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# EARLY LIFE INFLUENCES OF PHARMACOLOGICAL AND IMMUNE STRESSORS ON DEVELOPMENT

*by*

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# ABSTRACT OF THE DISSERTATION

## Early Life Influences of Pharmacological and Immune Stressors on Development

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Neurodevelopmental disorders manifest early and may impair function throughout life. Exposure to environmental challenges is thought to play a causal role in many such disorders, but a key unanswered question relates to the vulnerability window(s) during which there is an increased sensitivity to these challenges. In a mouse model, we found that administration of the GABAergic toxicant valproic acid (VPA) differentially disrupted neurobehavioral development depending on the precise timing of exposure, and that the resulting behavioral profiles were sex-dependent. Specifically, neonatal C57BL/6 mice were challenged with 400 mg/kg VPA or saline on postnatal day 14 (P14) or 300 mg/kg VPA or saline on postnatal day 7 (P7). Animals were

then assessed on a battery of behavioral tests chosen to assess social, emotional, and cognitive functioning. While VPA exposure at P14 resulted in a behaviorally disinhibited phenotype in males, VPA exposure at P7 resulted in social abnormalities in females. In addition, a single VPA challenge at either P7 or P14 affected dendritic spine plasticity in brain regions thought to regulate behaviors that reflect impulsivity. Finally, the behavioral consequences of VPA challenge were compared with an immunogenic challenge using the T cell superantigen, staphylococcal enterotoxin A (SEA). Challenge with SEA at P7 and P14 revealed that interleukin-2 (IL-2) production was lacking at P7, but not P14. Therefore, behavior was investigated after P14 treatment. There were no major differences in social, emotional or cognitive behaviors between SEA- and saline-treated mice during adolescence or adulthood. Overall, this investigation revealed that pharmacological challenges that focus on disruption of the GABAergic system influenced neurodevelopment in a sex- and time-dependent manner. However, T cell immune challenge was less disruptive, possibly due to the absence of key neuromodulatory cytokines that are normally induced by SEA in adult rodents and by other proinflammatory stimuli that target non-T cell populations. In conclusion, identifying the approximate vulnerability windows during postnatal development may allow for a better understanding of underlying mechanisms resulting in toxicant-induced deficits and provide a focus for prevention efforts.

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# Chapter 1

# Introduction

## 1.1 General Overview of the Project

Neurodevelopment is affected by a variety of perinatal pharmacological, environmental, and psychosocial factors. Agents such as lead, methyl mercury, thalidomide, influenza virus, cigarettes, and alcohol all produce neurobehavioral abnormalities during development if exposure occurs during the perinatal period (Rice & Barone, 2000). For example, maternal smoking during pregnancy is associated with IQ deficits, increased aggression, increased impulsivity, and increased incidence of conduct disorders and depression (Rice & Barone, 2000). Interestingly, in some cases, the behavioral abnormalities associated with prenatal insult to a toxicant may not become apparent until late adolescence or adulthood. A classic example of this delayed effect is that virus exposure during gestation has been associated with increased risk of schizophrenia in the adult offspring (Brown, 2011).

There are several animal models of early life environmental challenges; however, these largely focus on one prenatal time point: midgestation (or embryonic day 11.5 – 12.5), or on psychological stressors, such as maternal separation (Bale et al., 2010; Brunson et al., 2005; Neigh, Gillespie, & Nemeroff, 2009). Therefore, although there has been a great deal of research on the effects of *prenatal* exposure to several specific environmental challenges, the effects of these environmental insults during

the *postnatal* period – the first few years of life in humans or the first few weeks of life in rodents – remains poorly investigated. Therefore, a key unanswered question relates to the critical window of risk, during which an environmental insult increases risk for neurodevelopmental abnormalities.

Some evidence suggests that the precise timing of environmental insults is a contributing factor to the specificity of the long-term brain and behavioral pathology (Mac Giollabhui et al., 2019; Meyer, Yee, & Feldon, 2007; Meyer et al., 2006; Meyer, Nyffeler, Yee, Knuesel, & Feldon, 2008). However, there is a lack of research on the importance of timing on behavioral profile following early postnatal exposures. The present dissertation will examine the role of early life environmental insults on social, emotional, and cognitive functioning in adolescence and adulthood, as well as neuroanatomical, neurochemical, and immune outcomes. To achieve this goal, two agents will be utilized: valproic acid (VPA) and staphylococcal enterotoxin A (SEA). Although VPA has been studied extensively as a teratogen, less is known about SEA. The present dissertation will focus on understanding the effects of these agents when exposure occurs during the first few weeks of postnatal life.

## 1.2 Valproic acid (VPA)

Valproic acid is primarily used in the treatment of seizure disorders, but may also be prescribed for migraines, bipolar disorders, and mood disorders (Chateauvieux, Morceau, Dicato, & Diederich, 2010) VPA has several mechanisms of action, including acting as an ion channel blocker and a histone deacetylase 1 (HDAC1) inhibitor to increase gene expression. Highly relevant to its anticonvulsant properties, VPA also acts as a GABA agonist by inhibiting the enzymes *ABAT* (4-aminobutyrate aminotransferase) and *ALDH5A1* (aldehyde dehydrogenase 5 family member A1), which are involved in the degradation of GABA (Ghodke-Puranik et al., 2013). If

taken during pregnancy, VPA may increase the risk of autism spectrum disorders (ASD), known as fetal valproate syndrome (FVS) in the offspring. It has been reported that the rate of ASD in children of VPA-treated mothers is many fold greater than in the general population (Christensen et al., 2013; Moore et al., 2000; Rasalam et al., 2005).

In animal models, prenatal VPA exposure results in postnatal behavioral and neuroanatomical deficits similar to human ASD. Midgestational VPA exposure is one of the most common and well-characterized pharmacological animal models of ASD (for reviews see Chomiak, Turner, and Hu, 2013 and Roullet, Lai, and Foster, 2013). The behavioral profile in this model includes the core features of ASD, specifically reduced social interaction (Bambini-Junior et al., 2011; Gandal et al., 2010; Markram, Rinaldi, Mendola, Sandi, & Markram, 2008; Schneider & Przewłocki, 2005), repetitive behaviors (Gandal et al., 2010; Markram et al., 2008; Schneider & Przewłocki, 2005), and differences in pup ultrasonic vocalizations (Gandal et al., 2010), as well as several of the associated symptoms of ASD, such as increased anxiety (Markram et al., 2008; Schneider et al., 2008; Schneider, Ziółkowska, Gieryk, Tyminska, & Przewłocki, 2007). In addition, neuroanatomical abnormalities are observed, such as reduced Purkinje cell number and cerebellar cell volume (Ingram, Peckham, Tisdale, & Rodier, 2000). Furthermore, the VPA model of ASD has been used to investigate potential interventions for ASD, such as environmental enrichment (Schneider, Turczak, & Przewłocki, 2006; Woo & Leon, 2013) and antioxidant treatment (Al-Askar, Bhat, Selim, Al-Ayadhi, & El-Ansary, 2017).

Previous studies published by the Wagner lab have demonstrated that early *postnatal* exposure to VPA in mice led to neurodevelopmental deficits similar to the motor and cognitive deficits observed in ASD (Wagner, Reuhl, Cheh, McRae, & Halladay, 2006; Yochum, Dowling, Kenneth, Wagner, & Ming, 2008). Specifically, VPA treatment at P14 led to a regression of previously acquired mid-air righting, work-

ing memory deficits in the Morris water maze task, and delayed passive-avoidance acquisition (Wagner et al., 2006). Although early-life insults with VPA have been used previously to model ASD, most work to date has been done using the P14 time point. Therefore, few conclusions can be drawn regarding the role of timing of an insult on development. The present dissertation will extend findings with the P14 time point to create a more complete later-life behavioral profile (see Chapter 3), as well as determine the effects of P7 exposure on adolescent and adult behavior (see Chapter 4). Furthermore, this dissertation will seek to determine if observed behavioral differences are associated with neuroanatomical abnormalities in the layer V pyramidal cells of brain regions thought to be associated with human ASD (see Chapter 5).

## 1.3 Staphylococcal enterotoxin A (SEA)

The presence of immune dysregulation in neurodevelopmental disorders such as ASD suggests that compromised immune status may play a role in the development of such disorders (Mead & Ashwood, 2015; Michel, Schmidt, & Mirnics, 2012; Onore, Careaga, & Ashwood, 2012; Patterson, 2011). Epidemiological evidence points to maternal infection during pregnancy as contributing to an increased risk of neurodevelopmental abnormalities in offspring (Brown & Derkits, 2010; Chess, 1971; Clarke, Tanskanen, Huttunen, Whittaker, & Cannon, 2009; Mednick, Machon, Huttunen, & Bonett, 1988; O'Callaghan, Sham, Takei, Glover, & Murray, 1991; Penner & Brown, 2007). Although initially the link between maternal immune activation (MIA) and offspring developmental disorders was retrospective (e.g. Mednick et al., 1988), more recent studies have confirmed the association in humans using a prospective approach (e.g. Brown and Derkits, 2010). Furthermore, MIA has been used to model neurodevelopmental disorders, most commonly ASD and schizophrenia,

in rodents (Malkova, Yu, Hsiao, Moore, & Patterson, 2012; Meyer, 2014; Nyffeler, Meyer, Yee, Feldon, & Knuese, 2006; Patterson, 2011). The first epidemiological studies to associate maternal infection with increased risk of schizophrenia in the offspring identified the second trimester as a critical period of risk, and this has been the focus in animal models of MIA. However, recent studies have also implicated later environmental exposures, such as the third trimester (Brown & Derkits, 2010), or even the time of birth, with increased schizophrenia risk (Byrne, M, Agerbo, E, Bennedsen, B, Eaton, WW, & Mortensen, PB, 2007; Cannon et al., 2000; Fineberg & Ellman, 2013; Mittal et al., 2009).

Several recent studies in animals have demonstrated that immune activation may continue to be a threat to neurodevelopment even after birth. Early postnatal exposure to agents such as poly(I:C) and LPS can result in similar behavioral and neurochemical abnormalities as those seen in MIA models. This includes increased anxiety-like behavior, decreased prepulse inhibition (PPI), decreased sociability, and abnormalities in glutamate neurotransmission in the hippocampus. It appears that results differ based on timing of immune activation and the specific immune stimulus (see Chapter 6). However, there are still very few studies which examine the effect of immune insults during the early postnatal period on later-life behavioral outcomes. This is a surprising and critical oversight, given that following birth, the neonate is exposed to a threatening new microbial world to which an immature immune system must rapidly adapt. Furthermore, no study has characterized the effect of the T cell superantigen, staphylococcal enterotoxin A (SEA), during the early postnatal period.

The immune system responds to any challenge via two distinct but interdependent branches of immune functioning. First, there is a generalized neutralizing of invading pathogens via the innate immune system. The innate immune system is comprised of both physical and biological barriers, such as the skin or mucous mem-

branes, as well as a cellular component, wherein receptors on immune cells identify pathogens, induce inflammation, and signal to the cells of the adaptive immune system. The second, slower, aspect of the immune response is coordinated by the adaptive immune system, activation of which results in a highly specialized defense against the pathogen. The T cells of the adaptive immune system recognize antigens – fragments of pathogen – presented to them by the host's antigen presenting cells (APCs), such as dendritic cells or macrophages. The adaptive immune system also has the capability of creating memory cells – T or B cells which will rapidly mature and proliferate upon a repeat encounter with an antigen, resulting in a more rapid and abundant immune response to a second exposure to a pathogen (Abbas, Lichtman, & Pillai, 2011).

Superantigens (SAgs) are proteins that cause robust and prolonged T cell activation and proliferation, as well as a rapid production of T cell-derived cytokines. This T cell response to SAgs is not seen in response to other immune stimuli, such as LPS or poly(I:C), which activate the innate branch of the immune system, rather than the T cells of the adaptive immune system. Unlike conventional antigens, SAgs bind outside of the peptide-binding groove of MHC class II molecules (Dellabona et al., 1990) and outside of the variable region of the beta chain (v$\beta$) on T cell receptors (Fraser & Proft, 2008; Torres, Kominsky, Perrin, Hobeika, & Johnson, 2001). This unique binding configuration of SAgs bypasses the need for specific antigen recognition. Therefore, many more T cells are activated by SAgs than conventional antigens, as T cells with different peptide antigen specificities will react to SAg. Specifically, when a conventional antigen is present, about 0.01% of the body's T cells are activated (Abbas et al, 2011). In contrast, a superantigen will activate about 20% of the body's T cells (Fraser & Proft, 2008).

One major source of SAgs is *staphylococcus aureus* (*S. aureus*), a bacterium that colonizes in both humans and animals. Infection with *S. aureus* can cause food poi-

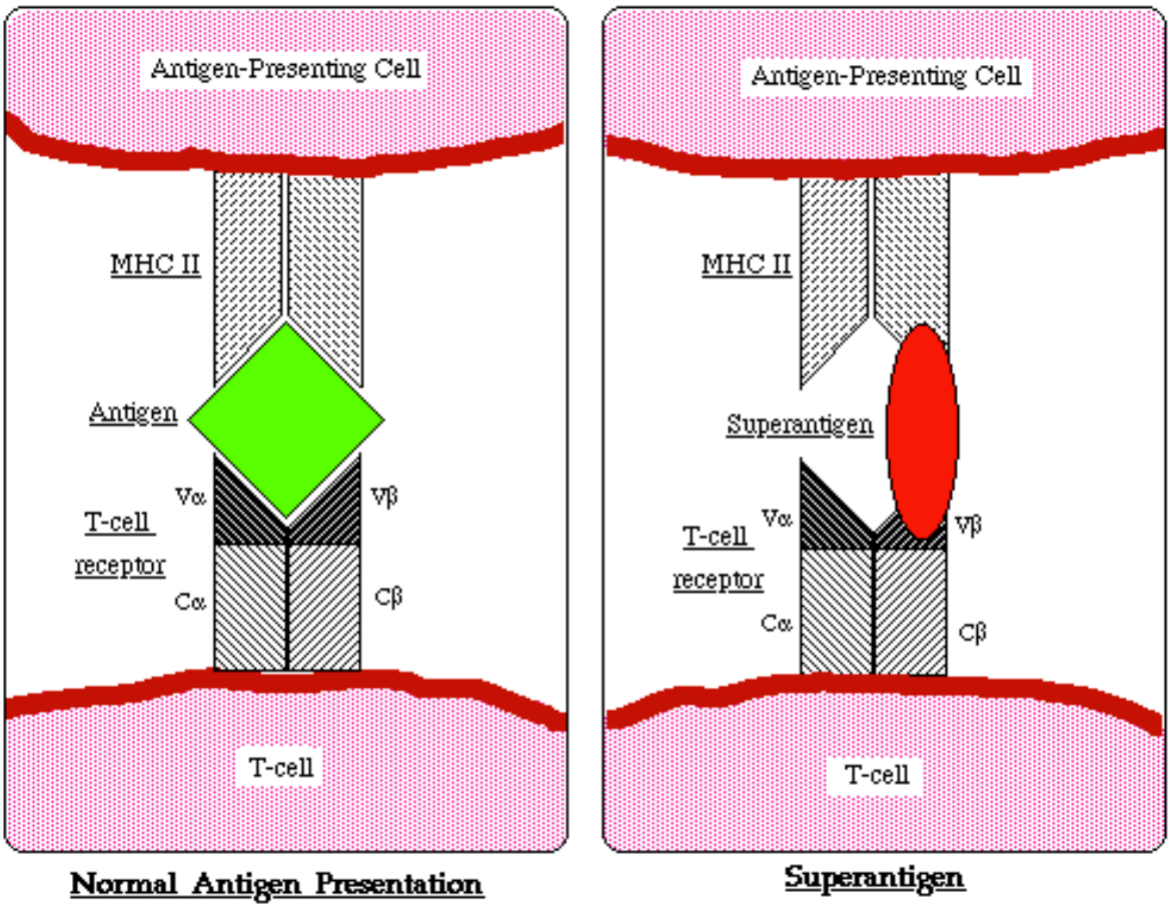



Figure 1.1: In conventional antigen presentation, T cells specifically recognize only antigens presented by antigen presenting cells (APCs) that fit into the antigen-binding groove of the v$\beta$ region of the T cell receptor. Superantigens, however, bind outside this region, thereby bypassing the need for specific antigen recognition. For this reason, many more T cells are activated by superantigens than conventional antigens. Image credit: Ruthy Glass

soning, skin infections, pharyngitis, and toxic shock, with the potential for infection to be lethal. *S. aureus* produces exotoxins (known as staphylococcal enterotoxins, or SEs) which cause a number of illnesses. SEs are powerful inducers of gastrointestinal symptoms, such as stomach pain, diarrhea, nausea, and vomiting, and are a leading cause of food-borne illness, nosocomial infections, and gastrointestinal inflammatory injuries, resulting in millions of infections and hospitalizations each year (Pinchuk, Beswick, & Reyes, 2010). The most widely studied SEs are SEA and SEB, although SEA is the SE most associated with food-borne illness (Hu et al., 2007). SEA was

chosen for the studies described in this dissertation because it is believed to be more potent in C57BL/6 mice (Stiles, Campbell, Castle, & Grove, 1999).

Enterotoxins, such as SEA, represent a useful model for studying the role of T cells in the effects of early life immune activation. Current studies have mostly used LPS or poly(I:C), and this literature is reviewed in Chapter 6. These agents do not engage T cells, being restricted in activating cells of the innate immune system. In choosing to use SEA, the current dissertation will build on these previous studies, and determine whether T cell activation is equally behaviorally disruptive as activated macrophages, the main targets of LPS and poly(I:C).

## 1.4 Relevance

The studies described in this dissertation will evaluate the effects of early life pharmacological (VPA) or immune (SEA) challenge on a battery of behavioral, neurochemical, and neuroanatomical assays related to human neurodevelopmental disorders, such as ASD. Although there has been a great deal of research on the effects of prenatal exposure to these particular challenges, the effects of these insults in the early postnatal period (P7 – P14) remains poorly understood.

In Study 1 (see Chapter 3), the effects of VPA exposure at postnatal day 14 (P14) on adolescent and adult social, emotional, and cognitive functioning are evaluated. In addition, levels of serotonin, 5HIAA, and dopamine are assessed in post-mortem brain samples. In Study 2 (see Chapter 4), the effects of VPA exposure at postnatal day 7 (P7) were assessed on a similar battery of behavioral tests. Study 3 (see Chapter 5) sought to determine the effects of P7 or P14 VPA exposure on the density and morphology of layer V pyramidal cell dendritic spines. And lastly, Study 4 (see Chapter 6) examined the acute immune response of neonates at P7 and P14 to SEA, as well as the long-term behavioral and immunological consequences of P14

SEA exposure.

In conducting these studies, it is hoped that identification of critical developmental windows of sensitivity, during which environmental exposures can increase risk of developmental disorders, can help provide insight into the underlying mechanisms though which toxicants disrupt the developing brain. This has the potential to inform public policy and provide a target for focusing prevention efforts which, in turn, could improve public health via reduced incidence of these disorders.

# Chapter 2

# General Methodology

## 2.1 Animals

Adult male and female C57BL6/J mice were originally ordered from Jackson Laboratories and used to establish an ongoing colony in our laboratory. Pregnancies were generated in females using the following standardized protocol: two adult female mice were introduced into the cage of a single adult male C57 mouse for 8-10 days, after which female mice were removed and checked each morning for signs of pregnancy. The day of birth was recorded as postnatal day 0 (P0), with litters maintained with their mothers until weaning at 4 weeks. During the pre-weaning period, cages were cleaned twice per week, and upon weaning, mice were separated into same-sex cages (3 – 4 per cage). Animals received ad lib access to food and water, under a 12:12h light:dark cycle, with lights on at 6:00am. All procedures were in accordance with AAALAC guidelines and Rutgers Institutional Animal Care and Use Committee.

Treatment with valproic acid (VPA) at P14. Mice were treated subcutaneously (s.c.) with either saline or VPA 400 mg/kg on postnatal day 14 (P14). This dose is consistent with those used previously (Wagner et al., 2006; Yochum et al., 2008). Each

litter consisted of both saline- and VPA-treated pups.

Treatment with VPA at P7. A dose of 400 mg/kg administered at postnatal day 7 (P7) resulted in over 75% mortality during preliminary tests, and so was not used. Instead, mice were treated s.c. with either saline or VPA 300 mg/kg. This resulted in 10% mortality, which was the equivalent mortality rate of 400 mg/kg in P14 pups. Each litter consisted of both saline- and VPA-treated pups.

Treatment with Staphylococcal Enterotoxin A (SEA). For pups aged P7 or P14, SEA was diluted in saline, and given i.p. at a dose of 5 $\mu$g in a volume of 100 $\mu$L at P14 or 50 $\mu$l at P7. Control pups received injections of saline in an equivalent volume of saline. To confirm that SEA elicited robust pro-inflammatory response in the mature immune system, adult mice 6 – 8 months old, were administered saline or 5 $\mu$g SEA in a volume of 200 $\mu$L. All injections were completed between 10:00am and 12:00pm. Because initial assays indicated that SEA did not result in a robust IL-2 response in P7 pups, this group was dropped from behavioral testing (see Chapter 6).

## 2.2 Behavioral Testing

After weaning, all VPA and saline-treated animals were subjected to a sequence of behavioral tests of social, cognitive, emotional and sensorimotor behavior. Each test was introduced at a specific age, with some tests repeated at later ages (eg., social approach). A full timeline of behavioral testing is shown in Figure 2.1.

Surface Righting (P7 – P9). Mouse pups were placed on their backs with all four limbs extended outwards. Time to right such that all four paws were touching the surface was recorded, with a maximum time of 30 seconds. This test was repeated three times for each mouse.

Mid-air Righting (P14 – P15). Ability to right in mid-air was assessed by holding the mouse pup by the scruff of the neck ventral side up with all four paws extended upward 30 cm above a padded surface. Ability to right was scored as a success if the mouse landed on all four paws on the surface. This test was repeated three times for each mouse, with the score for the day being an average of successes (1) and failures (0).

Social approach (P30, P90). Social testing took place in an open field containing two diagonally opposed cylindrical wire chambers (called S1 and S2), into which was placed a conspecific mouse (see Figure 2.2). The apparatus measured 38 cm x 38 cm, enclosed by 60 cm high plexiglass walls. The actual procedure consisted of three phases: habituation phase, single target phase (Phase A), and two-target phase (Phase B). The habituation phase consisted of an initial five-minute period, during which the experimental mouse was habituated to the experimental chamber, with the wire chambers empty. During this habituation period, ANY-Maze tracking software (Stoelting Co., Wood Dale, IL) was used to record the distance travelled (in meters) and the preferred side of the apparatus.

This habituation period was followed by the single target phase (Phase A), during which a target mouse was placed into the wire chambers (S1) on the least preferred side of the apparatus, leaving the second wire chamber (S2) empty. The experimental mouse was then placed in the center of the open field and contacts with either chamber were automatically recorded over a ten-minute period. This was then followed by the two-target phase (Phase B), which involved a second novel mouse being introduced to S2. The number of contacts with the original target mouse and the new target mouse were recorded for ten minutes. During each phase, videos were recorded using an overhead camera connected to a laptop computer running ANY-Maze tracking software. The videos were manually reviewed after testing to determine time spent in each of four quadrants (see Figure 2.2), and overall distance

travelled.

Social play (P32). A separate cohort of mice was run through the social play paradigm. The critical time period during which pup social behavior (play behavior) first appears ranges from postnatal day 30 to 40 (Thor & Holloway, 1984). Naive mice between P30 – 40 were individually-housed 48 hours prior to the test session. After the test session, mice were group-housed with same-sex littermates. The experimental C57 mouse was paired with an age- and sex-matched BALB/c mouse. The pair was then video-recorded and videos were later scored by a trained observer for the number of times that the experimental mouse initiated a behavior. Observers scored pairs of animals blind to treatment condition. During testing, the behaviors recorded were: aggression, ano-genital sniffs, face sniffs, crawl-under/over behaviors, self-grooming, and allogrooming (allogroom behaviors were defined as one mouse rising up on its hind legs to touch paws and snout to the other mouse to perform grooming motions).

Elevated plus maze (EPM) (P91-P94). To determine anxiety-like behavior, the elevated plus maze (EPM) test was conducted over two five-minute sessions separated by 72 hours. Testing took place over two sessions because of previous findings that a single exposure to the EPM reduces open arm activity on retest and that the anxiolytic response to benzodiazepines is abolished on second exposure to EPM (Albrechet-Souza, Cristina de Carvalho, Rodrigues Franci, & Brandão, 2007; File, 2001; Rodgers & Shepherd, 1993). This suggests that first and second exposures to the EPM may be associated with different emotional states and mediation by alternate neurochemical mechanisms.

The EPM consisted of a cross-shaped elevated apparatus with four arms, and elevated 82 cm above the floor. All arms were 25cm long and 7.62 cm wide. Two opposing arms were enclosed by high walls (21.6 cm high), and were designated as 'closed,' while the remaining two opposing arms had no walls, and were designated

as 'open.' A center area, simply designated 'center,' represented the access region to all four arms. Testing in the EPM lasted for 5 minutes, and commenced with the subject being placed in the center zone. The amount of time the animal spent exploring the closed and open arms was recorded, as well as the distance travelled in open and closed arms. Exploration was recorded using ANY-Maze software.

Water Y maze (P108). The Y maze consisted of three 37 cm long arms filled with water approximately 7 cm deep. A water version of the Y maze was used instead of an appetitive (food reward) version to eliminate the confounds of odor cues and food deprivation (Hyde, Hoplight, & Denenberg, 1998). In the first phase (acquisition phase), a mouse was placed in the start arm and was required to choose between the two remaining arms, only one of which contained a platform that allowed escape from the water. After the mouse made a choice, the door at the entrance of the arm (whether it contained the platform or not) was closed, trapping the mouse in the chosen arm for 10 seconds. After 10 seconds, the trial ended, and the mouse was returned to a cage on a heating pad for a 5-minute inter-trial interval (ITI), after which the mouse was returned to the start arm. The location of the escape platform did not change until the mouse reached criterion performance of correctly selecting the escape arm 11 out of 12 trials on two consecutive days. Once criterion was reached, the location of the escape platform was changed to the opposite arm of the maze. This represented the reversal phase. During the reversal phase, mice needed to consistently choose the opposite arm than they had initially learned, and difficulty with this task may represent a resistance to change in routine that is characteristic of ASD (Crawley, 2007). Criterion parameters were the same for the acquisition and reversal phases. Both number of days to reach criterion and number of errors made were considered as dependent variables for this tests.

Prepulse inhibition (PPI) (3 – 5 months). Prepulse inhibition (PPI) of the acoustic startle response is considered a test of sensorimotor gating in both humans and

rodents. Testing was conducted in four SR-Lab Systems startle chambers (San Diego Instruments, San Diego, California, USA). Each chamber contained a 5.1 cm (outside diameter) plexiglass cylinder mounted on a platform with a piezoelectric accelerometer unit attached below the plexiglass cylinder. The piezoelectric unit transduces vibrations into signals that are rectified and stored by a microcomputer interface. Each chamber was sound-attenuated and contained with a loudspeaker fitted into the ceiling of the chamber. Each test session began by placing a subject in the cylinder where it was left undisturbed for 2 minutes. After the 2-minute acclimation period, each subject was presented with 80 acoustic noise trials given over a 45-minute period. The noise trials were of seven distinct types. These included three different acoustic prepulse+pulse stimulus trials, with each having the onset of a prepulse stimulus 100 ms before the onset of the startle pulse stimulus. The 40 ms prepulse stimuli were sounds of 2, 4, and 8 db above background (60 db), while the startle-inducing pulse was 50db above background. Each of the prepulse intensities were presented alone to measure baseline startle to the prepulse stimulus. Finally, there were trials where startle pulse alone was presented to measure the maximal startle response. The seven trial types were presented in pseudo-random order. The average inter-trial interval was 15 seconds (ranging from 10 to 20 seconds). The startle response was recorded for 250 ms (measuring the response every 1 ms) starting with the onset of the startle stimulus. The maximum startle amplitude recorded during the 250 ms sampling window was used as the startle response of the subject for that trial. The formula for calculating prepulse inhibition was:

$$\frac{\text{pulse alone - (prepulse+pulse)}}{\text{pulse alone}} * 100$$

Data analysis. All data was analyzed using SPSS software. A combination of tests, including t-tests, ANOVAs, and chi-square tables of frequency, were used throughout

the analyses as appropriate for the data. All outliers were removed a priori to analysis if a given score was more than two standard deviations away from the mean of each group. In no instance were more than 2% of animals excluded from any group.

For EPM testing, the arcsine transformation of the proportion of time spent and distance travelled were used in all analyses. This was done to normalize the data, which was expressed as a proportion.

## 2.3 Brain Dopamine and Serotonin

High-performance liquid chromatography (HPLC) (6 months). After behavioral testing was complete, mice were rested until 6-months of age, when they were sacrificed by decapitation for analysis of brain monoamine concentration. The brain was dissected into regions corresponding to the frontal cortex, hippocampus, and cerebellum, and then snap frozen in liquid nitrogen, and stored at -80$^{o}$ C until homogenization. All tissue was homogenized in 0.3 ml of 0.4 N perchloric acid with 0.1 mM EDTA. Homogenized samples were centrifuged at 20,000g for 20 minutes at 4$^{o}$ C and the supernatant frozen at -80$^{o}$ C until the time of analysis. Supernatant was assayed for dopamine (DA), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5HIAA) using an electrochemical detector. Quantification was measured against 62.5 pmol monoamine external standards (Sigma-Aldrich). Serotonin turnover was calculated as 5-HT/5HIAA.

## 2.4 Neonatal and Adult Immune Response

Spleen extraction. Two hours after injection with either SEA or saline, mice were sacrificed in a standard $CO_2$ chamber. Spleens were removed and homogenized with 1 mL (or 0.5 mL for neonatal spleens) of phosphate-buffered saline (PBS),


(a)


(b)

Figure 2.1: Behavioral testing timelines. Behavioral tests described in (a) and (b) were run on separate cohorts of mice.

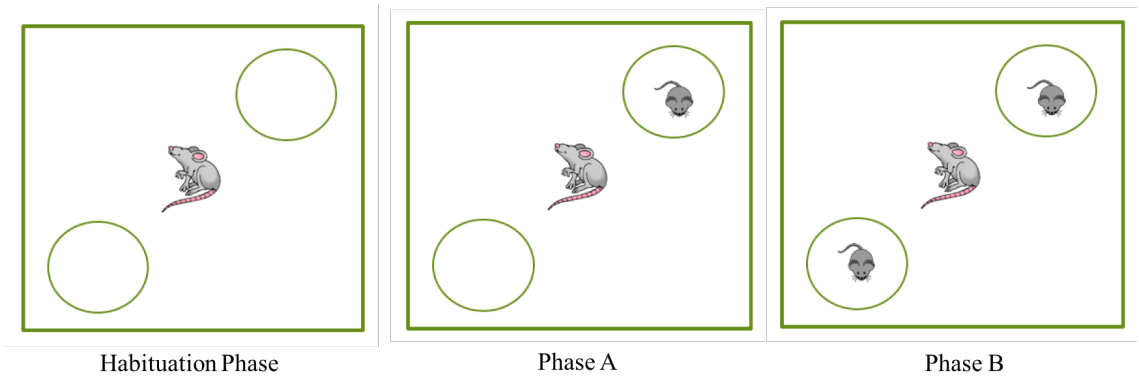



Figure 2.2: The social approach test consisted of three phases: a 5-minute habituation phase and two 10-minute test phases. During Phase A, a novel age- and sex-matched mouse was placed in S1 while S2 remained empty. During Phase B, another novel age- and sex-matched mouse was placed in S2, while the S1 mouse from Phase A remained in S1.

centrifuged for 10 minutes at 10,000 rpm, and the supernatant collected and stored at -80° C until immunoassay for cytokines.

ELISAs. An enzyme-linked immunosorbent assay (ELISA) was used to assess cytokine concentrations in the spleens. The spleen, a secondary lymphoid organ, is the body's largest filter of blood and contains lymphoid tissue that is made up of specialized macrophages, T-cells and B-cells that respond to infection (Mebius & Kraal, 2005). In addition, superantigens, such as SEA, have been shown to elicit T-cell cytokine responses in the spleen (Glass, Norton, Fox, & Kusnecov, 2019). Assays were performed for the cytokines IL-2, IL-6, and TNF$\alpha$. Assay reagents were obtained from Invitrogen and were performed with slight modifications to manufacturer's instructions. To perform the ELISAs, a 96-well flat-bottom microtiter plate (Nunc Maxisorp plates) was coated with capture antibody specific for the cytokine of interest and left at 4° C overnight. Plates were then washed five times with wash buffer (9L distilled water, 5mL Tween 20, and 1L 10x PBS), and samples and cytokine standards were added to the plate. Cytokine standards (Invitrogen) were established as serial two-fold dilutions, beginning with 200 pg/mL for IL-2, 500 pg/mL for IL-6, and 1000 pg/mL for TNF$\alpha$. Samples were diluted 1:10 for IL-2

detection and 1:4 for IL-6 and TNF$\alpha$ detection. The standards and samples were added in duplicate. After addition of samples and standards, the plates were left at 4$^{o}$ C overnight. The plate was then washed five times and a biotinylated capture antibody specific for the cytokine of interest was mixed with streptavidin-conjugated horseradish peroxidase (HRP) and added to the wells. The plate was left at room temperature for 90 minutes, the plates washed five times, and then a substrate solution (for HRP) consisting of 3,3',5,5'-Tetramethylbenzidine (TMB) was added in order to activate the HRP enzyme. The subsequent chromogenic reaction produced a blue color which was converted to yellow with the addition of 50 $\mu$L of sulfuric acid (1M H2SO4) to each well, and which terminated the reaction. The optical densities (expressed as absorbance units) of the wells were then read at a wavelength of 450 nm using a universal microplate reader (Bio-Tek Industries, ELx800) and accompanying software (KC Junior software, BioTek). For each ELISA a standard curve was established and the concentration of the target cytokine in each sample was calculated.

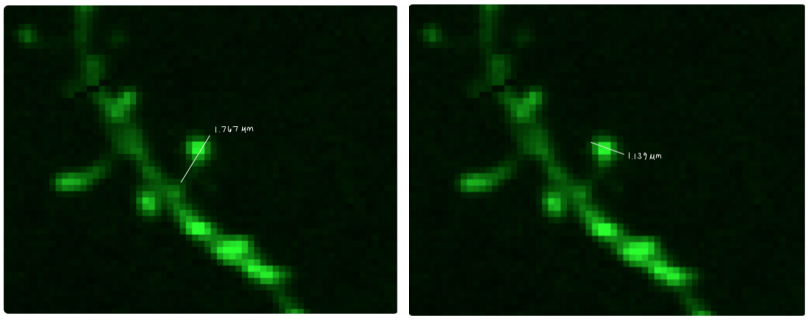
Protein assays. The cytokine concentrations that were determined by ELISA were corrected for the total protein concentration of the sample, such that cytokine concentrations were expressed per $\mu$g of total protein. Total spleen protein was quantified using the BCA protein assay kit as per manufacturer's instructions (Pierce, Rockford, IL, USA). Samples were diluted 1:20 and concentrations were compared to a standard curve prepared in the same plate using bovine serum albumin (BSA). The optical densities of the wells were read at a wavelength of 562 nm as described above.

Data analysis. For the ELISA data, 2 x 2 (Treatment x Sex) ANOVAs and correlations were used to look for significant main effects, interactions, and correlations in the data.

## 2.5 Dendritic Spine Density and Morphology

Thy1 mice. A colony of Thy1-yFP mice (kindly provided by Dr. Huaye Zhang, RBHS; JAX stock 003782; Feng et al., 2000) was maintained that express YFP in dendrites within layer 5 of the cerebral cortex. The yellow fluorescent protein (eYFP) is under the control of the Thy1 promoter region, which is expressed in neuronal and non-neuronal cells. This allowed for the visualization of neurons via fluorescence microscopy.

Dendritic spine morphology analysis. Spines vary in morphology (Nimchinsky, Sabatini, & Svoboda, 2002), which is thought to reflect their functional properties. For example, filopodia are transient fluctuations in dendritic membrane plasticity, whereas stubby or mushroom spines represent more stable forms that likely represent the existence of a synapse (Nimchinsky et al., 2002; Risher, Ustunkaya, Alvarado, & Eroglu, 2014; Ziv & Smith, 1996). To analyse the number and types of spines, 1 – 3 segments of 10 – 20 microns length were selected per dendrite. This was accomplished by importing Z-stack images into ImageJ, after which a frame was chosen where the dendrite could be seen the clearest and without other dendrites crossing over. The length and width of each spine were recorded by measuring the longest (corresponding to height) and widest part of each spine of each dendrite segment in the frame of the Z-stack and where the spine was clearest. The length-width ratio (LWR) of each dendritic spine was calculated. The unit of measurement was in microns and the data were imported into an Excel sheet. Length-width ratio was calculated and shape was determined based on the following parameters: Filopodia if length is $>2$ $\mu$m, long/thin if length is $<2$ $\mu$m, thin if length is $<1$ $\mu$m, stubby if length:width ratio is $<1$, mushroom if width $>0.6$ $\mu$m and branched if it contains two or more heads (Risher et al., 2014).



(a) (b)

Figure 2.3: Example images of dendritic spine with height (a) and width (b) measurements.

# Chapter 3

# Behavioral and Neurochemical Profile Following Valproic Acid Exposure on Postnatal Day 14

## 3.1 Introduction

A neurodevelopmental disorder that has received prominent attention in the past decade is autism spectrum disorder (ASD), which is characterized by impaired social communication and repetitive behaviors (American Psychiatric Association, 2013). The etiology of ASD remains unknown, but it is thought to involve a genetic predisposition interacting with exposure to environmental insults during critical periods of development. However, while the concept of a critical or sensitive period is prevalent in the literature (Meredith, 2015; Rice & Barone, 2000), little is known about the nature of the critical period: how long it may last, the types of insults that are most disruptive to a particular period, and whether different phenotypes develop based on the timing or intensity of a given insult (e.g., early versus late exposure).

Valproic acid (VPA) is a suspected GABAergic agonist (Winterer, 2003) that is

used as an anticonvulsant and, if taken during pregnancy, may result in "fetal valproate syndrome" with phenotypic similarities to ASD, including language deficits and stereotypical behavior (Chomiak et al., 2013; Singh et al., 2014). Therefore, prenatal VPA-treatment has been proposed as a useful animal model for investigating ASD-like symptomatology. Administration of VPA has occurred around midgestation, a critical time for neural tube closure and rapid neurogenesis (Pressler & Auvin, 2013; Rice & Barone, 2000; Semple, Blomgren, Gimlin, Ferriero, & Noble-Haeusslein, 2013), and which may be disrupted by VPA, given that it crosses the placental barrier and may be metabolized more slowly by the embryo (Ishizaki, Yokochi, Chiba, Tabuchi, & Wagatsuma, 1981; Nau, Rating, Koch, Häuser, & Helge, 1981). Indeed, in animal models, prenatal VPA exposure results in postnatal behavioral and neuroanatomical deficits similar to human ASD, with offspring phenotypes including decreased social interaction, increased anxiety, and increased repetitive behaviors (Markram et al., 2008; Rodier, Ingram, Tisdale, & Croog, 1997; Roullet et al., 2013; Schneider & Przewłocki, 2005; Schneider et al., 2008; Wagner et al., 2006). Therefore, VPA can be used to identify neurobiological mechanisms that drive the development of normal social and emotional functions.

In humans, synaptogenesis increases dramatically after birth and peaks at around two years of age (Semple et al., 2013). Similarly, murine synaptogenesis occurs rapidly during the first three weeks of postnatal life and peaks around week two (Pressler & Auvin, 2013; Semple et al., 2013). Recent animal studies have shown that VPA exposure at postnatal day 14 (P14) leads to neurodevelopmental deficits similar to the motor and cognitive deficits seen in ASD (Wagner et al., 2006). Specifically, VPA treatment on P14 led to a regression of previously acquired mid-air righting, working memory deficits in the Morris water maze task, and delayed passive avoidance acquisition. Furthermore, VPA treatment on P14 led to fewer social behaviors during an observation period with a novel mouse, as well as apoptosis

in the hippocampus and cerebellum at 12 and 24 hours post-treatment (Yochum et al., 2008). The deficits in mid-air righting suggests that early postnatal exposure to VPA may act as a model of autistic regression, in which normal development is halted and some acquired skills are lost. These data represent a much later time-point than what is typically used in the VPA model of ASD; however, it is unclear whether P14 VPA exposure may also result in intrusions that affect functioning in adulthood.

Hyperserotonemia is reported in about 30% of individuals with ASD (Anderson et al., 1987; Hranilovic et al., 2007). In addition, SSRIs are often used therapeutically to treat some of the symptoms of ASD. Therefore, serotonin (5-HT) may play a role in the pathogenesis of ASD, and is of interest in assessing animal models of ASD. Narita et al (2002) demonstrated increased 5-HT concentrations in the hippocampus following prenatal VPA exposure, suggesting that prenatal VPA exposure might result in the dysregulation of monoamine systems, which may contribute to the ASD-like phenotype observed in prenatally exposed mice (Narita et al., 2002). However, it is currently not known if early postnatal VPA exposures can also lead to serotonergic abnormalities.

Previous studies on the effects of VPA in the early postnatal period have largely focused on early life behavioral deficits, with the majority of behavioral testing confined to the juvenile period of life. As ASD is a life-long condition, the present study seeks to extend our understanding of the VPA model of ASD-like behavior into adulthood. In addition, although there is some evidence that prenatal VPA exposure can result in dysregulated serotonin systems, it is unclear if postnatal treatment will have a similar effect. The goal of the present study, therefore, is to determine whether early VPA-treatment leads to behavioral or neurochemical deficits that persist through adolescence and into adulthood.

## 3.2 Methods

Pregnancies were generated using the standardized protocol described in Chapter 2. The day of birth was recorded as postnatal day 0 (P0). Pups were maintained with their mothers until weaning at 4 weeks, at which time mice were separated into same-sex cages (3 – 4 per cage).

Treatment with VPA at P14. Mice were treated subcutaneously (s.c.) with either saline or VPA 400 mg/kg on postnatal day 14 (P14). This dose is consistent with those used previously (Wagner et al., 2006; Yochum et al., 2008). Animals used in the behavioral component of this study were taken from 12 litters. Each litter consisted of both saline- and VPA-treated pups.

Overview of Behavioral Testing. After weaning, all experimental animals underwent a sequence of behavioral tests of social, cognitive, emotional, and sensorimotor behavior (see Figure 3.1). Details about behavioral testing apparatuses can be found in Chapter 2.

Social approach (P30, P90). The social approach procedure consisted of three phases: habituation phase, single target phase (Phase A), and two-target phase (Phase B). The habituation phase consisted of an initial five-minute period, during which the experimental mouse was habituated to the experimental chamber, with the wire chambers empty. During this habituation period, ANY-Maze tracking software was used to record the distance travelled (in meters) and the preferred side of the apparatus.

This habituation period was followed by the single target phase (Phase A), during which a target mouse was placed into the wire chambers (S1) on the least preferred side of the apparatus, leaving the second wire chamber (S2) empty. This was then followed by the two-target phase (Phase B), which involved a second novel mouse


(a)


(b)

Figure 3.1: Behavioral testing timelines. Behavioral tests described in (a) and (b) were run on separate cohorts of mice.

being introduced to S2. For both Phase A and Phase B contacts with the S1 and S2 chamber were automatically recorded for ten minutes.

Social play (P32). A separate cohort of mice was run through the social play paradigm. Naive mice between P30 – 40 were individually-housed 48 hours prior to the test session. The experimental C57 mouse was paired with an age- and sex-matched BALB/c mouse. The pair was then video-recorded and videos were later scored by a trained observer for the number of times that the experimental mouse initiated a behavior. Observers scored pairs of animals blind to treatment condition. During testing, the behaviors recorded were: aggression, ano-genital sniffs, face sniffs, crawl-under/over behaviors, self-grooming, and allogrooming (allogroom behaviors were defined as one mouse rising up on its hind legs to touch paws and snout to the other mouse to perform grooming motions).

Elevated plus maze (EPM) (P91-P94). The elevated plus maze (EPM) test was conducted over two five-minute sessions separated by 72 hours. Testing in the EPM lasted for 5 minutes, and the amount of time the animal spent exploring the closed and open arms was recorded, as well as the distance travelled in open and closed arms.

Water Y maze (P108). In the first phase (acquisition phase), a mouse was placed in the start arm and was required to choose between the two remaining arms, only one of which contained a platform that allowed escape from the water. After the mouse made a choice, the door at the entrance of the arm (whether it contained the platform or not) was closed, trapping the mouse in the chosen arm for 10 seconds. After 10 seconds, the trial ended, and the mouse was returned to a cage on a heating pad for a 5-minute inter-trial interval (ITI), after which the mouse was returned to the start arm. The location of the escape platform did not change until the mouse reached criterion performance of correctly selecting the escape arm 11 out of 12

trials on two consecutive days. Once criterion was reached, the location of the escape platform was changed to the opposite arm of the maze. This represented the reversal phase. Criterion parameters were the same for the acquisition and reversal phases. Both number of days to reach criterion and number of errors made were considered as dependent variables for both phases.

Prepulse inhibition (PPI) (3 – 5 months). After the 2-minute acclimation period, each subject was presented with 80 acoustic noise trials given over a 45-minute period. The noise trials were of seven distinct types, described in Chapter 2. The three different acoustic prepulse+pulse stimulus trials were sounds of 2, 4, and 8 db above background (60 db), while the startle-inducing pulse was 50db above background. Each of the prepulse intensities were presented alone to measure baseline startle to the prepulse stimulus. The formula for calculating prepulse inhibition was:

$$\frac{\text{pulse alone - (prepulse+pulse)}}{\text{pulse alone}} * 100$$

High-performance liquid chromatography (HPLC) (6 months). After behavioral testing was complete, mice were rested until 6-months of age, when they were sacrificed by decapitation for analysis of brain monoamine concentration. The brain was dissected into regions corresponding to the frontal cortex, hypothalamus, striatum, hippocampus, and cerebellum, and then snap frozen in liquid nitrogen, homogenized, and stored at -80$^{o}$ C until analysis. Details on homogenization procedures can be found in Chapter 2. Quantification was measured against 62.5 pmol monoamine external standards (Sigma-Aldrich). Serotonin turnover was calculated as $\frac{\text{5-HT}}{\text{5HIAA}}$.

## 3.3 Results

### 3.3.1 Body Weight

*VPA-treated males weighed more than saline-treated males.*

Mice were weighed at the beginning of behavioral testing (P30) and again at P90. A 2 x 2 x 2 (Treatment x Sex x Age) repeated measures ANOVA was performed for body weight. There was a main effect of Age ($F_{(1, 34)}$=445.830, p<.001), such that mice weighed more at P90 than P30. There was a main effect of Sex ($F_{(1, 34)}$=75.664, p<.001), and an Age x Sex interaction ($F_{(1, 34)}$=14.944, p<.001), such that male mice weighed more than female mice at both P30 and P90, but this difference was greater at P90.

In addition, there was a Treatment x Sex interaction ($F_{(1, 34)}$=6.202, p=.018). Pairwise comparisons revealed that there was an effect of treatment in males only, such that male VPA-treated mice weighed more than their saline-treated counterparts (see Figure 3.2).

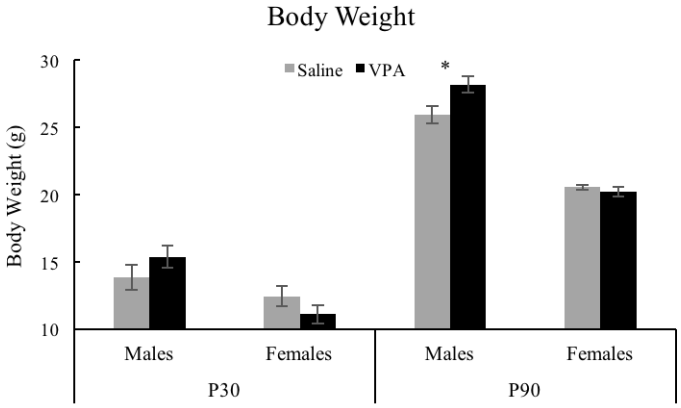



Figure 3.2: Average weight of male and female mice on P30 and P90. Male VPA-treated mice weighed more than male saline-treated mice. There was no treatment effect on body weight for females. *N, males=12 saline, 8 VPA; N, females=6 saline, 12 VPA.*

### 3.3.2 Social Approach

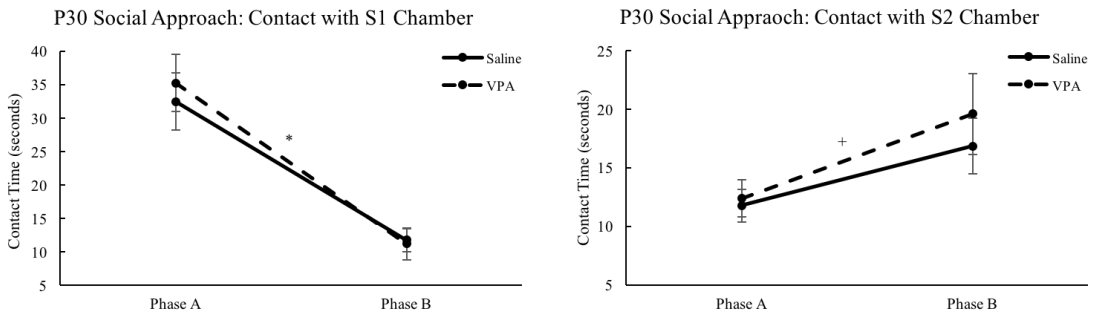
*Treatment had no effect on distance travelled during the 5-minute habituation period of the social approach test.*

A 2 x 2 (Treatment x Sex) ANOVA was run for distance travelled during the 5-minute habituation period at P30 and P90. There were no differences between saline- and VPA-treated mice (P30: $F_{(1, 31)}$=2.715, *ns*; P90: $F_{(1, 35)}$=.010, *ns*), nor between male and female mice at either time point (P30: $F_{(1, 31)}$=.704, *ns*; P90: $F_{(1, 35)}$=.032, *ns*). Therefore, overall locomotor activity was not affected by VPA treatment on P14.

*Treatment had no effect of number of contacts with either chamber during either phase of the test.*

During the social approach test, time spent in contact with each wire chamber (S1 and S2) was automatically recorded. During Phase A, S1 housed a novel weight- and sex-matched peer. During Phase B, the same peer remained in S1, and another peer was housed in S2.

A 2 x 2 x 2 (Treatment x Sex x Phase) repeated measures ANOVA was run with Phase as the within-subjects variable for time spent in contact with each of the wire chambers. At P30, there was a main effect of Phase ($F_{(1, 49)}$=64.863, p<.001) for time spent in contact with S1, such that animals spent less time in contact with S1 during Phase B (see Figure 3.3a). There was a trend toward an effect of Phase for time spent in contact with S2 ($F_{(1, 48)}$=3.316, p=.075), such that animals tended to spend more time in contact with S2 during the second phase of testing (when a mouse was present in this chamber) (see Figure 3.3b). There was no effect of Treatment. At P90, there was a main effect of Phase for time spent in contact with S1 ($F_{(1, 41)}$=23.565, p<.001), such that mice spent less time in contact with the S1 chamber during the second phase. There was also an effect of Phase for time spent in contact with S2 ($F_{(1, 40)}$=4.635, p=.037), such that mice spent more time in contact




(a) (b)

Figure 3.3: Time spent in contact with S1 (a) and S2 (b) chambers during the social approach task at P30. Mice explored S1 less and S2 more during Phase B. *N=25 saline, 28 VPA.*

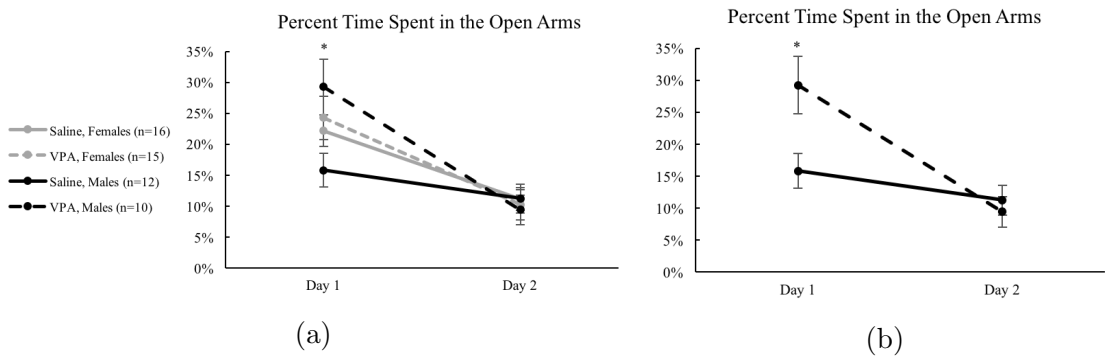
with the S2 chamber during the second phase. There was no effect of Treatment.

### 3.3.3 Elevated Plus Maze (EPM)

*VPA-treated male mice spent more time and travelled more in the open arms of the maze on Day 1, but not on Day 2.*

Since animals were given the EPM on two separate days, a repeated-measures ANOVA was performed for the variables time spent and distance travelled in the open arms. Proportions of time spent and distance travelled in the open arms were calculated by dividing the time spent or distance travelled in the open arms of the maze by the time spent or distance travelled in both the open and closed arms of the maze. The arcsine transformation of these proportions was used in all analyses.

For time spent in the open arms, there was a main effect of Day ($F_{(1,47)}$=88.737, p<.001), such that animals spent less time in the open arms on the second day of testing as compared to Day 1 (see Figure 3.4a). There was also a main effect of Day for distance travelled in the open arms ($F_{(1.45)}$=27.394, p<.001), such that animals travelled less in the open arms on the second day of testing as compared to Day 1. Post-hoc analysis revealed that saline-treated male mice were the only group that did not show a decrease in time spent or distance travelled in the open arms from




(a) (b)

Figure 3.4: Average percent time spent in the open arms of the EPM on Days 1 and 2 of testing. (a) Overall, male and female mice spent less time in the open arms of the maze on Day 2 testing, as compared to Day 1. (b) VPA-treated male mice spent more time in the open arms of the maze than saline-treated male mice on Day 1 of testing, but not on Day 2. There were no differences between VPA- and saline-treated female mice on either day of testing. *N, males=11 saline, 10 VPA; N, females=16 saline, 14 VPA.*

Day 1 to Day 2. There was no effect of day on total distance travelled.

For time spent in the open arms, there was a significant Day x Treatment interaction ($F_{(1,47)}$=12.308, p=.001), as well as a significant Day x Sex x Treatment interaction ($F_{(1,57)}$= 4.705), p=.035). Post-hoc analysis revealed that VPA-treated males spent more time in the open arms than saline-treated males on Day 1, but not on Day 2 (see Figure 3.4b). VPA-treated female mice did not differ from saline-treated female mice on either day. Saline-treated males and females did not differ on Day 1. For distance travelled in the open arms, there was a significant Day x Treatment interaction. Post-hoc analysis revealed that VPA-treated mice travelled more in the open arms than saline treated-mice on Day 1, but not on Day 2.

### 3.3.4 Water Y Maze

*VPA-treated male mice took longer and made more errors during the reversal phase.*

Animals commenced acquisition of escape from the Y-maze on P108. A 2 x 2 (Treatment x Sex) ANOVA was performed for number of days to reach criterion and number of errors made for the acquisition phase and the reversal phase. For the

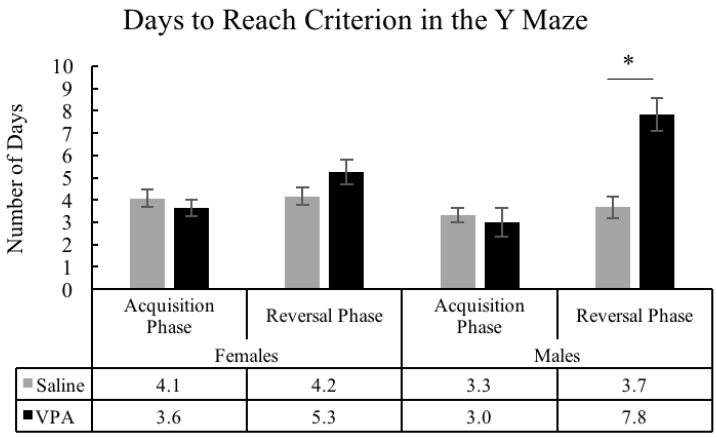
acquisition phase, there were no differences between saline- and VPA-treated mice, nor between males and females, in number of days to reach criterion. There was, however, a trend for females to make more errors ($F_{(1, 31)}$=3.670, p=.065).

For the reversal phase, in which animals needed to learn to alternate and escape from the opposite arm, there was an effect of Treatment ($F_{(1, 31)}$=21.765, p<.001) and a Treatment x Sex interaction ($F_{(1, 31)}$=7.333, p=. 011) for number of days to reach criterion. Male VPA-treated mice took significantly longer to reach criterion than saline-treated males (see Figure 3.5a). There was also an effect of Treatment ($F_{(1, 31)}$=23.354, p<.001), an effect of Sex ($F_{(1, 31)}$=6.418, p=.017), and a Treatment x Sex interaction ($F_{(}$1, 31)=14.256, p=.001) for number of errors made during the reversal phase. These effects were due to VPA-treated male mice making significantly more errors than any other group (see Figure 3.5b).

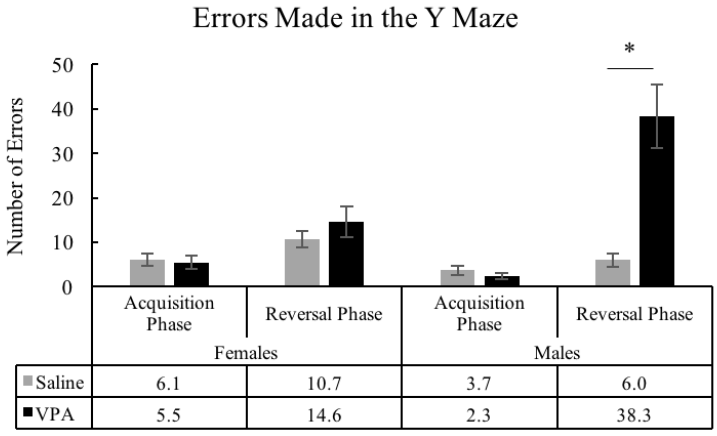
### 3.3.5 Social Play

*VPA-treated male mice displayed more aggressive behaviors.*

A 2 x 2 (Treatment x Sex) ANOVA was run for the following play behaviors: allogrooming, ano-genital sniffing, face sniffing, chasing, self-grooming, crawl over/under, and aggression. Of these behaviors, aggression was the only behavior that was found to be significantly different between saline and VPA-treated mice, or males and females. For aggression, there was a main effect of sex ($F_{(1, 29)}$=5.131, p=.001), in that females did not display any instances of aggression. There was also a main effect of Treatment ($F_{(1, 29)}$=5.355, p=.028) and a Treatment x Sex interaction ($F_{(1, 29)}$=5.355, p=.028), such that VPA-treated males had more instances of aggression than saline-treated males (see Figure 3.6). When instances of aggression were analyzed as nominal data – occurrence of aggression at least once during the 30-minute observation period – a chi-squared analysis revealed that there was a trend for treatment to be associated with aggression ($x^2$=2.951, p=.086). Specifically, 75%




(a)




(b)

Figure 3.5: Performance in the water Y maze in VPA- and saline-treated male and female mice. (a) VPA-treated male mice took longer to reach criterion when compared with similarly treated females and saline-treated male and female mice. (b) VPA-treated males also made more errors during the reversal phase of Y maze testing, but not during the acquisition phase. *N, males=6 saline, 6 VPA; N, females=12 saline, 11 VPA.*

of VPA-treated male mice displayed at least one instance of aggression, compared to only 33.3% of saline-treated male mice. Further analysis revealed that of the 53 instances of aggression, 34% were targeting the rump, whereas 66% were targeting the face. Only aggression initiated by the experimental mouse was included in this analysis.

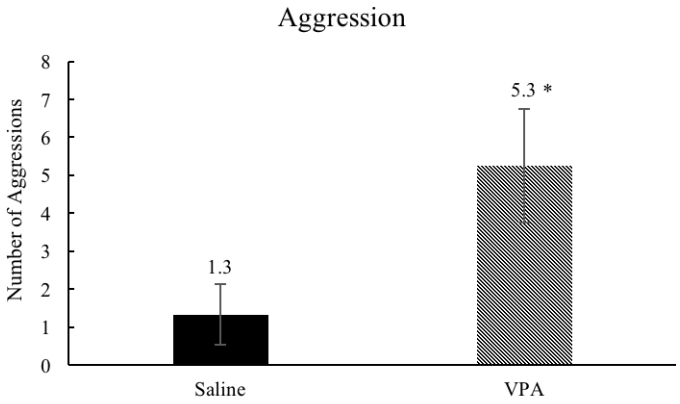
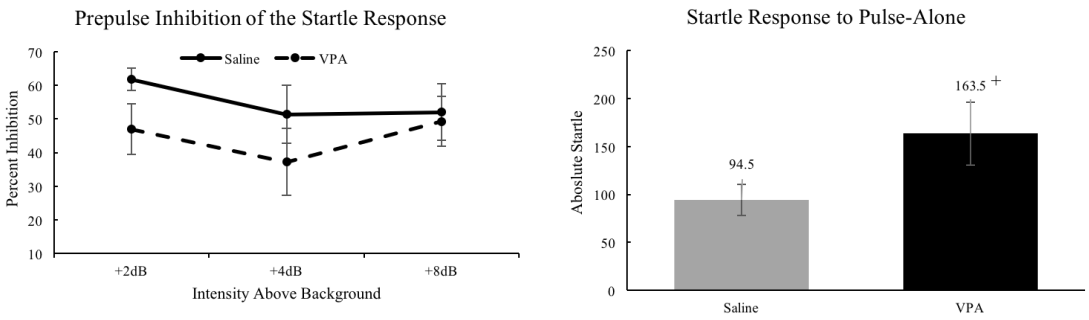



Figure 3.6: VPA-treated male mice initiated more acts of aggression than saline-treated male mice. *N=9 saline, 8 VPA.* Female mice did not display any aggression and therefore are not shown.

### 3.3.6 Prepulse Inhibition (PPI)

*There was a trend for VPA-treated mice to have a greater startle response to pulse-alone trials.*

The data in this test provides startle response to pulse alone, as well as inhibition (PPI) of this startle response in the presence of a prepulse (the prepulse+pulse trials, see Chapter 2). The primary measure was PPI, since this has been linked to schizophrenia and early life manipulations have affected PPI levels (Romero, Guaza, Castellano, & Borrell, 2010; Wolff & Bilkey, 2008). In the present case, there were no effects of sex or VPA treatment on PPI, as male and female mice treated with VPA did not differ in PPI magnitude when compared to corresponding saline-injected controls (see Figure 3.7a). For the pulse-alone trials, the first five




(a) (b)

Figure 3.7: Prepulse inhibition and absolute startle response results for VPA- and saline-treated mice. (a) Mean percent inhibition at each prepulse+pulse intensity. There was no effect of sex or treatment. (b) Mean absolute startle responses to the pulse alone trials. There was a trend toward VPA-treated mice having a greater startle response to the pulse-alone trials than saline-treated mice. *N=13 saline; 14 VPA.*

trials were considered habituation trails and were excluded from analysis. A 2 x 2 (Treatment x Sex) ANOVA revealed a trend for VPA-treated mice to have a larger startle response to the pulse-alone trials ($F_{(1, 25)}$=3.419, p=.076, see Figure 3.7b). There were no differences between males and females.

### 3.3.7 Neurochemical Analysis

*There were no treatment differences in levels of serotonin, 5HIAA, or dopamine for any of the brain regions assessed.*

The Bonferroni correction for multiple comparisons was used for the five brain regions studied. Therefore, the adjusted level was set to .01 (.05/5). There were no differences for levels of serotonin, 5HIAA, or for rates of serotonin turnover (5HIAA/5-HT) between VPA- and saline-treated mice for any brain region, as assessed by one-way ANOVA. In addition, there were no differences for levels of dopamine between VPA- and saline-treated mice for any brain region.

Table 3.1: Final concentrations of serotonin, 5HIAA, and dopamine for five brain regions, based on intial tissue weight. Serotonin turnover was calculated as 5HIAA/5-HT.

| | | Frontal Cortex | Hypothalamus | Striatum | Hippocampus | Cerebellum |
|---|---|---|---|---|---|---|
| Tissue Weight | Saline | 0.04 (.012) | 0.01 (.004) | 0.03 (.006) | 0.02 (.011) | 0.04 (.004) |
| | VPA | 0.03 (.007) | 0.01 (.003) | 0.03 (.004) | 0.03 (.008) | 0.04 (.013) |
| | F ($p$) | 3.530 (.077) | 0.089 (.77) | 0.02 (.89) | 1.02 (.331) | .001 (.975) |
| Serotonin | Saline | 0.67 (.333) | 1.54 (.636) | 0.93 (.278) | 0.83 (.409) | 0.39 (.181) |
| | VPA | 0.68 (.233) | 1.56 (.37) | 1.03 (.272) | 0.91 (.317) | 0.53 (.273) |
| | F ($p$) | .313 (.583) | 0.007 (.933) | 0.662 (.427) | 0.24 (.63) | 1.501 (.239) |
| 5HIAA | Saline | 0.37 (.267) | 1.14 (.824) | 0.59 (.314) | 0.82 (.496) | 0.35 (.154) |
| | VPA | 0.33 (.194) | 0.98 (.554) | 0.68 (.465) | 0.71 (.288) | 0.44 (.232) |
| | F ($p$) | .232 (.636) | 0.176 (.682) | 0.275 (.607) | 0.342 (.567) | .785 (.39) |
| Serotonin Turnover | Saline | 0.59 (.302) | 0.72 (.355) | 0.63 (.265) | 1.09 (.508) | 1.01 (.424) |
| | VPA | 0.52 (.275) | 0.62 (.309) | 0.58 (.351) | 0.80 (.215) | 0.86 (.307) |
| | F ($p$) | .017 (.898) | 0.333 (.574) | 0.12 (.733) | 2.507 (.132) | .718 (.41) |
| Dopamine | Saline | 1.51 (.767) | 1.42 (.635) | 8.01 (2.73) | 0.85 (.534) | 0.5 (.330) |
| | VPA | 1.27 (1.00) | 1.43 (.751) | 10.07 (2.33) | 1.05 (.382) | 0.86 (.325) |
| | F ($p$) | .673 (.423) | 0.001 (.981) | 2.958 (.104) | 0.869 (.364) | 5.055 (.04) |
| *N* | *Saline* | *11* | *9* | *12* | *10* | *13* |
| | *VPA* | *9* | *6* | *8* | *9* | *9* |

## 3.4 Discussion

Early life effects of valproic acid (VPA) have typically involved prenatal treatment as a model of ASD (Roullet et al., 2013). However, some evidence exists on postnatal treatment with VPA, especially at age P14, where it was found that VPA treatment led to regression in a developmental task, working memory deficits, and fewer social behaviors (Wagner et al., 2006; Yochum et al., 2008). However, while this previous behavioral testing was primarily conducted during the juvenile period, the present study extended these prior studies into adulthood. The results demonstrated that early postnatal exposure to VPA at age P14, can result in deficits that are evident in adulthood. However, these behavioral deficits are not necessarily suggestive of autism, and may represent a more general model of neurodevelopmental dysfunction.

Firstly, VPA-treated males were observed to weigh more than saline-treated males. This is inconsistent with previous work that showed no body weight differences in VPA-treated mice after P22 (Wagner et al., 2006). However, previous work has used Balb/c mice, whereas the present study used C57BL/6 mice. It is very

well established that C57BL/6 and Balb/C mice vary dramatically along a range of behavioral, neurochemical and endocrine parameters (Caldji, Diorio, Anisman, & Meaney, 2004; Deroche et al., 1997; Griebel, Belzung, Perrault, & Sanger, 2000). Therefore, the observed inconsistency may be the result of strain differences. Furthermore, the Wagner study consisted of several developmental tests between P14 and P22, whereas in the present study no behavioral testing was conducted between the time of injection and the first behavioral test and body weight measurement at P30. These inclusions of developmental testing in the Wagner study may have been stressful for the experimental mice, which may have interacted with treatment effects to counteract the increased weight observed in VPA-treated males.

In the social approach test, differences were not seen between saline- and VPA-treated mice on any variable at either P30 or P90. Therefore, the social play test was conducted in a separate experiment, on a separate cohort of mice treated with VPA or saline at P14, and then tested for social play between P30 and P40. Whereas the social approach test measures an animal's motivation to approach a novel peer, the social play test scores the structure of play behavior and may therefore be a more sensitive measure of disrupted social behavior. Although VPA-treated male mice initiated more instances of aggression, there were no differences between saline- and VPA-treated mice in allogrooming, ano-genital sniffing, face-sniffing, chasing, self-grooming, or crawl over/under behaviors. This was surprising, as previous work demonstrated differences in ano-genital sniffing, allogrooming, and crawl under/over (Yochum et al., 2008). The lack of VPA effect in the present study may be due to several key differences between the current and previous studies. Firstly, social play behavior in the Yochum et al study was conducted at the start of the dark cycle, whereas the present study conducted behavioral testing during the light cycle. Given that nocturnal behavior varies from that in the diurnal phase, this may account for the lack of social play differences between saline and VPA treated mice. Secondly,

and most importantly, the Yochum et al study paired treatment-matched Balb/c mice for play observation, whereas the present study paired experimental C57BL/6 mice with untreated Balb/c mice. In addition to potential strain differences, the use of Balb/c mice as strangers allowed the mice to be differentiated on camera, which facilitated scoring of experimental (C57) mouse-initiated behaviors. The counting of only experimental mouse-initiated behavior may have further accounted for the lower number of overall play behavior scores. It is also important to note that Balb/c mice are more sensitive to the effects of VPA (unpublished data), and that a higher dose of VPA may be needed to result in social behavior deficits in C57BL/6 mice. Ongoing studies in the Wagner lab are seeking to quantify potential sensitivity differences between Balb/c and C57BL/6 mice to VPA.

Anxiety disorders are highly comorbid with ASD, and therefore animals were tested for anxiety-like behavior in the EPM. Indeed, evidence suggests that prenatal exposure to VPA may result in a more anxious phenotype in the offspring, although less is known about postnatal VPA effects (Markram et al., 2008; Roullet et al., 2013; Schneider et al., 2008). The current results showed that VPA-treated males (but not females) spent more time and travelled a greater distance in the open arms of an elevated plus maze on day 1 of testing, when compared to saline-treated males. This was surprising, given that increased time spent in the open arms of the EPM is considered indicative of a less anxious phenotype. Importantly, it also contrasts with the increased anxiety that was observed in the prenatal VPA model of ASD (Markram et al., 2008; Schneider et al., 2008; Schneider et al., 2007) suggesting that VPA exposure at different neurodevelopmental stages shifts anxiety-like behaviors in opposing directions. However, the mechanisms ultimately affected will require further investigation.

The fact that only VPA-treated males were affected in the EPM implies that there may be sex differences in how VPA affects specific behaviors. In one study,

which looked at both male and female offspring of VPA-treated mothers, increased anxiety was present only in males (Schneider et al., 2008). While the effect after postnatal treatment is opposite to this prenatal treatment, the effects do seem to be confined to males. This could reflect sex differences in response to VPA treatment or sex differences in response to elevated plus maze exposure. However, in the present study, differences between saline-treated males and females did not reach statistical significance, in contrast to others who have reported increased open arm exploration in female rodents when compared to their male counterparts (Johnston & File, 1991; Walf & Frye, 2007). The reasons for this discrepancy is not known, but may be due to handling and manipulation for preceding behavioral testing (eg., social behavior).

Our EPM procedure involved two days of testing, which is not commonly conducted, but can reveal potentially important effects, since behavior on day 1 varies dramatically from day 2. For males, the VPA effects of increased time spent and distance travelled in the open arms of the EPM were only apparent on day 1 of testing. On day 2 of testing, which took place 72 hours after day 1, there were no differences between VPA- and saline-treated male mice. Consistent with previous findings, a single exposure to the EPM reduces open arm activity on retest, a phenomenon known as one-trial tolerance (OTT). Interestingly, OTT also abolishes the anxiolytic response of benzodiazepines on a second EPM test (Albrechet-Souza et al., 2007; File, 2001; Rodgers & Shepherd, 1993). This suggests that GABAergic mechanisms may not be operating during retest, and implies that first and second exposures to the EPM may also be associated with different emotional states.

Individuals with autism are often highly resistant to change, and can display perseveration of behavior, a core feature of autism. In the present study, VPA-treated male mice, but not female mice, took longer and made significantly more errors to reverse learned behavior in the Y maze task. Acquisition in the Y maze was not affected. Therefore, when taken together with decreased avoidance of the open

arms in the EPM and increased aggression in the presence of a novel peer (social play test), impaired reversal learning in the Y maze task points to the hypothesis that a disinhibited or "impulsive" phenotype develops in males treated with VPA on P14. Increased impulsivity is often observed in children with ASD; although impulsivity is by no means unique to ASD. Other disorders associated with disinhibition include ADHD, which is highly comorbid with ASD (Gargaro, Rinehart, Bradshaw, Tonge, & Sheppard, 2011; Lopez, Lincoln, Ozonoff, & Lai, 2005; Simonoff et al., 2008). Our findings are therefore consistent with a recent epidemiological report which found that prenatal exposure to VPA, but not other antiepileptic drugs, was associated with an increased risk of developing ADHD (Christensen et al., 2019). It is possible that an increase in impulsivity related to early life exposure to VPA may reflect dysfunction of a neurobiological domain that is common to several developmental disorders, including ASD and ADHD. A strong candidate for this may be fronto-striatal circuitry, as dysfunction of these pathways has been observed in both ASD and ADHD (Gargaro et al., 2011). As the frontal lobes are the slowest region of the brain to develop, they may remain susceptible to toxicant-induced damage well into the postnatal period, beyond the time points that are typically studied.

Prepulse inhibition of the acoustic startle response is considered a test of sensorimotor gating in both humans and rodents. And sensorimotor gating deficits have been reported in individuals with ASD (McAlonan et al., 2002; Perry, Minassian, Lopez, Maron, and Lincoln, 2007; although no differences have also been reported, see Kohl et al., 2014; Oranje, Lahuis, van Engeland, Jan van der Gaag, and Kemner, 2013; Yuhas et al., 2011). The present study found no differences between saline- and VPA-treated males and females in PPI. However, there was a trend toward VPA-treated mice to have an increased startle response to the pulse-alone trials. Interestingly, this increased startle response did not display the male-bias observed in several of the other behavioral tests. The reasons for this may be due to the PPI

test involving a sensorimotor component. Most importantly, however, given that deficits in PPI are observed in schizophrenia (Mena et al., 2016), the current data are also not consistent with P14 VPA treatment influencing the development of a schizophrenia-like phenotype. Although caution should be exercised in completely discounting this notion, since impulsivity is a prominent feature of schizophrenia (Bellgrove et al., 2006; Heerey, Robinson, McMahon, & Gold, 2007; Kaladjian, Jeanningros, Azorin, Anton, & Mazzola-Pomietto, 2011).

In conclusion, the current study found increased aggression in a social play test, decreased anxiety in the elevated plus maze, and impaired reversal learning in the Y maze in male mice treated with VPA at P14. The abnormalities suggest a disinhibited behavioral profile associated with early postnatal VPA exposure in male rodents. Increased impulsivity is associated with a number of neurodevelopmental disorders, including ASD, ADHD, and conduct disorders. Future studies may wish to explore the mechanism by which VPA affects fronto-striatal circuitry, and how timing of VPA exposure in the postnatal period affects the observed behavioral profile.

# Chapter 4

# Behavioral Profile Following Valproic Acid Exposure on Postnatal Day 7

## 4.1 Introduction

Valproic acid (VPA) has been linked to autism spectrum disorder (ASD)-like phenotypes following midgestational exposure. Furthermore, VPA may also be associated with abnormal developmental following postnatal exposure (see Chapter 3). However, as the majority of studies examining the effects of early postnatal exposure to VPA have only used one time point, P14, it is difficult to draw conclusions about the role of timing of postnatal VPA exposure on behavioral outcomes.

Recent epidemiological evidence has suggested that environmental insults during late gestation, i.e. the third trimester, and during birth may be important risk factors for disorders such as ASD and schizophrenia. For example, third trimester CMV infection may be associated with increased risk for ASD (Yamashita, Fujimoto, Nakajima, Isagai, & Matsuishi, 2003). Furthermore, birth complications such

as planned or emergency cesarean section, fetal distress, and maternal hemorrhage have been associated with increased risk for both ASD and schizophrenia (Brimacombe, Ming, & Lamendola, 2007; Brown, 2011; Fineberg, Ellman, Buka, Yolken, & Cannon, 2013; Gardener, Spiegelman, & Buka, 2011; Hultman, Sparén, & Cnattingius, 2002; Mittal et al., 2009). Several studies have shown that multiple birth complications are a risk factor for later psychiatric conditions. Thus, while there is not enough evidence to implicate specific birth complications with increased risk, exposure to a broad class of events at around the time of birth may increase risk. However, it remains unclear whether these events are causal in nature, or if they represent additional consequences of other prenatal or genetic events that increase risk for psychiatric illness. Therefore, an animal model of environmental challenge representing this period of developmental vulnerability would be useful.

Although the timescale of CNS development varies across species, the sequence of key events is largely conserved between humans and rodents. Therefore, it is possible to equate developmental time points between humans and rodents. Traditionally, P7 has been used as a time point representative of a full-term (37 – 42 weeks) human infant. This equivalence was based on measurement done in the 1970s on post-mortem brain weights, which showed that the brain growth spurt peaks in the rat at P7 and in the human at about the time of birth. However, this method of equating CNS development does not account for the heterogeneous development of different brain regions, which mature at different rates.

In the present study, P7 was used to represent a term infant based on the development of the GABA system. Neurotransmitter systems also develop at different rates across species, and therefore have different age-related milestones. Studies have shown that levels of the GABA-synthesizing enzyme, glutamate decarboxylase, are equivalent at 7.4 days in the rat and 40 weeks in the human (Semple et al., 2013).

## 4.2 Methods

Pregnancies were generated using the standardized protocol described in Chapter 2. The day of birth was recorded as postnatal day 0 (P0). Pups were maintained with their mothers until weaning at 4 weeks, at which time mice were separated into same-sex cages (3 – 4 per cage).

A detailed description of the behavioral tests can be found in Chapter 2, and a brief description of the schedule can be found in Chapter 3. In addition to the behaviors tests described in Chapter 3, the present study also assessed motor development in the days following VPA-treatment with the surface righting test, described below (see Figure 4.1. Lastly, in this study, social play behavior was not assessed as a follow-up to social approach testing.

Treatment with VPA at P7. A dose of 400 mg/kg administered at postnatal day 7 (P7) resulted in over 75% mortality during preliminary tests, and so was not used. Instead, mice were treated s.c. with either saline or VPA 300 mg/kg. This resulted in 10% mortality, which was the equivalent mortality rate of 400 mg/kg in P14 pups. Each litter consisted of both saline- and VPA-treated pups.

Body Weight Measurements (P7 - P9, P30, P90) Body weight measurements were taken immediately before mid-air righting and social approach testing.

Surface Righting (P7 – P9). Mouse pups were placed on their backs with all four limbs extended outwards. Time to right such that all four paws were touching the surface was recorded, with a maximum time of 30 seconds. This test was repeated three times for each mouse.


(a)


(b)

Figure 4.1: Behavioral testing timelines. Behavioral tests described in (a) and (b) were run on separate cohorts of mice.

## 4.3 Results

### 4.3.1 Body Weight

*VPA-treatment reduced body weight for up to 30 days.*

Treatment with VPA on P7 produced a rapid change in body weight, as determined by a 2 x 2 x 3 (Treatment x Sex x Day) repeated-measures ANOVA run for body weight data collected on days P7 – P9. This showed a main effect of Day ($F_{(2, 18)}$=31.689, p<.001), and a Day x Treatment interaction ($F_{(2, 18)}$=62.920, p<.001). Post-hoc simple main effects showed that there were no differences between treatment groups on P7 (immediately before treatment). However, there was a significant difference between VPA- and saline-treated pups on P8 ($F_{(1, 25)}$=23.454, p<.001) and P9 ($F_{(}$1, 27)=30.887, p<.001), such that VPA-treated pups weighed less than their saline-treated counterparts (see Figure 4.2).

Later-life body weight was analyzed by a 2 x 2 (Treatment x Sex) ANOVA on weight measures collected on days P30 and P90. On P30, there was a main effect of Treatment ($F_{(1, 23)}$=5.118, p =.033), such that VPA-treated mice weighed less than their saline-treated counterparts. On P90, there was a main effect of Sex ($F_{(1, 26)}$=7.330, p=.012), such that male mice weighed more than female mice at P90. However, there was no longer an effect of Treatment ($F_{(1, 26)}$=.262, *ns*) on P90.

Thus, VPA-treated mice weighed less than their saline-treated counterparts 24 and 48 hours post-treatment, and remained underweight at P30. However, experimental animals recovered by P90 when there were no longer differences between VPA- and saline-treated mice (see Figure 4.2).

### 4.3.2 Surface Righting

*VPA-treatment led to increased latency to surface right.*

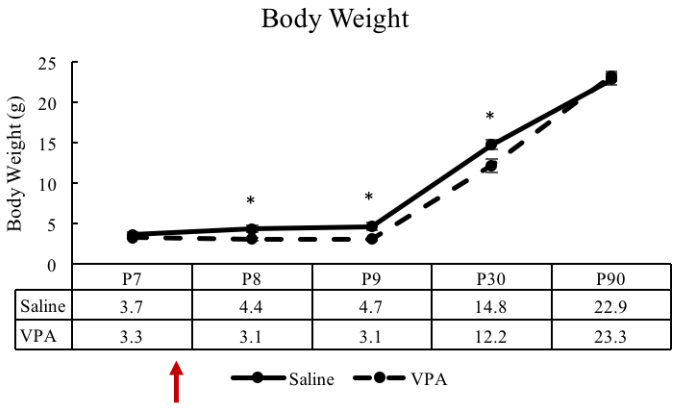



Figure 4.2: Body weight changes in mice given VPA or saline on P7. VPA-treated mice weighed less than their saline-treated counterparts 24 and 48-hours post-treatment (timing of treatment indicated by red arrow) and remained underweight at P30. By P90, there were no differences in body weight between VPA- and saline-treated mice. *N=7 saline; 6 VPA.*

During the surface righting test, pups were turned onto their dorsal surface and latency to completely right (stand on all four paws) was recorded. A 2 x 2 x 3 (Treatment x Sex x Day) repeated-measures ANOVA was conducted for latency to right on days P7 – P9. There was a main effect of Day ($F_{(2, 56)}$=26.181, p<.001), as well as a Day x Treatment interaction ($F_{(2, 56)}$=25.435, p<.001). Post-hoc simple main effects showed that there were no differences between treatment groups on P7 (immediately before treatment, $F_{(1, 33)}$=.189, *ns*). However, there was a significant difference between VPA- and saline-treated pups on P8 ($F_{(1, 33)}$=90.022, p<.001), with VPA-treated pups taking longer to right than their saline-treated counterparts. In addition, there was also a trend for VPA-treated pups to take longer to right on P9 ($F_{(1, 33)}$=3.533, p=.069) (see Figure 4.3).

### 4.3.3 Social Approach

*Treatment had no effect on distance travelled during the 5-minute habituation period of the social approach test.*

The social approach test was given twice, once at P30 and again at P90. The test

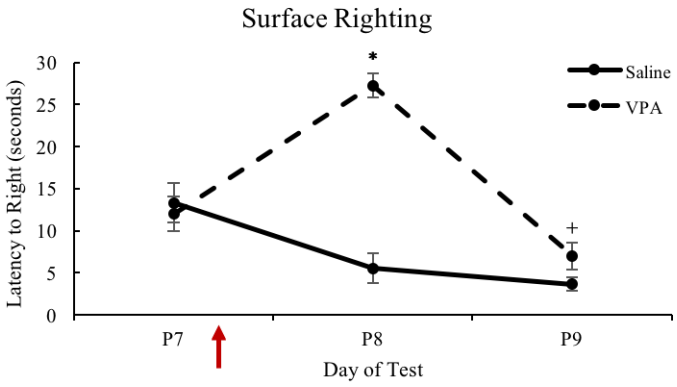



Figure 4.3: Latency to surface right in mice given VPA or saline on P7. VPA-treated pups took longer to surface right 24-hours post-treatment (timing of treatment indicated by arrow). On P9 (48-hours post-treatment) this had dramatically recovered, although there was still a trend toward VPA-treated mice taking longer to surface right. *N=7 saline; 6 VPA.*

is divided into three phases, as described in Chapter 2, and initially, a five-minute habituation phase was given, during which animals explored the apparatus and the empty wire chambers. Distance travelled during habituation was analyzed by a 2 x 2 (Treatment x Sex) ANOVA. At P30, there was a main effect of Sex ($F_{(1, 25)}$=4.666, p=.041), as well as a borderline Sex x Treatment interaction ($F_{(1, 25)}$=3.543, p=.071). Pairwise comparisons revealed that females travelled more during the 5-minute habituation period than males in the VPA-treated group only (see Figure 4.4). However, at P90, there was no effect of Sex on distance travelled during the habituation period.

*Juvenile VPA-treated females spent more time in contact with S2 during Phase B than any other group.*

During the social approach test, time spent in contact with each wire chamber (S1 and S2) was automatically recorded. During Phase A, S1 housed a novel weight- and sex-matched peer. During Phase B, the same peer (from Phase A) remained in S1, and another (novel) peer was housed in S2.

A 2 x 2 x 2 (Treatment x Sex x Phase) repeated-measures (across Phase) ANOVA

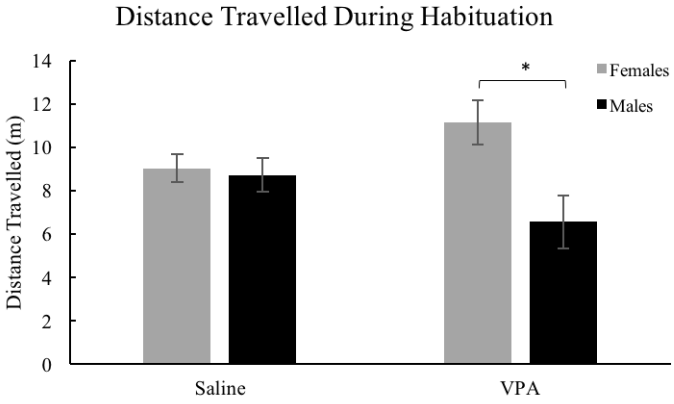



Figure 4.4: Distance travelled during the 5-minute habituation period of the social approach task at P30. Females travelled farther than males in the VPA-treated group only. *N, females=5 saline, 4 VPA; N, males=11 saline; 9 VPA.*

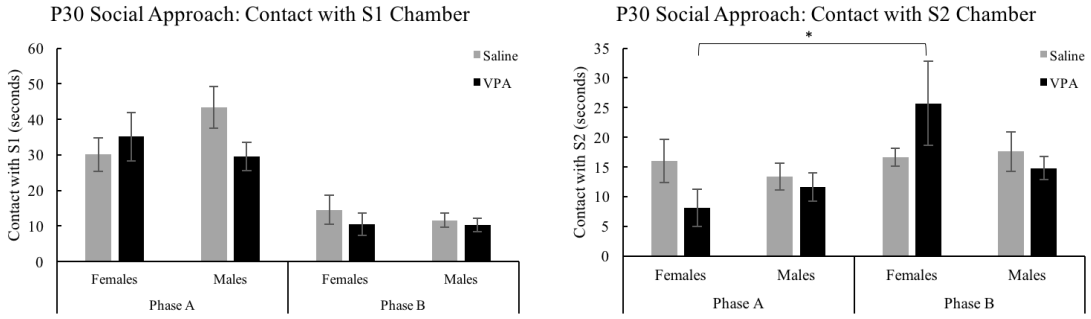
was run for time spent in contact with S1 and time spent in contact with S2. At P30, for time spent in contact with the S1 chamber, there was a main effect of Phase ($F_{(1, 25)}$=62.422, p<.001), such that mice spent less time in contact with S1 during Phase B (see Figure 4.5a).

For time spent in contact with the S2 chamber, there was a main effect of Phase ($F_{(1, 24)}$=9.8, p=.005), and a Phase x Treatment x Sex interaction ($F_{(1, 24)}$=4.890, p=.037). VPA-treated female mice spent more time in contact with S2 during Phase B (when there was a novel peer present in this chamber) as compared with Phase A (when this chamber was empty) (see Figure 4.5b).

*Adult VPA-treated females spent less time in contact with S2 than any other group.*

At P90, there was a main effect of Phase on time spent in contact with the S1 chamber ($F_{(1, 23)}$=49.318, p<.001), such that mice spent less time in contact with the S1 chamber during Phase B. There was also a main effect of Phase on time spent in contact with the S2 chamber ($F_{(1, 24)}$=6.071, p=.021), such that mice spent more time in contact with the S2 chamber during Phase B.

There was no effect of Treatment or Sex on time spent in contact with S1 (see Figure 4.6a). However, there was a main effect of Treatment ($F_{(1, 24)}$=.034, p=.034),




(a) (b)

Figure 4.5: Contact time with each chamber during the social approach test at P30. (a) Mice spent more time in contact with S1 during Phase A. There was no effect of Treatment or Sex. (b) For VPA-treated female mice, time spent in contact with S2 increased during Phase B, when a novel peer was present in this chamber. This was not true for any other group. *N, males=11 saline, 9 VPA; N, females=5 saline; 3 VPA.*

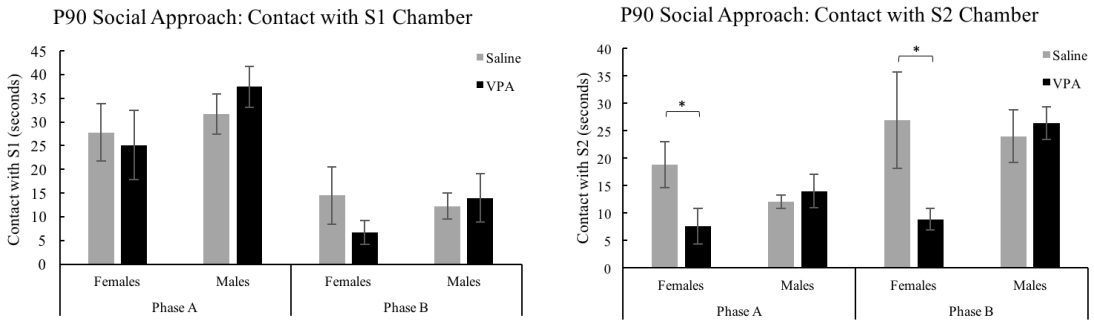
and a Treatment x Sex interaction ($F_{(1, 24)}$=9.310, p=.005) for time spent in contact with S2. VPA-treated female mice spent less time in contact with the S2 chamber overall (i.e., across both phases) (see Figure 4.6b).

### 4.3.4 Elevated Plus Maze (EPM)

*There was no effect of VPA-treatment on exploration of the open arms in the elevated plus maze.*

Animals were given the EPM on two separate days; therefore, a 2 x 2 x 2 (Treatment x Sex x Day) repeated-measures ANOVA was performed for the variables time spent and distance travelled in the open arms. Proportion of time spent and distance travelled in the open arms were calculated by dividing the time spent or distance travelled in the open arms of the maze by the time spent or distance travelled in both the open and closed arms of the maze. The arcsine transformation of these proportions were used in all analyses.

For time spent in the open arms, there was a main effect of Day ($F_{(1, 26)}$=65.522, p<.001), such that mice spent less time in the open arms on Day 2. There was no




(a) (b)

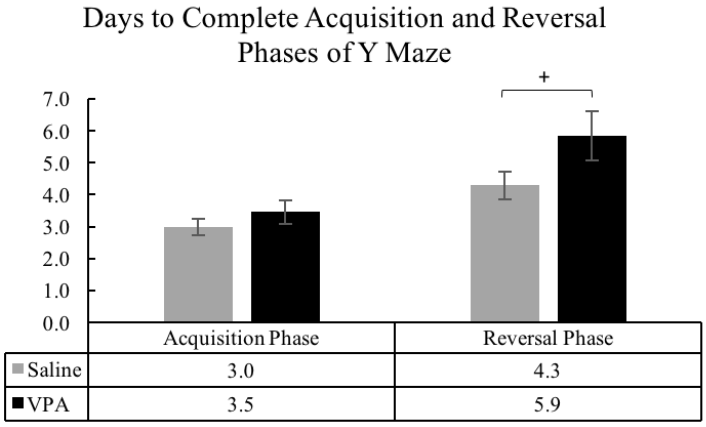
Figure 4.6: Contact time with each chamber during the social approach test at P90. (a) Mice spent more time in contact with S1 during Phase A. (b) VPA-treated female mice spent less time in contact with the S2 chamber overall than saline-treated female mice. *N, males=10 saline, 7 VPA; females=6 saline; 5 VPA.*

effect of Treatment or Sex. For distance travelled in the open arms, there was a main effect of Day ($F_{(1, 26)}$=34.630, p<.001), such that mice travelled less on the open arms on Day 2. There was no effect of Treatment or Sex.

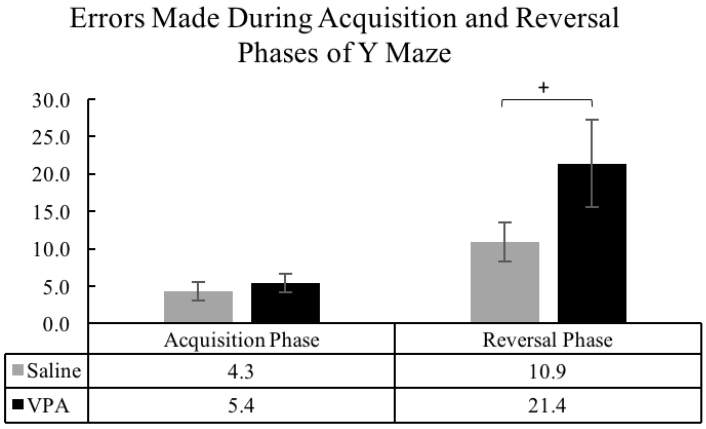
### 4.3.5 Water Y Maze

*VPA-treated mice displayed longer times to reach criterion and made more errors during the reversal phase of Y maze.*

A 2 x 2 (Treatment x Sex) ANOVA was performed for number of days to reach criterion and number of errors made for the acquisition phase and the reversal phase. There was no effect of either Treatment or Sex on either days or errors to acquire the task. There was a trend for an effect of Treatment on number of days to reach criterion for reversal learning ($F_{(1, 23)}$=3.851, p=.062), such that VPA-treated animals took longer to complete the reversal phase (see Figure 4.7a. There was also a trend for an effect of Treatment on errors to reverse ($F_{(1, 23)}$=3.875, p=.061), such that VPA-treated animals tended to make more errors during the reversal phase (see Figure 4.7b).




(a)




(b)

Figure 4.7: Performance in the water Y maze in VPA- and saline-treated mice. There was a trend for VPA-treated mice to take longer to reach criterion during the reversal phase (a) and to make more errors during the reversal phase (b). *N=14 saline, 13 VPA.*

### 4.3.6 Prepulse Inhibition (PPI)

*VPA-treated mice had greater PPI at an intermediate prepulse intensity.*

As described in detail in Chapter 2, the PPI procedure involved an initial train of five pulse-alone trials, which served to establish as habituation trials and were excluded from analysis. Startle amplitude for all other pulse-alone trials were analyzed using a 2 x 2 (Treatment x Sex) ANOVA. Results did not reveal any Treatment or Sex effects.

For the prepulse-pulse trials, a 2 x 2 x 3 (Treatment x Sex x Prepulse Intensity) repeated measures ANOVA was conducted, with the three prepulse intensities (2, 4, and 8 dB above background) as the within-subjects variable. This revealed a significant Intensity x Treatment interaction ($F_{(2, 50)}$=3.337, p=.048), such that VPA-treated mice had greater prepulse inhibition than saline-treated mice when prepulse intensity was set at 4 dB above background noise level (see Figure 4.8).

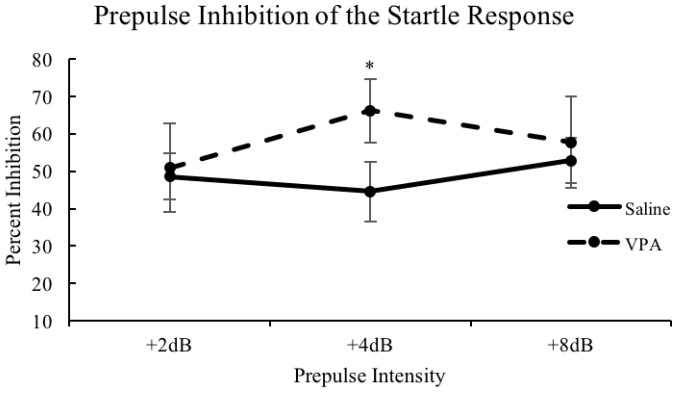



Figure 4.8: Percent prepulse inhibition in mice treated with VPA or saline at P7. A significant interaction between treatment and prepulse intensity was observed. Specifically, VPA-treated mice appeared to have greater prepulse inhibition when the prepulse intensity was 4 dB above background. *N=18 saline; 11 VPA.*

## 4.4 Discussion

The present study found that P7 exposure to VPA resulted in abnormalities in several behavioral outcomes. VPA-treated mice were significantly underweight compared to their saline-treated peers beginning 24-hours post-treatment and continuing into the juvenile period. However, by P90, there were no differences in body weight between VPA- and saline-treated mice. In addition, VPA-treated mice displayed a regression in surface righting ability 24-hours post-treatment. That is, latency to right increased from P7 (before treatment) to P8 (24-hours post-treatment) in VPA-treated pups, whereas latency to right decreased for saline-treated pups. Although there was dramatic functional recovery by P9 (48-hours post-treatment), there remained a trend for VPA-treated pups to take longer to right than saline-treated pups.

In the social approach test, there were no differences between VPA- and saline-treated mice for time spent with Stranger 1 (S1) during Phase A. Typically, behavior in Phase A of the social approach task is used as a measure of general sociability (Yang, Silverman, & Crawley, 2011). In Phase B of the social approach task, experimental mice are given a choice between the now-familiar peer from Phase A (Stranger 1, S1) and a novel peer (Stranger 2, S2). A preference for social novelty is expected (Yang et al., 2011), indicated by a preference for S2 over S1. In the present study, female VPA-treated mice were significantly different than their saline-treated counterparts for Phase B, but, surprisingly, this difference was in the opposite direction during juvenile testing and adult testing. Juvenile VPA-treated females spent an increased amount of time in contact with the S2 chamber, indicating increased preference for social novelty. In contrast, at P90, VPA-treated females spent a decreased amount of time in contact with the S2 chamber, indicated decreased preference for social novelty. Therefore, it appears that some effect of early life VPA-treatment is delayed until the post-juvenile period, with decreased social

behavior only present in adulthood.

There was a trend toward VPA-treated mice taking longer and making more errors during the reversal phase of the Y maze task. This may indicate subtle deficits in reversal learning. As there were no differences in the acquisition phase of the task, spatial learning appears to be intact.

Lastly, there increased PPI at an intermediate prepulse intensity, with no differences in the startle response to the pulse alone. Prepulse inhibition assesses sensorimotor gating, by which irrelevant information is filtered out in the early stages of processing. In the case of the prepulse inhibition procedure, the prepulse is the initial stimulus to which the nervous system attends, thereby necessitating a "gating out" of the pulse stimulus that closely follows the prepulse to allow for processing of the initial stimulus without disruption from the stimulus which immediately follows. Failure to do this will result in a persistent response to the pulse (in a prepulse-pulse trial), and evidence of potential abdication of prepulse processing. The clinical significance of retaining PPI (i.e. holding on to the prepulse, and therefore, attenuating the response to the closely following pulse) has been demonstrated through observation of decreased PPI in a number of psychiatric conditions, including schizophrenia, obsessive compulsive disorder, Tourette's syndrome, and post-traumatic stress disorder (Braff, Geyer, & Swerdlow, 2001). Alternatively, increased PPI may be related to positive outcomes in healthy subjects – for example, one study found that increased PPI was associated with superior strategy formation and execution times in males (Bitsios, Giakoumaki, Theou, & Frangou, 2006). However, increased PPI has also been observed in rodents following physical stress, such as foot shock (Grillon & Davis, 1997; Pijlman, Herremans, van de Kieft, Kruse, & van Ree, 2003) suggesting that increased PPI may represent, in some cases, a deviation from normal functioning.

The neurochemical basis of PPI is thought to reside in dopaminergic limbic

circuitry, including the nucleus accumbens and hippocampus (Swerdlow, Geyer, & Braff, 2001). For example, PPI is sensitive to changes in dopamine levels, with dopamine agonists typically disrupting PPI (Li, Olsen, Vestergaard, & Obel, 2011). However, other neurotransmitter systems may also be involved. In humans, low doses of ketamine increase PPI in healthy men (Abel, Allin, Hemsley, & Geyer, 2003; Duncan et al., 2001). Although at high doses ketamine is an NMDA receptor antagonist, at low doses it may actually enhance glutamatergic neurotransmission in the PFC (Moghaddam, Adams, Verma, & Daly, 1997). Furthermore, although decreased PPI is a consistent finding in schizophrenia populations, there have been mixed results regarding correlations between PPI deficits and positive and negative symptom profiles (Kumari, Soni, Mathew, & Sharma, 2000; Weike, Bauer, & Hamm, 2000). High doses of ketamine are associated with the full spectrum of schizophrenia symptoms, whereas low doses are associated with primarily negative symptoms, such as avolition and flattened affect. Therefore, a low dose of ketamine may cause enhanced glutamatergic neurotransmission and increased central inhibition that may manifest behaviorally as abnormal insensitivity to environmental stimuli. Other measures of increased central inhibition, such as latent inhibition (Cohen et al., 2004; Rascle et al., 2001), skin conductance hyporesponsiveness (Mirkin, 1985), and orienting response non-response (Bernstein, 1987), are seen in some individuals with schizophrenia who have predominantly negative symptoms.

Another possible mechanism through which PPI may become increased is through interactions with steroid sex hormones. One study found that estrogen treatment increased prepulse inhibition, and that the increase was similar when PPI was disrupted in rats with treatment of either apomorphine (a dopamine agonist) or MK-801 (a NMDA receptor antagonist), suggesting that estrogen treatment's effects on PPI are mediated by a separate central mechanism than dopaminergic or glutamatergic drugs (Van den Buuse & Eikelis, 2001). One candidate is the serotonin

system, as a 5-HT1A antagonist attenuate PPI disruption caused by administration of MK-801 (Wedzony, 2000).

In conclusion, P7 administration of VPA resulted in immediate failure to meet developmental and body weight milestones, as well as subtle social, reversal learning, and PPI differences. Social deficits were observed only in females, suggesting that some effects of early life VPA exposure are sex-specific.

# Chapter 5

# Effect of Postnatal Valproic Acid Treatment on Dendritic Spine Density and Morphology

## 5.1 Introduction

Autism spectrum disorder (ASD) is associated with a number of structural and functional alterations of the CNS in both human and animal models. For example, in humans, one of the most consistently reported alterations associated with ASD is increased average brain size in young children with ASD (Courchesne, Campbell, & Solso, 2011; Redcay & Courchesne, 2005). In adults with ASD, average brain size does not differ from that of controls, although there is a larger degree of variance in individuals with ASD (Redcay & Courchesne, 2005). Other structural differences observed in individuals with ASD include increased cortical thickness (Bailey et al., 1998), increased neuronal density (Bailey et al., 1998), enlarged cerebellum (Piven, Saliba, Bailey, & Arndt, 1997), and abnormal amygdala development (Munson et al., 2006; Schumann et al., 2004; Sparks et al., 2002) (for a review of neuroanatomical

abnormalities in ASD, see Amaral, Schumann, and Nordahl, 2008).

The disrupted connectivity hypothesis of ASD suggests that connectivity changes may relate to the behavioral presentation of the disorder. For example, in the cerebral cortex, individuals with ASD may have increased local, short-range connections, which may behaviorally manifest as hyper-arousal and reduced selectivity (Markram & Markram, 2010). This local hyperconnectivity may also explain occasional superior abilities in certain tasks which require local information processing. However, local hyperconnectivity may also be accompanied by hypoconnectivity over long-range connections. This is supported by evidence from imaging studies, which have shown abnormal activity patterns between distal cortical areas.

Pyramidal cell (PC) abnormalities have been observed in animal models of ASD, including the prenatal valproic acid (VPA) model. A single prenatal exposure to VPA at embryonic day 11.5 results in significant connectivity changes in a number of brain regions, including the prefrontal cortex (PFC; Rinaldi, Perrodin, and Markram, 2008), somatosensory cortex (Rinaldi, Silberberg, & Markram, 2008), and amygdala (Markram et al., 2008). For example, layer V PCs in the PFC of VPA-exposed animals are connected to significantly more neighboring neurons than in controls. However, these connections are weaker in VPA-treated animals as compared to controls, with greater current required to achieve the same number of action potentials. Furthermore, these excitatory connections are more plastic, displaying enhanced long-term potentiation (LTP) following Hebbian pairing (Rinaldi, Perrodin, & Markram, 2008). Overall, these changes may result in hypersensitive neocortical circuits that may be difficult to control once activated, and could provide inadequate top-down executive control over multiple brain regions, including those involved in emotionality (eg., amygdala).

One method of assessing the quantity and distribution of connections is to examine the expression of dendritic spines on neurons, as higher synaptic connectivity

occurs between neurons with higher numbers of spines. The dendritic spines of pyramidal cells are thought to support excitatory connections, with over 95% of excitatory synapses on PCs occurring on spines (Fiala, Spacek, & Harris, 2002). Therefore, spine analysis can be used as an index of excitatory connectivity in the cortex. Furthermore, spines vary in shape and size, suggesting different states of maturity, as the formation, plasticity, and maintenance of spine morphology is dependent on synaptic activity and experience (Fiala et al., 2002). As such, examination of spine morphology, in addition to spine density, may provide insight into the functioning of the system. For example, filopodia, long/thin, thick, and stubby spines are thought to be less mature compared to mushroom-shaped spines (Fiala et al., 2002).

Notably, human postmortem studies have shown that spine density varies in brains taken from ASD individuals (Hutsler & Zhang, 2010). Brain tissue of individuals with ASD were compared to age-matched typically-developing controls, and found to have higher average spine densities, with this being greatest on oblique dendrites as compared to apical or basilar dendrites. Furthermore, preliminary analysis revealed that the spine length in the ASD group was also shorter, due to an increased number of compact spine types. In further analysis of the apical dendrites, greater spine densities were found in layer II of the frontal, parietal, and temporal lobes, as well as layer V of the temporal lobe. These higher spine densities were associated with decreased brain weight in the ASD group, but not the control group, and higher spine densities were more common in ASD subjects with lower levels of cognitive functioning (Hutsler & Zhang, 2010).

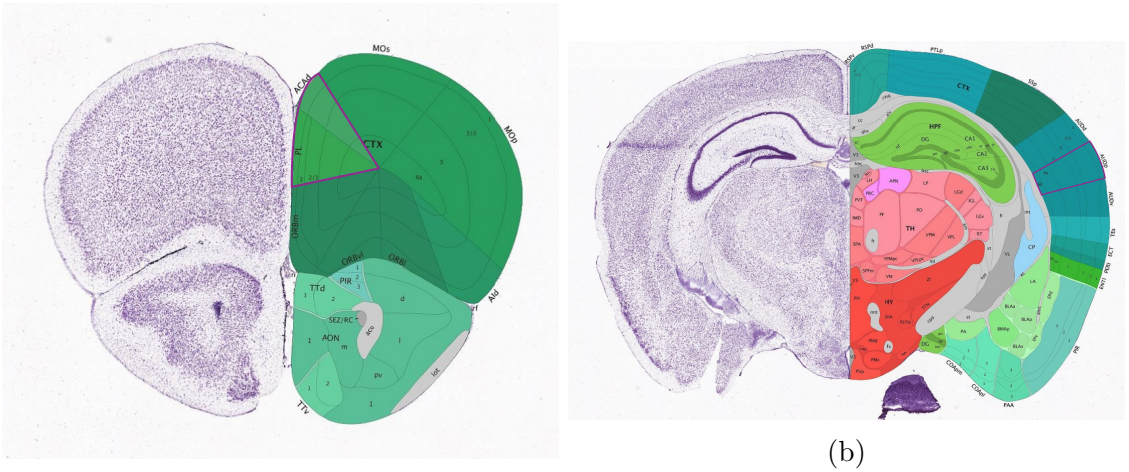
In the current study, it was sought to determine if differences in layer V PCs exist following postnatal exposure to VPA. Mice were exposed to VPA or saline on either P7 or P14. Dendritic spine density and morphology were then analyzed post-mortem on P30 – 35 in the medial prefrontal cortex (mPFC) and the primary

auditory cortex (AUDp) of the temporal lobe. These regions were chosen because of their association with the behavioral symptoms of ASD in both human and animal models of the disorder.

## 5.2 Methods

Animals and Treatment. Transgenic mice (Thy1-yFP-H) that express yellow fluorescence protein (yFP) in dendrites under the control of the Thy-1 promoter, were bred in-house from original breeding pairs supplied by Dr. Huaye Zhang. These mice (Feng et al., 2000) were previously obtained from Jackson Laboratories (JAX stock 003782). Expression of yFP is preferentially found in the neurons of layer V of the cortex, and not in other cortical layers. Moreover, non-neuronal cells do not express yFP. Treatment of mice with either VPA or saline occurred on P7 or P14, in both male and female Thy1-yFP pups, as described in Chapter 2.

Collection of brain tissue. Animals were sacrificed at 30 – 35 days. Whole brains were placed in PFA for 48 hours, then transferred to a 30% sucrose solution until slicing. Brains were then transferred to the cryostat, in which coronal sections 70 microns thick were sliced using a disposable microtome blade. Slices from the prefrontal cortex and auditory cortex were taken, mounted onto glass slides, left to dry for 24 hours, then cover-slipped. Select slides containing the medial prefrontal cortex and primary auditory cortex were visualized using a confocal microscope with a 60x objective and analyzed with ImageJ software (Schindelin et al, 2012). Brain regions were identified using the Allen Mouse Brain Atlas (©2004 Allen Institute for Brain Science. Allen Mouse Brain Atlas. Available from: https://mouse.brain-map.org/static/atlas). Example images from the atlas that were used to select brain regions are shown in Figure 5.1.




(a) (b)

Figure 5.1: Example images from the Allen Mouse Brain Atlas of the mPFC (a) and AUDp (b) that were used to select sections for image analysis. Image credit: Allen Institute

Spine density analysis. Independent samples t tests were run comparing VPA- and saline-treated animals for both the mPFC and the AUDp. Dendritic spines of any shape were counted and expressed per length (in microns) of the dendrite. In addition, analysis was run on length of dendrites included in the analysis.

Dendritic morphology analysis. A Z-stack image was imported into ImageJ and 1 – 3 segments of 10 – 20 microns were selected per dendrite. The length and width (in microns) were recorded for the longest and widest part, respectively, of each dendrite in the frame of the Z-stack where the spine was clearest. Length-width ratio (LWR) of each dendritic spine was calculated. Independent samples t tests were run comparing VPA- and saline-treated animals for width, height, and LWR. The alpha level was set to .0167 for multiple comparisons (.05/3 = .0167).

## 5.3 Results

### 5.3.1 Spine Density following P7 VPA-treatment

For animals that had been treated at P7, there was no difference between VPA- and saline-treated mice in either brain region for dendritic length (mPFC: t(84)=.813, *ns*; AUDp: t(102)=.327, *ns*) or number of dendritic spines per micron (mPFC: t(84)=.757, *ns*; AUDp: t(102)=.826, *ns*). Analysis was based on 29 dendrites in the mPFC and 61 dendrites in the AUDp from 3 saline-treated animals and 57 dendrites in the mPFC and 43 dendrites in the AUDP from 2 VPA-treated animals.

### 5.3.2 Spine Morphology following P7 VPA-treatment

For animals that had been treated at P7, there was no difference in the average width of dendritic spines (t(447.123)=-1.963, *ns*), or the LWR (t(447.123)=-1.679, *ns*) in the mPFC. However, there was a significant effect of Treatment on average length of dendritic spines in the mPFC, with spines from VPA-treated animals being longer (t(447.123)=-2.398, p=.017). Furthermore, equal variances could not be assumed for this data, as the spines from the VPA-treated group had a larger degree of variance than spines from the saline-treated group. Analysis was based on 176 dendritic spines from 3 saline-treated animals and 294 dendritic spines from 2 VPA-treated animals.

In the AUDp, there was no difference in the average length (t(1041)=1.295, *ns*) or the LWR (t(1041)=1,291, *ns*) of VPA- and saline-treated animals. There was a significant effect of Treatment for the average width of dendritic spines, with spines from VPA-treated animals being, on average, thinner (t(1041)=5.804, p<.001). Analysis was based on 558 dendritic spines from 4 saline-treated animals and 485 dendritic spines from 4 VPA-treated animals.

### 5.3.3 Spine Density following P14 VPA-treatment

For animals treated at P14, there was no difference in dendritic length between groups in the mPFC (t(49)=.336, *ns*). However, VPA-treated animals had significantly less dendritic spine density than saline-treated animals (t(49)=2.532, p=.015; see Figure 5.2). Analysis was based on 27 dendrites from 3 saline-treated animals and 24 dendrites from 3 VPA-treated animals.

In the AUDp, there was again no difference in dendritic length between groups (t(58)=1.663, *ns*). Interestingly, there was a trend for VPA-treated animals to have *greater* spine density than saline-treated animals in this brain region (t(58)=-1.725, p=.090; see Figure 5.3). Analysis was based on 35 dendrites from 2 saline-treated animals and 25 dendrites from 2 VPA-treated animals.

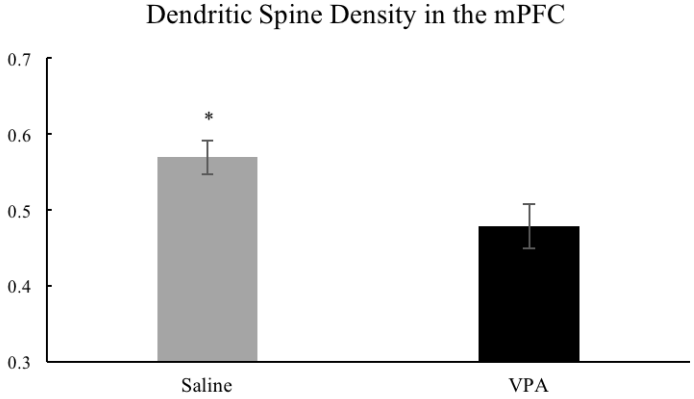



Figure 5.2: Mean dendritic spine density was reduced in the mPFC of mice treated with VPA at P14, as compared to saline controls.

### 5.3.4 Spine Morphology following P14 VPA-treatment

For animals that had been treated at P14, dendritic spines from VPA-treated animals were, on average, shorter (t(813)=2.435, p=.015), and had a smaller LWR ratio (t(813)=2.916, p=.004), although there was no difference in average width (t(813)=-.781, *ns*). Analysis was based on 331 dendritic spines from 4 saline treated

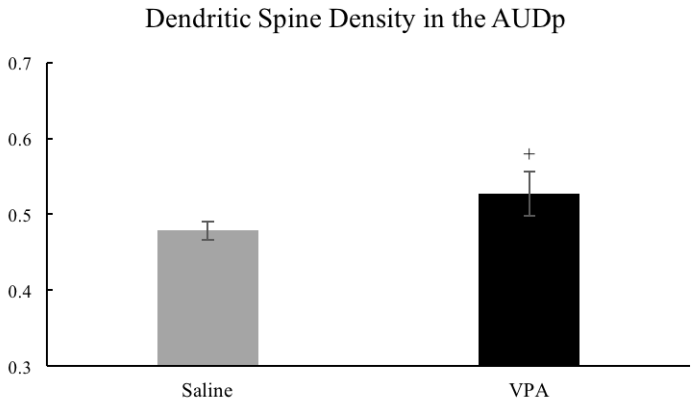



Figure 5.3: There was a trend toward mean dendritic spine density being greater in the AUDp of mice treated with VPA at P14, as compared to saline controls.

animals and 484 dendritic spines from 6 VPA-treated animals.

In the AUDp, dendritic spines from VPA-treated animals were, on average, thinner ($t(1137.495)=3.129$, $p=.002$), shorter ($t(1137.495)=4.736$, $p<.001$), and had a smaller LWR ($t(1137.495)=2.759$, $p=.006$). Equal variances could not be assumed for this data, as spines from the VPA-treated group had a *lesser* degree of variance than spines from the saline-treated group in the AUDp. Analysis was based on 749 dendritic spines from 4 saline-treated animals and 484 dendritic spines from 5 VPA-treated animals.

## 5.4 Discussion

In the present study, there were no differences in dendritic spine density between VPA- and saline-treated animals that had been treated at P7. However, there were more subtle differences in the dendritic spine morphology, presenting as longer dendritic spines in the mPFC of VPA-treated animals and thinner spines in the AUDp of VPA-treated animals. In addition, there was a greater degree of variability in the morphology of dendritic spines from VPA-treated animals in the mPFC.

Taller or thinner spines are present in a number of conditions involving mental

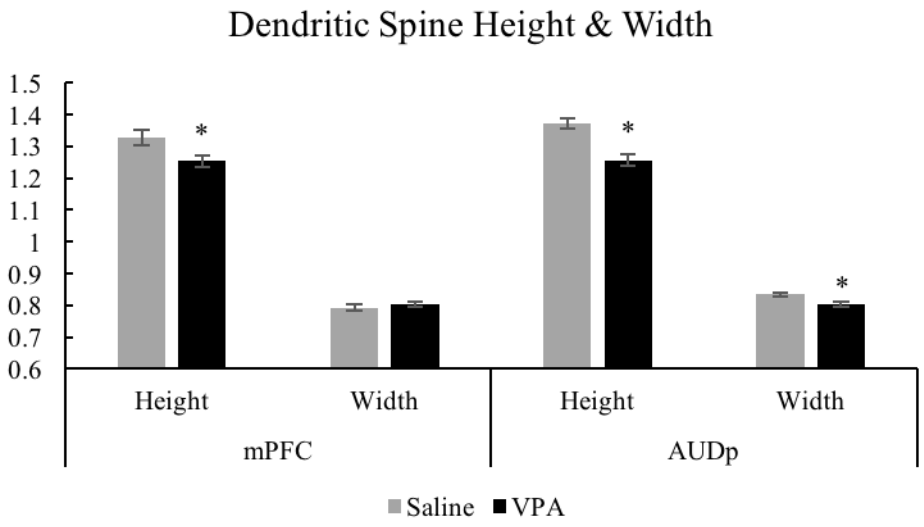
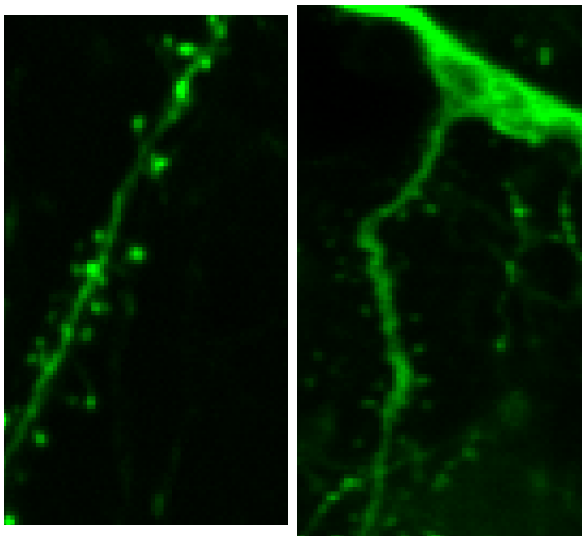



Figure 5.4: Height and width of dendritic spines from the mPFC and AUDp of mice treated with VPA or saline at P14. Dendritic spines from VPA-treated mice were shorter in the mPFC and shorter and thinner in the AUDp.



(a) (b)

Figure 5.5: Example images of dendritic spines in the mPFC of a saline (a) and VPA-treated (b) mouse.

retardation (Fiala et al., 2002). Thinner spines, along with the observed greater degree of variability in spine shape, are reminiscent of the filopodia which are present during early dendritic spine development. Filopodia are highly motile and are much longer than mature dendritic spines. The observed morphological changes may therefore represent a failure of the dendritic spines to stabilize as the brain matures. Furthermore, taller spines may have decreased synaptic efficacy, as longer spine necks appear to be correlated with weaker somatic depolarizations (Araya, Vogels, & Yuste, 2014).

For animals that had been treated at P14, there was a decrease in dendritic spine density in the mPFC. This is consistent with the behavioral profile observed following P14 VPA-exposure (see Chapter 3). Specifically, mice treated with VPA at P14 showed a behavioral profile indicative of reduced behavioral inhibition, including increased time spent and distance travelled in the open arms of the elevated plus maze, increased aggression, and impaired reversal learning in the water Y maze. This attenuation of behavioral inhibition may be explained by PFC hypofunction. Interestingly, there was a trend for an *increase* in dendritic spine density in the AUDp of mice that had been treated with VPA at P14. This also appears to be consistent with the observed behavioral profile following P14 VPA-exposure, specifically, the trend towards an exaggerated startle response observed in VPA-treated mice. Interestingly, several of the behaviors observed following P14 VPA exposure are only seen in male mice, indicating that males may be particularly vulnerable to VPA insult during the second week of life. In the present study, sex was not included in the analysis, as there were too few animals in the study. However, it would be interesting to see if males are also particularly vulnerable to the effects of P14 VPA exposure on dendritic spine density and morphology.

Across both brain regions, dendritic spines from VPA-treated animals were shorter and had a decreased LWR than dendritic spines analyzed from saline-treated

animals. Furthermore, in the AUDp, dendritic spines from VPA-treated animals were also thinner. Dendritic spines being, on average, smaller than spines from saline-treated animals likely has functional consequences for receptor expression and efficacy. Larger spines are thought to be more stable and less plastic than smaller spines (Kasai, Matsuzaki, Noguchi, Yasumatsu, and Nakahara, 2003; although, paradoxically, there was less variance in the morphology of dendritic spines in the AUDp from VPA-treated animals). Furthermore, smaller dendritic spines are thought to express fewer AMPA receptors and contribute to weaker synaptic connections (Kasai et al., 2003). Decreased spine volume is also observed in certain pathological conditions, even when the density of spines remains unchanged, such as in the visual cortex following visual deprivation (Freire, 1978; Globus & Scheibel, 1967; Tieman, 1985), in the stiatum of individuals with schizophrenia (Roberts, Conley, Kung, Peretti, & Chute, 1996), and in the motor cortex of individuals with Down's syndrome (Marin-Padilla, 1972). In the present study, smaller spine size may represent a failure of dendritic spines to mature and stabilize, and may therefore have functional consequences, such as altering specific brain region-dependent processes (e.g. impaired mPFC-dependent reversal learning).

# Chapter 6

# Effect of Early Postnatal T cell Activation on Cytokine Responses and Behavior

## 6.1 Introduction

Early life stress (ELS) is a risk factor for a number of different psychopathologies. One form of ELS is bacterial or viral infection. Although the focus of the immune system as a source of ELS has largely been on exposure during the prenatal period - known as maternal immune activation (MIA) - a number of recent studies have demonstrated that early postnatal immune activation can affect later behavioral, neurochemical, endocrine, and immune outcomes. However, many of the specific findings are mixed and therefore difficult to interpret. Results may depend on the timing of the exposure, the immune stimulus used, and the test used to assess the behavioral state.

### 6.1.1 Measures of Anxiety

Whereas some studies have found that neonatal immune activation (NIA) with poly(I:C) increases anxiety behavior in tests such as the EPM (Ibi et al., 2009) and the open field (OF) (Konat, Lally, Toth, & Salm, 2011), reports of the effects of NIA with LPS on anxiety-like behaviors are more varied. Three studies, which together span time points of NIA from P3 to P28, found no evidence of increased anxiety-like behavior in the EPM or OF (Spencer, Heida, & Pittman, 2005; Spencer, Martin, Mouihate, & Pittman, 2006; Walker, Knott, & Hodgson, 2008). However, two studies, which induced NIA at P3 and again at P5, did find evidence of increased anxiety. Interestingly, in one study, increased anxiety-like behavior in the EPM was only observed in adulthood and senescence, but not during adolescent testing, suggesting that the effects of NIA on the development of mood pathology may be delayed until later in life (Walker, March, & Hodgson, 2004). In another study by the same group, and one of the few studies to include female animals, the majority of increased anxiety-like behavior were observed only following three days of 30-minute restraint stress, and this effect was only observed in male animals (Walker et al., 2009). The observation that this effect was exclusive to males is interesting given that other studies on the physiological effects of NIA have revealed lasting and long-term sex differences in the responsivity of the HPA axis. For example, rats neonatally treated with LPS had a prolonged corticosterone response to 30-minute restraint stress in adulthood, but LPS-treated males took significantly longer to return to baseline than LPS-treated females (Walker, Nakamura, & Hodgson, 2010). Furthermore, neonatal LPS treatment resulted in increased CRH mRNA expression in the paraventricular nucleus (PVN) of the hypothalamus in males only (Shanks, Larocque, & Meaney, 1995). Taken together, these studies suggest that males may be particularly vulnerable to HPA axis hyper-reactivity following NIA, which may manifest behaviorally as increased anxiety-like behaviors following stress

in adulthood.

Of note, one study found decreased fear-associated behaviors in the OF following LPS exposure on P3 and P5 (Breivik et al., 2002). However, this same study also found no difference in anxiety-like behaviors in the EPM unless animals were food deprived, again suggesting that NIA may interact with stress in adulthood to produce a specific pattern of behaviors. Furthermore, animals in this study also demonstrated increased self-grooming behavior during a social interaction test, which may be related to increased anxiety.

### 6.1.2 Measures of Exploration

NIA with poly(I:C) results in decreased exploration of a novel object placed into an open field (Ibi et al., 2009). Although the authors interpret this as impaired recognition memory, this could also be the result of decreased preference for novelty, or even neophobia, given the same study found evidence of increased anxiety-like behavior following NIA. Another study which employed NIA with LPS also found decreased exploration of a novel object when placed in an OF (Spencer et al., 2005). Again, it is unclear whether this is an index of increased anxiety or decreased preference for or recognition of the novel object.

The hole-board test is another measure of exploratory behavior, while head-dipping behavior during the hole-board test may reflect the anxiolytic state of the animal (Takeda, Tsuji, & Matsumiya, 1998). However, the use of head-dipping as a measure of anxiety-like behavior after NIA has yielded mixed results, with one study finding decreased head-dipping (Breivik et al., 2002), and another finding increased head-dipping and exploratory behavior following NIA (Walker et al., 2008).

### 6.1.3 Measures of Memory

Similar to the effects of NIA on anxiety-like behaviors, NIA may cause memory impairments only following an acute stressor. One study found no deficits in MWM learning in NIA animals (Harré, Galic, Mouihate, Noorbakhsh, & Pittman, 2008). In contrast, two other studies found impaired memory in a modified version of the fear contextual conditioning task known as the context preexposure facilitation task in rats neonatally exposed to *E. coli*. In these studies, however, NIA rats displayed impaired memory for the recently explored context, as measured by freezing behavior, only following a second immune activation event with LPS (Bilbo, Biedenkapp, et al., 2005; Bilbo, Levkoff, et al., 2005) The effect of NIA on memory impairment was completely reversed by a capsase 1 inhibitor, which selectively prevents the synthesis of IL-1$\beta$, suggesting that IL-1$\beta$ plays a critical role in NIA-induced cognitive changes (Bilbo, Biedenkapp, et al., 2005).

### 6.1.4 Measures of Social Behavior

A handful of studies have examined the effects of NIA on social behavior, although a wide variety of social interaction tests have been used. While this has the potential of making comparisons difficult, similar results seem to be observed across different tests and different neonatal immune activators (i.e., poly(I:C) or LPS). NIA with poly(I:C) resulted in decreased social interaction with a non-aggressive intruder in the home cage (Ibi et al., 2009). Similar effects have been observed in LPS-induced NIA. One study observed a decrease in time spent in social interaction while simultaneously observing an increase in self-grooming behavior during the social interaction test (Breivik et al., 2002). Although the authors interpret these behaviors through the lens of mood disorders, the concurrent decrease in social behavior and increase in self-grooming is a pattern of behavior often observed in rodent models of autism spectrum disorder (ASD). Another study found the NIA

resulted in a decrease in socially reactive behaviors, which was defined as a startle response, jump, or kick in response to social stimulation from the partner mouse (Granger, Hood, Ikeda, Reed, & Block, 1996). Furthermore, in a mouse strain bred for high aggression, NIA increased freezing behavior during the social interaction test while decreasing acts of aggression (attacks). This was not seen in a mouse strain bred for low aggression. Importantly, the two mouse strains differed not only in their later-life behavioral responses to NIA, but also in acute response to NIA, as the high-aggression line had a greater increase in LPS-induced levels of IL-1$\beta$ and corticosterone. Therefore, individual differences between strains could be the result of differences in the biological response to NIA. Given this, strain may be an important factor to consider when selecting animals for a NIA study, and may account for some of the differences noted between studies cited in this review.

Although the results of the Granger et al study might suggest that IL-1$\beta$ plays a large role in mediating the effects of NIA on later-life social differences, IL-1$\alpha$ may have the opposite effect. In one study, rats treated neonatally with IL-1$\alpha$ had increased social interaction scores when tested during the juvenile period. However, IL-2, IL-6, and IFN-$\gamma$ had no effect on social interaction. The authors suggest that this decrease in social interaction may have been a failure to recognize the stranger rat as unfamiliar. This is possible, given that NIA-induced memory impairment in adulthood appears to be dependent on IL-1$\beta$ (Bilbo, Biedenkapp, et al., 2005), and that both IL-1$\alpha$ and IL-1$\beta$ bind to the IL-1R1 receptor to initiate a pro-inflammatory response (Dinarello, 2018). However, it is also possible that the observed effects of IL-1$\alpha$ represent a true decrease in social interest, which would be in contrast to other NIA findings. However, the Granger et al study tested social play during the juvenile period, and NIA may have differential effects on juvenile and adult social interaction scores. Some psychopathologies, such as schizophrenia, involve social disinterest that does not manifest until post-adolescence. Additionally, social play

behavior peaks during the juvenile period and declines in adulthood, while in males, aggressive behaviors towards peers increases in adulthood.

### 6.1.5 Measures of Startle and Prepulse Inhibition

Prepulse inhibition of the acoustic startle response is considered a test of sensorimotor gating in both humans and rodents. Sensorimotor gating deficits and/or potentiation of the baseline startle response are reported in a number of psychiatric conditions, including schizophrenia, ASD, obsessive-compulsive disorder (OCD) and post-traumatic stress disorder (PTSD) (McAlonan et al., 2002; Perry et al., 2007; Swerdlow et al., 2001). PPI deficits have been observed following NIA with poly(I:C) (Ibi et al., 2009) and LPS (Feleder, Tseng, Calhoon, & O'Donnell, 2010), although in both cases baseline startle was unaffected. Alternatively, another study found that mice neonatally exposed to LPS did have an exaggerated acoustic startle response, although this was only observed following exposure to restraint stress. This is consistent with other observations by this group that some behavioral abnormalities resulting from NIA are only observable in response to a later-life stressor. As with the effects of NIA on memory, IL-1 may be particularly important to the effects of NIA on PPI. Although IL-2, IL-6, and IFN-$\gamma$ had no effect on PPI, neonatally IL-1$\alpha$ treated rats showed decreased PPI in adulthood, but not during the juvenile period, and this effect was reversed by clozapine, mimicking both the temporal and pharmacological features of schizophrenia (Tohmi, Tsuda, Watanabe, Kakita, & Nawa, 2004).

### 6.1.6 Conclusions

In conclusion, NIA seems to result in a number of emotional, cognitive, and social behavioral outcomes. The most consistent behavioral effects seem to result from NIA with poly(I:C) on multiple consecutive days, which resulted in increased anx-

iety, decreased exploration of a novel object, impaired PPI, and decreased social interaction (Ibi et al., 2009). However, these results have not been replicated by another group, and should be interpreted with appropriate caution, especially given that NIA with LPS at similar time points (P3 and again at P5) has resulted in inconsistent outcomes or behavioral differences only following an adulthood stressor (Breivik et al., 2002; Shanks et al., 1995; Walker et al., 2009; Walker et al., 2008; Walker et al., 2004). Although it appears that NIA alone can be sufficient to produce differences in behavior in adulthood, these alterations do seem to be amplified in the face of an adulthood stressor. This is consistent with the two-hit hypothesis of a number of psychiatric conditions, including schizophrenia and depression.

Although there has been some work on the effects of NIA with poly(I:C), LPS, *E. coli*, and some specific cytokines, there is no evidence to date regarding the behavioral effects of NIA immune activation with a selective T cell stimulus. Staphylococcal enterotoxin A (SEA), which targets T cells, has recently been shown to affect social behavior, anxiety, locomotion, interest in a novel object, and learning in the MWM following MIA (Glass et al., 2019). However, it is unclear whether SEA has similar effects when administered during the early postnatal period.

Unlike poly(I:C) and LPS, which act through the innate immune system (i.e. monocytes, neutrophils, and macrophages), SEA causes robust and prolonged T cell activation and proliferation, resulting in both a quick and extreme release of cytokines by T cells (Urbach-Ross & Kusnecov, 2009). Because of their unique effect on the adaptive branch of the immune system, resulting in the direct activation of T cells and high production of T cell-derived cytokines, SEA represents an interesting and yet-unexplored area of study in regards to NIA.

## 6.2 Experiment 1: Determination of the cytokine response to SEA administered at P7, P14 and in adulthood.

There is no evidence to date regarding the impact of SEA on the neonatal immune response. Since the overall goal of this study is test the effects of T cell activation on later life behavioral development, immune status needed to be confirmed. Therefore, in Experiment 1, animals were administered SEA at P7 or P14. Spleens were collected 2 hours later for quantification of total protein, IL-2, and TNF$\alpha$.

### 6.2.1 Methods

Treatment with Staphylococcal Enterotoxin A (SEA). For pups aged P7 or P14, SEA was diluted in saline, and given i.p. at a dose of 5 $\mu$g in a volume of 100 $\mu$L at P14 or 50 $\mu$l at P7. Control pups received injections of saline in an equivalent volume of saline. To confirm that SEA elicited robust pro-inflammatory response in the mature immune system, adult mice 6 – 8 months old, were administered saline or 5 $\mu$g SEA in a volume of 200 $\mu$L.

Spleen extraction. Two hours after injection with either SEA or saline, mice were sacrificed in a standard $CO_2$ chamber. Spleens were removed and homogenized with 1mL of phosphate-buffered saline (PBS) for adult spleen or 0.5 mL PBS for neonatal spleens, centrifuged for 10 minutes at 10,000 rpm, and the supernatant collected and stored at -80$^o$ C until immunoassay for cytokines.

ELISAs. An enzyme-linked immunosorbent assay (ELISA) was used to assess cytokine concentrations in the spleens as superantigens, such as SEA, have been shown to elicit T-cell cytokine responses in the spleen (Glass et al., 2019). Assays were

performed for the cytokines IL-2 and TNF$\alpha$. Assay reagents were obtained from Invitrogen and were performed with slight modifications to manufacturer's instructions, as described in Chapter 2. Samples were diluted 1:10 for IL-2 detection and 1:4 for TNF$\alpha$ detection. For each ELISA a standard curve was established and the concentration of the target cytokine in each sample was calculated.

Protein assays. The cytokine concentrations that were determined by ELISA were corrected for the total protein concentration of the sample, such that cytokine concentrations were expressed per $\mu$g of total protein. Total spleen protein was quantified using the BCA protein assay kit as per manufacturer's instructions (Pierce, Rockford, IL, USA). Samples were diluted 1:20 and concentrations were compared to a standard curve prepared in the same plate using bovine serum albumin (BSA).

Data analysis. For the ELISA data, 2x2 (Treatment x Age) ANOVAs were used to look for significant main effects and interactions in the data.

### 6.2.2 Results

At ages P7 or P14, mice were treated i.p. with 5 $\mu$g SEA or saline. The mice were sacrificed 2 hours after injection and the spleens processed for measures of total protein and the cytokines IL-2 and TNF$\alpha$. The responses at P7 and P14 were compared with adult animals also treated with 5 $\mu$g SEA or saline.

#### Total Protein

There was no effect of Treatment on total protein quantified from the spleen homogenate. However, there was a main effect of Age ($F_{(1, 12)}$=5.355, p=.022), such that P7 pups had significantly less total protein per mL of sample than adults. As there were differences in the amount of total protein in the age groups, all cytokine concentrations were normalized per $\mu$g of protein for statistical analysis.

**Interleukin-2 (IL-2)**

The IL-2 data was normalized to total protein (pg/$\mu$g protein), and this was analyzed by ANOVA. There was a main effect of Treatment ($F_{(1, 12)}$= 39.237, p<.001), with saline-treated mice having no detectable IL-2 responses at any age. Moreover, there was a main effect of Age ($F_{(1, 12)}$=6.237, p=.013), and a Treatment x Age interaction ($F_{(1, 12)}$=6.237, p=.013). Interestingly, at P7, the difference in IL-2 response to saline and to SEA did not reach statistical significance. On the other hand, P14 and adult animals showed significantly greater IL-2 concentrations after SEA treatment, when compared to respective saline treatments and IL-2 concentrations in P7 pups. The IL-2 levels between P14 and adult mice did not differ significantly from each other (see Figure 6.1).

**TNF$\alpha$**

Analysis of TNF$\alpha$ concentrations (pg/$\mu$g protein) showed a main effect of Treatment ($F_{(1, 12)}$=36.105, p<.001), since saline-treated mice had no detectable TNF$\alpha$ response at any age. There was also a main effect of Age ($F_{(1, 12)}$=482, p<.001) and a Treatment x Age interaction ($F_{(1, 12)}$=34.482, p<.001). At P7 and P14, there was no detectable TNF$\alpha$ response and therefore no differences between saline- and SEA-treated mice at these ages. For adults, however, there was a significant difference between saline and SEA treatment (see Figure 6.1).

### 6.2.3 Discussion

At P7, there was no detectable TNF$\alpha$ response 2-hours after SEA treatment, and only a modest IL-2 response that was not significantly different than 0. Although an IL-2 response was observed by P14, no TNF$\alpha$ response to SEA was detectable until adulthood. The diminished immune response at P7, and to a lesser extent at P14, may have resulted from the anti-inflammatory properties of breast milk. In

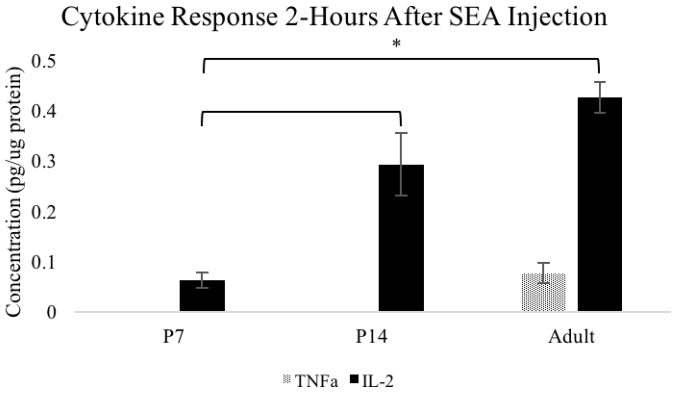



Figure 6.1: IL-2 and TNF$\alpha$ response 2-hours post-treatment with SEA. Cytokine concentrations were normalized to total protein concentration. There was no detectable cytokine response to saline at any age; therefore, saline is not shown. At P7, there was no detectable TNF$\alpha$ response, with only a modest increase in IL-2 concentration. At P14, there was still no detectable TNF$\alpha$ response in SEA-treated animals, but the IL-2 response was significantly elevated and not significantly different than adult levels. *N= 4 P7, 5 P14, 2 Adult.*

humans, TNF$\alpha$ and IL-2 concentrations were significantly reduced in breast-fed as compared to formula-fed infants during the first year of life (Kainonen, Rautava, & Isolauri, 2013). Furthermore, two bacteria isolated from human breast milk both individually suppressed the LPS-induced TNF$\alpha$ response in murine bone marrow derived macrophages (Díaz-Ropero et al., 2007). Although neither P7 nor P14 pups had been weaned by the age of injection, the third week of life is roughly when eyes are opening and pups begin to transition to solid food (Enzmann, 1933).

Other studies have demonstrated a pro-inflammatory immune response in the first week of life to *E. coli* (Bilbo, Levkoff, et al., 2005), LPS (Feleder et al., 2010), and poly(I:C) (Ibi et al., 2009), suggesting that SEA may have failed to illicit a robust pro-inflammatory response due to the T cell specific nature of the toxin. Therefore, an alternative, although not contradictory, explanation is that the diminished immune response may have been the result of an immature adaptive immune system. Unlike in humans, where the immune system is more or less fully established (although inexperienced) at birth, very few T or B cells are present at birth

in the rodent. These cell lines do not fully develop until 2 – 3 weeks of age, and T cell numbers are still reduced at postnatal day 21 (P21) (Semple et al., 2013). As SEA exerts its inflammatory effects via activation of T cells and antigen presenting cells (APCs), it is possible that no robust immune response was observed due to a lack of these cells. Moreover, TNF$\alpha$ induction by SEA specifically may be strictly T cell-dependent (Krakauer, 1999).

In addition to quantitative differences in the number of T cells, the quality of the immune response may be different in the immature vs mature immune system, resulting in different levels of specific cytokine production (Semple et al., 2013). Therefore, the lack of IL-2 or TNF$\alpha$ response may also have been due to qualitative differences of these cells in the immature system, resulting in lower levels of these cytokines but normal (or even above normal) levels of others.

At P7, there did not appear to be a significant IL-2 or TNF$\alpha$ response to SEA 2-hour post-treatment. As there was no evidence of a robust immune response at this age, the decision was made not to run behavioral testing on this group. However, post-treatment behavioral testing was conducted for the P14 animals (see Experiment 2), given that they showed a pronounced IL-2 response in response to SEA.

## 6.3 Experiment 2: Effect of SEA treatment at P14 on development of mouse behavior

In Experiment 1, we established that SEA can cause an immune response in P14 pups. However, the effect of this immune response on later-life behavior remains unknown. Therefore, Experiment 2 sought to determine the effect of NIA with SEA on the development of mouse behavior.

### 6.3.1 Methods

Pregnancies were generated using the standardized protocol described in Chapter 2. The day of birth was recorded as postnatal day 0 (P0). Pups were maintained with their mothers until weaning at 4 weeks, at which time mice were separated into same-sex cages (3 – 4 per cage).

Treatment with SEA at P14. SEA was diluted in saline, and given i.p. at a dose of 5 $\mu$g in a volume of 100 $\mu$l at P14. Control pups received injections of an equivalent volume of saline. All injections were completed between 10:00am and 12:00pm. Because initial assays indicated that SEA did not result in a robust IL-2 response response in P7 pups, this group was dropped from behavioral testing.

Overview of Behavioral Testing. After weaning, all experimental animals underwent a sequence of behavioral tests of social, cognitive, emotional, and sensorimotor behavior (see Figure 6.2). Details about behavioral testing apparatuses can be found in Chapter 2.

Mid-air Righting (P14 – P15). In the mid-air righting task animals were dropped dorsal side down from 18 cm above a padded surface and their ability to right themselves (land on all four paws) was assessed. The timing of this test is based on known developmental milestones (Wagner et al., 2006), with mid-air righting taking place on P14 (before treatment) – P15. Three trials were performed per day, with the total day's score being an average of the three trials.

Social approach (P30, P90). The social approach procedure consisted of three phases: habituation phase, single target phase (Phase A), and two-target phase (Phase B). The habituation phase consisted of an initial five-minute period, during which the experimental mouse was habituated to the experimental chamber, with the wire chambers empty. During this habituation period, ANY-Maze tracking software was


(a)


(b)

Figure 6.2: Behavioral testing timelines. Behavioral tests described in (a) and (b) were run on separate cohorts of mice.

used to record the distance travelled (in meters) and the preferred side of the apparatus.

This habituation period was followed by the single target phase (Phase A), during which a target mouse was placed into the wire chambers (S1) on the least preferred side of the apparatus, leaving the second wire chamber (S2) empty. This was then followed by the two-target phase (Phase B), which involved a second novel mouse being introduced to S2. For both Phase A and Phase B contacts with the S1 and S2 chamber were automatically recorded for ten minutes.

Elevated plus maze (EPM) (P91-P94). The elevated plus maze (EPM) test was conducted over two five-minute sessions separated by 72 hours. Testing in the EPM lasted for 5 minutes, and the amount of time the animal spent exploring the closed and open arms was recorded, as well as the distance travelled in open and closed arms.

Water Y maze (P108). In the first phase (acquisition phase), a mouse was placed in the start arm and was required to choose between the two remaining arms, only one of which contained a platform that allowed escape from the water. After the mouse made a choice, the door at the entrance of the arm (whether it contained the platform or not) was closed, trapping the mouse in the chosen arm for 10 seconds. After 10 seconds, the trial ended, and the mouse was returned to a cage on a heating pad for a 5-minute inter-trial interval (ITI), after which the mouse was returned to the start arm. The location of the escape platform did not change until the mouse reached criterion performance of correctly selecting the escape arm 11 out of 12 trials on two consecutive days. Once criterion was reached, the location of the escape platform was changed to the opposite arm of the maze. This represented the reversal phase. Criterion parameters were the same for the acquisition and reversal phases. Both number of days to reach criterion and number of errors made were considered as dependent variables for both phases.

Prepulse inhibition (PPI) (3 – 5 months). After the 2-minute acclimation period, each subject was presented with 80 acoustic noise trials given over a 45-minute period. The noise trials were of seven distinct types, described in Chapter X. The three different acoustic prepulse+pulse stimulus trials were sounds of 2, 4, and 8 db above background (60 db), while the startle-inducing pulse was 50db above background. Each of the prepulse intensities were presented alone to measure baseline startle to the prepulse stimulus. The formula for calculating prepulse inhibition was:

$$\frac{\text{pulse alone - (prepulse+pulse)}}{\text{pulse alone}} * 100$$

Second-hit immune response. At least two weeks after the end of behavioral testing, animals were administered 5 $\mu$g SEA. Two hours post-treatment, animals were sacrificed and spleens were collected. Homogenization of spleens and ELISAs were performed for IL-2 and IL-6, as described in Experiment 1.

### 6.3.2 Results

#### Mid-air Righting

*SEA treatment did not impact ability to mid-air right 24 hours later.*

A 2 x 2 x 2 (Treatment x Sex x Day) repeated-measures ANOVA was run with Day as the within-subjects factor for the number of mid-air righting successes on P14 and P15. There was a main effect of Day ($F_{(1, 26)}$=5.377, p=.029), such that pups had more successes on P15 than P14. There was no effect of Sex or Treatment on number of successes on either day.

#### Social Approach

*Treatment had no effect on distance travelled during the 5-minute habituation period of the social approach test.*

A 2 x 2 (Treatment x Sex) ANOVA was run for distance travelled during the 5-minute habituation period at P30 and P90. There was no effect of Sex or Treatment for either day of testing.

*SEA treated mice spent more time in contact with S1 during Phase B.*

During the social approach test, time spent in contact with each wire chamber (S1 and S2) was automatically recorded. During Phase A, S1 housed a novel weight- and sex-matched peer. During Phase B, the same peer remained in S1, and another peer was housed in S2.

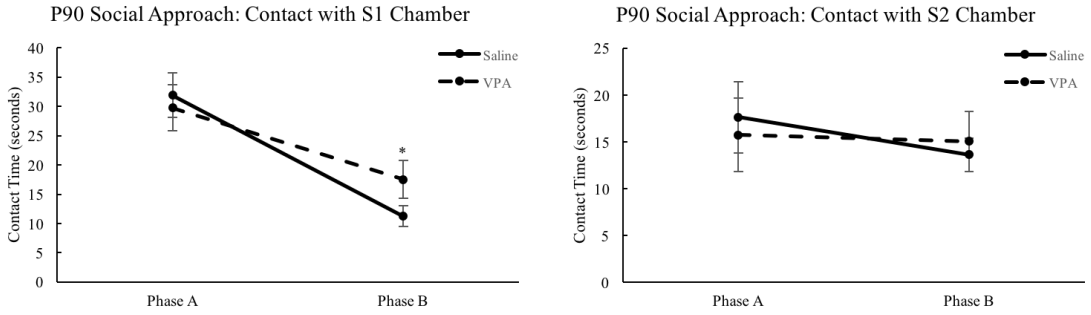
A 2 x 2 x 2 (Treatment x Sex x Phase) repeated measures ANOVA was run with Phase as the within-subjects variable for time spent in contact with each of the wire chambers. At P30, there was a main effect of Phase ($F_{(1, 32)}$=18.769, p<.001), such that animals spent less time in contact with S1 during Phase B. There was no effect of Treatment or Sex on amount of time spent in contact with S1. There was no effect of Treatment, Sex, or Phase on amount of time spent in contact with S2.

At P90, there was also a main effect of Phase ($F_{(1, 27)}$=37.642, p<.001), such that animals spent less time in contact with S1 during Phase B. Additionally, there was a Phase x Treatment interaction ($F_{(1, 27)}$=4.618, p=.041). Pairwise comparisons revealed that during Phase B only, there was a difference between saline- and SEA-treated mice, such that SEA treated mice spent more time in contact with S1 (see Figure 6.3a). There was no effect of Treatment, Sex, or Phase on time spent in contact with S2 (see Figure 6.3b).

**Elevated Plus Maze**

*Treatment had no effect on time spent or distance travelled in the open arms of the maze.*

A 2 x 2 x 2 (Treatment x Sex x Day) repeated-measures ANOVA was performed for the variables time spent and distance travelled in the open arms. Proportions




(a) (b)

Figure 6.3: Time spent in contact with S1 (a) and S2 (b) chambers during the social approach task at P30. Mice spent less time in contact with S1 during Phase B. Furthermore, SEA-treated mice spent more time in contact with S1 than saline-treated mice during Phase B. *N=17 saline, 13 VPA.*

of time spent and distance travelled in the open arms was calculated by dividing the time spent or distance travelled in the open arms of the maze by the time spent or distance travelled in both the open and closed arms of the maze. The arcsine transformation of these proportions was used in all analyses.

For both time spent and distance travelled in the open arms, there was a main effect of Day ($F_{(1,25)}$=40.354, p<.001 for time spent; $F_{(1, 25)}$=24.411, p<.001 for distance travelled), such that animals spent less time and travelled less in the open arms on the second day of testing. There was no effect of Treatment or Sex on either variable.

### Water Y Maze

*Treatment had no effect on either acquisition or reversal in the Y maze.*

Animals commenced acquisition of escape from the Y-maze on P108. A 2 x 2 (Treatment x Sex) ANOVA was performed for number of days to reach criterion and number of errors made for the acquisition phase and the reversal phase. There was no effect of Treatment or Sex for either dependent variable for either phase of the task.

**Prepulse Inhibition (PPI)**

*Treatment has no effect on either startle response or prepulse inhibition.*

The data in this test provides startle response to pulse alone, as well as inhibition (PPI) of this startle response in the presence of a prepulse (the prepulse+pulse trials, see Chapter 2). The pulse-alone trials were analyzed to determine each animal's baseline startle response to the pulse. For these trials, the first five trials were considered habituation trials and were excluded from analysis. A 2 x 2 (Treatment x Sex) ANOVA did not reveal any effect of Treatment ($F_{(1, 11)}$=.791, *ns*) or Sex ($F_{(1,11)}$=.251, *ns*). Prepulse inhibition was calculated as described above for the three prepulse intensities. A 2 x 2 x 2 (Treatment x Sex x Intensity) ANOVA did not reveal any effect of Intensity ($F_{(1, 10)}$=.202, *ns*), Treatment ($F_{(1,5)}$=.011, *ns*), or Sex ($F_{(1, 5)}$=1.011, *ns*). There was a trend toward an Intensity x Treatment interaction ($F_{(1, 10)}$=3.359, p=.099), such that the +4dB prepulse intensity produced the most PPI in SEA-treated mice and the least PPI in saline-treated mice. This failure of this effect to reach significance may be related to the extremely small *n* (4 saline, 5 SEA) for this part of the study. [1]

Second-hit immune response. A 2 x 2 x 2 (Treatment 1 [SEA or saline as neonate] x Treatment 2 [SEA or saline as adult] x Sex) multivariate ANOVA was run for the dependent variables IL-2 response and IL-6 response. As expected, there was a significant main effect of Treatment 2 ($F_{(2, 7)}$=12.515, p=.005), such that mice that mice that received saline 2 hours before the spleens were collected had much lower levels of IL-2 ($F_{(1, 8)}$=25.327, p=.001) and IL-6 ($F_{(1.8)}$=9.434, p=.015). However, there was no effect of either Treatment 1 ($F_{(1,2)}$=.136, *ns*) or Sex ($F_{(2, 7)}$=1.406, *ns*), nor any Treatment x Sex or Treatment x Treatment interactions. In other words,

[1] The small *n* for this part of the study was due to an outbreak of mouse parvovirus (MPV) in our colony rooms that resulted in a quarantine. The quarantine necessitated a cessation of behavioral testing for several months, after which point animals were too old to participate in PPI testing.

there was no difference in immune response to SEA between adult mice that had been treated with SEA as neonates and those that had been treated with saline as pups. Results of the IL-2 and IL-6 assays are displayed below in Table 6.1.

Table 6.1: IL-2 and IL-6 concentrations (pg/$\mu$g) 2 hours post-treatment with SEA or saline.

| Treatment 1 (Neonate) | Treatment 2 (Adult) | IL-2 | IL-6 | *N* |
|---|---|---|---|---|
| Saline | SEA | .2338 (.0523) | .355 (.0144) | *6* |
| SEA | | .1975 (.1422) | .0261 (.0208) | *5* |
| Saline | Saline | 0 (0) | .0027 (.0024) | *3* |
| SEA | | .0051 (-) | .0069 (-) | *1* |

### 6.3.3 Discussion

Other than a small increase in time spent with a familiar peer during Phase B of the social approach test, which was not accompanied by a decrease in time spent with the novel peer during this same phase, neonatal SEA treatment did not affect any of the behaviors assayed in this study. This is in contrast to what has been observed following prenatal exposure to SEA. Maternal immune activation with SEA has resulted in offspring with decreased social approach behavior, increased anxiety, increased locomotion, increased interest in a novel object placed within an open field, and impaired learning in the Morris water maze task (Glass et al., 2019).

One possible explanation for the discrepancy between pre- and postnatal exposure to SEA may lie in the failure of postnatal SEA to elicit a robust TNF$\alpha$ response. Whereas TNF$\alpha$ was potently induced in maternal spleens by 2 hours post-injection, the present study was unable to detect any TNF$\alpha$ in neonatal spleens. The lack of TNF$\alpha$ in the neonatal samples may have been a result of an immature adaptive immune system, as discussed above. TNF$\alpha$ has been shown to be a necessary condition for several of the behvioral and endocrine effects of SEA in adult mice.

For example, SEA-induced neophobia and HPA-axis activation are not observed in TNF$\alpha$ null mice and in wildtype mice who have been administered TNF$\alpha$ antibody (Rossi-George, LeBlanc, Kaneta, Urbach, & Kusnecov, 2004). Endogenous TNF$\alpha$ is also a necessary condition for SEA-induced recruitment of several brain areas, as observed by increased c-Fos expression, such as the paraventricular nucleus (PVN) and arcuate nucleus (Rossi-George et al., 2004).

The present study's findings are also in contrast with findings that neonatal immune activation with agents such as LPS or poly(I:C), which have shown differences in anxiety-like behaviors, social behaviors, memory, and prepulse inhibition. Although TNF$\alpha$ seems to be a critical mediator of SEA-induced neuromodulation and behavioral effects, this may not extend to other immune activators. For example, LPS is a potent inducer of TNF$\alpha$, but the CNS effects of LPS do not appear to be entirely dependent on TNF$\alpha$. Blocking TNF$\alpha$ only partially attenuates the immune activation-induced corticosterone response (Ebisui et al., 1994; Perlstein, Whitnall, Abrams, Mougey, & Neta, 1993; Turnbull & Rivier, 1998), and TNF$\alpha$ null mice retain the anorexic response following LPS exposure (Rossi-George et al., 2004). Possibly, the effects of TNF$\alpha$ depend on the presence or absence of other cytokines, such as IL-1$\beta$. IL-1$\beta$ is induced by LPS, but is not produced in substantial quantities following SEA exposure (Kawashima & Kusnecov, 2002). In contrast, SEA potently and rapidly induces an IL-2 response, whereas LPS does not (De Groote et al., 1992). Therefore, the distinct pattern of cytokine production following an immune stimulus may be just as critical as any individual cytokine and may determine the primacy of certain cytokines in influencing the behavioral and endocrine response.

# Chapter 7

# General Discussion

The studies described in this dissertation evaluated the effects of early postnatal exposure to two environmental challenges, which have previously been well-characterized regarding their teratogenic effects. The effects of the GABA agonist valproic acid (VPA) and the T cell superantigen staphylococcal enterotoxin A (SEA) were observed on a battery of behavioral tests designed to assess social, emotional, and cognitive functioning in ways relevant to human neurodevelopmental disorders. In addition to behavioral testing, neurochemical and neuroanatomical assessments were performed for mice that had been treated with VPA, and immunological assays were performed to determine the acute and adult immune response to early life SEA exposure.

## 7.1 Study 1: Behavioral and Neurochemical Profile Following Valproic Acid Exposure on Postnatal Day 14

*VPA exposure at P14 resulted in a behavioral profile indicative of reduced behavioral inhibition, although this effect was largely restricted to male mice.*

In the first study, male mice treated with VPA at P14 demonstrated more aggressive behaviors than saline-treated mice during the 30-minute social play observation period. In addition, VPA-treated male mice demonstrated differences in the elevated plus maze (EPM), spending more time and travelling more distance in the open arms of the EPM, which is usually considered to be indicative of a less anxious phenotype. This was unexpected, as the prenatal model of ASD is often characterized by increased anxiety. This was only observed in males; VPA-treated females did not differ from saline-treated females in their anxiety-like EPM behavior, seemingly maintaining a normal response in this test. Therefore, early VPA treatment may precipitate a sexual dichotomy for anxiety-like behavior. Male mice appeared to be sensitive at P14 to VPA treatment, which appeared to render them less anxious, however, this may have been driven by a more impulsive-like behavioral style. Indeed, these same VPA-treated male mice took longer to reverse, but not to acquire, a water Y maze task. This suggests the presence of a perseveration, which may also be related to an inability so inhibit a maladaptive behavior.

Finally, VPA-treated mice had an increased startle response to an acoustic stimulus. Taken together, these data suggest a sex-specific effect of VPA on the development of a behaviorally disinhibited phenotype. Although increased impulsivity is often observed in children with ASD, this phenotype is by no means exclusive to ASD and is in fact observed in a number of other neurodevelopmental disorders, such as ADHD. It is therefore possible that early life exposure to VPA may disrupt neurobiological mechanisms of behavioral regulation (eg., fronto-striatal circuitry) common to several developmental disorders, including ASD and ADHD.

## 7.2 Study 2: Behavioral Profile Following Valproic Acid Exposure on Postnatal Day 7

*Females treated at P7 with VPA demonstrated decreased preference for social novelty, a symptom of disrupted social behavior.*

Both male and female pups treated with VPA at P7 displayed a regression in mid-air righting ability by 24 hours post-treatment. Earlier VPA treatment of males at P7 did not produce a similar sexually dimorphic disruption of behavioral inhibition, as was seen after treatment on P14, but did reveal an alteration of social behavior in females. Specifically, juvenile VPA-treated female mice showed an increased preference for social novelty in the social approach test. However, when the social approach test was repeated in adulthood (2 months later), VPA-treated female mice showed a *decreased* preference for social novelty. VPA therefore differentially affected the ontogeny of social behavior, producing opposing effects depending on stage of development.

In the water Y maze task, there was a trend toward VPA-treated mice having delayed reversal learning, although acquisition learning was unaffected by treatment. Although in Study 1 impaired reversal learning was seen only in male mice, that was not the case for this study, when VPA was given earlier. Lastly, a trend for increased prepulse inhibition (PPI) at an intermediate prepulse intensity was observed. This was unexpected, as decreased PPI is associated with a number of psychiatric conditions. However, increased PPI may in some cases reflect a deviation from normal functioning. For example, enhanced PPI is observed following physical stress in rodents, as well as following administration of low doses of ketamine (high doses of ketamine, however, impair PPI). Interestingly, although high doses of ketamine are associated with the full spectrum of symptoms of schizophrenia, low doses may mimic only the negative symptoms, including asociality. In this study, disrupted

social behavior was observed in female VPA-treated mice only during adult testing, which matches the temporal features of schizophrenia (specifically, post-adolescent onset). An alternative explanation for the observation of enhanced PPI is through interactions with sex hormones, as estrogen treatment has been noted to enhance PPI.

## 7.3 Sex differences observed in Studies 1 and 2

*Males and females are vulnerable to developmental disruption by VPA at different times of exposure.*

As already noted, in Study 1 (P14 treatment), male mice were primarily affected by VPA treatment, with weight differences, increased aggression, decreased anxiety, and impaired reversal learning only being observed in treated males. In contrast, in Study 2, females seemed to be more affected by VPA treatment at P7, with only treated females displaying differences in the social approach test. This suggests not only that the precise timing of an environmental challenge is a contributing factor to the specificity of the long-term behavioral pathology, but also that biological factors influenced by or relating to sex, create different periods of vulnerability during development.

One possible explanation for this differential vulnerability may be via an interaction with sex hormones, such as testosterone. Prenatal exposure to VPA can affect the expression of the androgen receptor (AR) in both males and females in a region, sex, and age specific manner. In males, prenatal VPA-treatment decreases AR density in the Purkinje cells of the cerebellum at P7, but increases AR density at P14. In females, there was no effect of treatment on AR density at P7, but at P14 AR density was increased in some lobules of the cerebellum and decreased in others (Perez-Pouchoulen et al., 2016). Although the function of ARs in the cere-

bellum remains unclear, testosterone is known to play an important role in brain development (McEwen, 1992).

Both ASD and ADHD have a male bias, with ASD occurring in boys at four times the rate seen girls, while the incidence of ADHD in boys is two times that of girls (American Psychiatric Association, 2013). Furthermore, androgens may play a role in the development of autistic traits, and it has been suggested that ASD may be the consequence of a so-called "extreme male brain," as some of the behavioral and neuroanatomical symptoms of ASD can be interpreted as extreme manifestations of male-typical behavior (Baron-Cohen, 2002). In fact, it has been suggested that prenatal androgen exposure may play a role in the etiology of ASD. For example, fetal testosterone levels have been positively associated with higher scores on the Childhood Autism Spectrum Test (CAST) and Child Autism Spectrum Quotient (AQ-Child) (Auyeung et al., 2009). In this study, fetal testosterone was measured during the prenatal testosterone surge, during the second trimester; however, a second surge of testosterone occurs shortly after birth, known as the neonatal testosterone surge. In humans, the neonatal testosterone surge occurs within a few days of birth and remains elevated for several months before dropping to minimal levels (Forest, Cathiard, & Bertrand, 1973). In rodents, the neonatal testosterone surge occurs within hours of birth, and testosterone levels begin to drop within a day. Testosterone levels remain low for the first two weeks, begin to rise during the 2nd and 3rd postnatal week, then rise sharply with the onset of puberty around the 4th week and continue to rise into adulthood (Lee, de Kretser, Hudson, & Wang, 1975). Future studies should explore whether VPA exposure at P7 or P14 affects AR expression in a way similar to prenatal VPA exposure, and whether decreased AR expression at P7 is protective against the disrupting effects of VPA.

## 7.4 Study 3: Effect of Postnatal Valproic Acid Treatment on Dendritic Spine Plasticity

*Dendritic spine density was altered in the mPFC and AUDp following P14 VPA treatment in a manner consistent with the behavioral profile observed in Study 1. Dendritic spine morphology was altered following VPA treatment at either time point.*

Study 3 sought to determine the effect of VPA-treatment at P7 or P14 on dendritic spine density and morphology in the media prefrontal cortex (mPFC) and primary auditory cortex in the temporal lobe (AUDp), as dendritic spine abnormalities have been observed in both human ASD and in the prenatal VPA model of ASD. For animals treated at P7, there were no differences in spine density in either brain region, although dendritic spines were, on average, taller in treated mice in the mPFC and thinner in treated mice in the AUDp. These morphological changes may represent a failure of the dendritic spines to stabilize as the brain matures.

For animals treated at P14, there was decreased spine density in the mPFC and a trend toward decreased spine density in the AUDp. These observations are consistent with the behavioral profile observed in Study 1. Specifically, decreased behavioral inhibition may be the result of frontal cortex hypofunction, and increased connectivity in the AUDp could be associated with an exaggerated acoustic startle response. Unfortunately, this study did not have the power necessary to determine if these spine abnormalities demonstrated the same sex-dependent effects as observed in Study 1. However, future studies could determine if these observations are restricted to male mice.

In addition to dendritic spine density changes, there were also morphological differences between spines in animals that had been treated with VPA at P14 across both brain regions. In the mPFC, spines were shorter and had a lesser length:width

ratio (LWR), and in the AUDp, spines were shorter, thinner, and had a lesser LWR. The observation of smaller dendritic spines likely has functional consequences, as larger spines are thought to be more stable and express more AMPA receptors, thereby contributing to stronger synaptic connections.

## 7.5 Study 4: Effect of Early Postnatal T cell Activation on Cytokine Responses and Behavior

*Experiment 1: SEA elicited a diminished immune response in the neonate as compared to adults.*

To determine whether an immunogenic T cell stimulus would mimic the effects of a pharmacological disruptor, such as VPA, experiments were conducted using the strong T cell stimulus, SEA. Confirmation of cytokine induction was necessary, as little evidence exists for the neonatal response to staphylococcal enterotoxins. Treatment at P7 produced only a modest IL-2 response that was significantly lower than the IL-2 response observed in adults, and not significantly different than saline-treated mice. Development of an IL-2 response was evident on P14, as SEA treatment elevated concentrations of IL-2 at this time, but other cytokine responses were deficient, such as a failure to elicit a TNF$\alpha$ response at either neonatal time point. As other studies have noted an immune response following other immune stimuli, such as LPS or poly(I:C), it is possible that SEA may have failed to elicit a robust immune response due to its T cell specific nature, given that T cells do not fully develop until 2 to 3 weeks of age in rodents.

*Experiment 2: Neonatal SEA treatment did not have large behavioral or long-lasting immunological effects.*

Given that IL-2 has been shown to have neural and behavioral effects (Rankin, Zalcman, Zhu, & Siegel, 2013) the IL-2 response to SEA may potentially be neuro-

modulatory. Therefore, behavioral testing and adulthood immune assays to further SEA challenge were carried out with animals treated at P14. The findings from this experiment, however, did not show major behavioral effects, other than a small increase in time spent with a familiar peer during the social approach test, and which was not accompanied by a decrease in time spent with the novel peer. The reasons for this are not immediately apparent. Furthermore, SEA-treated mice did not have an altered immune response following a second exposure to SEA in adulthood.

The general lack of behavioral effects are in contrast to studies that have evaluated the effects of prenatal SEA exposure (Glass et al., 2019), as well as studies that have shown behavioral effects following neonatal immune activation (NIA) with other immunological agents (see Chapter 6 for a summary of findings). One possible explanation for this discrepancy is that SEA was not observed to generate a TNF$\alpha$ response after either P7 or P14 injection. TNF$\alpha$ has been shown to be a necessary condition for several of the behavioral and endocrine effects of SEA in adult mice (Rossi-George et al., 2004), and may therefore also be a necessary condition for long-lasting developmental disruption following NIA with SEA. This should be investigated in future studies, by determining if TNF$\alpha$ antagonism during maternal immune activation with SEA eliminates the development of behavioral deficits. If proven to be the case, it would suggest that the maturation of T cell responses during early life restricts the development of cytokines that might be disruptive to neural development.

## 7.6 Concluding Comments on Valproic Acid

In conducting these studies, we hoped to identify whether environmental exposures to two known teratogens during the neonatal period could have long term consequences for neurodevelopment. In the case of VPA, neonatal exposure had outcomes

that were dependent on both the timing of the exposure and the sex of the animal. At both P7 and P14, the observed behavioral profile differed from prenatal exposure studies in key ways. Prenatal VPA exposure in rodents is a common model of ASD; however, at P14 we observed decreased anxiety-like behaviors, rather than the increased anxiety observed in human ASD and in the prenatal VPA model. Furthermore, we did not observe differences in the social approach test, as is seen following prenatal VPA exposure, although we did see disrupted social play behavior in the form of increased aggression. This behavioral profile does not suggest ASD *per se*, but rather a broader behaviorally disinhibited phenotype that does overlap with some associated symptoms of ASD. This phenotype, when combined with other risk factors (e.g. genetic risk factors, other environmental exposures), may increase risk for a group of disorders characterized or associated with increased impulsivity. As these effects were only observed in males, males may be more at risk following exposure at this time point, and the mechanism by which VPA selectively disrupts male neurodevelopment may provide insight into the male bias seen in disorders such as ASD and ADHD.

In contrast to the behavioral profile observed after P14 VPA exposure, after P7 exposure differences were observed in the social approach test. However, this effect was different depending on whether the test took place during the juvenile period or during adulthood. Although prenatal VPA exposure is a model of ASD, this temporal profile of social abnormalities is more consistent with a model of asociality in schizophrenia, which also has a post-adolescent onset. Unlike at P14, P7 VPA-treatment preferentially affected female mice, as only females showed social abnormalities. These sex differences may be accounted for by VPA's interaction with androgen receptor expression in the brain, or via an interaction with the neonatal testosterone surge.

Although VPA is not recommended to be taken by pregnant women due to its

known teratogenic effects, its effects during the neonatal period have not been well documented, and VPA is still prescribed to children (Malamiri et al., 2012). More importantly, however, is the mechanism by which VPA disrupts neurodevelopment, and whether this mechanism is common to other environmental toxicants. The GABA-shift hypothesis proposes that GABA agonist action is excitatory early in development and transitions to inhibitory later in life (Ben-Ari, 2002). VPA acts as an indirect GABA agonist as it non-specifically blocks the enzymatic degradation of GABA, resulting in GABA build-up. It is therefore possible that VPA, as a GABA agonist, may play an excitatory role during development and that an excess of GABA will therefore result in excitotoxicity. This excitotoxicity may then result in oxidative stress-induced neural damage. Similar damage may be sustained not only by other environmental toxicants that act to increase excitatory neurotransmission in the brain, but also by means that decrease the body's ability to deal with oxidative damage, such as specific nutrient deficiency. To confirm this hypothesis, future studies could seek to replicate these findings with another GABA agonist, or to determine if co-administration of VPA with a GABA antagonist mediates the observed effects.

## 7.7 Concluding Comments on SEA

The present dissertation found that treatment with SEA at P7 did not produce a robust immune response and that, although treatment at P14 produced a IL-2 response, there was no TNF$\alpha$ response and no strong behavioral or later-life immunological effects of treatment. This was surprising, given that studies have found behavioral and immunological differences following NIA with LPS and poly(I:C). TNF$\alpha$ may be a necessary condition for behavioral effects following SEA exposure; however, in this dissertation only levels of IL-2 and TNF$\alpha$ were assessed. A future

study could seek to determine if levels of other pro-inflammatory cytokines known to be secreted by T cells, such as IL-6, IL-17, and IFN-$\gamma$, are also undetectable or secreted at lower levels in the neonate. It would also be interesting to see how anti-inflammatory cytokines normally secreted by T cells, such as IL-4, are expressed following SEA stimulation in the neonate.

Regardless, given that NIA with SEA does not produce long-term behavioral differences as do challenges which exert their immune effects via the innate immune system, T cells may play a minimal role in neurodevelopmental disruption following illness. Understanding the role of the different cells of the immune system in causing neurodevelopmental damage following illness is crucial to our ability to mitigate this damage, as well as to understanding the relative risks of immune-stimulating agents. A practical consideration here is that the development of vaccines and effective immunity relies on optimal interplay between T cells, B cells and antigen-presenting cells. If T cell function is restricted to the assistance of B cell antibody responses, therefore, this can limit the impact T cell-derived cytokines, like IL-2, on the developing nervous system. In the current dissertation, it appears to be the case that a robust IL-2 response does not appear to predict disruptions in behavioral development. Consequently, if harm occurs from some aspect of the immune system, innate immune processes may very well be the most likely source.

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