-65.

Walker FR, Brogan A, Smith R, Hodgson DM (2004b) A profile of the immediate endocrine, metabolic and behavioural responses following a dual exposure to endotoxin in early life. Physiology & behavior 83:495-504.

Wang DD, Kriegstein AR (2009) Defining the role of GABA in cortical development. J Physiol 587:1873-1879.

Weinstock LS (1999) Gender differences in the presentation and management of social anxiety disorder. The Journal of clinical psychiatry 60 Suppl 9:9-13.

Weiss SM, Wadsworth G, Fletcher A, Dourish CT (1998) Utility of ethological analysis to overcome locomotor confounds in elevated maze models of anxiety. Neuroscience and biobehavioral reviews 23:265-271.

Whiteside MB, Quan N, Herkenharn M (1999) Induction of pituitary cytokine transcripts by peripheral lipopolysaccharide. J Neuroendocrinol 11:115-120.

Wigger A, Neumann ID (1999) Periodic maternal deprivation induces gender-dependent alterations in behavioral and neuroendocrine responses to emotional stress in adult rats. Physiol Behav 66:293-302.

Winocur G, Wojtowicz JM, Sekeres M, Snyder JS, Wang S (2006) Inhibition of neurogenesis interferes with hippocampus-dependent memory function. Hippocampus 16:296-304.

Woolley DW (1963) The Biochemical Bases of Psychoses or the Serotonin Hypothesis about Mental Illness. New York, NY: John Wiley and Sons, Inc.

Yan W, Wilson CC, Haring JH (1997) 5-HT1a receptors mediate the neurotrophic effect of serotonin on developing dentate granule cells. Brain Res Dev Brain Res 98:185-190.

Yoshikawa T, Watanabe A, Ishitsuka Y, Nakaya A, Nakatani N (2002) Identification of multiple genetic loci linked to the propensity for "behavioral despair" in mice. Genome Res 12:357-366.

Young R, Johnson DN (1991) A fully automated light/dark apparatus useful for comparing anxiolytic agents. Pharmacol Biochem Behav 40:739-743.

Zanettini C, Carola V, Lo Iacono L, Moles A, Gross C, D'Amato FR (2009) Postnatal handling reverses social anxiety in serotonin receptor 1A knockout mice. Genes Brain Behav (Aug 8; ahead of print).

APPENDIX.

Validation of an automated system for measuring anxiety-related behaviours in the elevated plus maze

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Abstract

The elevated plus maze (EPM) is one of the most widely used tests to assess anxiety-related behaviours in rodents. Although relatively quick and simple to conduct, there always exists the potential for observer bias during data collection. A number of behavioural tests have been automated with the advantage of rapidity of data collection and circumvention of observer bias in obtaining measures. The KinderScientific EPM system uses a series of apparatus embedded photobeams to collect spatiotemporal measures such as the amount of time spent in each zone of the maze (centre, open and closed arms), and the frequency of arm entries. Risk assessment behaviours, such as head dips and protected stretches, are also measured which represents a unique feature of this system over other automated EPM systems. We compared observer spatiotemporal and risk assessment measures with automated generated data to test the reliability and accuracy of the automated system. Data was manually collected using different zone entry/exit criteria (2 vs. 4 paws). Automated data was generated using both the default zone map provided with the system and a user-modified zone map. We show that the automated EPM provides accurate and reliable measurements of spatiotemporal and risk assessment behaviours. In addition, we show that the default zone map overestimated visually observed arm entries while our modified map generated data comparable to manual data using a 4 paws open arm entry criteria which is most consistently used to define arm entry in the literature. The KinderScientific automated EPM system represents a reliable tool for collection of a wide range of anxiety-related measures.

1. Introduction

Since its inception over twenty years ago (Handley and Mithani, 1984) the elevated plus maze (EPM) has become a widely used behavioural tool to assess the efficacy of anxiety modifying interventions (Handley and Mithani, 1984; Pellow and File, 1986; Lister, 1987) and to explore the neurobiological basis of anxiety (File et al., 1998; Lacroix et al., 2000; Kjelstrup et al., 2002; Holmes et al., 2003; Bissiere et al., 2006). The original Y-maze formation (Montgomery, 1955) was modified to include two opposing open arms perpendicular to two closed arms (Handley and Mithani, 1984) and has since been validated for use in both rats (Pellow et al., 1985; Pellow and File, 1986) and mice (Lister, 1987). The plus-maze is purported to be an ethologically relevant rodent test that creates a conflict between the innate curiousity to explore novel environments and natural aversion to potentially threatening areas, such as the unprotected open arms of the plus-maze (Lister, 1990; Rodgers and Dalvi, 1997). Accordingly, anxiety is measured by the extent to which an animal explores the aversive open arms, with decreased exploration indicating enhanced anxiety-like behaviour. Indeed, pharmacological treatments that modify anxiety alter time spent in, and entries into, the open arms (Pellow and File, 1986; Lister, 1987). Various other spatiotemporal and ethological measures can be obtained during testing (Rodgers and Johnson, 1995). Closed arm or total arm entries are often reported as an index of protected exploration and locomotor activity (Handley and Mithani, 1984; Lister, 1987). Risk assessment behaviours such as open arm head dips and protected stretches into the open arms can also be measured (Cruz et al., 1994; Rodgers and Johnson, 1995; Fernandes and File,

1996; Fernandez Espejo, 1997). These latter 'non-traditional' behavioural measures are important to consider given their proposed utility as a more sensitive index of emotional-reactivity (Cole and Rodgers, 1994; Weiss et al., 1998; Roy and Chapillon, 2004).

A number of variables have been identified that can influence the results obtained during plus-maze testing. Such variables can be defined rather broadly into organismic and procedural (Carobrez and Bertoglio, 2005). Organism variables such as species, strain, and age are, ostensibly, more easily controlled for than certain procedural variables that rely heavily on the subjective scoring of behaviour. Indeed, observer-related variability in the definition of what constitutes a particular behaviour, and the technique used to score them, can contribute to inconsistent reporting both within and across laboratories. Automated systems have been introduced for a wide range of rodent behavioural tests, with the advantage of circumventing the subjective nature of behavioural scoring (Young and Johnson, 1991; Noldus et al., 2001; Spink et al., 2001; Torres and Escarabajal, 2002; Kurtuncu et al., 2005). These systems rely on either video tracking of a rodent's behaviour or use a series of apparatus embedded lasers that translate beam breaks into spatiotemporal measures. Even with the advent of these automated systems, however, non-traditional risk assessment behaviours are typically not measured. Given that such measures have been proposed as a more sensitive index of anxiety-like behaviour (Cole and Rodgers, 1994; Roy and Chapillon, 2004), an automated system that provides their objective measurement would undoubtedly offer an advantage over other systems. KinderScientific's automated plus maze system uses a series of infrared photobeams to obtain both traditional spatiotemporal measures of time and entry,

and more non-traditional risk assessment behaviours such as open arm head dips and protected stretches into the open arms.

We tested the automated system by comparing data generated by manual scoring what that generated by the automated system. Outcome measures compared were time spent in zone (open arm, closed arm, centre), entries into arms, poke arounds, and head dips. Manual scoring included tabulating and comparing time and entry based on different zone entry/exit criteria. This was performed in order to determine the criteria used by the automated system and the provided default zone map settings. A user-modified zone map was created and values obtained with this, and the default zone map, were then compared against manually generated data. We also show additional automated generated data such as distance travelled and rest time for each zone.

2. Methods

2.1 Animals. Twenty adult C57BL/6 mice (12 males, 8 females) 14-16 weeks in age were housed in groups of 2-4 per cage with food and water available *ad libitum.* All animals were kept under a 12 h light-12 h dark cycle, with lights on at 7 AM. Housing room temperature was maintained at 20°C and humidity at 60-70%. All experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Research Ethics Board, McMaster University, Hamilton, Ontario, Canada.

2.2. Elevated plus maze apparatus. The automated elevated plus maze apparatus from KinderScientific (Poway, CA) was used to assess anxiety-like behaviour. This apparatus is elevated 76.2 cm off the ground and consists of four black plexiglas arms in the shape of a plus (Fig. 1). Two of the four arms are considered enclosed arms as they contain 15.2 cm black plexiglas sides. The two open arms have small raised ledges (0.5cm) situated along the perimeter. The automated EPM operates with infrared photobeams designed to detect movement throughout the maze. Thirty-two electronic photobeams are situated along the extent of the maze with eight beams located on each the four arms. Two of these eight beams are used to detect intersection activity. On each open arm there are two pairs of censors placed on either side of the arm just below the edge. These censors detect head dips over the edge of the open arm.

2.3. Testing procedure. Behavioural testing occurred during the light phase between 10:00-3:00 h. Animals were brought into the non-colony behavioural room in their home cage and testing commenced when placed into the centre of the elevated plus maze facing the closed arm. The apparatus was situated in the middle of the testing room under standard overhead fluorescent lighting. The apparatus was thoroughly cleaned with water and dried between each animal. Behavioural data were collected using the MotorMonitor software package on a PC computer connected to the EPM. Each test session was simultaneously videotaped using a Canon Elura100 camcorder.

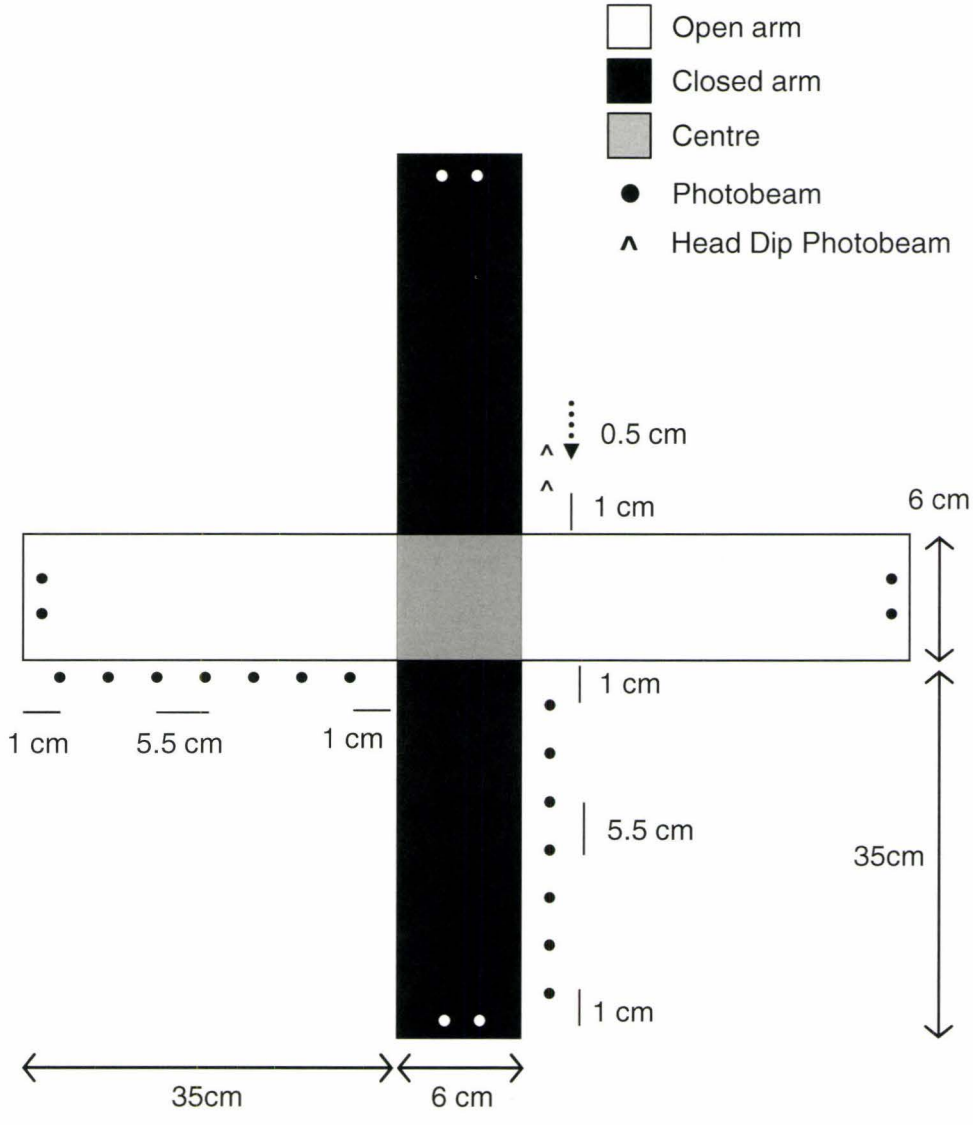

Figure 1. Schematic representation of the KinderScientific elevated plus maze system detailing apparatus dimensions and photobeam locations.

2.4. Automated scoring. Data collected were reduced using the MotorMonitor software (supplied by KinderScientific). This software offers users the flexibility of using preloaded zone maps or generating user-specific zone maps. The default zone map settings are depicted in Figure 2. Data from the open/closed arms and center were generated along the following parameters: (1) Time, which measures total time spent in a given zone, (2) Distance travelled within a zone, (3) Entries into open and closed arms (4) Poke arounds (protected stretches) which measures extension of the head into and around the corner of the open arms with the body primarily situated in the intersection or enclosed arms, (5) Head dips, which involves extension of the head and shoulders over the edge of the open arms, (6) Rest time, which occurs when no beam breaks are made within a user-defined amount of time in a particular zone. The data set generated for any given zone map based on the above parameters was transferred to an excel spreadsheet for user interface.

2.5. Manual scoring. Videotapes were analyzed by two trained observers who recorded both conventional spatiotemporal measures in addition to risk assessment behaviours. Spatiotemporal measures included the frequency of open and closed arm entries and the time spent in the open, closed and centre zones of the maze. Observers scored arm entry based on two definitions: two forepaws (two-paw) required versus all four paws required for entry into a given zone. Risk assessment behaviours such as head dipping (exploratory movement of head/shoulders over side of open arm) and protected stretches (a forward extension of the head into, and around the corner of, the open arms with the body primarily situated in the intersection or enclosed arms) were also measured.

Both the closed arm and central platform were considered "protected" areas of the maze whereas the open arm was considered unprotected (Rodgers and Johnson, 1995).

2.6. Statistical analysis. Data generated by manual scoring and automated scoring for different outcome measures were compared using one-way ANOVA followed by Bonferroni's Multiple comparison test. Data generated by each observer (manual) were tested for normality using the D'Agostino & Pearson omnibus normality test. Inter-rater reliability for manual scoring was determined using the Pearson correlation coefficient. A p-value less than 0.05 was considered statistically significant. All analyses were performed using the Prism 5.0b software for Macintosh.

3. Results

3.1. Analysis of time spent in the open arms, centre, and closed arms

Manual scoring data was generated via videotape analysis by 2 trained observers. Open arm and closed arm time were determined using 4 different criteria: 1) 2 paws in = entry, 2 paws out = exit; 2) 2 paws in = entry, 4 paws out = exit; 3) 4 paws in = entry, 2 paws out = exit; 4) 4 paws in = entry, 4 paws out = exit. Data for each observer (n=20) using the above criteria was confirmed to have a normal distribution using the D'Agostino & Pearson omnibus normality test ($p>0.05$). Inter-rater reliability was calculated using Pearson correlation and is shown in Table 1. Automated scoring was generated by

Table 1. Inter-rater reliability determined by Pearson's correlation

Manual scoring criteria used				
Entry	2 paws in	2 paws in	4 paws in	4 paws in
Exit	2 paws out	4 paws out	2 paws out	4 paws out
	Correlation of Observer 1 and 2 (p value)			
Open Arm Time	0.986* (<0.00001)	0.987* (<0.00001)	0.918* (<0.00001)	0.937* (<0.00001)
Closed Arm Time	0.916* (<0.00001)	0.898* (<0.00001)	0.925* (<0.00001)	0.947* (<0.00001)
Centre Time	0.835* (<0.00001)	0.470* (0.036)	0.788* (<0.00001)	0.634* (0.0027)

	2 paw entry	4 paw entry
Open Arm	0.967* (<0.00001)	0.914* (<0.00001)
Closed Arm	0.977* (<0.00001)	0.995* (<0.00001)
Head Dips	0.916* (<0.00001)	
Poke Arounds	0.579* (0.0094)	

Pearson correlation *r* values are given; *p<0.05.

*significant correlation between observers

MotorMonitor software using the default zone map (Fig. 2A) and using a modified zone map (Fig. 2B).

Open arm time and closed arm time for manual and automated scoring are shown in Figure 3. A significant main effect of criteria used was observed for open arm time ($F_{4,99}$=11.3, $p<0.0001$). Post-hoc analysis showed a significant difference in open arm time between manual scoring using criteria 1/2 (2 paws in/2 paws out and 2 paws in/4 paws out) and criteria 3/4 ($p<0.05$, 4 paws in/2 paws out and 4 paws in/4 paws out) demonstrating that entry criteria was the key determinant to this outcome measure. A significant difference in open arm time was observed between the default and modified zone maps ($p<0.05$). Qualitatively and statistically, it was determined that the default zone map produced open arm time data that was the same as manual scoring data generated using 2 paws in = entry (criteria 1 and 2). The modified zone map generated open arm time data that was the same as manual scoring data using criteria 3 and 4 (4 paws in). A main effect of criteria was observed for closed arm time ($F_{4,99}$=4.62, $p=0.002$). Posthoc analysis showed a significant difference between the default and modified zone maps for closed arm time ($p<0.05$) but no significant differences between manual scoring criteria. Manual scoring for closed arm time using criteria 2 and 4 (4 paws out) matched the automated scoring using the default zone map.

As entries and exits into the centre zone are essentially returns from the open or closed arms, centre time was scored manually using the inverse of the 4 criteria used above, that is, 1) 2 paws in = entry, 2 paws out = exit, 2) 4 paws in = entry, 2 paws out =

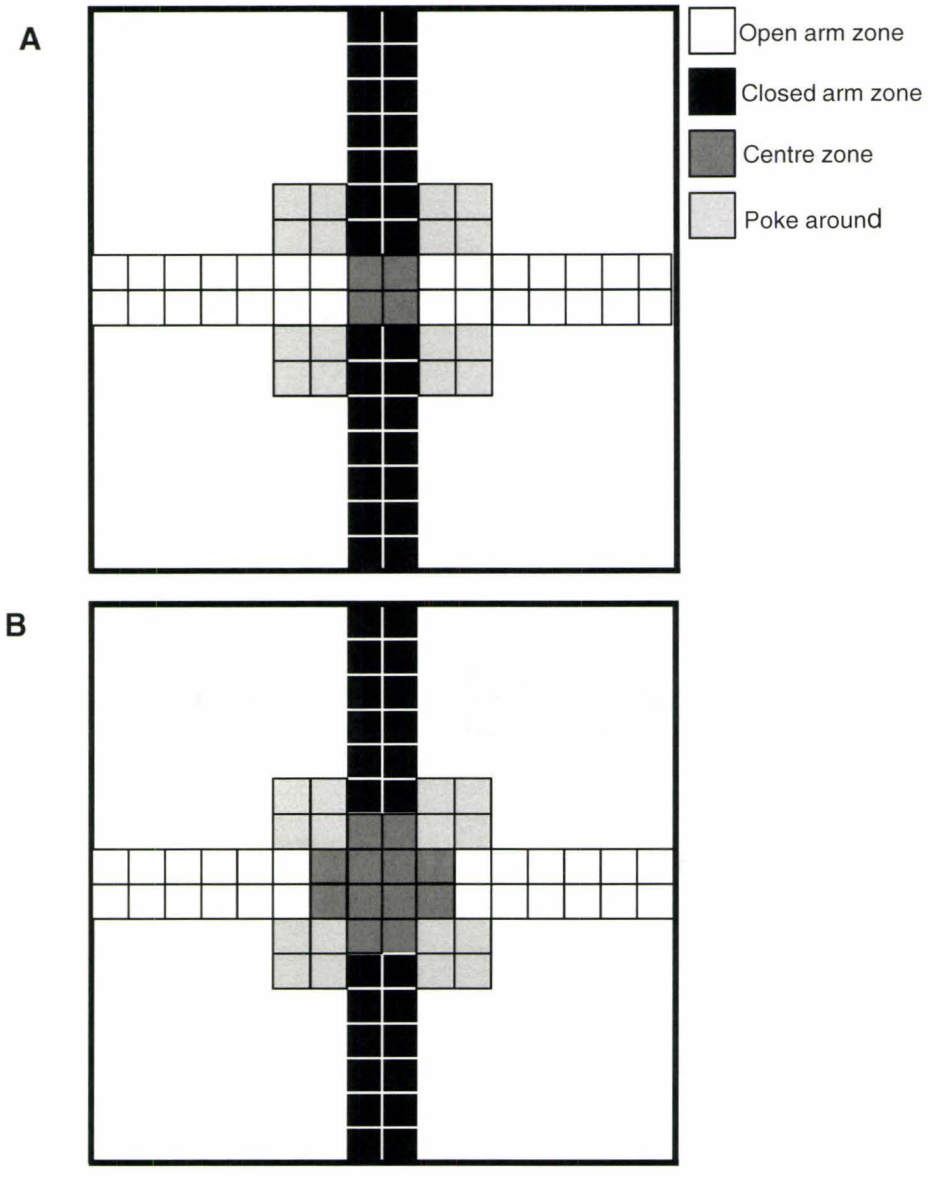

Figure 2. Zone maps are virtual representations of the EPM that the software uses to generate data. Both the default zone map (**A**) and a user-modified zone map (**B**) are depicted. The modified zone map virtually extends the centre zone one segment into the open and closed arms.

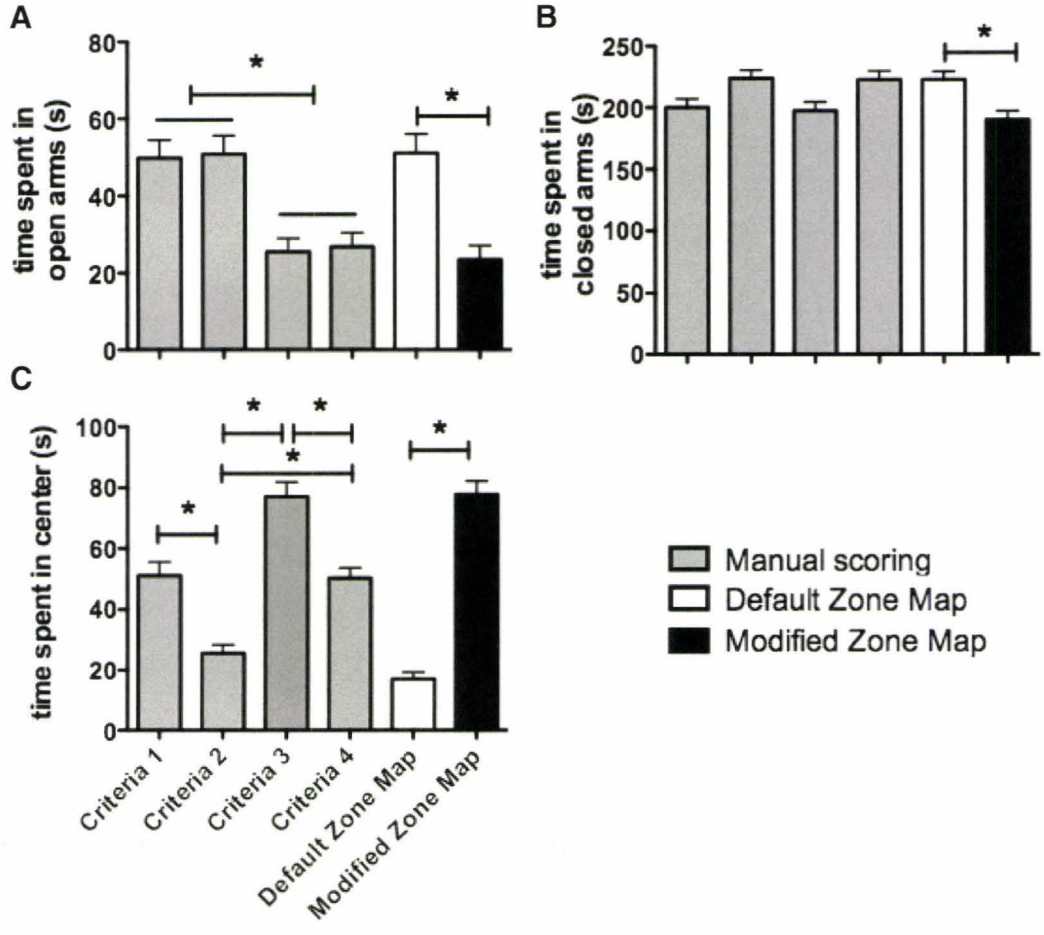

Figure 3. Comparison of manual and automated (default and modified zone map) data for time spent in the open arms (A) closed arms (B) and centre (C). Two observers scored data (n=20) using different zone arm/exit criteria (below). (**A**) Open arm time was sensitive to the entry/exit criterion used: open time 2-paw > 4-paw. Open arm time generated using the default ZM > modified ZM. Manual scores using a 2-paw entry criterion (1/2) were similar to the default ZM; manual scores using a 4-paw criterion (3/4) were similar to the modified ZM. (**B**) The default ZM generated significantly greater closed arm time values than the modified ZM. (**C**) Centre time was similar between a 4-paw entry criterion (#2) and the default ZM, and between a 2-paw entry (#3) and the modified ZM. Note that centre entry criterion is the inverse of open/closed arm. Open/closed arm entry/exit criteria: 1) 2 paws in = entry, 2 paws out = exit; 2) 2 paws in = entry, 4 paws out = exit; 3) 4 paws in = entry, 2 paws out = exit; 4) 4 paws in = entry, 4 paws out = exit. ZM=zone map. *$p<0.05$.

exit, 3) 2 paws in = entry, 4 paws out = exit, 4) 4 paws in = entry, 4 paws out = exit. By inverting the criteria, total time for each mouse was equal to the total time of the test (5 min). A main effect of criteria was observed for centre time ($F_{5,119}$=43.5, $p<0.001$). Manual scoring showed statistically different centre time values for criteria 2 vs. 3 vs. 1 and 4 ($p<0.05$). A significant difference was observed between the default and modified zone map ($p<0.05$). A qualitative and statistical match was observed between the default zone map score and the manual score using criteria 2; between the modified zone map and criteria 3.

3.2. Analysis of open and closed arm entries

Manual scoring of arm entries used 2 criteria; 2 paw entry and 4 paw entry. Data for each observer using each criteria was determined to have a normal distribution using the D'Agostino & Pearson omnibus normality test ($p>0.05$). Inter-rater reliability was calculated using Pearson correlation and is shown in Table 1. Automated scoring was generated by MotorMonitor software as above. Data generated using manual and automatic scoring for open and closed arm entries are shown in Figure 4. A significant main effect of criteria was observed for open arm entries ($F_{3,79}$=39.5, $p<0.0001$). Posthoc analysis showed a significant difference between 2 paw entry and 4 paw entry criteria for manual scoring ($p<0.05$) and a significant difference between the default and modified zone map ($p<0.05$). Qualitatively and statistically, manual scoring of open arm entries as 4 paw entry was comparable to open arm entries provided by the modified zone map. The

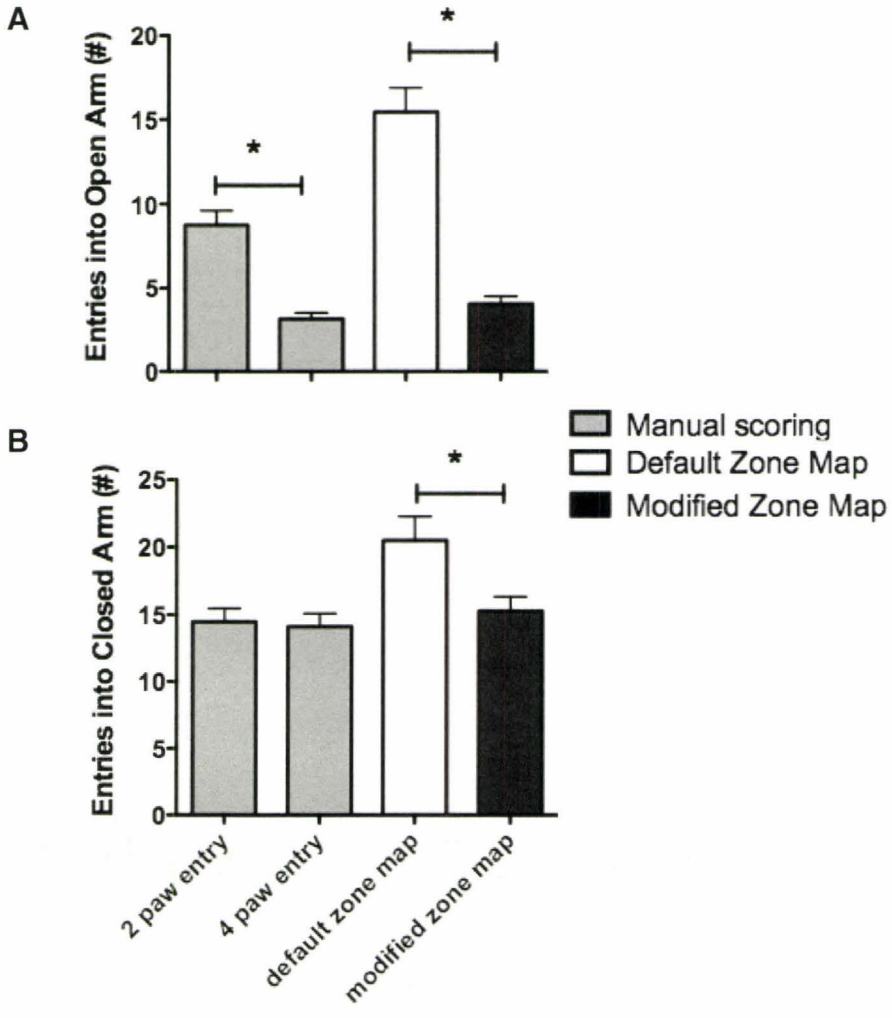

Figure 4. Manual and automated (default and modified zone map) generated data for entries into the open (A) and closed (B) arms. Two observers scored data (n=20) using a 2 vs. 4 paw entry criterion. (**A**) Entries into open arm were sensitive to the entry definition used as manual values using a 2-paw entry > than a 4-paw entry. Default ZM values are qualitatively similar to a manual 2-paw open arm entry criteria; modified ZM values are similar to a manual 4-paw criteria. (**B**) Values for closed arm entry were inflated when using the default ZM relative to the modified ZM and either of the manual entry criteria. ZM=zone map. *$p<0.05$.

default zone map detected significantly more open arm entries than manual scoring using 2 paw entry as a criteria ($p<0.05$). The basis of this difference is not clear.

Data generated for closed arm entries using manual and automated scoring is presented in Figure 4B. A significant main effect of criteria used for closed arm entries was observed ($F_{3,79}=5.7$, $p=0.0014$). Posthoc analysis showed a significant difference between the default and modified zone map ($p<0.05$). No difference was observed in closed arm entries using a 2 paw or 4 paw entry. Qualitatively and statistically the modified zone map provided closed arm entry values that matched the manual scoring values. The default zone map generated closed arm entry values that were significantly higher than both manual scoring criteria. This increase in closed arm entries using the default zone map is consistent with the observations for open arm entries, suggesting that automated scoring using the default zone map gives an overestimate of visually observed zone entries.

3.3. Analysis of risk assessment behaviours

Given the placement of the photobeams associated with head dips and poke arounds, either zone map provided identical automated generated values (data not shown). Head dips were manually defined as a dip of the animal's head and shoulders over the sides of the open arm. For automated scoring, the software generates values for both sides of each open arm, these values were summed to get the total number of head dips per animal. Protected stretches, or poke arounds as termed by the plus-maze software, were manually scored as an extension of the head into and around the corner of

the open arm with the animal's body primarily situated in either the protected closed arms or centre. Manual and automated values for head dips are given in Figure 5A. Inter-rater reliability was calculated using Pearson correlation and is shown in Table 1. No significant difference was observed between the manual score and automated score generated for head dips ($t=1.08$, $df=38$, $p=0.286$). Manual and automated values for poke arounds are given in Figure 5B. Inter-rater reliability for poke arounds was calculated using Pearson correlation and is shown in Table 1. No significant difference was observed between the manual score and automated score generated for poke arounds ($t=0.681$, $df=36$, $p=0.500$). These data demonstrate that automated scoring methods provide an accurate and reliable measure of risk assessment behaviours.

3.4. Additional outcome measures generated by automated scoring

Several additional data measures are provided by the MotorMonitor software including basic movements, immobility, fine movements, x ambulation, and y ambulation; these measures are not specific to the different zones but can be used to assess general activity. Total distance traveled is a measure of activity and is generated by the automated system for each zone. In addition, rest time, a measure of immobility, is generated for each zone. Data for these measures are presented in Figure 6. No difference was observed between the distance travelled using the default and modified zone maps (Fig.6A, $p<0.05$). As expected, the values generated by the modified zone

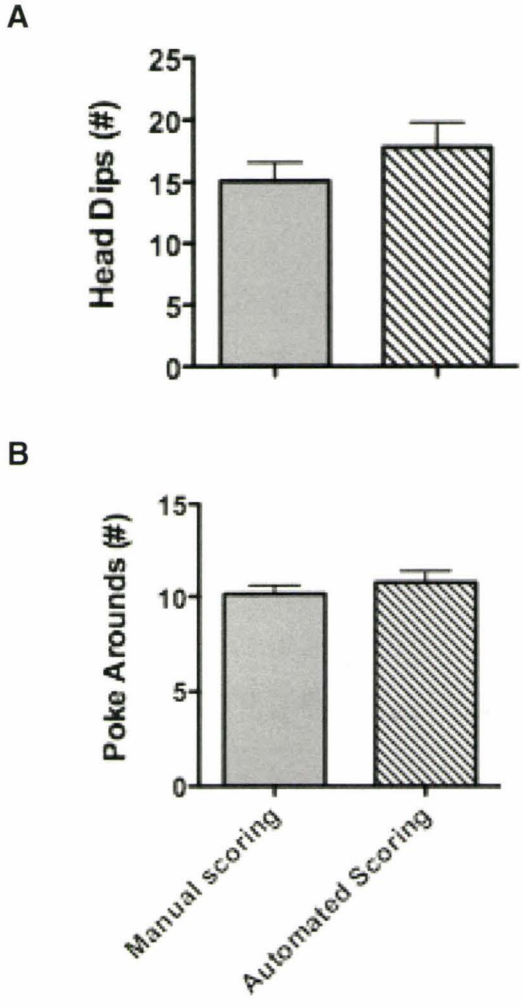

Figure 5. Manual and automated scores of risk assessment behaviours. The automated system provides a reliable measure of risk assessment as there were no qualitative or statistically significant differences between manual and automated generated values for number of head dips (**A**) or poke arounds (**B**).

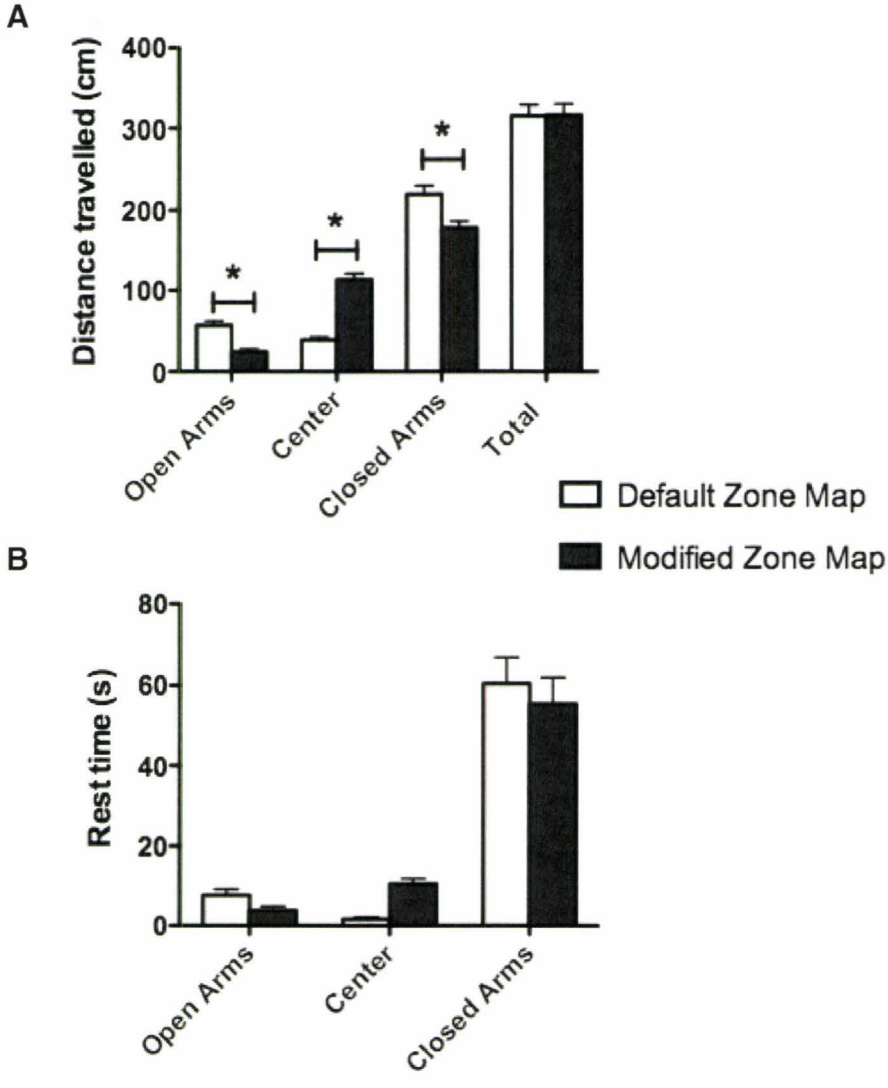

Figure 6. Automated data for indices of locomotor activity. Distance travelled (A) and rest time (B) are calculated for each zone of the plus-maze. (**A**) Compared with the modified ZM, the default zone map generates higher values for distance travelled in the open/closed arm and decreased values for centre distance. (**B**) Rest time in each zone was not sensitive to the choice of zone map. *$p<0.05$.

map showed a significant decrease in open and closed arm distance with a corresponding significant increase in centre distance ($p<0.05$). Rest time (Fig. 6B) was greatest in the closed arms. There was no significant difference between values generated for rest time using the default and modified zone maps (ANOVA, Bonferroni, $p<0.05$).

4. Discussion

The data presented above demonstrate that the automated elevated plus maze system provides an accurate and reliable method for obtaining spatiotemporal measures such as time spent in a given zone and arm entries. A unique and strong advantage is the ability of the automated system to accurately measure risk assessment behaviours such as head dips and protected stretches. The system also provides activity data such as distance travelled and rest time in each zone which is useful for determining general locomotion during testing.

A potential problem with automated systems is their lack of sensitivity in detecting the position of a rodent's forepaws for arm entry in the plus-maze (Torres and Escarabajal, 2002). This is important to consider given that using a four paw versus two paw requirement for arm entry, will undoubtedly change the spatiotemporal values provided by the automated system. Demarcations between different areas of the KinderScientific plus-maze (open/closed arm and centre) are dependant upon virtual zones that are preset by the software and provided as the default setting. Entry into these zones is highly sensitive to the distance that an animal travels into them. Since the

definition of what constitutes entry into a zone depends on the placement and triggering of a photobeam, the first step in validating the system was to compare and correlate time and entries based on different manual scoring criteria (two versus four paw entry) with the data generated by the automated EPM. This enabled determination of the preset default criteria for arm entry used by the automated software. The literature often cites that all four paws are required to constitute arm entry (Walf and Frye, 2007). To enable the automated system to accurately reflect this definition, a modified zone map was generated to capture this entry definition. The modified zone map is depicted in Figure 2B. This modified map extends the center zone one segment into each of the arms. By virtually extending the centre zone, an animal must travel farther into an arm, with all four paws required to trigger the photobeams associated with the newly defined open/closed zone. Indeed, the results show that the modified zone map generated data that was comparable to manually generated data using 4 paws in as criteria for zone entry into the open arms. In addition, the modified zone map generated arm entry data that matched the manual scoring data, while the default zone map resulted in an overestimate of visually observed arm entries. Given that the software offers the flexibility of virtually changing the default zone maps, we were able to create a zone map sensitive to a four paw entry criteria which is most consistently used to define arm entry (Walf and Frye, 2007).

Risk assessment behaviours considered were head dips and poke arounds or protected stretches. The manual scoring data and the automated scoring data did not

differ on these measures indicating that the automated system provides an accurate measurement of risk assessment behaviours. There is a growing awareness that risk assessment be measured as it provides a more complete profile of anxiety-related behaviours and may be a more sensitive index of emotional-reactivity (Cole and Rodgers, 1994; Rodgers and Dalvi, 1997; Roy and Chapillon, 2004). Indeed, the efficacy of the EPM in screening anxiety modifying interventions has increased with the use of ethological measures such as head dipping and protected stretches (Cruz et al., 1994; Rodgers and Johnson, 1995; Weiss et al., 1998). The subjective nature of manually scoring risk assessment behaviours, however, can lead to variability between observers, within a group, and between labs. Our data show that the automated system can provide an accurate measure of risk assessment behaviours and eliminates user-bias in scoring.

One additional advantage of the automated system is the ability to collect activity data including distance travelled and rest time in a zone which is important in ascertaining the general mobility or locomotor capacity of an animal during testing. This also adds another dimension to interpreting time spent in a given arm. For instance, although time spent in a zone may be similar between two animals, there is a qualitative difference in an animal that spends most of its time resting in an arm versus one that actively explores it.

One caveat is that our system was not validated for use with juvenile mice. Young mice are invariably smaller than the adults tested here and may have to travel farther into an arm to trigger a photobeam. In this case, the photobeams associated with our modified zone map may extend too far into an arm and the default zone map may

suffice. Individual labs planning on using juvenile rodents should test the sensitivity of the default zone map against our modified zone map for detection of arm entry.

In addition to accurate collection of traditional spatiotemporal measures, we demonstrated the accuracy of the automated system to collect risk assessment behaviours. This is the first report validating an automated EPM system that reliably measures both spatiotemporal and risk assessment behaviours. The current study demonstrates the advantage of the KinderScientific automated system in terms of rapidity, accuracy, and reliability and represents a high-throughput method by which to collect a full repertoire of anxiety-related behavioural measures.

5. References

Bissiere S, McAllister KH, Olpe HR, Cryan JF (2006) The rostral anterior cingulate cortex modulates depression but not anxiety-related behaviour in the rat. Behav Brain Res 175:195-199.

Carobrez AP, Bertoglio LJ (2005) Ethological and temporal analyses of anxiety-like behavior: the elevated plus-maze model 20 years on. Neuroscience and biobehavioral reviews 29:1193-1205.

Cole JC, Rodgers RJ (1994) Ethological evaluation of the effects of acute and chronic buspirone treatment in the murine elevated plus-maze test: comparison with haloperidol. Psychopharmacology 114:288-296.

Cruz AP, Frei F, Graeff FG (1994) Ethopharmacological analysis of rat behavior on the elevated plus-maze. Pharmacology, biochemistry, and behavior 49:171-176.

Fernandes C, File SE (1996) The influence of open arm ledges and maze experience in the elevated plus-maze. Pharmacology, biochemistry, and behavior 54:31-40.

Fernandez Espejo E (1997) Structure of the mouse behaviour on the elevated plus-maze test of anxiety. Behavioural brain research 86:105-112.

File SE, Gonzalez LE, Gallant R (1998) Role of the basolateral nucleus of the amygdala in the formation of a phobia. Neuropsychopharmacology 19:397-405.

Handley SL, Mithani S (1984) Effects of alpha-adrenoceptor agonists and antagonists in a maze-exploration model of 'fear'-motivated behaviour. Naunyn-Schmiedeberg's archives of pharmacology 327:1-5.

Holmes A, Kinney JW, Wrenn CC, Li Q, Yang RJ, Ma L, Vishwanath J, Saavedra MC, Innerfield CE, Jacoby AS, Shine J, Iismaa TP, Crawley JN (2003) Galanin GAL-R1 receptor null mutant mice display increased anxiety-like behavior specific to the elevated plus-maze. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 28:1031-1044.

Kjelstrup KG, Tuvnes FA, Steffenach HA, Murison R, Moser EI, Moser MB (2002) Reduced fear expression after lesions of the ventral hippocampus. Proceedings of the National Academy of Sciences of the United States of America 99:10825-10830.

Kurtuncu M, Luka LJ, Dimitrijevic N, Uz T, Manev H (2005) Reliability assessment of an automated forced swim test device using two mouse strains. Journal of neuroscience methods 149:26-30.

Lacroix L, Spinelli S, Heidbreder CA, Feldon J (2000) Differential role of the medial and lateral prefrontal cortices in fear and anxiety. Behav Neurosci 114:1119-1130.

Lister RG (1987) The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology 92:180-185.

Lister RG (1990) Ethologically-based animal models of anxiety disorders. Pharmacol Ther 46:321-340.

Montgomery KC (1955) The relation between fear induced by novel stimulation and exploratory behavior. Journal of comparative and physiological psychology 48:254-260.

Noldus LP, Spink AJ, Tegelenbosch RA (2001) EthoVision: a versatile video tracking system for automation of behavioral experiments. Behavior research methods, instruments, & computers : a journal of the Psychonomic Society, Inc 33:398-414.

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. Pharmacology, biochemistry, and behavior 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. Journal of neuroscience methods 14:149-167.

Rodgers RJ, Johnson NJ (1995) Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety. Pharmacology, biochemistry, and behavior 52:297-303.

Rodgers RJ, Dalvi A (1997) Anxiety, defence and the elevated plus-maze. Neurosci Biobehav Rev 21:801-810.

Roy V, Chapillon P (2004) Further evidences that risk assessment and object exploration behaviours are useful to evaluate emotional reactivity in rodents. Behavioural brain research 154:439-448.

Spink AJ, Tegelenbosch RA, Buma MO, Noldus LP (2001) The EthoVision video tracking system--a tool for behavioral phenotyping of transgenic mice. Physiology & behavior 73:731-744.

Torres C, Escarabajal MD (2002) Validation of a behavioral recording automated system in the elevated plus-maze test. Life Sciences 70:1751-1762.

Walf AA, Frye CA (2007) The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nature protocols 2:322-328.

Weiss SM, Wadsworth G, Fletcher A, Dourish CT (1998) Utility of ethological analysis to overcome locomotor confounds in elevated maze models of anxiety. Neuroscience and biobehavioral reviews 23:265-271.

Young R, Johnson DN (1991) A fully automated light/dark apparatus useful for comparing anxiolytic agents. Pharmacol Biochem Behav 40:739-743.