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Anxiety and the Amygdala in the Prenatal Valproic Acid Exposure Model of Autism

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Anxiety and the Amygdala in the Prenatal Valproic Acid Exposure Model of Autism

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An abstract of
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2017

Abstract

Anxiety and the Amygdala in the Prenatal Valproic Acid Exposure Model of Autism
By Thomas M. Hennessey

Confusion endures as to the exact role of the amygdala in relation to autism. The amygdala controls socioemotional behavior and has consistently been implicated in the etiology of Autism Spectrum Disorder (ASD). In addition, there is a strong association of anxiety disorders with both the amygdala and ASD. Precocious amygdala development is commonly reported in ASD youth with the degree of overgrowth positively correlated to the severity of ASD symptoms. Prenatal exposure to valproic acid (VPA) leads to an ASD phenotype in both humans and rats, and has become a commonly used tool to model the complexity of ASD symptoms in the laboratory. In this dissertation, we first examined abnormalities in gene expression in the amygdala and socioemotional behavior across development, then how VPA exposure predisposes rats to anxiety-like behavior after stress.

Effects of VPA on time spent in social proximity and anxiety were sex dependent, with increased social abnormalities, as well as increased grooming, presenting in males after chronic stress. Adolescent VPA animals did not show a reduction in social behaviors. At postnatal day 10, gene pathways involved in nervous system and cellular development displayed predicted activations in prenatally exposed VPA amygdala samples. By juvenile age, however, transcriptomic and proteomic pathways displayed reductions in cellular growth and neural development. Alterations in immune pathways, calcium signaling, Rho GTPases, and protein kinase A signaling were also observed.

As behavioral, developmental, and genomic alterations are similar to those reported in ASD, these results lend support to prenatal exposure to VPA as a useful tool for understanding how developmental insults to molecular pathways in the amygdala give rise to ASD-related syndromes. In addition, our results support the hypothesis that elevated rates of anxiety disorders in individuals with autism may be the result of a greater innate vulnerability to stress.

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Supplementary Material

Chapter 1

RDoC-based categorization of amygdala functions and its implications in autism

Abstract

Confusion endures as to the exact role of the amygdala in relation to autism. To help resolve this we turned to the NIMH's Research Domain Criteria (RDoC) which provides a classification schema that identifies different categories of behaviors that can turn pathologic in mental health disorders, e.g. autism. While RDoC incorporates all the known neurobiological substrates for each domain this review will focus primarily on the amygdala. We first consider the amygdala from an anatomical, historical, and developmental perspective. Next, we examine the different domains and constructs of RDoC that the amygdala is involved in: Negative Valence Systems, Positive Valence Systems, Cognitive Systems, Social Processes, and Arousal and Regulatory Systems. Then the evidence for a dysfunctional amygdala in autism is presented, with a focus on alterations in development, prenatal valproic acid exposure as a model for ASD, and changes in the oxytocin system. Finally, a synthesis of RDoC, the amygdala, and autism is offered, emphasizing the task of disambiguation and suggestions for future research.

1. Introduction

The principal focus of the field of social neuroscience in recent decades has been the elucidation of social brain networks capable of detecting cues, empathizing, interacting with others, and responding adaptively in ambiguous contexts (Dunbar, 2009; Frith, 2007; Stanley and Adolphs, 2013). An important aspect of this work has been uncovering how psychiatric diseases impact these networks, particularly in autism spectrum disorder (ASD). One region consistently highlighted by this research as important in social functioning is the amygdala (Bickart et al., 2014).

The amygdala is a prime example of how neuroscience has shifted from a phrenological perspective mapping one function—fear—to one brain region and towards a network paradigm where actions arise from a dynamic affiliation of neural assemblages communicating with one another (Pessoa, 2014; Weiskrantz, 1956). The amygdala is now seen as a hub in several other distinct networks: establishing valence or salience, cognition, reward, and social learning (Rutishauser et al., 2015). Despite its small size, the amygdala's anatomy and functioning is complex, including disparate nuclei with dense connectivity to cortical and subcortical brain regions.

Increasing knowledge of the amygdala is paralleled by new investigations of the social brain and autism. Since the original descriptions of Kanner (1943) and Asperger (1944), substantial research has been done to expand the understanding of autism leading, along with better awareness, to greatly increased rates of diagnosis (Fombonne, 2009; U.S. Department of Health and Human Services, 2014; Volkmar and Pauls, 2003; Wing, 2005, 1993, 1981; Wing and Potter, 2002). ASD's etiology remains obscure and has been posited to arise from the breakdown of particular genes, networks, or brain regions (Anney et al., 2012; Baron-Cohen et al., 2000; Markram et al., 2007; Poelmans et al., 2013; Voineagu et al., 2011). Presently, it is thought to be the product of altered expression in hundreds of genes, highly heritable, influenced by epigenetic factors, and deficits in connectivity between many different networks and brain regions, including the amygdala (Eapen et al., 2013; Hallmayer et al., 2011; Kosmicki et al., 2017; Mintz, 2017).

Based on the 5th revision of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5, American Psychiatric Association, 2013), ASD is characterized by: "a) persistent deficits in social interaction and communication as well as b) restricted, repetitive patterns of behaviors, interests, or activities with an early onset of deficits." The detection of ASD is predicated on clinical measures that rely on parental reports, and on observations of the child's social aptitude during interactive tasks. These measures are crucial for diagnosing autism and categorizing the severity of symptoms. However, the DSM does not account for the vast heterogeneity of ASD and consequently it may not be the best predictor for treatment outcomes; it is thought that only 50% of individuals with ASD who receive treatment achieve significant gains as a result (Stahmer et al., 2010). In addition to improved clinical diagnosis, we need objective tools that acknowledge biological markers to demarcate variation in ASD. Towards this end the NIMH is adopting a new methodology, Research Domain Criteria (RDoC), to better characterize variation from typical behavior and enable precision medicine to improve psychiatric outcomes as it has in oncology and cardiology (Insel et al., 2010; Insel and Cuthbert, 2009). RDoC looks at psychological constructs dimensionally: from healthy to abnormal, examining the underlying genetics, neuropeptides, circuits, and physiology that ultimately gives rise to behavior (Cuthbert and Insel, 2013).

Along with renewed interest in nosology, there are questions regarding the amygdala and its contribution to neuropsychiatric disorders, including autism. Here we propose that by using RDoC to classify amygdala functions into constructs and dimensions, we can better characterize its role in normal functioning and explicate how it is altered in autism. Of the five currently defined RDoC domains—**Negative Valence Systems, Positive Valence Systems, Cognitive Systems, Social Processes,** and **Arousal and Regulatory Systems**—the amygdala is represented in each, within one or more constructs. The amygdala is not a unitary structure and we will decorticate its role in these domains and constructs by drawing on studies of lesions, animal behavior, in vivo and ex vivo electrophysiology, genetics, functional imaging, and optogenetics.

In this review, we will highlight: how conceptualizations of the amygdala's anatomy, development, and function within the social brain have changed over time; amygdala functions in RDoC; dysfunction of the amygdala in autism throughout development; linkages to comorbid psychiatric disorders; amygdala alterations seen in the valproic acid (VPA) model of autism; and the effects of oxytocin (OT) on the amygdala and behavior. By understanding the diversity of amygdala functions, and the myriad ways it can be altered in autism, the route to improved therapies and outcomes for people with autism can be illuminated.

2. The Amygdala: anatomy, history, development

2.1 Anatomy

The human amygdala (**Fig. 1.1**) comprises 13 nuclei, distinguished by cytoarchitectonics, histochemistry, and connectivity with other brain regions. To greatly simplify the anatomical layout of the amygdala (see Duvarci & Pare, 2014; and Sah, et. al, 2003 for review), there are two main divisions 1) the **basolateral complex** (**BLA**) encompassing the **lateral** (**LA**), **basal** (**BA**) and **accessory basal** (**AB**) nuclei with extensive connections to sensory and cortical brain regions and 2) the **central amygdala** (**CeA**) and its two sub-nuclei, the **central lateral** (**CeL**), connected with the BLA, and **central medial** (**CeM**), connected with brainstem areas related to the expression of innate behaviors (e.g. fear), along with the **medial amygdala** (**MeA**) linked to the olfactory system (Janak and Tye, 2015; LeDoux, 2008). The BLA is heavily innervated by sensory systems and the thalamus. The LA, and the BA also receives information from higher-order sensory and associative cortices

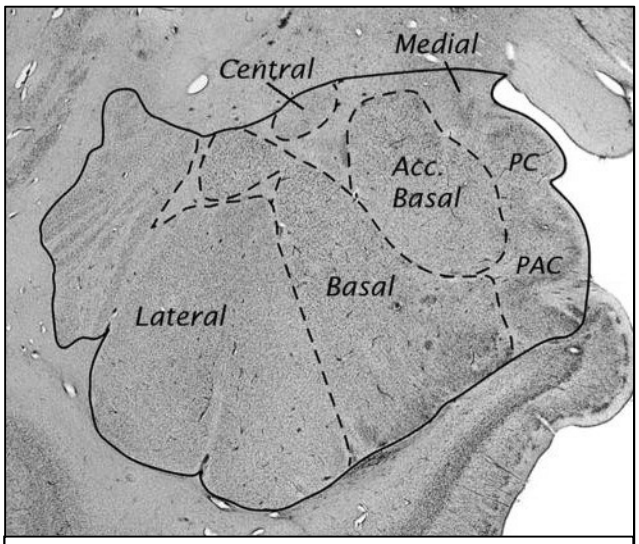

Figure 1.1. The human amygdala, *Schumann 2011.*

(Adolphs, 2009; Amaral et al., 2008). The BLA has strong efferent projections to reward regions like the nucleus accumbens (NAc), the ventral pallidum in the basal forebrain, and reciprocal connections with memory processing areas, associative and executive cortical sectors, and with the CeA (**Fig. 1.2**).

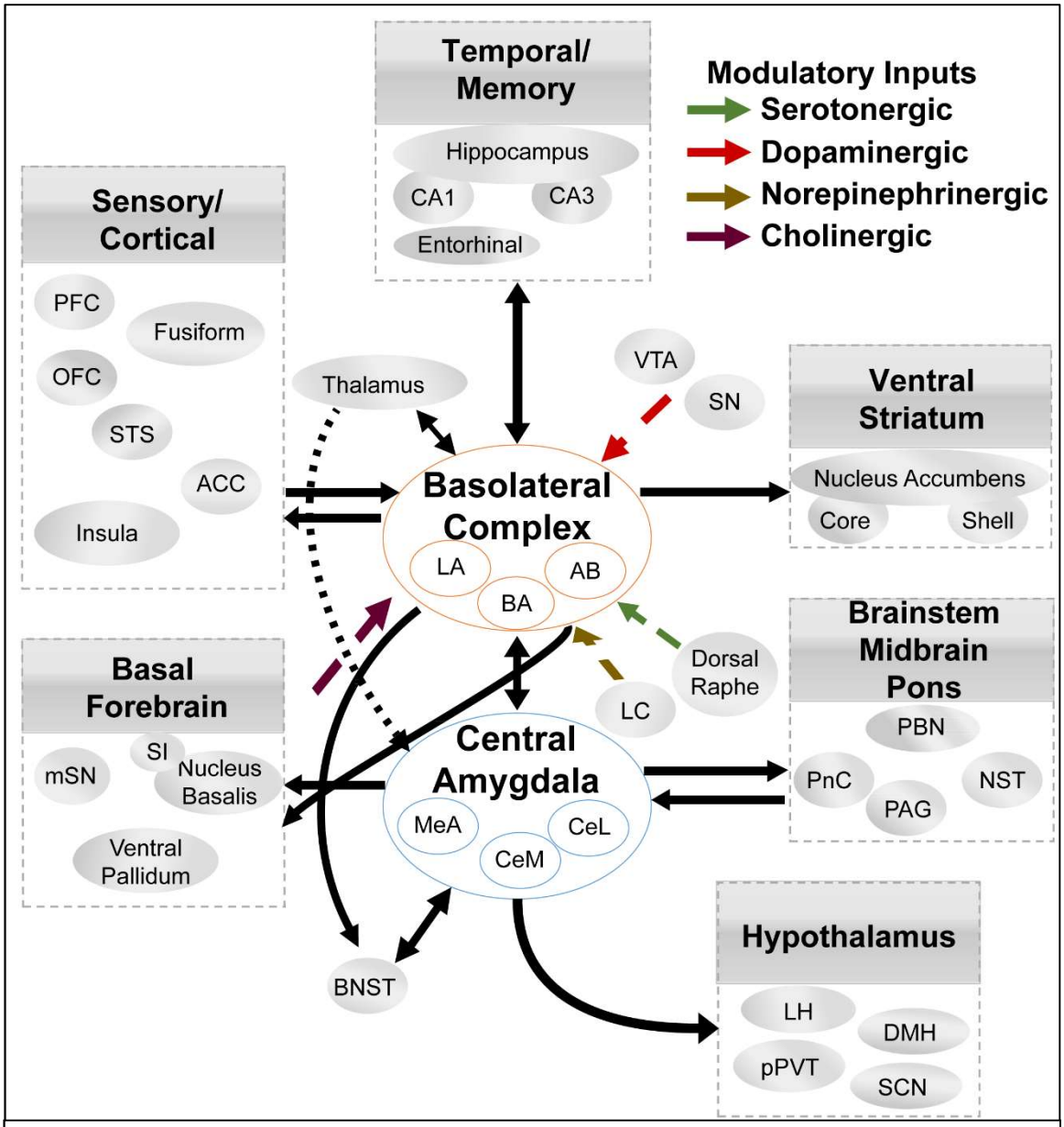

Figure 1.2. Components, inputs, and outputs of the amygdala. The BLA has bidirectional connections to sensory and cortical areas including the Insula, Fusiform gyrus, Prefrontal Cortex (PFC), Orbital Frontal Cortex (OFC), Anterior Cingulate Cortex (ACC), and Superior Temporal Sulcus (STS), as well as temporal memory areas including CA1 and 3 of the Hippocampus, and Entorhinal cortex. The BLA innervates the Ventral Striatum, including the Nucleus Accumbens core (NAcc) and shell (NAcs), and the Bed Nucleus of the Stria Terminalis (BNST) which is also connected with the CeA. Monoaminergic modulation of the BLA comes from the Dorsal Raphe, Ventral Tegmental Area (VTA), Substantia Nigra (SN), and Locus Coeruleus (LC). The CeA is connected to the Basal Forebrain including the Medial Septal Nucleus (mSN) and the Substantia Inominata (SI), which contains the Nucleus Basalis and together with the Ventral Pallidum (innervated by the BLA) provides cholinergic input to the BA. The CeA also connects to regions in the brainstem, midbrain, and pons, such as the Parabrachial nucleus (PBN), Caudal Pontine Reticular Nucleus (PnC), Periaqueductal Gray (PAG), and Nucleus of the Solitary Tract (NTS). The CeA projects to regions of the hypothalamus including the Dorsomedial Hypothalamic Nucleus (DMH), Posterior Paraventricular Thalamus (pPVT), Lateral Hypothalamus (LH), and Superchiasmatic Nucleus (SCN). Dashed line from Thalamus to CeA indicates a small number of direct projections there vs. substantial innervation of the LA.

2.2 Historical context

The classic studies of Klüver and Bucy in the 1930s first described the involvement of the amygdala in emotional processing by elucidating the behavioral consequences of large, non-selective temporal lesions in monkeys (Klüver and Bucy, 1939, 1937). Lesioned monkeys had 'psychic blindness' stemming from a lack of access to the value of social cues. Subsequent studies with more precise lesions of the amygdala caused more selective behavioral disruptions: less caution and distrust in approaching novel objects (such as snakes) or human strangers, in line with the known role of the amygdala in resolving unpredictability or ambiguity (Adolphs, 2010; Bauman et al., 2006; Machado et al., 2009).

The most selective lesions of the human amygdala result from vascular damage or from Urbach-Wiethe disease (UWD), a rare autosomal disorder that causes the progressive calcification of the amygdala (Adolphs et al., 1994; Cinaz et al., 1993; Urbach and Wiethe, 1929). SM, the best described case of UWD, and other such patients show impairments in fear recognition and autonomic conditioned responses in Pavlovian fear conditioning (Adolphs et al., 1994; Bechara et al., 1995; Cristinzio et al., 2010; Siebert et al., 2003). They also have deficits in social judgments from faces, more fixations on the mouth than the eyes during conversations, and general deficits in affective perspective taking (Adolphs et al., 1998; Hillis, 2014; Spezio et al., 2007). Individuals with UWD report experiencing no negative emotions in real life, exhibit no sensitivity to personal space, and fail to tag emotionally charged words as important to remember (Hampton et al., 2007; Hurlemann et al., 2007; Kennedy et al., 2010; Tranel et al., 2006). Research into UWD confirms the role of the amygdala not only in fear processing but also processing salient or unpredictable stimuli, and in social settings.

Historically the amygdala and the rest of the limbic system was thought of as 'evolutionarily primitive', and resistant to change (MacLean, 1955, 1952). However, components of the limbic system, principally the BLA, are in fact quite plastic, with evolutionary dynamism that has been

attributed to the influence of social factors (Barger et al., 2007). The volume of the monkey's BLA is about 30-40 times that of the rat, while the CeA and MeA are only four and eight times larger respectively (Chareyron et al., 2011). Humans in turn have a BLA and, primarily, a lateral division, several times the size of any other ape or monkey (Barger et al., 2007). The LA's neuronal density is substantially higher in the rat (99,000 neurons/mm^3), compared to the monkey (42,000 neurons/mm^3), and lowest in the human (9,000 neurons/mm^3). This lower density reflects expanded dendritic arbors and glial populations, and greater opportunity for connections with many networks, particularly the social (Pessoa, 2014). Volume, density, and other structural elements of the amygdala are all established during development from fertilization to adulthood.

2.3 Development

In humans the BLA shows synaptogenesis from the first trimester until the seventh month, the CeA is identifiable in the fifth month, and overall neuronal migration is complete by the end of the eighth month of gestation (Schumann et al., 2011; Setzer and Ulfig, 1999; Ulfig et al., 2003). The cellular structure of the amygdala and its neurotransmitter systems are well established at birth, with similar distribution to that seen in adults (Bauman and Amaral, 2005). While the number of neurons in the amygdala does not increase, the volume grows throughout childhood and into adolescence (Payne et al., 2010; Schumann et al., 2004). This is due to significant increases in both the dendritic arbor (**Fig. 1.3**) and number of oligodendrocytes that myelinate input to the amygdala, a recapitulation of the evolutionary forces that increased its size and functionality in primates (Chareyron et al., 2012; Ryan et al., 2016).

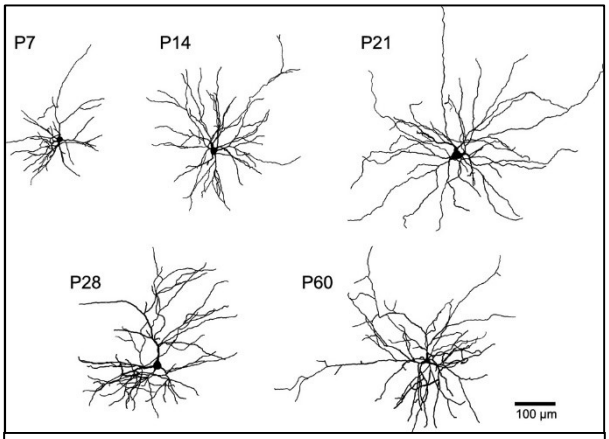

Figure 1.3. Developmental expansion of rat BLA dendritic arbors, *Ryan 2016.*

Simultaneously, more connections are made within the amygdala between the projecting principal and local inhibitory neurons that tune the network, filtering out extraneous information and binding together the input of different sensory modalities (Ryan et al., 2012). The functional role of the amygdala shifts dramatically at these stages, especially in terms of its receptivity to external input and internal modulation. Not until the second week of rat development, (corresponding to a newborn human, Quinn, 2005), are they able to learn associations between painful shocks and odors, and to avoid them (King et al., 2014). This is just one facet of the development of the amygdala, a process that links its different nuclei to the rest of the brain and to each other.

Understanding the underlying neurobiology of the amygdala is essential to seeing how it is involved in both normal and pathological behaviors, and a cornerstone of RDoC (Cuthbert and Insel, 2013). With RDoC as a framework, we will identify the domains and circuitry that include the amygdala and are altered in autism.

3. Amygdala functions and RDoC

Using RDoC, we have here classified amygdala functions in the domains of negative valence, positive valence, cognition, social functions, and arousal. We are interested in both the behaviors and neural circuitry of these different constructs and the relative importance of the amygdala within them.

3.1 Negative Valence Systems (NVS)

Defined as "primarily responsible for responses to aversive situations or contexts such as fear, anxiety and loss," NVS have a very strong link to the amygdala.

3.1.1 Acute Threat

The role of the amygdala in Acute Threat, i.e. fear learning, may be one of the most studied relationships in all of neuroscience. Weiskrantz (1956) first demonstrated that a lesion of the

amygdala prevented monkeys from pairing an aversive unconditioned stimulus, such as a shock, with a neutral cue like a tone, the conditioned stimulus. More detailed studies in rodent models, including seminal papers by LeDoux and others, explicated which sub-regions of the amygdala (**Fig. 1.2**) are required: the LA for input, BA for associative learning, and CeM for expression (Clugnet and LeDoux, 1990; Kalin et al., 2004; Keifer et al., 2015; LeDoux et al., 1990, 1988; LeDoux, 1992; Madarasz et al., 2016; Romanski and LeDoux, 1992). Structural and functional imaging studies in humans support a strong amygdala response to fearful faces. Recordings from implanted electrodes in epileptic individuals viewing faces reveal an extremely rapid response to fear, preceding a general response to any expression (Fullana et al., 2015; Méndez-Bértolo et al., 2016). Within the BLA, inhibition and disinhibition of principal neurons by specific populations of interneurons is required for conditioned and unconditioned stimulus association learning to occur (Wolff et al., 2014).

In terms of neuromodulatory factors (**Fig. 1.2**), there are substantial serotonin (5-HT) inputs to the BLA from the dorsal raphe which are sensitive to the presence of stressors (Vertes, 1991). During non-stressful conditions, 5-HT release in the BLA decreases the excitability of neurons presumably by increasing inhibition and depolarizing GABAergic interneurons (Rainnie, 1999). Conversely, the selective depletion of 5-HT in the LA results in significantly enhanced fear potentiated startle and increased expression of excitatory glutamate receptors (Tran et al., 2013). The role of 5-HT in the amygdala, in the expression of fear and anxiety, as well as the clinical implications of SSRIs are still not fully understood (for review see Burghardt & Bauer, 2013).

Dopamine is also released in the BLA during stressful situations and D1 receptors in the BLA seem to be necessary for the acquisition and expression of Pavlovian fear conditioning (Fadok et al., 2009; Muller et al., 2009). D1 receptors can have effects on long-term potentiation (LTP) induction through the modulation of NMDA receptors, and facilitate the transition of salient sensory inputs and fear learning (Li et al., 2011). Dopamine also suppresses certain GABAergic interneurons of

the BLA via D2 receptor activation to further boost relevant signals and promote learning (Chu et al., 2012).

3.1.2 Potential Threat and Sustained Threat

Potential Threat (or anxiety) and Sustained Threat (chronic stress) are closely related to each other, and to Acute Threat, differing in the physical or mental proximity to the perceived danger and in the time course (Avery et al., 2015; Davis et al., 2010).

Anxiety can be considered a generalization of fear learning and is an adaptive process from a survival perspective. However, overgeneralization of fear to harmless stimuli can become pathological and result in posttraumatic stress disorder (PTSD), phobias, obsessive compulsive disorder, panic disorder, and generalized anxiety disorder (Lissek et al., 2014). A delicate neuronal balance within the CeA (**Fig. 1.2**) regulates fear expression: The CeM is tonically inhibited by the GABAergic output of the CeL, which in turn is innervated by the BLA. If this balance is disrupted, and the CeM disinhibited, fear expression can generalize from conditioned, to non-conditioned stimuli as well (Ciocchi et al., 2010; Keifer et al., 2015).

The Stoop lab (2008) has shown that two neuropeptides, oxytocin (OT) and vasopressin (VP), have opposite effects on fear expression at the neuronal level in the amygdala. The CeM is excited by VP but fear responses are inhibited by the CeL, which is in turn excited by OT (Huber et al., 2005; Knobloch et al., 2012). Hence, an absence, reduction, or desensitization of oxytocin receptors (OTRs) in the CeL can lead to an imbalance at the neuronal level within the CeA and to generalized fear responses and anxiety. In rodents, chronic treatment with OT has been found to be anxiogenic at high doses (10ng/hr) but at low doses (1 ng/hr) prevents psychosocial stress induced anxious behaviors (Peters et al., 2014).

There is increasing evidence for OT reducing fear, anxiety, and stress, in part by lowering amygdala activity (Bethlehem et al., 2013; Domes et al., 2007; Labuschagne et al., 2010; Meyer-Lindenberg

et al., 2011; Petrovic et al., 2008). Researchers have found that intranasal OT facilitates the extinction of learned fear and reduces anxiety in patients with anxiety disorders (Acheson et al., 2013; Dodhia et al., 2014; Eckstein et al., 2015; Gorka et al., 2015; Labuschagne et al., 2010). It has also been shown that men with PTSD show diminished functional connectivity between the CeM and the ventromedial PFC, while in women with PTSD there is enhanced functional connectivity between the BLA and dorsal anterior cingulate cortex (ACC). OT administration normalized the functional connectivity between the amygdala and frontal areas during resting state in both sexes (Koch et al., 2016). In healthy controls though, Grillon (2013) found that intranasal OT enhanced startle response after unpredictable shock stress, which suggests that care should be taken in translating OT, it may be most efficacious specifically for anxiety related to social behaviors (Neumann and Slattery, 2016).

In terms of neuromodulatory factors within the amygdala involved in stress and anxiety, Norepinephrine is released into the BLA immediately following a stressful event and enhances memory of emotionally salient events while 5-HT is released during prolonged, uncontrollable stress (Roozendaal et al., 2009). During stressful conditions there is reduced expression of serotonergic receptors (5-HT2A) leading to a down-regulation in activation of GABAergic BLA interneurons, and increased excitability of BLA neurons (Jiang et al., 2009). Other 5-HT receptors can increase BLA excitability and lead to anxiety related behaviors, but infusion of the 5-HT2C receptor antagonist SB242,084 into the BLA prevents the display of anxiety related behaviors in response to prolonged stressors (Christianson et al., 2010; Pockros-Burgess et al., 2014; Zangrossi and Graeff, 2014).

3.1.3 Frustrative Nonreward

The last construct in NVS linked to the amygdala is Frustrative Non-Reward, reactions elicited in response to the withdrawal or prevention of reward (Amsel, 1958; Gallup, 1965; Machado et al., 2009). Frustrative nonreward is distinct from both offensive aggression which, as an expression of

dominance or competition for resources, is included in the social domain, and defensive aggression, one potential response to acute threat (NVS Working Group, 2011). Tye (2010) and Badrinarayan (2012) demonstrated that there was a distinct population of cells in the amygdala recruited after 'frustrative events' which predicted the intensity of response, and that they were unlike those neurons activated in response to freezing inducing shocks (Purgert et al., 2012).

Frustration, harm avoidance, and fear are all powerful motivators of behavior, and the amygdala is involved in each construct, processing early autonomic and nonconscious stimuli that signal danger for instance, but it is also vital for the opposite mode of influence in **Positive Valence Systems**.

3.2 Positive Valence Systems (PVS)

In a world of ambiguous rewards and outcomes, the amygdala is vital for updating the value of stimuli based on the current state of the organism. The PVS domain "primarily responsible for responses to positive motivational situations or contexts, such as reward seeking, consummatory behavior, and reward/habit learning" is linked to the amygdala by two related constructs.

3.2.1 Approach Motivation

Approach motivation is the instigation and direction of behavior and generally entails approach when the valence is positive (Elliot and Covington, 2001; Harmon-Jones et al., 2013). Making decisions with incomplete information is a common experience, especially so in ambiguous social encounters. Key for these circumstances is awareness of what the internal state of the individual in that moment is and, thus, the relative value of incentives; information supplied by the amygdala and areas, like the OFC, that it is connected with (Gottfried et al., 2003; Janak and Tye, 2015). In terms of motivated behavior, the medial prefrontal cortex (mPFC) seems to play an important role in balancing the relative contributions of the NAcc and the amygdala (Ernst et al., 2006).

3.2.2 Reward Learning

The differential response of the BLA as a result of positive or negative learning (or valence encoding) can be seen in human fMRI studies as well as electrophysiology studies of non-human primates discriminating between cues; when those cues are reversed, some 'positive' or 'negative' neurons stopped responding, or were inhibited, demonstrating that it was not the sensory features of the cue that were triggering them, but the values they represented (Gottfried et al., 2003; Paton et al., 2006; Shabel and Janak, 2009). In rodents, BLA neurons can be distinguished not only based on where they connect to, e.g. the NAc, part of the reward network, but also by what genes they express, allowing more fine grained dissections of how positive and negative signals are encoded in the brain (Jennings et al., 2013; Namburi et al., 2015).

OTR expression is an important modulator of the reward network. During reward anticipation, healthy individuals carrying the OTR rs2268493 autism risk allele showed decreased activation of the right amygdala and other parts of the reward network compared to non-carriers and heterozygotes (Damiano et al., 2014). Importantly, lesioning the amygdala does not impair all reward based behaviors, only those specifically related to changing reward value (Machado and Bachevalier, 2007). There is evidence of increased BOLD activity in the amygdala during monetary reward-learning and decision-making tasks. For example, the framing effect from economics where a participant's decisions about which option to select is manipulated by presenting the choice in terms of either potential loss or gain; this effect is correlated with activation of the amygdala (De Martino et al., 2006).

The amygdala's function in reward processing is part of a network of brain regions (**Fig. 1.2**) including the orbital frontal cortex (OFC) and NAc (Montague and Berns, 2002). Lesions that disconnect the amygdala from the OFC result in deficits on reward learning tasks (Baxter et al., 2000). It appears that the amygdala acquires information about the value of stimuli and the OFC uses this to guide choices (Happé and Frith, 2014). Disrupting the connection between the

amygdala and NAc can lead to deficits in instrumental behavior toward rewards (Ambroggi et al., 2008). Following the acquisition of information, the amygdala communicates the sensory aspects of stimuli to the NAc to guide approach behavior and reward learning by modulating dopaminergic neurons (Jackson and Moghaddam, 2001; Stuber et al., 2011).

The PVS domain, and within it the amygdala, has a fundamental role in initiating, and shaping activities, but command of thoughts and actions requires input from **Cognitive Systems**.

3.3 Cognitive Systems

In addition to the primary role of the amygdala in modulating emotional states, it has a crucial function at the perceptual level in integrating sensory information about stimuli. This domain, "responsible for various cognitive processes" covers a very large range of behaviors, one of which is the construct of attention, or salience.

3.3.1 Attention

The process by which the brain assigns attention to various stimuli depends on careful coordination of the top-down, value assigning areas, such as the dorsolateral PFC and inferior parietal cortex, associated respectively with appraisal and reappraisal (Ochsner et al., 2012); and the bottom-up middle frontal gyrus and superior parietal cortex, responsible for directing the visual gaze (Ferri et al., 2016). This coordination takes place within the amygdala in conjunction with the anterior insula and ACC (**Fig. 1.2**) as part of the larger saliency network (Jacobs et al., 2012; Menon, 2015). Interestingly, there is increased functional connectivity between the amygdala and the precuneus and dorsal ACC when resisting distraction, particularly from arousing, negative images (Ferri et al., 2016; Kanske et al., 2011). This balancing of top-down control and bottom-up input requires the precise functioning of the inhibitory circuits within the amygdala that regulate its activity (Rainnie et al., 1991a, 1991b; Truitt et al., 2007).

Perception of fearful stimuli could be considered one highly specialized and conserved task of the amygdala within the larger context of salience assigning (Jacobs et al., 2012). To that end, some inputs from sensory thalami, and nociceptive signals from the pontine parabrachial nucleus (PBN), bypass the LA and project directly to the CeA (**Fig. 1.2**), allowing for very rapid and adaptable responses to danger signals (Keifer et al., 2015). The amygdala also has a role in gating connections between the cortex to the hippocampus and striatum, tagging experiences with emotional marks, of any valence, which increases their salience and improves recall and learning (Hurlemann et al., 2007; Paz and Pare, 2013; Vuilleumier and Driver, 2007).

Attention control is vitally important for navigating any number of situations, but especially those involving **Social Processes**.

3.4 The Social Processes

These constructs "mediate responses to interpersonal settings of various types, including perception and interpretation of others' actions."

3.4.1 Affiliation and Attachment

The first construct in the social domain, Affiliation and Attachment, concerns both the initial engagement in positive interactions as well as the development of bonds that reinforce and motivate the communication constructs. In both non-human primates and humans, amygdala volume is positively correlated with the size of an individual's social network (Bickart et al., 2011; Zhang et al., 2012). The enlargement of the BLA (Section **2.2**) is correlated with, and may be the result of, a concomitant expansion in the cortical regions related to social behavior that it is connected with (Janak and Tye, 2015; Stephan and Andy, 1977). Direct evidence for the role of the amygdala in social motivation can be seen by lesioning the BLA which causes rats to stop preferring prosocial rewards (Hernandez-Lallement et al., 2016).

Particularly important in the discussion of attachment and the amygdala is the function of OT. This neuropeptide is released in key socio-emotional brain regions, including the amygdala, which is rich in OTRs. OT is known to modulate a wide range of social behaviors, such as maternal attachment, pair bonding, social recognition and empathy-based behaviors (Burkett et al., 2016; Ferguson et al., 2001; Johnson et al., 2016; Rilling and Young, 2014). OT acting on the amygdala also enhances social attachment, possibly by reducing fear and anxiety (Section **3.1**).

In accordance with the animal literature, there is substantial evidence in humans showing that OT plays a crucial role in maternal care and maternal motivation. Though the circuitry of human parental care is more elaborate than in rodent species, the reward and sub-cortical components involved in the motivational and emotional aspects of parenting are common to both. This circuitry includes (**Fig. 1.2**) the hypothalamus, ventral striatum, and amygdala (Feldman, 2015).

3.4.2 Social Communication

The next construct in Social Processes, Social Communication, and the sub-construct Reception of Facial Communication (including either implicit or explicit communication such as affect recognition, facial recognition and characterization) clearly involves the amygdala, particularly in the perception of emotions that signal danger, e.g. fear or anger (Adolphs et al., 1999, 1998; Gamer and Buchel, 2009). The amygdala's role was formerly thought to be only in automatic and non-conscious rapid processing of such stimuli (LeDoux, 1996; Morris et al., 1999; Öhman et al., 2007). Today, we know that the purview of the amygdala is broad, neuroimaging studies show that the amygdala is activated in response to all faces, and not only to fearful expressions (Fitzgerald et al., 2006). These studies also show that the involvement of the amygdala in processing information from faces is complex, can show inter-individual variability, and is dependent on context (Adams et al., 2003; Kim et al., 2004).

The involvement of the amygdala in controlling attention and ambiguity resolution extends to this construct: SM's fear recognition impairment (Section **2.2**) appears to arise from a failure to make spontaneous use of the eye region of faces, a potent source of disambiguating social information (Adolphs et al., 2005). In addition, it has been shown that when interpreting equivocal expressions positive interpretations are associated with decreased amygdala activation, and negative with increased (Kim et al., 2003; M. J. Kim et al., 2011). The amygdala's response to faces is also dependent on the motivational state of the perceiving individual; Radke (2015) discovered that testosterone administration biased individuals towards threat approach and increased amygdala activation, away from threat avoidance and decreased activity. Anatomical differences affect facial reception as well; Zhang (2012) found a strong correlation in monkeys between larger amygdala and longer periods of gazing into the eye region of other monkeys. There is also evidence that the function of the amygdala in interpreting gaze changes throughout development, with amygdala size in children positively correlated to *cognitive* mental state inferences and in adults to *emotional* ones (Rice et al., 2014).

Clearly the amygdala has a significant role in social bonding and communication, how important is it for the last domain in RDoC, **Arousal and Regulatory Systems**?

3.5 Arousal and Regulatory Systems

These constructs "are responsible for generating activation of neural systems as appropriate for various contexts, and providing appropriate homeostatic regulation of such systems as energy balance and sleep."

3.5.1 Arousal

In studies of arousal**,** which is distinct from both salience and anxiety, activation of the amygdala is linked with increased vigilance (Pessoa, 2011). After electrostimulation of a cat's amygdala Ursin and Kaada (1960) noted an "attention response" and orienting around the environment.

Stimulation of the CeA's connection to the basal forebrain results in more activation of cholinergic neurons, including those that innervate the BLA (**Fig. 1.2**), increasing the signal-to-noise ratio therein (Davis and Whalen, 2001; Unal et al., 2015). In monkeys that have undergone neonatal removal of the amygdala there is an enhancement of CRF activity and HPA axis activation suggesting that early in development the amygdala acts as a brake on the arousal system but switches to an activator in maturity (Raper et al., 2014).

3.5.2 Circadian Rhythms

Coordinating biological activity to times of day and the light-dark cycle is a function of the superchiasmatic nucleus (SCN) and the rhythmic expression of 'clock' genes that serve as transcription factors (Reppert and Weaver, 2002). These same genes are expressed in other subordinate sites throughout the brain and body, including the amygdala (Harbour et al., 2014).

4. Amygdala dysfunction in Autism

The separation of a heterogeneous clinical population by biomarkers holds great promise in the treatment of many complex psychiatric disorders but particularly in autism (Clementz et al., 2016; Loth et al., 2016). Using biomarkers, such as characteristics of the amygdala, to parcellate ASD allows the development of targeted therapies and facilitates overall understanding of autism.

4.1 Alterations in Development and Neurobiology

One of the hallmarks of autism is that it is a developmental disorder; though diagnosis typically occurs between 2-7 years, precursor symptoms can be identified even in the first six months of infancy (Hazlett et al., 2017; Jones and Klin, 2013; Mandell et al., 2005; Mazurek et al., 2014). In healthy subjects the amygdala continues to elaborate throughout childhood and adolescence, some connections with forebrain regions are still being strengthened even in adulthood, most notably with the mPFC (Chareyron et al., 2012; Ehrlich et al., 2012; Ernst et al., 2006; Lenroot and Giedd, 2006; Schumann et al., 2011). Indeed, the amygdala has more connections with distinct parts of the

brain than almost any other region (Pessoa, 2014, 2008; Petrovich et al., 2001; Young et al., 1994). It is important then to see how exactly prominent characteristics of autism can be linked with altered amygdala development.

While many studies have found enlargement of the amygdala in autism, others have found no change, or a decrease in volume (Aylward et al., 1999; Haar et al., 2016; Nordahl et al., 2012; summary in Allely et al., 2013). We believe this is evidence of subgroups of individuals with autism, and that improvements in care and treatment require a close examination of these seemingly discordant findings, and replication with larger groups (Müller and Amaral, 2017). In addition to altered development in the amygdala, we will look at changes in connectivity with major neural networks, and how these variations compare symptomatically with damage to the amygdala that can come from UWD (section **2.2**) or other sources.

4.1.1 Volume differences

Enlarged amygdala were among the first brain anomalies to be identified in individuals with autism (Bauman and Kemper, 1985; Kemper and Bauman, 1993; Schumann et al., 2004; Sparks et al., 2002). This increase is age dependent; Nordhal et al. (2012) found the amygdala was enlarged from 2-4 years and that the rate of increase year over year was higher in children with autism. An enlarged amygdala was also found to correlate with more severe symptoms in toddlers (Schumann et al., 2009). But from 8-14 years and older the increase slowed or reversed, no differences are seen between adolescents with typical development vs. those with autism (Barnea-Goraly et al., 2014).

This early overgrowth in the amygdala in children with autism could be related to later excessive pruning, accounting for volume differences seen between children, but not adults, with autism (Amaral et al., 2008). Dziobek et al. (2010) and Eilam-Stock et al. (2016) found no size differences in adult individuals with autism, although Murphy et al. (2012) found significant increases in amygdala volume in their comparison of individuals with Asperger's vs. typically developing

controls. One important proviso is that there is a significant degree of heterogeneity. While Nordhal et al. (2012) found that on average children with autism had enlarged amygdala, there were a considerable number whose amygdala development was on par or decreased relative to typically developing individuals.

Does normalization of amygdala volumes in older adolescents and adult individuals with autism correlate with improvements in social abilities? Although adolescent subjects with autism's amygdala growth does not differ on the whole from typically developing subjects, increases in eye contact in those with autism correlates with increased amygdala size (Barnea-Goraly et al., 2014). In children with autism there is a positive relationship between amygdala volume, eye gaze, and response to joint attention, with smaller amygdala predicting less eye gaze, stronger symptoms, and non-response to bids for attention (Mosconi et al., 2009; Nacewicz et al., 2006). Unaffected siblings also have smaller amygdala and decreased eye gaze but they, like typically developing controls, do not show a relationship between amygdala volume and eye gaze (Dalton et al., 2007). Interestingly, Williams syndrome, a rare autosomal disorder characterized by mild to moderate cognitive impairments and hypersociability, is associated with an *enlarged* amygdala, along with an overall decrease in brain volume (Schumann et al., 2011). Ultimately, the change in size could be a consequence, rather than cause, of social interaction (Zalla and Sperduti, 2013). When the number of individuals in a monkey's social group was altered, the volume of the amygdala also changed (Sallet et al., 2011). If this translates to humans, then amygdala size during development could serve as a biomarker for effective therapeutic intervention.

A stereological comparison of cell types in the amygdala between those with autism and typically developing controls found that before 20 years of age there were no differences, but afterwards there was a significant drop in myelinating oligodendrocytes (Morgan et al., 2014). Thus, it is essential to look beyond gross anatomical differences of the amygdala to alterations in its activity,

how it connects with other areas of the social brain, and what significance this has for the pathogenesis of autism.

4.1.2 Abnormal amygdala activity and social cognition

Social deficits are another hallmark of ASD. The amygdala, along with regions like the superior temporal sulcus (STS) and OFC (**Fig. 1.2**), are an essential part of the neurobiological substrate of social cognition. Early neuroimaging of ASD individuals revealed decreased activation in the amygdala and frontal areas during social tasks such as reading the mind in the eyes (Baron-Cohen et al., 1997). Other studies revealed a lack of amygdala activity during the implicit processing of emotional facial stimuli (Critchley et al., 2000). Functional neuroimaging studies have found that abnormal amygdala activation in ASD is, reminiscent of SM, related to decreased eye fixations to faces (Dalton et al., 2005; Kliemann et al., 2012).

In addition to a lack of amygdala activation, individuals with autism show a significant reduction in functional connectivity between the amygdala, principally the BLA, with cortical, visual, and parietal regions involved in perception during resting state (Rausch et al., 2016). This reduction in functional connectivity was also seen between the amygdala and the salience network including the insula (Von Dem Hagen et al., 2014). However, there are a number of attentional and social deficits in ASD, independent of the amygdala, and it's bilateral destruction is insufficient to produce autism, as determined by clinical measures (Paul et al., 2010; Wang et al., 2014).

4.1.3 Damage to the amygdala

In comparative studies between people with autism and focal amygdala lesions (Section **2.2**) there are several similarities in their behavioral phenotypes: abnormal social judgment regarding the rating of trustworthiness and approachability in faces, difficulties in identifying emotional features from facial expressions, impairments in judging faces with negative affect such as fear and anger, and abnormalities in attributing social meaning to ambiguous and moving shapes (Adolphs et al.,

2001; Klin, 2000; Pelphrey et al., 2002). What's more, individuals whose amygdala are removed due to a tumor or refractory epilepsy also display significant deficits in Theory of Mind tasks, but only if the damage occurred in their childhood (<16 years old, Shaw et al., 2004). Loss of the amygdala from UWD appears to impair the saliency network's (Section **3.3.1**) ability to prioritize emotionally charged inputs (Hurlemann et al., 2007). This phenomenon is similar to the weak central coherence model of autism from Happé and Frith (2006): a focus on details rather than the whole.

This suggests autism does not arise exclusively from the functioning of the amygdala, as a complete lesion would be expected to result in the most severe social dysfunction (Paul et al., 2010). It suggests instead a change in *how* the amygdala develops and functions is responsible, and that modeling these developmental disruptions can be a useful way to understand autism.

4.2 Valproic acid and autism

Valproic acid (VPA) is the most widely prescribed anti-epileptic drug in the world, as well as a potent teratogen (Perucca, 2002). VPA exposure during pregnancy is linked with fetal valproate syndrome (FVS) characterized by dysmorphic facial features, spina bifida, lower IQ, delayed language, and autism in approximately 9% of those exposed (Ardinger et al., 1988; Clayton-Smith and Donnai, 1995; Jäger-Roman et al., 1986; Rasalam et al., 2005; Williams et al., 2001; Williams and Hersh, 1997). FVS is thought to result from the effects of VPA on the developing central nervous system in the 1st trimester of pregnancy (Binkerd et al., 1988; Christianson et al., 1994; but see Roullet et al., 2013). VPA is to date the only environmental agent so strongly linked to autism (Bromley et al., 2013; Christensen et al., 2013; Ornoy et al., 2015).

How exactly the epigenetic changes wrought by VPA contribute to FVS and autism is still unknown. By promoting the acetylation of histones, VPA makes DNA more accessible to transcription factors, altering the course of development, and ultimately the neural systems

underlying cognitive and social domains (for review see Chomiak et al., 2013). Much of the work in explicating this process has been done in animal models: As originally developed by Rodier (1997) fetal exposure to VPA resulted in rats that were less sociable and more anxious, a finding that has been replicated many times (K. C. Kim et al., 2011; Mabunga et al., 2015; Markram et al., 2008; Roullet et al., 2013 but see Cohen et al., 2013; Štefánik et al., 2015). VPA exposed rats also display hyperserotonemia, one of the oldest known biomarkers of autism (Anderson et al., 2011, 1990; Hranilovic et al., 2007; Narita et al., 2002; Takahashi et al., 1976).

The amygdala is significantly altered along several dimensions following prenatal VPA exposure. In rats, VPA changes the volume of the amygdala and drastically alters dendritic arbors of principal neurons, following a progression that is comparable to humans, with initial overgrowth of spines at the time of weaning followed by a retraction in the fully-grown rat (Amaral et al., 2008; Bringas et al., 2013; Sosa-Díaz et al., 2014). This may be related to increases in the excitability of LA neurons, facilitating LTP, and contributing to behavioral alterations in the model (Lin et al., 2013). VPA exposure changes the expression of many amygdala genes including Homer1, a notable finding as overexpression of Homer1 in the amygdala leads to a reduction in social interaction and impaired fear conditioning (Banerjee et al., 2016; Oguchi-Katayama et al., 2013). We have also observed that VPA exposure in the rat significantly increased OTR expression in the female, but not male, CeA (authors, unpublished).

Two caveats: First, though rodent models of autism are crucial for better understanding the neurobiological mechanisms of social functioning, most of the behavioral tests that are used in these experiments do not mirror the complexity of social dysfunction seen in ASD. The autism phenotype is heterogeneous and not restricted to a lack of social approach, the gold standard measure in the majority of animal studies (Moy et al., 2004; Tordjman et al., 2007). Second, the use of a teratogen as the agent for creating autism-like symptoms must be reconciled with autism's high heritability (Hallmayer et al., 2011). But the fact that this model is not the outcome of a single

gene mutation makes it in many ways more representative of the broad swath of individuals whose autism is not related to any known monogenic cause. Autism is associated with higher overall rates of de novo protein truncating mutations, and more severe autistic symptoms and intellectual disability are associated with the highest rates of mutations (Kosmicki et al., 2017).

4.3 Autism, anxiety, and oxytocin

Besides the core social symptoms in ASD, there are several medical, neurological, and psychiatric comorbidities, including disorders of: anxiety, depression, obsessive-compulsiveness, sleep, irritability, and hyperactivity. Anxiety disorders in particular are much more common in individuals with autism than in the general population, with estimates ranging from 40-55% of young people with autism also exhibiting one or more anxiety disorders (van Steensel et al., 2011; White et al., 2009). Currently, there are two FDA approved drugs (risperidone and aripiprazole) for comorbid symptoms of irritability. There are no approved treatments for the social dysfunctions of ASD or for anxiety disorders in these individuals.

In addition to reducing fear and anxiety (Section **3.1.2**) intranasal OT has been shown to promote social functioning, gaze to the eyes, and theory of mind, in both typically developing individuals, and those with autism, as well as activity in, and functional connectivity between, key brain regions involved in social and emotional processing, such as the amygdala (Andari et al., 2016, 2010; Aoki et al., 2014; Auyeung et al., 2015; Gordon, 2014; Guastella et al., 2010; Watanabe et al., 2016, 2015, 2014). A recent meta-analysis reported significant associations between ASD and the OXTR SNPs rs7632287, rs237887, rs2268491 and rs2254298 (LoParo and Waldman, 2015). Importantly, one of these SNPs (rs237887) was found to be strongly associated with recognition memory in individuals with ASD, their parents and their siblings, suggesting a critical role of the OT system in social recognition (Skuse et al., 2014). We have also demonstrated that the administration of intranasal OT triggered reciprocal cooperation between individuals with ASD and partners in an interactive social game (Andari et al., 2010). Finally, OT differentially altered BOLD activity in

the amygdala during an interactive social game, reducing it with an equitable partner but increasing it with an untrustworthy one (Andari et al., 2016).

More research is needed to better understand the mechanism by which OT affects social cognition in these individuals, and whether it can also be a treatment for co-occurring anxiety (Andari, 2016).

5. Conclusion: synthesis of RDoC, the amygdala, and autism

While we have described each of the domains and constructs of RDoC, and their associated neural networks, as independent systems, they exist in dynamic equilibrium. Evaluating competing needs: fear of punishment vs. possibility of reward, or interest in complex systems vs. in conspecifics, is one of the essential tasks of the brain across all species and all levels of development. Disruption of this mechanism, manifested in one construct or many is, in a sense, the definition of mental illness; especially so in autism.

Consider an amygdala dependent ability that cuts across several RDoC domains: disambiguation. It is a difficult task, though one that typically developing individuals accomplish with relative ease: **Attention** must be switched across multiple contextual levels, salient details extracted and judged. The challenge increases if the information is **social** in nature, given the unwritten mores and subtleties of that domain. Imagine how, **stressful,** and **frustrating** that task would be, and how little **motivation** to pursue it, were it not intrinsically **rewarding**. Mental bandwidth is not limitless and, once exhausted, individuals with autism might retreat to spaces where cause and effect are clear, and where they have control over the stimuli they experience i.e. stereotypies.

This line of thinking about autism has several implications. It explains some discordant fMRI findings in the amygdala: hypoactivation when bandwidth is low and potent stimuli such as the eyes are unengaged, hyperactivation otherwise, such as when the subject is specifically instructed to look at the eyes (Baron-Cohen et al., 1999; Critchley et al., 2000; Dalton et al., 2005; Kleinhans

et al., 2010; Kliemann et al., 2012; Schultz, 2005; Zürcher et al., 2013). The highest degree of amygdala activation, and association with symptom severity, occurs when judging ambiguous expressions (Swartz et al., 2013; Tottenham et al., 2014). Madarasz (2016) directly studied the role of the amygdala in disambiguation by examining changing cue contingencies; neurons in the LA not only store sensory associations, they update dynamically when new information is presented.

As the classification of the amygdala as a single brain structure was questioned decades ago, there are calls now to abandon ASD as unsuitable for neurobiological research (Davis and Whalen, 2001; Swanson and Petrovich, 1998; Waterhouse et al., 2016). We, like Müller and Amaral (2017), think this is unwarranted. Instead, by embracing RDoC, grasping how variations from neuron to brain are reflected dimensionally across domains of behavior, in typically developing and individuals with autism, we can fulfil the promise of individualized medicine, finding optimal outcomes for everyone with this disorder. We conclude that there are likely dysfunctions in the amygdala of many, but not all, individuals with autism, and that investigation of this relationship remains a very fruitful area of research.

In addition to adoption of RDoC, four specific issues in this field call out for further study: 1) Much of autism research (and biomedical research generally) is done in males, and while a gender disparity in autism does exist, bias in subject selection may serve to exaggerate it (Beery and Zucker, 2011; Giarelli et al., 2010; Mandy et al., 2012; Robinson et al., 2013; Stone et al., 2004; Werling and Geschwind, 2013a, 2013b). Evidence strongly suggests the amygdala is sexually dimorphic, with larger volume and more variance in males, but differences in connectivity, development, and functioning of the amygdala between sexes are poorly understood (Ritchie et al., 2017). Amygdala dimorphism may be related to the higher incidences of ASD in males, but more studies in females with ASD are needed to explore this possibility (Baron-Cohen et al., 2011, 2005).

2) Most neuroimaging studies of individuals with autism look at only a single time point while the few longitudinal studies have only 1-4 years between measurements (Courchesne et al., 2011; Hazlett et al., 2017; Mosconi et al., 2009; Nordahl et al., 2012). Since amygdala connections are developed well into the second decade of life, imaging studies are clearly needed on this timescale, especially if monitoring the size and growth trajectory of the amygdala could serve as a biomarker for effective therapeutic intervention (Ernst et al., 2006; Lenroot and Giedd, 2006; Ruggeri et al., 2014; Uddin et al., 2011).

3) While fMRI studies are useful for finding large scale changes in activity and connectivity, experiments using implanted electrodes would reveal precisely how information flow between the amygdala and social brain is altered (Rutishauser et al., 2013). Since electrophysiological studies in humans are necessarily in populations with significant comorbidities, such as epilepsy, this is one area that animal models, e.g. VPA, would prove useful (Jeffrey et al., 2013; MacFabe et al., 2007; Meletti et al., 2012; Mormann et al., 2015; Rutishauser et al., 2015; Sato et al., 2011).

4) Promising therapies, such as oxytocin, can be fully realized only through a detailed understanding of how exactly they do, or do not, work in the brains of individuals with autism, especially in critical areas like the amygdala (Bales et al., 2013; Young and Barrett, 2015). To translate research on the effects of acute administration of intranasal OT into clinical therapeutics, it will be necessary to adopt a precision medicine approach to determine OT's targets in autism. More studies and replications of OT treatment's effect on functioning and development, particularly with chronic vs. acute exposure, are needed.

It is our hope that researchers can move to address these and other significant gaps in knowledge, deepening our understanding of a remarkable brain area, and improving the wellbeing of millions of individuals with neuropsychiatric disorders.

While many of the studies considered and proposed in this chapter are clinical, there is still much to be gained from preclinical research into autism and the amygdala. In **Chapter 2** we will show how prenatal VPA exposure not only recapitulates much of the ASD phenotype in juvenile rats, but also drastically shifts the expression of key developmental pathways in the amygdala. Then in **Chapter 3** we will further expand the scope of this research by adding an environmental stressor to the VPA model, exploring how co-occurring anxiety disorders can be modeled in addition to ASD. Our research offers new opportunities for clarifying the genes and circuits that underlie RDoC, and for improved comprehension of both the amygdala and autism.

Chapter 2

Developmental disruption of amygdala transcriptome and socioemotional behavior in rats exposed to valproic acid prenatally

Content in Chapter 2 adapted from:

Developmental disruption of amygdala transcriptome and socioemotional behavior in rats exposed to valproic acid prenatally. Catherine E. Barrett, **Thomas Hennessey**, Katelyn M. Gordon, Steve J. Ryan, Morgan L. McNair, Kerry J. Ressler, Donald G. Rainnie. *Molecular Autism,* 2017.

Abstract

Background: The amygdala controls socioemotional behavior and has consistently been implicated in the etiology of Autism Spectrum Disorder (ASD). Precocious amygdala development is commonly reported in ASD youth with the degree of overgrowth positively correlated to the severity of ASD symptoms. Prenatal exposure to VPA leads to an ASD phenotype in both humans and rats, and has become a commonly used tool to model the complexity of ASD symptoms in the laboratory. Here, we examined abnormalities in gene expression in the amygdala and socioemotional behavior across development in the valproic acid (VPA) rat model of ASD.

Methods: Rat dams received oral gavage of VPA (500mg/kg) or saline daily between E11-13. Socioemotional behavior was tracked across development in both sexes. RNA sequencing and proteomics were performed on amygdala samples from male rats across development.

Results: Effects of VPA on time spent in social proximity and anxiety were sex dependent, with social abnormalities presenting in males and heightened anxiety in females. At postnatal day 10, gene pathways involved in nervous system and cellular development displayed predicted activations in prenatally exposed VPA amygdala samples. By juvenile age, however, transcriptomic and proteomic pathways displayed reductions in cellular growth and neural development. Alterations in immune pathways, calcium signaling, Rho GTPases, and protein kinase A signaling were also observed.

Conclusions: As behavioral, developmental, and genomic alterations are similar to those reported in ASD, these results lend support to prenatal exposure to VPA as a useful tool for understanding how developmental insults to molecular pathways in the amygdala give rise to ASD-related syndromes.

1. Background

The etiology of Autism Spectrum Disorder (ASD) likely involves complex interplay between genetic and environmental factors. As over 800 genes are implicated in the etiology of ASD (Simons SFARI database), any one knockout will not entirely represent the complexity of core abnormalities. Moreover, although genetic heritability plays an important role in the etiology of ASD, a number of early environmental exposures have also been linked to ASD risk (Ornoy et al., 2015). The antiepileptic drug and mood stabilizer, valproic acid (VPA), has a well-documented history of increasing the susceptibility to ASD. Children exposed to VPA during the first trimester of pregnancy are at increased risk of developing ASD, with estimates varying between 2.9 to 18-fold greater risk compared to the general population (Christensen et al., 2013; Moore et al., 2000; Rasalam et al., 2005).

Rats exposed to VPA prenatally around embryonic day (E) 12.5 display abnormalities in neurological and behavioral development. Notably, the VPA rat model recapitulates many of the core symptoms of ASD, including impaired social behaviors, increased repetitive behaviors, and communication impairments, as well as hyperserotonemia, heightened dopamine levels, increased ratio of excitatory to inhibitory neurotransmission, elevated physiological and behavioral measures of anxiety, and enhanced responsivity to sensory stimulation (American Psychiatric Association, 2013; Arndt et al., 2005; Banerjee et al., 2013; Roullet et al., 2013; Schneider and Przewłocki, 2005).

VPA alters neural circuits in brain regions implicated in ASD, such as the amygdala, and thus is a useful tool to investigate how the disruption of these circuits can lead to emotional and behavioral abnormalities. Abnormal functioning of the amygdala has long been implicated in the etiology of ASD (Baron-Cohen et al., 2000). Amygdala abnormalities in structure (Aylward et al., 1999; Nacewicz et al., 2006; Rojas et al., 2004), neuronal density (Palmen et al., 2004; Schumann and Amaral, 2006), and functional MRI activity during social tasks (Baron-Cohen et al., 1999; Dalton

et al., 2005; Hadjikhani et al., 2007; Monk et al., 2010; Schultz, 2005; Wang et al., 2004; Weng et al., 2011) have been reported in patients with ASD. Prenatal VPA exposure induces hyper-excitability, enhanced long-term potentiation, and hyper-plasticity of neurons in the amygdala, a reduction in inhibitory synaptic transmission (Lin et al., 2013; Markram et al., 2008), and local hyper-, but distal hypo-connectivity of neural microcircuits (Bringas et al., 2013). Local amygdala hyperactivity contributes to enhanced fear memories, over-generalized fear, reduced fear extinction, and enhanced anxiety in rats prenatally exposed to VPA (Banerjee et al., 2014; Markram et al., 2008; Sui and Chen, 2012). The impact of VPA on local hyperactivity and hyperplasticity is in line with the "intense world" theory of ASD, which postulates that excessive neuronal processing leads to a hyper-functionality underlying ASD symptomatology (Markram et al., 2007). Impairment in GABAergic circuitry (Rubenstein and Merzenich, 2003) and under-connectivity between brain regions (Just et al., 2004; Rippon et al., 2007) are also similarly implicated in ASD etiology.

Premature amygdala development is commonly reported in youth with ASD (Nordahl et al., 2012; Schumann et al., 2004; Sparks et al., 2002), potentially contributing to the observation of clinically-significant anxiety in 40% of children and adolescents with ASD, twice that seen in typically developing children (Costello et al., 2005; van Steensel et al., 2011; White et al., 2010). The basolateral amygdala (BLA) is an important center for multimodal sensory information processing in the control of emotional arousal and social behavior (LeDoux, 2007). We have previously shown that the rat amygdala undergoes phenomenal changes in the first few weeks of life in GABA and glutamatergic signaling, structure, and function (Ehrlich et al., 2013, 2012; Ryan et al., 2016). At postnatal (P) day 10, the ability to learn fear association emerges, along with functional, molecular, and physiological changes in the amygdala (Ehrlich et al., 2013; Ryan et al., 2016; Sullivan et al., 2000; Thompson et al., 2008). Between P7-P28, extensive changes occur in molecular and electrophysiological properties of BLA principal neurons (Ehrlich et al., 2012) and in GABAergic

fibers and cell body densities (Brummelte et al., 2007). Behaviorally, amygdala-mediated fear learning and extinction undergo dynamic changes between juvenile and adolescent stages (Kim et al., 2006). Plasticity in BLA neurons is bidirectional and under very tight control by second messenger systems like cAMP-Protein Kinase A (PKA) (Li and Rainnie, 2014; Ryan et al., 2012). However, surprisingly little is known about the cellular, molecular, or genetic changes that occur in the amygdala over development in ASD individuals or rodent models. In order to develop appropriate early interventions and treatments for neurodevelopmental disorders, it is important to investigate changes across development. Appropriate treatments may differ greatly across time, depending on the natural biological state of neurons in the amygdala. Here, we investigated the gene expression in the BLA across development in prenatally VPA exposed rats. This is the first comprehensive analysis of genetic changes in this region over development.

2. Methods

2.1 Animals

Pregnant Sprague-Dawley dams (Charles River, Wilmington, MA) arrived at our animal facilities at 5-6 days of gestation, and were maintained on a 12:12h light-dark schedule with access to food and water *ad libitum*. Day of birth was considered postnatal (P) day 0. Pups were weaned at P21 and group housed with same-sex, same-treatment siblings. No more than two animals of the same sex and litter were used per behavioral experiment. No more than one animal per sex and litter were used in genomic analyses. A timeline of experiments and cohorts can be found in **Table 2.1**. All protocols strictly conformed to the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health, and were approved by the Emory University Institutional Animal Care and Use Committee.

Table 2.1. Outline of experiments.

Age	Measure	Cohort 1	Cohort 2	Cohort 3	Subjects
	Behavior				
P1-6	Maternal behavior		X		Dams
P7, 11	Ultrasonic vocalizations		X		Males, Females
P9	Maternal approach	X			Males, Females
P8-10	Fear conditioning			X	Males, Females
P13-18	Odor-potentiated startle		X		Males
P14	Eye opening	X			Males, Females
P32-35	Open field, Social Interaction	X			Males, Females
P36	Startle	X			Males, Females
	Genomic analysis				
P10	RNA Seq	X			Males
P21	RNA Seq, Proteomics	X			Males

2.2 Drug administration

As intraperitoneal VPA administration leads to high rates of fetal resorption (Favre et al., 2013; Nau, 1986), an oral route of administration was chosen, which also mimics clinical usage. Pregnant dams received oral gavage, with either 500 mg/kg of VPA (Sigma-Aldrich, St. Louis, Missouri; ~ 800-900 ul) diluted in 0.9% saline or saline alone on embryonic (E) days 11-13. Offspring were weighed between P3-50 and assessed for eye opening on P14. Preliminary studies in our lab demonstrated this dose was sufficient to produce the previously described features of the VPA model (Schneider and Przewłocki, 2005).

2.3 Behavior

2.3.1 Maternal behavior

Home cage observations were conducted daily from P1-6 (16:00-17:00) on VPA (n = 6) and saline (n = 4) treated dams. Point observations of position of dams on the litter (on or off), nursing style (arched-back, blanket, passive), and licking/grooming pups were made every 4 minutes (15 obs / hour).

2.3.2 Ultrasonic vocalizations (USVs)

On P7 and 11, pups (n = 10 / grp) were removed from the home cage and individually placed into sound-proofed cages (31.7 × 17.2 × 14.2 cm) for 5 min. USVs were recorded and analyzed for the number, frequency, and duration using Sonotrack software (Metris, Netherlands).

2.3.3 Nest-seeking response

On P9, the latency to approach maternal bedding was assessed (n = 14-16 / grp). Bedding from the maternal home cage and clean bedding was placed on filter paper at either corner of the testing cage (31.7 × 17.2 × 14.2 cm) and a live observer recorded latency to approach either bedding.

2.3.4 Infant fear learning.

The impact of VPA on the development of fear learning was assessed by light-shock conditioning infant rats (P8-10; n = 20-23 / grp). Heating pads were placed underneath the shock grid as young rats are not able to regulate body temperature. On P7, rats were habituated for 20 min to chambers and exposed to 30 noise bursts (95 dB). On P8-10, rats received 2-3 days of conditioning consisting of a 5 min acclimation, followed by 15 startle leaders (95 dB), and 10 light-shock pairing (1s light terminating with 0.6 mA shock). Half of each group received 2 days and half received 3 days of conditioning, but data was combined for analysis as both paradigms yielded similar results. After 12 days (P21-22), animals were returned to the original chambers for a 5 min acclimation, followed by exposure to 30 light-tone (95 dB) and 30-tone alone trials. Fear-potentiated startle was calculated by normalizing startle amplitudes to the acclimation period responses and calculating the percent increase from tone alone to light-tone trials.

2.3.5 Startle to maternal odor

In order to assess maternal attachment learning, we exposed pups to 10% acetophenone (Sigma) scented food from P0-10 to create a maternal odor as described in Todrank et al. (2011). Standard laboratory chow was mixed with 10% acetophenone (Sigma) in propylene glycol (1ml per 100g

chow) and allowed to air dry in a fume hood for 3 d. Scented food was provided as the sole food source from P0-10, and then regular laboratory chow was provided. Acetophenone odor potentiated startle (OPS) was assessed in male offspring (P17-20, n = 17 saline, 14 VPA) as previously described (Dias and Ressler, 2014). Testing consisted of 15 startle leaders (105 dB) followed by 10 odor-startle (10 s odor ending in 50 ms 105 dB noise burst) and 10 startle-alone trials randomly interspersed. A subset of the animals was also tested for OPS to a neutral odor (n = 10 saline, 8 VPA). The procedure was run on two consecutive days with the odor being acetophenone (maternal) or propanol (neutral) in a counter balanced fashion. Percent OPS was calculated by subtracting the startle amplitude during the last startle leader from the first odor-startle trial, dividing by the last leader startle amplitude, and multiplying by 100.

2.3.5 Open field and social behavior testing

At P35, subjects (n = 22-26 / grp) were habituated to the novel testing arena (27.3 x 90.2 x 91.1 cm) for 5 min under red light illumination, which also served as an open field test of basal anxiety levels. Subsequently, a cage containing a same-sex, same-age conspecific and an empty cage were placed at opposite corners of the arena to test the subject animal's preference to spend time in proximity to another rat or an object. After 5 min, a novel same-sex, same-age conspecific was put into the empty cage, and the preference for a novel or familiar animal was assessed over 5 min. For analysis, the arena was divided into four quadrants, each approximately 45 x 45 cm. The social preference portion contained a social zone, an object zone, and two empty zones. Subsequently, the social novel portion contained a familiar zone, a novel zone, and two empty zones. Durations in and entry bouts into the zones were analyzed using an automated system (Cleversys, TopScan).

2.3.6 Acoustic startle

At P36, rats (n = 22-26 / grp) were presented with 10 trials each of 95, 100, and 110dB acoustic stimuli, along with 10 prestimulation trials with no noise burst. A fast-rise-time (<1 msec) burst of noise presented for 40 msec was used, with intertrial intervals of 30 sec.

2.4 Brain collection

Subjects were deeply anaesthetized with isofluorane, decapitated, and brains were frozen on dry ice and stored at -80˚C. BLA punches (~1μm^3) were collected on a microtome.

2.5 Next generation RNA sequencing

Transcriptomics was assessed in male amygdala micropunches from P10 (n = 4 saline, 4 VPA) and P21 (n = 4 saline, 3 VPA). RNA sequencing libraries were prepared by the Yerkes Nonhuman Primate Genomics Core (NHPGC). Total RNA was prepared using the QIAGEN RNEasy Micro Kit. Libraries were generated from 5 ng of Total RNA using the CLONTECH SMARTer HV kit, and barcoding and sequencing primers were added using NexteraXT DNA kit. Libraries were validated by microelectrophoresis, quantified, pooled and clustered on Illumina TruSeq v3 flowcell. The clustered flowcell was sequenced on an Illumina HiSeq 1000 in 100-base single-read reactions. Sequenced reads were processed using the Illumina BaseSpace Cloud environments RNAExpress App Workflow version 1.0.0. The reads were aligned to the UCSC (http://genome.ucsc.edu/index.html) rn5 reference assembly using the STAR Aligner (Dobin et al., 2013). Gene abundance estimation was done by counting the number of aligned reads that overlap annotated genes in the reference assembly using a custom script based on the method of htseq-count (Anders et al., 2015). The per sample count files were loaded into the DESeq2 (Love et al., 2014) R package for normalization and differential expression analysis. Library size normalization was performed and differential expression was calculated with a two factor two level crossed model

using the negative binomial Wald test, and Benjamini-Hochberg False Discovery Rate (FDR) was use for multiple comparisons.

2.6 Proteomics

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) was performed by the Emory Integrated Proteomics Core using contralateral P21 amygdala micropunches from the same animals that were run for RNA sequencing (n = 4 saline, 3 VPA). Tissue samples were homogenized in lysis buffer (8M urea, 100 mM NaHPO4, pH 8.5), including HALT protease and phosphatase inhibitor cocktail (Pierce), using a Bullet Blender (Next Advance). Supernatants centrifuged, sonicated (Sonic Dismembrator, Fisher Scientific), and vortexed. Protein concentration was determined by the bicinchoninic acid (BCA) method. Protein homogenates (100 ug) were treated with 1 mM dithiothreitol (DTT), followed by 5 mM iodoacetimide (IAA), digested with 1:100 (w/w) lysyl endopeptidase (Wako), diluted with 50 mM NH4HCO3, and further digested overnight with 1:50 (w/w) trypsin (Promega). Resulting peptides were desalted with a Sep-Pak C18 column (Waters).

Peptides were resuspended in loading buffer (0.1% formic acid, 0.03% trifluoroacetic acid, 1% acetonitrile), separated on a self-packed C18 (1.9 um Dr. Maisch, Germany) fused silica column (25 cm x 75 uM internal diameter (ID); New Objective, Woburn, MA) by a Dionex Ultimate 3000 RSLCNano and monitored on a Fusion mass spectrometer (ThermoFisher Scientific, San Jose, CA). Elution was performed over a 120-minute gradient at a rate of 300nl/min with buffer B ranging from 3% to 80% (buffer A: 0.1% formic acid in water, buffer B: 0.1 % formic in acetonitrile). The mass spectrometer (MS) cycle was programmed to collect at the top speed for 3-second cycles. MS scans (400-1600 m/z range, 200,000 AGC, 50 ms maximum ion time) were collected at a resolution of 120,000 at m/z 200 in profile mode and the HCD MS/MS spectra (2 m/z isolation width, 30% collision energy, 10,000 AGC target, 35 ms maximum ion time) were detected in the ion trap. Dynamic exclusion was set to exclude previous sequenced precursor ions for 20

seconds within a 10 ppm window. Precursor ions with +1, and +8 or higher charge states were excluded from sequencing.

RAW data was analyzed using MaxQuant v1.5.2.8 with Thermo Foundation 2.0. The search engine Andromeda, integrated into MaxQuant 1, was used to build and search a concatenated target-decoy Uniprot rat reference protein database (retrieved April 20, 2015; 29370 target sequences), plus 245 contaminant proteins from the common repository of adventitious proteins (cRAP) built into MaxQuant. Quantitation of proteins was performed using summed peptide intensities given by MaxQuant. The full list of parameters used for MaxQuant are available as mqpar.xml. T-tests assuming unequal variances were performed on mean saline and VPA log label-free quantification (LFQ) intensity values.

2.7 Statistics

Statistics were analyzed in SPSS v.24 (IBM, Chicago, IL) and PRISM (GraphPad, La Jolla, CA) with an alpha level of 0.05. Outliers in behavioral data were removed using Grubbs' test. Repeated measures ANOVA were run on maternal behavior counts, with drug as a between-subjects factor and day as a within-subjects repeated measure, and planned t-tests with Bonferroni corrections were performed. USV counts, frequencies, and lengths were analyzed with t-tests with Holm-Bonferroni adjustments within each sex. Fisher's exact tests on eye opening and maternal approach counts were run. Student's t-tests on latency to approach and fear potentiated startle responses for males and females were performed. For odor potentiated startle data, a two-way drug X odor type (maternal vs neutral) ANOVA and planned t-tests with Bonferroni correction were performed. Duration and bouts in the center of the open field were analyzed with Student's t-tests. Two-way drug by zone ANOVAs and planned t-tests with Bonferroni correction were run separately for males and females for duration and bouts in the social preference (zones = social, object, empty) and social novelty (zones = novel, familiar, empty) tests. A two-way drug X decibel level ANOVA

was run for baseline startle amplitude data separately for males and females, with Bonferroni-corrected t-tests at each decibel level.

3. Results

3.1 Developmental disruption of social preference, fear expression, and anxiety-like behavior

VPA did not lead to changes in maternal behavior in treated dams, nor did it significantly impact weight or eye opening in pups. No significant main effect of drug or drug by day interaction effect was detected for frequencies on nest, nursing, or licking and grooming (**Fig. 2.1A-D**). On P1, saline-treated dams spent more time on the nest than did VPA-treated dams (t-test, $p = 0.05$). Weights did not differ between saline and VPA treated animals at any age examined when combining sexes (Student's t-tests, $p > 0.05$; P3, 7, 14, 21, 37, 50). However, VPA treated females weighed significantly less than their saline counterparts at P37 ($p = 0.025$). Fewer VPA females tended to open their eyes by P14 (saline 21 of 25; VPA 11 of 19; Fisher's exact test, $p = 0.088$; **Fig. 2.1E**), but there was no difference in eye openings in males ($p > 0.05$, saline 15 of 24, VPA, 10 of 19).

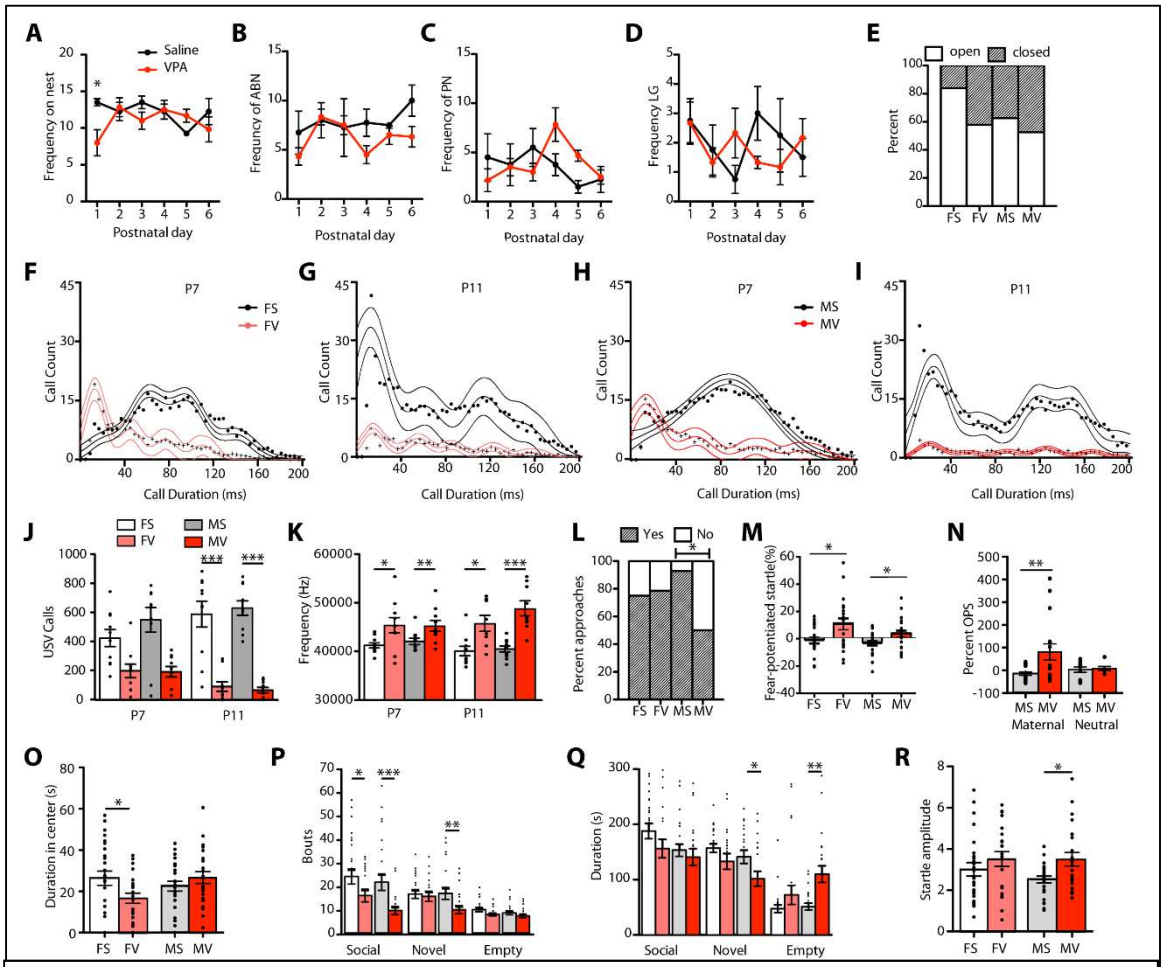

Figure 2.1. Behavioral phenotype of prenatally-exposed VPA animals. Frequencies on the nest, arch-back nursing (ABN), passive nursing (PN), and licking and grooming (LG) were largely unaltered between saline and VPA treated dams, with the exception of P1 frequencies on the nest (t-test with Bonferroni correction, n = 4-6/grp **A-D**). No differences in eye openings were detected at P14 (Fisher's exact test, females, p = 0.088; n=19-25/grp; **E**). Histograms of ultrasonic vocalization lengths display qualitative differences in calls of various length over time and between treatment groups of each sex (n = 10/grp; **F-I**), with females having significantly reduced mean call lengths at P7 (not displayed, t-test with Holm-Bonferroni correction, $p < 0.01$). VPA-treated males and females called significantly less than saline animals at P11 (**J**) and had higher frequency calls (**K**; t-tests with Holm-Bonferroni correction). VPA-treated males were less willing to approach maternal bedding at P9 (Fisher's exact test; n = 14-16/grp; **L**). After fear conditioning to light at P8-10, VPA treated animals of both sexes displayed heightened fear-potentiated startle responses (t-tests; n = 20-23/grp; **M**). Prenatal VPA treatment also enhanced startle amplitudes in the presence of maternal, but not neutral, odor (t-tests with Bonferroni corrections; n = 14-17/grp, only males **N**). Juvenile VPA-treated females spent less time in the center of the open field (t-test; n = 22-26/grp; **O**). Female and male VPA juveniles approached a social stimulus less than did saline males in the social preference test (post-hoc t-tests with Bonferroni corrections; n = 21-26/grp; **P**). Males also displayed lower approaches to and less time in proximity to a novel conspecific, as well as more time in an empty arena during the social novelty component (**P, Q**). Juvenile VPA males displayed enhanced baseline startle amplitudes (post-hoc t-test with Bonferroni correction; n = 22-25/grp; **R**). FS, female saline; FV, female VPA; MS, male saline; MV, male VPA. Group (grp) indicates each of the four drug/sex combinations. Asterisks indicate significant comparisons between VPA and saline groups *, $p < 0.05$; **, $p < 0.01$; ***, $p \leq 0.001$.

Prenatal VPA impaired early communication, responses to maternal odors, and enhanced fear expression in exposed pups. Mean call length of ultrasonic vocalizations was significantly shorter in prenatally exposed VPA females than in saline females at P7 (Student's t-test, p=0.004), but not between males at P7 or either sex at P11. At P7, VPA treated animals displayed peak call counts at 13.65 ms duration, compared to 76-97 ms in controls (**Fig. 2.1F-I**). VPA-treated animals called less than did saline at P11 (female p = 0.001, males p = 0.001; Fig **2.1J**) but not P7 (females p = 0.081, males p = 0.05), and VPA calls were of a higher frequency at both ages (P7 females p = 0.0028, males p = 0.004; P11 female p = 0.028, males p = 0.001; Fig **2.1K**).

In the nest-seeking test, saline-treated males approached maternal bedding at a significantly higher frequency than the VPA-treated males (13 of 14 saline; 7 of 14 VPA; Fisher's exact test, p = 0.033, **Fig. 2.1L**). A trend toward increased latency to approach was also observed in the VPA males (Student's t-test, p = 0.058). Females did not differ in the number that approached (saline, 12 of 16; VPA, 11 of 14). After fear conditioning to light at P8-10, VPA treated males and females both displayed enhanced fear-potentiated startle responses to light 12 d later (t-tests, males p = 0.0187; females, p = 0.0353, **Fig. 2.1M**). VPA males displayed enhanced startle amplitudes in the presence of the maternally-conditioned odor (acetophenone) as compared to saline treated males, but no differences were detected in responses to the neutral propanol odor (**Fig. 2.1N**; ANOVA, ns; Sal vs. VPA Aceto Student's t-test, p = 0.019). Reduced distress calls, disrupted maternal nest-seeking responses, enhanced fear expression, and amplified startle responses to maternal odor indicate impaired early social behavior and enhanced fear and anxiety-like behavior in VPA-exposed males.

Prenatal VPA also enhanced anxiety-like behavior and reduced social interaction in juvenile animals. Females exposed to VPA prenatally spent less time investigating the center of the open field (**Fig. 2.1O**, Student's t-test, p = 0.031). In the social preference test, saline males and females entered the social zone more than did VPA-treated counterparts, although durations did not differ (Males; drug X zone, $F_{2,128} = 4.652$, p = 0.0112; drug, $F_{1,128} = 9.682$, p = 0.0023; Social zone t-test,

$p < 0.001$; Females; drug X zone, $F_{2,132} = 4.155$, $p = 0.0178$; Social zone t-test, $p < 0.05$; **Fig. 2.1P-Q**). VPA-treated males differed significantly from saline males in durations (drug X zone, $F_{2,130} = 9.872$, $p = 0.0001$) and bouts (drug, $F_{2,133} = 8.78$, $p = 0.0036$) in the social novelty component, spending more time in the empty arena ($p < 0.01$), less time in the arena with the novel animal ($p < .05$), and less bouts entering the novel arena ($p < .01$; **Fig. 2.1P-Q**). In females, a main effect of drug was also detected for bouts in the social novelty test ($F_{2,134} = 4.156$, $p = 0.0435$), but no significant effect was detected within any zone. VPA-treated males exhibited significantly larger startle responses at 110dB noise bursts (ANOVA, Drug $F_{2,136} = 7.621$, $p = 0.006$ t-test, $p < 0.05$; Fig 1R) but no differences were detected in females. As VPA impaired social behavior and enhanced fear and anxiety-like behavior across development, and these effects were predominantly in males, genomic alterations in male neonatal and juvenile amygdala samples were examined.

3.2 RNA Sequencing

Separate Ingenuity Pathway Analyses (IPA) were run on genes with significant ($q<0.05$) effects of time in saline (n = 1498) and VPA (n = 1682 VPA). Treatment effects did not reach statistical significance after FDR multiple comparison correction, thus genes with uncorrected $p < 0.05$ treatment effects at P10 (n = 542) and P21 (n = 406), as well as time by treatment interaction effects (n = 390) were run through IPA. IPA reports p-values from a right-tailed Fisher's exact test of the ratio of number of genes altered in a given comparison (i.e., drug, time) within the total number of genes in that pathway. Additionally, z-scores represent predicted changes in gene regulation of given pathways, which are based on a literature-derived Ingenuity® Knowledge Base (Krämer et al., 2014). Here, we focus on canonical biological pathways with activation z-scores and diseases and functions categories with predicted activation states (z-scores $\geq |2|$). Additional canonical pathways with gene enrichment, without predicted activation states, can be found in the supplemental material.

From P10 to P21, 72 pathways, many of which are involved in synaptic plasticity and neurotransmission, changed similarly in both saline and VPA animals (Supplementary Table 2.1A, 2.4A). Exclusively in saline animals, 46 pathways were altered across time, and 91 pathways were altered only in the VPA group (**Fig. 2.2**, Supplementary Table 2.1B-C, 2.4B-C). Saline-treated amygdala samples displayed exclusive increases in pathways involved in cellular development, molecular transport, neurotransmission, and metabolism, and reductions in those involved in neurological disease. Changes from P10-21 unique to VPA treated animals included alterations in pathways involved in immune responses, reductions in a number of pathways involved in cellular and neural development, and increases in pathways involved in neurological disease.

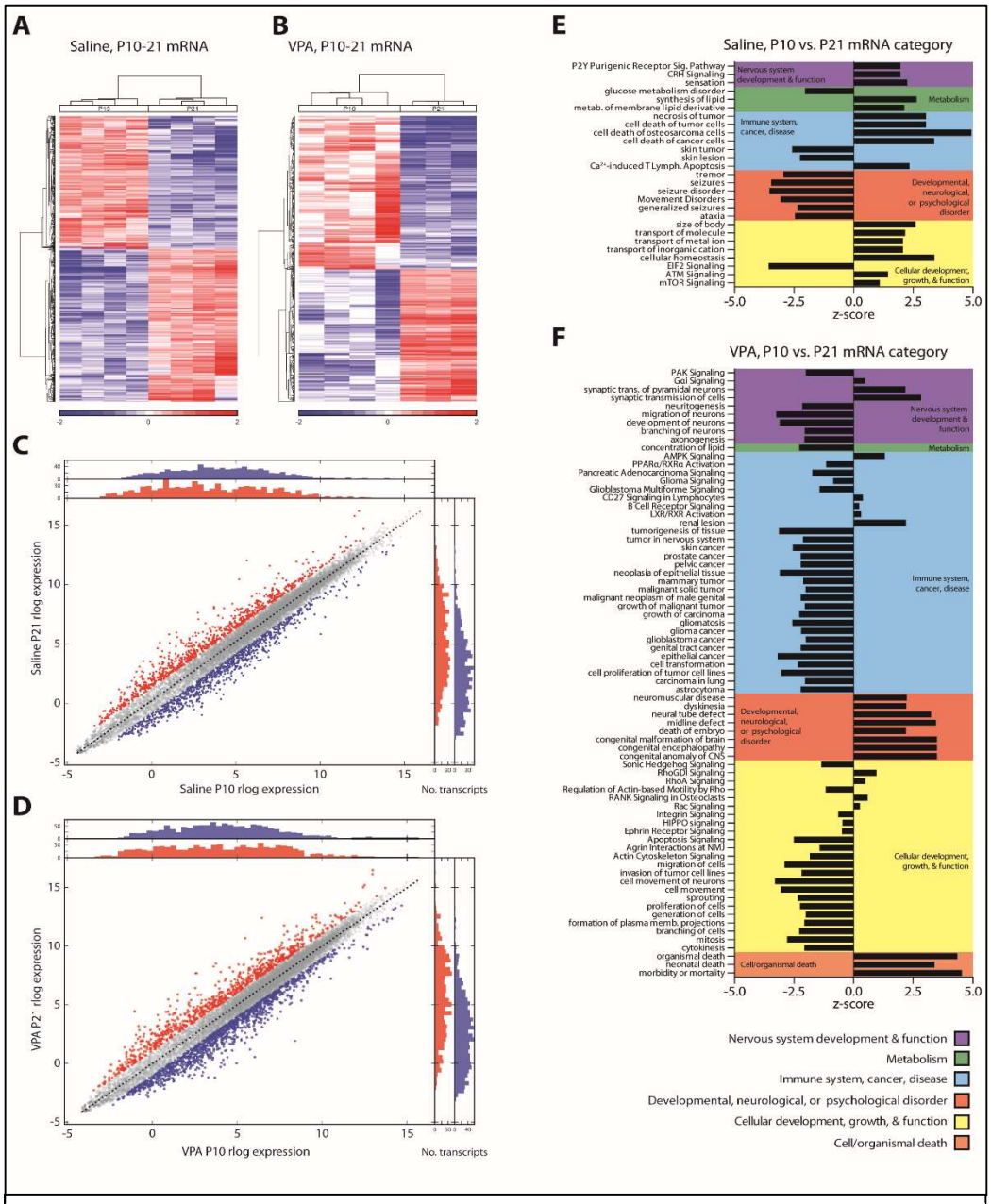

Figure 2.2. VPA disrupts cellular growth, neural development, and immune function in amygdala gene pathways from P10-21. Heat maps of median normalized gene expression in saline (A) and VPA (B) animals from P10-21 depict log2 fold changes of genes with $q < 0.05$. Differential expression scatterplots and transcript histograms of individual genes from P10-21 in saline (C) and VPA (D) exposed animals, where red and blue points represent genes with over 2-fold up- or down-regulation changes, respectively, in log expression across time. Ingenuity Pathway Analyses (IPA) were run on genes with significant ($q<0.05$) effects of time in saline (n = 1498) and VPA (n = 1682 VPA) samples. Pathways with predicted activation changes from P10 to P21 exclusive to either saline (E) or VPA (F). Canonical pathways and diseases and functions categories with predicted activation or inhibition were broadly categorized into the following groups: cellular development and growth; nervous system development and function; immune system, cancer, disease; cell/organismal death; metabolism; and developmental, neurological, or psychological disorder.

A time by treatment pathway analysis revealed a decrease in the magnitude of change in pathway activity from P10 to 21 in the VPA animals in pathways relevant to cellular development, immune function, and neurotransmission (Supplementary Fig 2.1).

Between treatments at P10, pathways, such as those involved in cellular development and function, nervous system development and function, and immune function, were increased in the VPA treated animals; **Fig. 2.3**, Supplementary Table 2.2A, 2.5A). P10 VPA samples displayed predicted decreases in pathways involved in neurological and developmental diseases. By P21, there were differences in pathways involved in neurotransmission between saline and VPA amygdala samples (Supplementary Table 2.2B, 2.5B). Pathways involved in cellular development and function displayed decreases and organismal death and growth failure pathways displayed increases (**Fig. 2.3**).

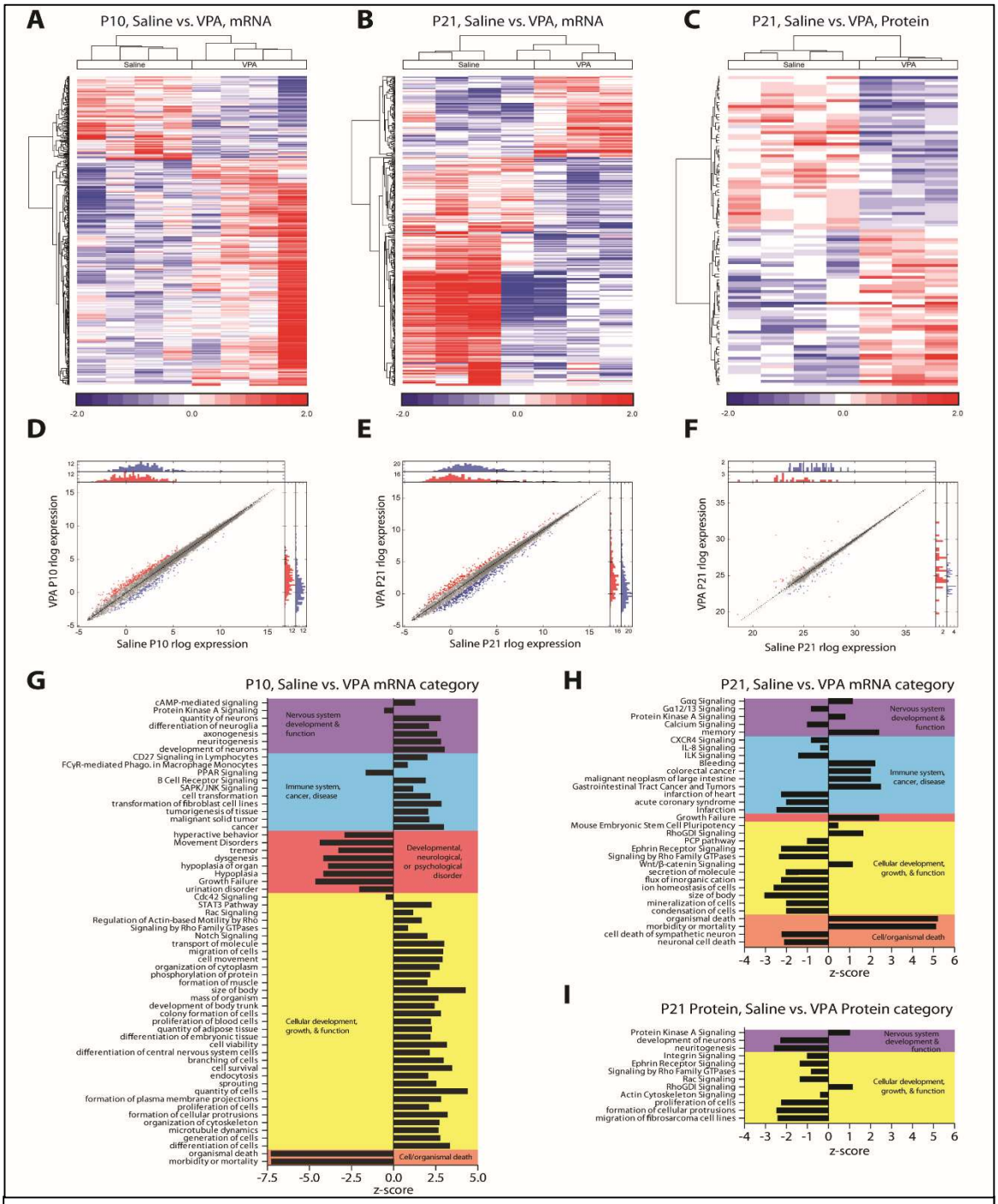

Figure 2.3. Early enhancement but later deficit in cellular growth and neural development in amygdala from prenatal VPA exposed animals. Heat maps of differential gene expression between saline and VPA at P10 (**A**), P21 (**B**), and of differential protein expression at P21 (**C**) depict log2 fold changes of genes with $p < 0.05$. Differential expression scatterplots and transcript histograms of individual genes at P10 (**D**), P21 (**E**), and of individual proteins at P21 (**F**), where red and blue points represent molecules with over 2-fold up- or down-regulation changes, respectively, in log expression across time. Treatment effects did not reach statistical significance after FDR multiple comparison correction, thus Ingenuity Pathway Analyses were run on genes with uncorrected $p < 0.05$ treatment effects at P10 ($n = 542$) and P21 ($n = 406$). Pathways with predicted activation changes between saline and VPA transcriptomic samples at P10 (**G**) and P21 (**H**) and protein samples at P21 (**I**).

3.3 Proteomics

A total of 103 proteins ($p < 0.05$, uncorrected) were run in IPA analyses from proteomics results from contralateral amygdala samples from the same animals as from RNA sequencing. At P21, proteomics also revealed predicted reductions in pathways involved in nervous system and cellular development; Supplementary Table 2.3, 2.6; **Fig. 2.3**). Across proteomic and transcriptomic samples from the same animals, three genes displayed alterations of $p < 0.05$ in both samples (*Ryr2, Slc7a14, Cacn2d1*). These genes also displayed the same direction of change in both assays (reductions in *Ryr2* and *Cacna2d1*, increase in *Slc7a14*). A number of pathways displayed predicted alterations across both transcriptomic and proteomic analyses at P21 (**Fig. 2.4**). Notably, PKA signaling, and signaling by Rho Family GTPases are the only pathways altered between saline and VPA animals at all time points (P10, 21) and in both proteomic and transcriptomic samples.

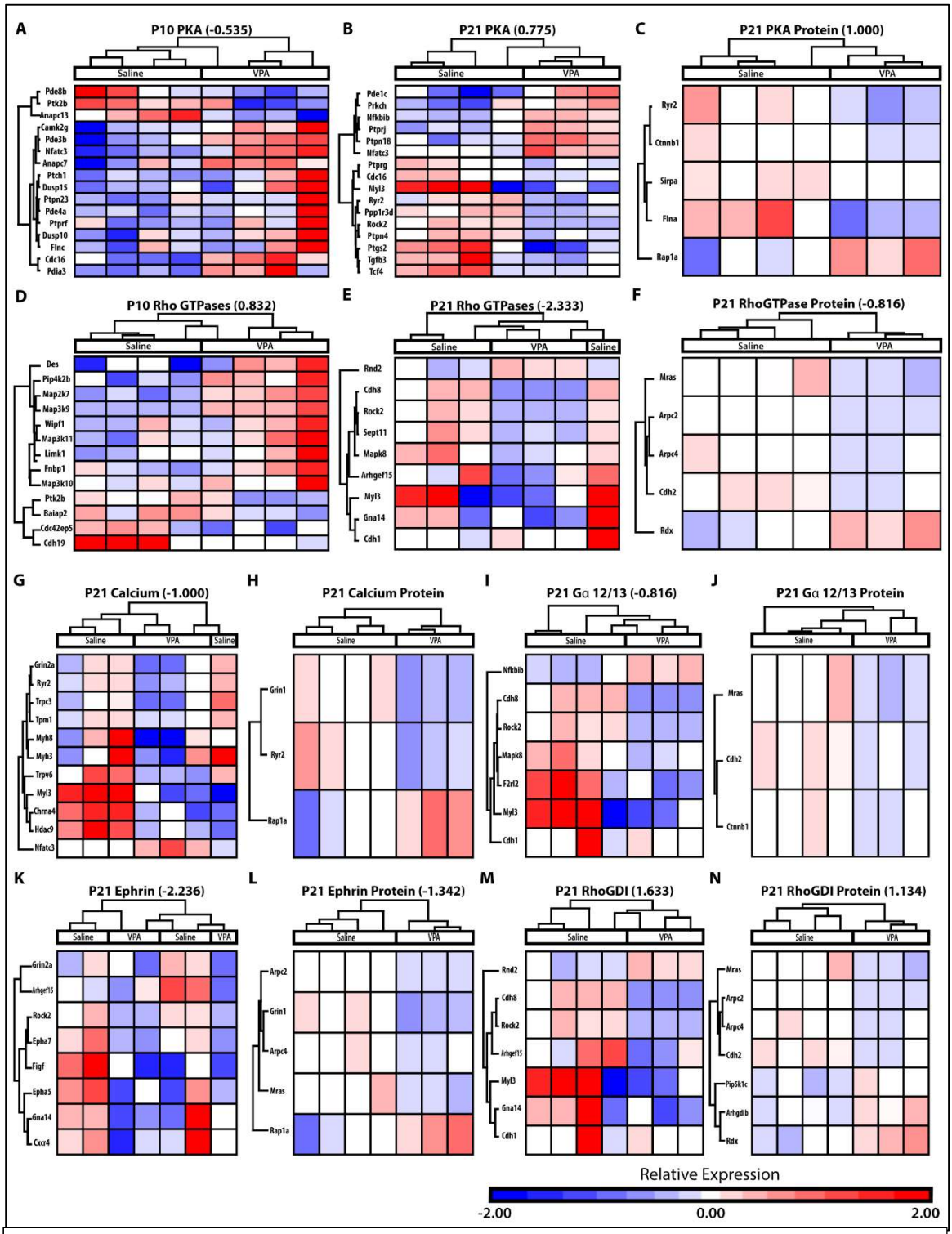

Figure 2.4. Pathways altered from prenatal VPA in both transcriptomic and proteomic samples. Individual pathway heat maps of differential gene expression between saline and VPA amygdala samples at P10, P21, and of differential protein expression at P21 depict log2 fold changes of genes with $p < 0.05$. Predicted changes (z-scores) in activation between groups are noted. PKA, Protein kinase A; GDI, guanine nucleotide dissociation inhibitor.

4. Discussion

Prenatal exposure to VPA disrupted later social behavior, fear expression, anxiety-like behavior, and amygdala gene expression in infant and juvenile animals, with many alterations corresponding to ASD-like symptomatology (**Table 2.2**). Maternal behavior was largely unaltered in dams given VPA, suggesting changes in offspring are not due to indirect maternal effects. A reduction in time spent in social proximity and enhancement in isolation were detected in the VPA-treated males. This reduction in sociality is in accordance with a number of previous reports of the VPA model (Kim et al., 2013; Markram et al., 2008; Moldrich et al., 2013; Roullet et al., 2010; Schneider et al., 2008, 2006; Schneider and Przewłocki, 2005). Prenatal VPA also lead to enhancements in basal anxiety with males displaying greater startle amplitudes and females investigating the center of the

Table 2.2. Overlap between VPA model and ASD.

ASD	*Prenatal VPA model*
Core Pathology (APA, 2013)	
Deficits in social interaction	Reduced social investigation; Impaired attraction to maternal bedding; Maternal odor-induced potentiation of startle
Impaired social communication	Reduced USVs and altered call structure
Repetitive/stereotypical behaviors	Increased stereotypic-like beam breaks (Schneider et al., 2008; Schneider and Przewłocki, 2005)
Additional Symptoms	
Comorbid anxiety disorders (van Steensel et al., 2011; White et al., 2010)	Reduced open field exploration in females Enhanced baseline startle amplitude in males
Early amygdala overgrowth (Nordahl et al., 2012; Schumann et al., 2004; Sparks et al., 2002)	Enhanced P10 nervous system and cellular development and function pathways
Reduced amygdala volume (Aylward et al., 1999; Nacewicz et al., 2006), neuron number (Schumann and Amaral, 2006), and activity (Baron-Cohen et al., 1999; Schultz, 2005) in adolescents and adults	Reduced P21 nervous system and cellular development and function, and enhanced cellular death and psychological disorder pathways
Gastrointestinal problems (Kuddo and Nelson, 2003)	Enhanced P21 gastrointestinal tract & colorectal cancer genes
Immune alterations (Estes and McAllister, 2015)	Altered P10 & P21 immune function and cancer pathways
Male predominance (Christensen et al., 2016)	Behavioral alterations primarily in males

open field less than saline counterparts. Enhanced baseline acoustic startle responses are correlated with anxiety disorders like PTSD (Grillon, 2002) as well as with ASD in adolescents (Chamberlain et al., 2013).

Notably, behavioral effects were found primarily in male rats, paralleling the male predominance of ASD and neurodevelopmental disorders resulting from prenatal VPA (Bromley et al., 2013; Christensen et al., 2016, 2013). Sex differences have been detected in some (Kataoka et al., 2011; Kim et al., 2013; Raza et al., 2015; Schneider et al., 2008) but not all (Roullet et al., 2010) previous studies of the VPA animal model in which sexes were compared. Impairments in social behavior that are enhanced or exclusive to male VPA-exposed offspring have been reported in rats and mice (Kim et al., 2013; Nordahl et al., 2012; Schneider et al., 2008). Reduced pain sensitivity, enhanced anxiety-like behavior in an elevated plus maze, and increased cortical and hippocampal post-synaptic marker proteins have also been detected exclusively in males (Kim et al., 2013; Schneider et al., 2008). Raza et al. (2015) found that prenatal VPA exposure impaired motor behavior and decreased closed-arm time exclusively in female rats. However, Roullet et al. (2010) reported male and female mice exposed prenatally to VPA were similarly impaired in sociability scores and nest-seeking responses. In some studies, sexes were combined in analyses with no reports of differences (Dufour-Rainfray et al., 2011; Markram et al., 2008) or only male behavioral deficits were examined (Moldrich et al., 2013; Nakasato et al., 2008; Schneider et al., 2007, 2006; Schneider and Przewłocki, 2005). Due to enhanced behavioral deficits observed in males in our sample and previous reports (Kim et al., 2013; Nordahl et al., 2012; Schneider et al., 2008), genomic analyses were run in male amygdala. However, future investigation into potential protective pathways in females that accounts for reduced deficits warrants future investigation.

The amygdala undergoes significant functional, morphological, and physiological maturation during the first two weeks of development, and insults to this region can dramatically alter later socioemotional behavior. By postnatal day 10, the ability to learn fear associations emerges, which

corresponds to changes in the amygdala including the differential responsivity of the amygdala to shock (Sullivan et al., 2000), enhanced synaptic plasticity (Thompson et al., 2008), an emergence of dendritic spines (Ryan et al., 2016) and a switch in the postsynaptic GABA response from excitation to inhibition (Ehrlich et al., 2013). Significantly, learned fear in young rats (P17) is no longer expressed after 10 days, but early maternal separations induce adult fear retention (Callaghan and Richardson, 2012). Prenatal VPA exposure may similarly have led to early maturation of fear responses, which is supported by the observed enhancements in fear expression after infant fear conditioning in our data. A hyperactive, precocious amygdala may lead to an early termination of the attachment-learning period and the beginning of fear learning (Landers and Sullivan, 2012).

Early in development, neonates experience a sensitive period for attachment learning and impaired fear learning, thought in part to be mediated via maternal suppression of corticosterone levels (Moriceau and Sullivan, 2006). VPA-treated males displayed reduced approaches to maternal bedding, increased startle amplitudes in the presence of the maternal odor, and both sexes displayed reduction in distress ultrasonic vocalizations upon separation from the mother. Reduced vocalizations and altered call structures (reduced length, increased frequency) are suggestive of impaired social communication and imply a functional difference in these calls, which may further exacerbate social impairments through development. The number and length of calls increases after an isolated pup is briefly reunited with the mother, known as maternal potentiation (Hofer et al., 1994; Scattoni et al., 2008). Furthermore, anxiolytics and antidepressants reduce call length in neonates during isolation (Hodgson et al., 2008), suggesting the decreased duration and rate of calling in VPA pups may indicate reduced distress from maternal separation. Increased infant call frequencies were also observed in VPA pups, a pattern previously associated with reduced fitness and increased risk for ASD (Esposito et al., 2014; Furlow, 1997).

Olfactory learning is particularly important for attachment during this time period, and the quality of the infant-mother bond and early olfactory learning is a salient predictor of later social relationships (Wilson and Sullivan, 1994). Learned maternal odors serve as safety signals later in life, reducing depressive-like behavior, attenuating fear conditioning, and enhancing social behavior in rats exposed to early life stress (Raineki et al., 2015; Sevelinges et al., 2007). Rather than attenuating, the presence of the maternal odor (acetophenone) enhanced baseline startle responses, while not impacting responses in control animals. It should be noted that female startle amplitudes in response to maternal odor were not measured, and warrant future investigation. The observed alterations in prenatal VPA-treated pup behavior may reflect abnormal amygdala functioning and maternal-infant bonding.

VPA, a nonselective histone deacetylase inhibitor, may transiently increase histone acetylation in the developing embryonic brain when exposed *in utero* (Kataoka et al., 2011), leading to widespread changes in the amygdala transcriptome. This is the first analysis of transcriptomic changes across development in the amygdala in both normative and VPA-exposed conditions. Many pathways involved in development, nervous system function, and the immune system were predicted to be activated in VPA amygdala at P10, whereas at P21 pathways involved in cell death and developmental disorders predominated (**Fig. 2.5A**). Across time, VPA appears to be stunting the normal developmental alterations in amygdala gene expression from P10 to P21 (**Fig. 2.2F**). A number of pathways involved in synaptic plasticity, neurotransmission, cellular growth, immune function, and metabolism are reduced in VPA exposed animals across time relative to saline-treated animals. Conversely, pathways involved in cell death and neurological and developmental disorders are increased across time exclusively in the VPA group. Previous microarray analyses of adult amygdala gene expression from prenatally exposed VPA animals pointed to alterations in similar pathways, such as neuronal projection, cell-cell signaling, synaptic transmission, vesicle, and calcium signaling pathways (Cohen et al., 2013). Moreover, a microarray study from 35 day

old rats exposed to VPA on E12 reported that the amygdala displayed alterations in many similar IPA gene pathways, including cell death, cell signaling, development, proliferation, movement, inflammatory disease, molecular transport, neurological disease, developmental, psychological disorder, and tissue development (Oguchi-Katayama et al., 2013). It should be noted that although changes in gene expression across development within each group survived multiple comparisons, differences between the groups did not, and thus these results should be considered exploratory analyses to identify targets for future investigation. Furthermore, the BLA was specifically targeted at P21, but P10 samples may include surrounding amygdala regions.

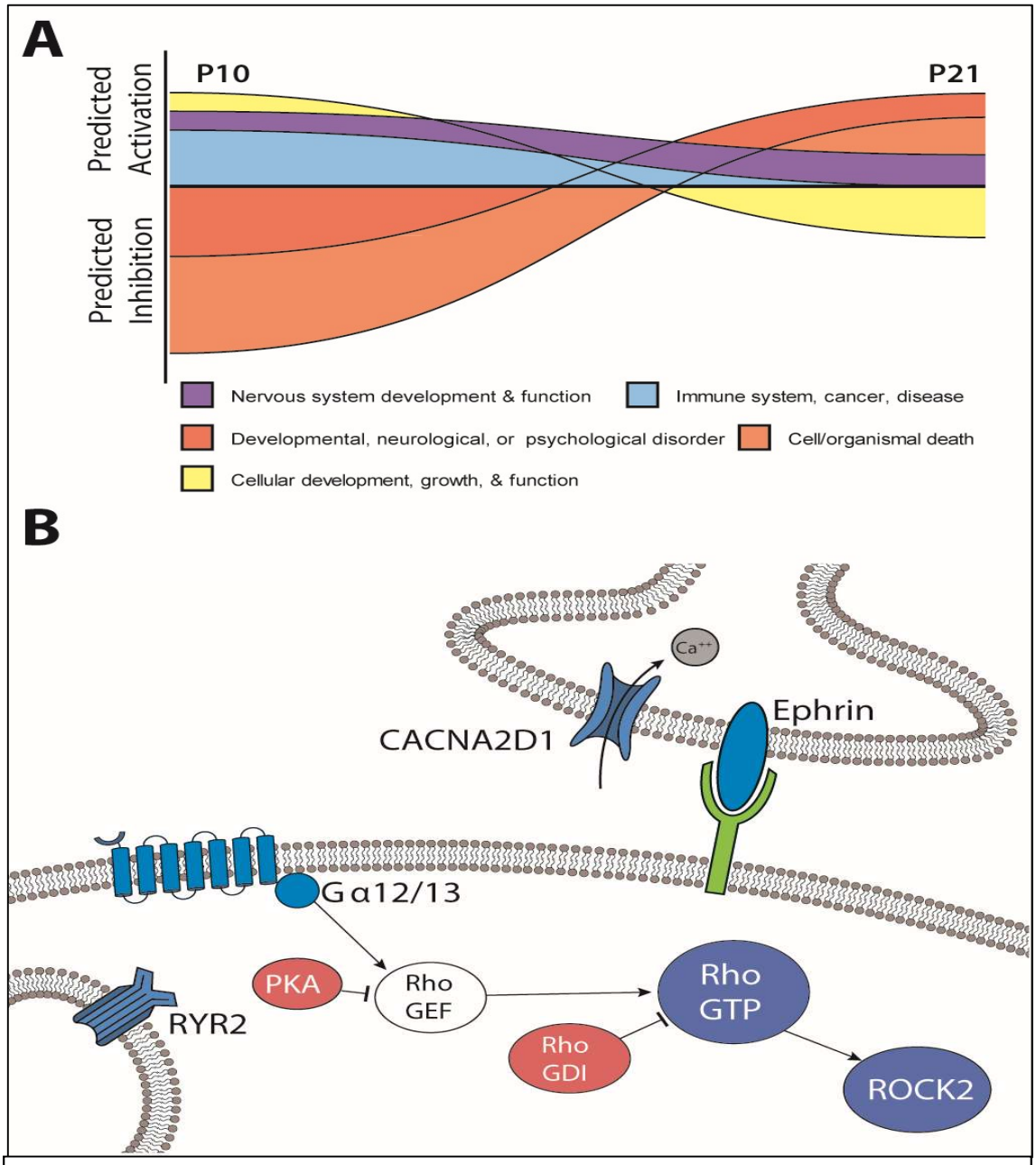

Figure 2.5. Alterations in amygdala genomic profile resulting from prenatal VPA exposure. Pathway changes predicted in animals exposed to VPA prenatally differed across time, with enhancements in developmental and growth pathways predicted early and reductions predicted later in development (**A**). Predicted reductions (blue) or enhancements (red) common to P21 proteomic and transcriptomic analyses were observed in pathways involved in neurotransmission and synaptic plasticity (**B**). G-protein coupled receptor activation of Gα12/13 proteins leads to activation of Rho GTPases by dissociating from the inhibitory RhoGDI, and through the release of the inhibitory PKA from RhoGEF (Siehler, 2009). Reductions in ROCK2, a downstream effector that inhibits dendritic remodeling, may lead to structural changes in dendritic spines in VPA animals. VPA exposed animals displayed impaired ephrin receptor and calcium signaling pathways, with reduced *Ryr2* and *Cacna2d1* expression in both transcriptomic and proteomic analyses. CACNA2D1, voltage-gated calcium channel auxiliary subunit; RYR2, Ryanodine receptor 2; PKA, Protein kinase A; GEF, guanine nucleotide exchange factor; GDI, guanine nucleotide dissociation inhibitor; *ROCK2*, Rho kinase 2.

These data are remarkably in line with transcriptomic analysis of the temporal and frontal cortex showing alterations in genes involved in immune function and synaptic plasticity between individuals with and without ASD: A gene co-expression module involved in synaptic function, vesicular transport, and neuronal projection was downregulated, whereas a module associated with astrocyte markers, activated microglia, immune and inflammatory responses was upregulated in ASD adult samples. The authors also observed an attenuation of gene expression differences between frontal and temporal cortices. Previous microarray-based analyses have revealed similar alterations in developmental or immune pathways in ASD (Birnbaum et al., 2014; Chow et al., 2012; Tebbenkamp et al., 2014). Chow et al. (2012) examined dorsolateral prefrontal cortex gene expression in both young and adult brains of individuals with ASD, and reported alterations in pathways involved in neurogenesis, neurodevelopment, cell cycle, DNA damage response, apoptosis, cytoskeleton remodeling, and the immune response. ASD susceptibility genes involved neurodevelopmental pathways such as neuritogenesis, axogenesis, quantity of axons, and formation of neural tube display prenatal enrichment in the dorsolateral prefrontal cortex (Birnbaum et al., 2014). Transcriptomic analyses have also found enrichment of ASD genes in cortical projection neurons during embryonic development (Parikshak et al., 2013; Willsey et al., 2013), suggesting future analyses of the cortex is warranted at P10, the embryonic equivalent of the third trimester in humans (Romijn et al., 1991). A previous microarray analyses in rats revealed differing effects of VPA on OFC, as compared to anterior amygdala and cerebellar vermis gene expression, which were more similar (Cohen et al., 2013). However, in a study of primate neuro-development using laser-capture microdissection and microarray profiling from early gestation (E50) through the first 48 months, the amygdala had both an earlier onset of genes related to synaptogenesis and myelination, and a quicker downregulation of those same processes compared to basal ganglia, hippocampus and neocortex (Bakken et al., 2016). Genes related to ASD were also enriched in early developing regions like the amygdala, as opposed to genes related to schizophrenia which

saw increased expression post birth. Together these data would suggest that gene disruption in response to fetal VPA exposure will be regulated not only by the process of normative cellular development, but also by the time at which individual components of circuits involved in affective regulation come “on-line”.

At P10, pathways involved in the development, morphology, proliferation and differentiation of cells displayed predicted increases in the amygdala of males exposed to VPA in utero. Increases in pathways involved in neuronal signaling and immune function, and decreases in those involved in developmental disorders were predicted in VPA animals. An early enhancement in cellular differentiation and proliferation is in line with an early overgrowth of the amygdala in ASD (Nordahl et al., 2012; Schumann et al., 2004; Sparks et al., 2002), and may underlie the observed enhancement in fear learning in infants exposed to VPA. Indeed, VPA promotes neurogenesis and cortical neuronal growth in primary cultures (Hao et al., 2004).

A number of pathways were similarly altered across P21 transcriptome and proteome samples (**Fig. 2.4**). Although more immediate effects of VPA in early development may appear beneficial, abnormalities appeared later in development. In juvenile P21 males exposed to VPA prenatally, a number of amygdala gene pathways involved in neurotransmission, cellular development, nervous system development, cell death, and immune function displayed alterations. Both proteomic and transcriptomic analyses of contralateral amygdala from the same animals at P21 revealed reductions in pathways involved in cellular development and proliferation, which is in contrast to the enhancement in these pathways found at P10. Both transcriptomic and proteomic analyses revealed reduced activity of the ephrin receptor signaling pathway, which regulates axonal guidance and cell migration early in development. Over time from P10-21, the ephrin pathway was reduced exclusively in the VPA animals. Knockouts in the ephrin pathway cause ASD-like symptoms (Wurzman et al., 2015). Interestingly, although early premature overgrowth of the amygdala is described in ASD (Nordahl et al., 2012; Schumann et al., 2004; Sparks et al., 2002),

reductions in amygdala volume (Aylward et al., 1999; Nacewicz et al., 2006), neuron number (Schumann and Amaral, 2006), and functional activity (Baron-Cohen et al., 1999; Dalton et al., 2005; Hadjikhani et al., 2007; Wang et al., 2004) are observed later in life.

A predicted reduction in activity of immune function pathways was detected in P21 proteomic and transcriptomic samples, as well as in transcriptomic samples from P10 to 21, in VPA animals, which is in line with previously reported immune alterations in VPA rats (Schneider et al., 2008). Interleukin function has been correlated to social behavior and ASD (Abdallah et al., 2012). Enhanced autoimmunity, allergies, asthma, and reduced immune function have been reported in individuals with ASD (Estes and McAllister, 2015). Enhancements in gastrointestinal tract and colorectal cancer were predicted at P21, which is in line with commonly reported gastrointestinal problems in ASD (Kuddo and Nelson, 2003). Reductions in natural killer cell function (Enstrom et al., 2010), increases in chemokines, and enhancements in pro-inflammatory cytokines have been associated with ASD (Ashwood et al., 2011a, 2011b). As cytokines serve dual functions in both the immune system and in fetal brain development (Goines and Ashwood, 2013), immune regulators may underlie impairments in both.

The calcium signaling pathway was significantly altered in proteomic and transcriptomic analyses, with reduced activity in VPA animals predicted by mRNA. Between P10-21 both saline and VPA amygdala displayed predicted increases in the calcium signaling pathway, however increases were of a greater magnitude in the saline group. Ryanodine receptor 2 (*Ryr2*) and the Calcium Voltage-Gated Channel Auxiliary Subunit α2δ 1 (*Cacna2d1*) were two of the three genes altered in both mRNA and protein analyses; and both displayed reduced expression across analyses. Notably, *RYR2*, which mediates sarcoplasmic calcium release, is a potential ASD risk gene in humans (Lu and Cantor, 2012; Soueid et al., 2016). *RYR2* mRNA is differentially expressed between the frontal and temporal cortices in postmortem tissue from control individuals, but not those with ASD (Voineagu et al., 2011). *Cacna2d1* promotes calcium-induced neurotransmitter exocytosis through

regulating voltage-gated calcium channel trafficking and increased efficiency of exocytosis (Hoppa et al., 2012). Significantly, functional mutations in several genes encoding voltage-gated calcium channels have also been linked to ASD (see Krey and Dolmetsch, 2007). Alteration in calcium signaling, and thus neuronal communication, may contribute to aberrant amygdala functioning and thus of behavior in VPA rats and individuals with ASD.

Pathways altered across proteomic and transcriptomic analyses at both developmental time points were Protein Kinase A (PKA) signaling and signaling by Rho family GTPases. A reduction in PKA signaling at P10 and an increase at P21 in VPA was predicted relative to saline amygdala samples. Overtime, both groups displayed predicted increases in the PKA pathway. PKA signaling regulates emotionality and social anxiety, and dysfunction in this pathway is implicated in ASD (Ji et al., 2011; Kitagishi et al., 2015). Alterations in PKA may underlie changes in synaptic plasticity and activity seen in the amygdala of VPA exposed animals. We have previously shown that PKA signaling in the amygdala is critical for dopamine D1 receptor facilitation of long-term potentiation (Li et al., 2011), enhances membrane potential oscillations in BLA principal neurons (Ryan et al., 2012), and bidirectionally controls synaptic strength (Li and Rainnie, 2014). Membrane potential oscillations enhance spike-timing precision and coordinated firing of BLA principal neurons (Ryan et al., 2012), thus VPA-induced changes in PKA activity may significantly alter functional activity of the amygdala.

An increase in signaling by Rho family GTPases at P10, but a reduction at P21, was predicted in VPA amygdala. Over time, this pathway displayed a predicted increased in saline animals but a decrease in VPA exposed males. RhoGTPases are essential regulators of neuronal motility and morphology, particularly in the development and maturation of dendritic spines, and alterations in these systems have been linked to ASD (Lin et al., 2016). GTP bound Rho activates Rho-kinase 2 (*ROCK2*), which ultimately inhibits neurite outgrowth and promotes cell contraction (for review see Shapiro et al., 2017; Siehler, 2009). Down-regulation of Rho pathways at P21 may contribute

to amygdala hyperplasticity in the VPA model (Lin et al., 2013; Markram et al., 2008). However, knockout of *Rock2* expression reduces dendritic spine density and synaptic transmission (Zhou et al., 2009). Thus, functional outcomes of altered Rho signaling in juvenile animals warrants future investigation. Interestingly, the Rho-Guanine nucleotide dissociation inhibitor (GDI) signaling pathway, which inhibits Rho family GTPases, displayed a corresponding predicted enhancement in activity at P21 in both transcriptomic and proteomic analyses. Additionally, the RhoGDI pathway displayed an exclusive increase in activity in VPA animals.

It should be noted that previous work has found very modest overlap between transcriptomic and proteomic samples, which may be due to posttranscriptional processing such as alternative splicing, modifications, translational efficacy, and degradation (Vogel and Marcotte, 2012). In a comprehensive genomic analysis of mouse liver tissue, an average correlation of only 0.27 was obtained between levels of transcripts and proteins (Ghazalpour et al., 2011). Similarly, an analysis of human brain tissue yielded only 0.25 mRNA-protein correlation (Seyfried et al., 2017). When controlling for neural cell type, a maximum of 0.47 correlation was reached (Sharma et al., 2015). We are also limited in mRNA-protein comparisons in that transcriptomic analyses mapped reads to ~17,000 genes, whereas significantly less proteins were assessed (~3,500). Future targeted quantification of differentially expressed genes proteins, especially ones highlighted in **Figure 2.5B** that overlapped in transcriptomic and proteomic assays, should be performed.

5. Conclusions

These exploratory genomic analyses provide potential targets of interest in the amygdala in the study of the molecular underpinnings of ASD. Furthermore, behavioral and genetic alterations observed support the use of prenatal VPA exposure as an effective tool for the study of pathways underlying social dysfunction relevant to ASD. Future studies should investigate gene expression and physiology of individual BLA neuronal populations throughout development in animals

prenatally exposed to VPA. An understanding of the genetic alterations occurring in BLA neurons could identify potential drug targets and critical windows for treatment interventions for anxiety in children with ASD.

As discussed in **Chapter 1**, evidence for amygdala involvement in many facets of autism is strong, and this research supports that contention. These experiments provide a foundation for understanding the altered neurobiology of the amygdala, suggesting that particular molecular pathways are responsible for the changes observed in social and anxiety-like behaviors. By P21 the expression of genes in the amygdala involved in cellular development, growth and function are reduced, while those implicated in psychiatric disorders and cell death are upregulated. Given the prenatally exposed VPA rat's altered amygdala development, the next logical step is to see if this inheritance predisposes them to respond differentially to stress. We would predict that rats prenatally exposed to VPA, and presumably individuals with autism, would be significantly more vulnerable to anxiety-provoking, stressful events. This is what we assessed in **Chapter 3** of my dissertation: Increased Stress Vulnerability in Rats Exposed to Valproic Acid Prenatally.

6. Supplemental Information

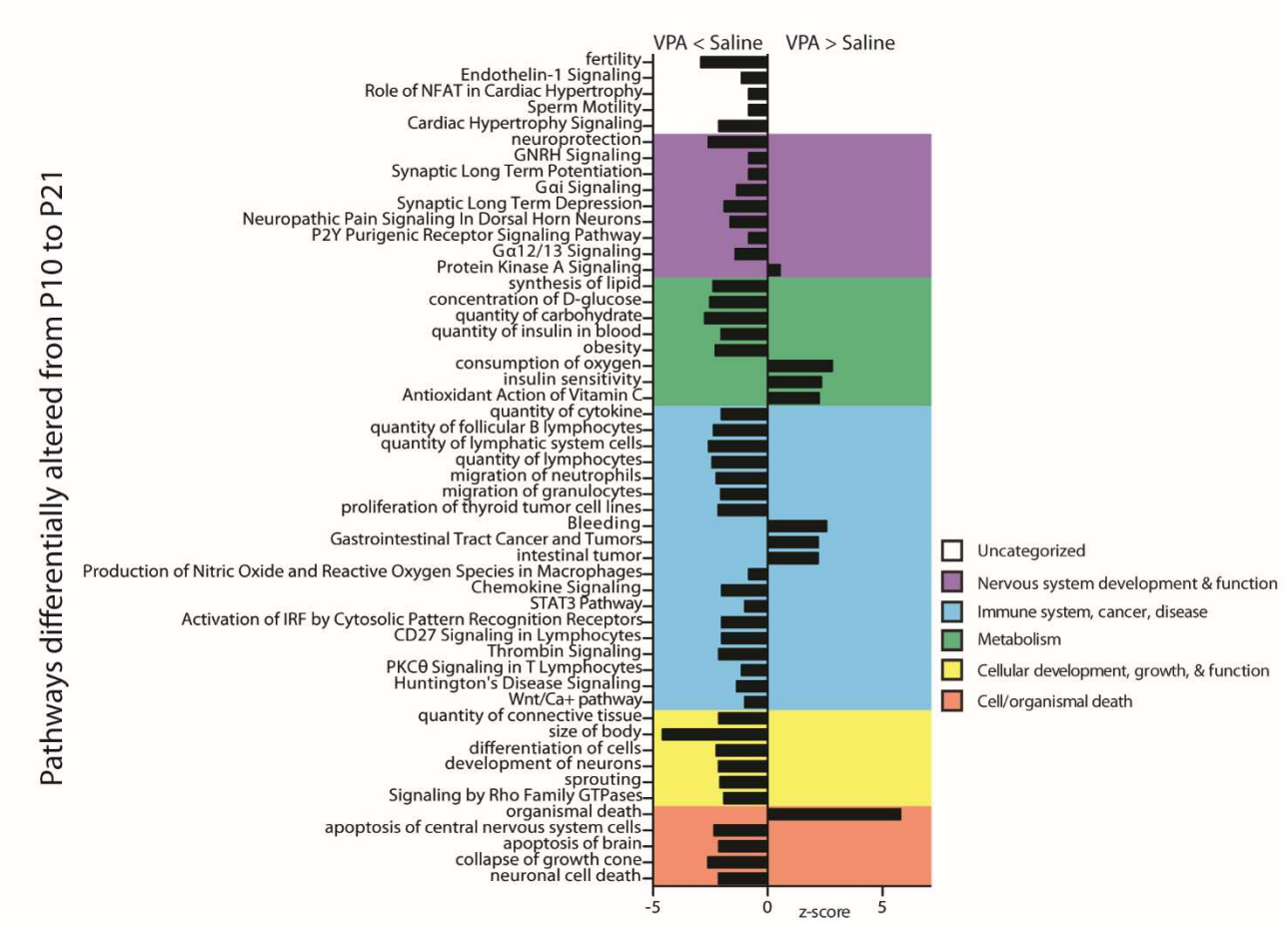

Figure S2.1. RNA sequencing pathways differentially altered across development between VPA and Saline amygdala.

Pathways with significant time (P10-21) by treatment (VPA/Saline) effects are displayed. Treatment effects did not reach statistical significance after FDR multiple comparison correction, thus Ingenuity Pathway Analyses were run on genes with uncorrected $p < 0.05$ treatment effects at P10

(n = 542) and P21 (n = 406). Canonical pathways and diseases and functions categories with predicted activation or inhibition differences were broadly categorized into the following groups: cellular development and growth; nervous system development and function; immune system, cancer, disease; cell/organismal death; metabolism; and developmental, neurological, or psychological disorder.

Table S2.1. Canonical RNA Sequencing Pathways differing from P10-21 in (A) both VPA and Saline amygdala, (B) exclusively in Saline amygdala or (C) exclusively in VPA amygdala.

Ratio represents the number of genes altered between treatments within the total number of genes in that pathway, and the p-value is from a right-tailed Fisher exact test of this ratio. The z-score represents the predicted change in gene regulation of that pathway.

A.

Group	*Comparison*	*Ingenuity Canonical Pathways*	*-log(p-value)*	*Ratio*	*z-score*	*Molecules*
VPA	P10-21	14-3-3-mediated Signaling	2.57	0.144		RAF1,TUBB3,MAPK1,TUBB2A,MAPK8,TUBB,PRKCZ,TUBB2B,SRPK2,PLCB4,TUBA1A,PLCE1,TUBB6,KL,MAPT,PLCB1,TUBA1C,TUBB4A,MAP2K1
Saline	P10-21		4.03	0.159		TUBB3,MAPK1,PDIA3,TUBB2A,TUBA4A,BAX,TUBB,PRKCZ,TUBB2B,PRKCG,SRPK2,PLCB4,TUBA1A,TUBB6,MAPT,MAPK10,PLCB1,TUBA1C,TUBB4A,PDCD6IP,MAP2K1
VPA	P10-21	Aldosterone Signaling in Epithelial Cells	1.57	0.114	0.277	RAF1,CRYAB,MAPK1,DNAJB4,HSPH1,DNAJC27,PIP4K2B,ITPR1,PRKCZ,PLCB4,PLCE1,KL,ASIC1,PLCB1,NR3C2,MAP2K1,PIP4K2C,DNAJB5,HSPB6,HSPA4L
Saline	P10-21		2.37	0.119	2.714	CRYAB,MAPK1,DNAJC9,PDIA3,DNAJB4,HSPH1,DNAJC27,PDPK1,ITPR1,PRKCZ,PRKCG,PLCB4,PIP5K1A,DUSP1,PLCB1,MAP2K1,DNAJC16,PIP4K2C,DNAJB5,HSPB6,HSPA4L
VPA	P10-21	Amyotrophic Lateral Sclerosis Signaling	4.42	0.183		PRPH,CAPN5,GRIN2A,CAPN6,CASP3,GRIN2D,GRIA2,APAF1,SOD1,GRIN3A,GRINA,BCL2L1,GRIK5,IGF1,KL,GLUL,SLC1A2,CAPN2,CASP7,PPP3CA,GRIA3,GRIK1
Saline	P10-21		2.39	0.133		GRIN2A,CAPN6,CASP3,GRIN2D,APAF1,BAX,GRINA,BCL2L1,IGF1,GRIK4,GLUL,SLC1A2,CASP7,PPP3CA,GRIA3,GRIK1
VPA	P10-21	Atherosclerosis Signaling	2.01	0.133		PLA2G16,ALOX15,PDGFA,COL2A1,F3,ALOXE3,SELPLG,IL33,ALB,LYZ,ALOX15B,IL1RN,ALOX5,CLU,RBP4,APOD,PAFAH1B3
Saline	P10-21		1.78	0.117		PLA2G16,COL2A1,CXCL12,F3,ALOXE3,SELPLG,PLA2G6,ALB,LYZ,IL1RN,COL11A2,ALOX5,RBP4,APOD,PAFAH1B3
VPA	P10-21	Axonal Guidance Signaling	6.84	0.141		DPYSL2,RAF1,RAC2,MAPK1,Wasl,GNB5,TUBB,LIMK1,ROCK2,PTK2,SEMA6D,PLCE1,ECE2,PPP3R1,ABLIM3,BAIAP2,WNT4,PLCB1,ABLIM2,TUBA1C,EFNB3,TUBB3,NGEF,PAPPA,STK36,SEMA5A,TUBB2A,PTCH1,L1CAM,HHIP,DPYSL5,ADAMTS9,HERC2,NRP1,ADAMTS7,PLXNA3,SLIT1,PDGFA,ARHGEF7,SEMA6C,PRKCZ,TUBB2B,IGF1,KL,SRGAP1,MKNK1,TUBB4A,SEMA3B,MAP2K1,PPP3CA,ITGB1,C9orf3,ROCK1,GNAI3,PLCB4,TUBA1A,TUBB6,GLIS2,PAK3,EPHA5,SEMA4G,EPHB3,SEMA3C,GLI1

Saline	P10-21		2.64	0.0991		SLIT3,ADAMTS7,PLXNA3,ECEL1,MAPK1,PDIA3,Wasl,CXCL12,GNB5,GNG13,TUBB,SEMA6C,PRKCZ,TUBB2B,ROCK2,PTK2,IGF1,PPP3R1,BAIAP2,ABLIM3,PRKAR1B,UNC5D,PLCB1,ABLIM2,TUBA1C,TUBB4A,SEMA3B,EFNB3,MAP2K1,PPP3CA,TUBB3,CRKL,C9orf3,TUBB2A,TUBA4A,L1CAM,HHIP,DPYSL5,PRKCG,GNAI3,PLCB4,SEMA4D,TUBA1A,TUBB6,SEMA3C
VPA	P10-21	Breast Cancer Regulation by Stathmin1	8.03	0.191		RAF1,CAMK4,MAPK1,ARHGEF7,GNB5,PPP1CB,TUBB,PPP1R14B,PRKCZ,LIMK1,TUBB2B,STMN1,ROCK2,CAMK2A,ADCY5,PPP1R7,KL,PLCB1,TUBB4A,TUBA1C,PPP2R2C,ARHGEF3,ADCY8,MAP2K1,PPP1R14C,TUBB3,TUBB2A,PPP1R11,ITPR1,CDK1,ROCK1,GNAI3,PLCB4,TUBA1A,TUBB6,PPP2R3A,ADCY10,ARHGEF9,CDK2,CAMK2G
Saline	P10-21		7.84	0.177		CAMK4,MAPK1,GNB5,GNG13,TUBB,PPP1R14B,PRKCZ,TUBB2B,ROCK2,STMN1,CAMK2A,ADCY5,PRKAR1B,PLCB1,TUBB4A,PPP2R2C,TUBA1C,ARHGEF3,ADCY8,MAP2K1,PPP1R14C,TUBB3,TUBB2A,TUBA4A,PPP1R14A,PPP1R11,ITPR1,CDK1,PRKCG,GNAI3,ADCY9,PLCB4,TUBA1A,TUBB6,PPP2R3A,ARHGEF9,CDK2
VPA	P10-21	Calcium Signaling	3.84	0.151	0.426	GRIN2A,CAMK4,MAPK1,GRIN2D,CHRNB1,Tpm1,TRPC3,GRINA,CAMK2A,HDAC11,PPP3R1,CHRNA3,PPP3CA,GRIK1,AKAP5,CHRNA4,TNNC2,SLC8A3,GRIA2,ITPR1,TRPC7,GRIN3A,MICU1,CAMKK1,MYH3,SLC8A1,CAMK2G,GRIA3
Saline	P10-21		1.81	0.108	2.828	AKAP5,GRIN2A,CAMK4,MAPK1,TNNC2,GRIN2D,RYR2,ITPR1,GRINA,TRPC7,MICU1,CAMK2A,PPP3R1,MEF2D,CAMKK1,PRKAR1B,PPP3CA,CAMKK2,GRIK1,GRIA3
VPA	P10-21	cAMP-mediated signaling	6.65	0.173	2.744	AKAP12,ADRA2B,GPR17,RAF1,CAMK4,AKAP8,HTR4,MAPK1,PDE1A,HRH3,CAMK2A,DRD1,ADCY5,PPP3R1,SMPDL3B,RGS14,PKIA,ADCY8,MAP2K1,HCAR2,PPP3CA,HTR6,AKAP5,RGS2,NPY1R,MC4R,PDE1C,GNAI3,GABBR2,LPAR1,CREM,PDE1B,S1PR1,PDE8B,HTR1F,ADRA2C,ADCY10,ADORA2A,CAMK2G
Saline	P10-21		3.97	0.133	2.041	AKAP12,CAMK4,AKAP8,MAPK1,CHRM4,PDE1A,HRH3,AKAP11,CAMK2A,ADCY5,PPP3R1,HTR7,PRKAR1B,PKIA,ADCY8,MAP2K1,PPP3CA,ADRB2,AKAP5,PDE2A,RGS4,CNGA1,GNAI3,ADCY9,TULP2,LPAR1,DUSP1,CREM,PDE1B,S1PR1
VPA	P10-21	Cardiac Hypertrophy Signaling	1.35	0.102	0.408	ADRA2B,MAP3K9,RAF1,CAMK4,MAPK1,MAPK8,GNB5,ROCK2,ROCK1,MAP3K10,GNAI3,PLCB4,PLCE1,RHOG,IGF1,RND3,KL,ADCY5,PPP3R1,PLCB1,ADRA2C,ADCY8,ADCY10,MAP2K1,PPP3CA
Saline	P10-21		1.4	0.0943	2.837	CAMK4,MAPK1,PDIA3,GNB5,GNG13,ATF6,ROCK2,ADCY9,GNAI3,PLCB4,RHOG,IGF1,RND3,ADCY5,PPP3R1,MEF2D,MAPK10,PRKAR1B,PLCB1,ADCY8,MAP2K1,PPP3CA,ADRB2
VPA	P10-21	Cardiac β-adrenergic Signaling	3.28	0.154	1.5	AKAP12,AKAP5,PPP1R14C,AKAP8,GNB5,SLC8A3,PPP1CB,PPP1R11,PDE1A,PPP1R14B,PDE1C,PPP1R7,ADCY5,PPP2R3A,PDE1B,PPP2R2C,PDE8B,SMPDL3B,PKIA,ADCY8,SLC8A1,ADCY10
Saline	P10-21		3.97	0.154	1.807	AKAP12,AKAP5,PPP1R14C,PDE2A,AKAP8,RYR2,GNB5,GNG13,PPP1R14A,PPP1R11,PDE1A,PPP1R14B,AKAP11,ADCY9,TULP2,ADCY5,PPP2R3A,PDE1B,PRKAR1B,PPP2R2C,PKIA,ADCY8
VPA	P10-21	CDK5 Signaling	5.52	0.214	0.655	ITGB1,RAF1,LAMA5,PPP1R14C,MAPK1,PPP1R1B,EGR1,MAPK6,MAPK8,PPP1CB,PPP1R11,PPP1R14B,DRD1,PPP1R7,ADCY5,PPP2R3A,MAPT,LAMA1,PPP2R2C,ADCY8,ADCY10,MAP2K1
Saline	P10-21		4.07	0.175	0.471	PPP1R14C,MAPK1,MAPK4,EGR1,MAPK6,PPP1R14A,PPP1R11,PPP1R14B,ADCY9,PPP2R3A,ADCY5,MAPT,PRKAR1B,MAPK10,LAMA1,PPP2R2C,ADCY8,MAP2K1
VPA	P10-21	Cell Cycle Control of Chromosomal Replication	5.09	0.316		MCM5,MCM3,TOP2B,LIG1,MCM6,MCM2,POLE,CDC6,ORC5,TOP2A,POLA2,CDK2

Saline	P10-21		5.6	0.316		MCM5,MCM3,TOP2B,LIG1,MCM6,MCM2,POLE,ORC5,ORC6,CDK4,TOP2A,CDK2
VPA	P10-21	Cell Cycle: G2/M DNA Damage Checkpoint Regulation	3.25	0.224	1.897	TOP2B,CDC25C,TOP2A,CCNB2,PKMYT1,RPRM,AURKA,CDK1,PRKCZ,SKP2,CCNB1
Saline	P10-21		3.67	0.224	2.53	TOP2B,CDC25C,WEE1,TOP2A,CCNB2,PLK1,CDK1,PRKCZ,SKP2,CHEK1,CCNB1
VPA	P10-21	Cellular Effects of Sildenafil (Viagra)	1.86	0.126		CACNG1,CAMK4,PPP1CB,ITPR1,PRKG2,PDE1A,PDE1C,CACNG2,PLCB4,PLCE1,GPR37,ADCY5,PDE1B,MYH3,PLCB1,ADCY10,ADCY8,SLC4A10
Saline	P10-21		1.4	0.105		PDE2A,CAMK4,PDIA3,SLC4A11,ITPR1,PDE1A,ADCY9,PLCB4,GPR37,ADCY5,PDE1B,PRKAR1B,PLCB1,ADCY8,SLC4A10
VPA	P10-21	Chemokine Signaling	3.63	0.2	0.258	RAF1,CAMK4,PTK2B,MAPK1,MAPK8,PPP1CB,LIMK1,ROCK2,PTK2,GNAI3,PLCB4,CAMK2A,PLCB1,MAP2K1,CAMK2G
Saline	P10-21		2.11	0.147	2.111	ROCK2,PTK2,GNAI3,PLCB4,CAMK4,CAMK2A,MAPK1,PTK2B,CXCL12,PLCB1,MAP2K1
VPA	P10-21	Cholecystokinin/Gastrin-mediated Signaling	2.86	0.16		RAF1,PTK2B,MAPK1,MAPK8,ITPR1,PRKCZ,IL33,ROCK2,PTK2,ROCK1,PLCB4,RHOG,RND3,IL1RN,CREM,PLCB1,MAP2K1
Saline	P10-21		3.42	0.16		PTK2B,MAPK1,ITPR1,PRKCZ,PRKCG,ROCK2,PTK2,PLCB4,RHOG,RND3,IL1RN,MEF2D,CREM,MAPK10,PLCB1,PTGS2,MAP2K1
VPA	P10-21	CREB Signaling in Neurons	1.98	0.12	0.426	RAF1,GRIN2A,CAMK4,MAPK1,GRIN2D,GRIA2,GNB5,ITPR1,PRKCZ,GNAI3,PLCB4,PLCE1,GRIK5,CAMK2A,KL,ADCY5,PLCB1,ADCY8,ADCY10,MAP2K1,GRIA3,GRIK1,CAMK2G
Saline	P10-21		2.57	0.12	1.789	GRIN2A,POLR2D,CAMK4,MAPK1,PDIA3,GRIN2D,GNB5,GNG13,ITPR1,PRKCZ,PRKCG,GNAI3,ADCY9,PLCB4,CAMK2A,GRIK4,ADCY5,PRKAR1B,PLCB1,ADCY8,MAP2K1,GRIA3,GRIK1
VPA	P10-21	CXCR4 Signaling	2.21	0.127	0	RAF1,MAPK1,EGR1,MAPK8,GNB5,ITPR1,PRKCZ,ROCK2,PTK2,ROCK1,GNAI3,PLCB4,RHOG,RND3,PAK3,KL,ADCY5,PLCB1,ADCY8,ADCY10,ELMO2,MAP2K1
Saline	P10-21		2.46	0.121	1.886	MAPK1,EGR1,GNB5,CXCL12,GNG13,ITPR1,PRKCZ,PRKCG,ROCK2,PTK2,ADCY9,GNAI3,PLCB4,RHOG,RND3,ADCY5,MAPK10,PLCB1,ADCY8,ELMO2,MAP2K1
VPA	P10-21	Cyclins and Cell Cycle Regulation	2.93	0.179	-1.897	RAF1,SUV39H1,CCNB2,CCND1,CDK1,SKP2,CCNB1,CCNA2,MYT1,PPP2R3A,HDAC11,PPP2R2C,CDK2,CDC25A
Saline	P10-21		1.98	0.141	-1.667	MYT1,PPP2R3A,WEE1,SUV39H1,CDK4,CCNB2,PPP2R2C,CDK1,CDK2,SKP2,CCNB1
VPA	P10-21	D-myo-inositol-5-phosphate Metabolism	2.42	0.135		CDC25C,PPP1R1B,PIP4K2B,PAWR,PPP4R1,PPM1K,PPP1R14B,DUSP14,DUSP2,PLCH1,ATP1A1,PLCB4,PLCE1,PPP1R7,PPP2R3A,PLCB1,THTPA,PIP4K2C,PPP3CA,CDC25A,NUDT1
Saline	P10-21		3.41	0.141		CDC25C,PPFIBP2,PPP1R14A,PPP4R1,PPM1K,PPP1R14B,DUSP14,DUSP2,PLCH1,SET,ATP1A1,PLCB4,SYNJ1,DUSP1,PPP2R3A,PTPRO,PLCB1,PTPRN,THTPA,PIP4K2C,PPP3CA,NUDT1
VPA	P10-21	DNA damage-induced 14-3-3σ Signaling	2.03	0.263		CCNB2,CDK1,CDK2,CCNB1,RAD1
Saline	P10-21		1.53	0.211		CCNB2,CDK1,CDK2,CCNB1
VPA	P10-21	Dopamine Receptor Signaling	1.9	0.143	0.632	PPP1R14C,PPP1R1B,PPP1CB,PPP1R11,PPP1R14B,DRD1,PPP2R3A,PPP1R7,ADCY5,SLC18A1,PPP2R2C,ADCY10,ADCY8
Saline	P10-21		1.52	0.121	0.707	PPP1R14C,ADCY9,ADCY5,PPP2R3A,PRKAR1B,PPP1R14A,SLC18A1,PPP1R11,PPP2R2C,ADCY8,PPP1R14B
VPA	P10-21	Dopamine-DARPP32 Feedback in cAMP Signaling	5.12	0.17	1.279	GRIN2A,CAMK4,KCNJ2,GRIN2D,PPP1CB,PRKG2,PPP1R14B,PRKCZ,GRINA,PLCE1,DRD1,PPP1R7,ADCY5,PPP3R1,PLCB1,PPP2R2C,ADCY8,PPP3CA,PPP1R14C,PPP1R1B,PPP1R11,PAWR,ITPR1,GRIN3A,GNAI3,PLCB4,PPP2R3A,CREM,CAMKK1,ADCY10

Saline	P10-21		5.63	0.165	1.877	GRIN2A,CAMK4,PDIA3,GRIN2D,PPP1R14B,PRKCZ,GRINA,KCNJ11,ADCY5,PPP3R1,PRKAR1B,PLCB1,PPP2R2C,ADCY8,PPP3CA,PPP1R14C,PPP1R14A,PPP1R11,ITPR1,PRKCG,GNAI3,ADCY9,PLCB4,KCNJ10,PPP2R3A,KCNJ9,CAMKK1,CREM,CAMKK2
VPA	P10-21	Endothelin-1 Signaling	1.49	0.109	-1.528	PLA2G16,RAF1,MAPK1,EDNRB,CASP3,MAPK6,MAPK8,ITPR1,PRKCZ,MYC,GNAI3,PLCB4,PLCE1,EDN1,KL,ECE2,ADCY5,PLCB1,ADCY10,ADCY8,CASP7,PAFAH1B3
Saline	P10-21		2.29	0.114	0.626	PLA2G16,MAPK1,EDNRB,CASP3,MAPK4,PDIA3,MAPK6,ITPR1,PRKCZ,PRKCG,MYC,GNAI3,PLA2G6,ADCY9,PLCB4,EDN1,ADCY5,MAPK10,PLCB1,PTGS2,ADCY8,CASP7,PAFAH1B3
VPA	P10-21	Ephrin B Signaling	2.17	0.16	-1.265	ROCK2,PTK2,ROCK1,VAV2,GNAI3,RAC2,MAPK1,GNB5,CAP1,EPHB3,EFNB3,LIMK1
Saline	P10-21		1.69	0.133	0	ROCK2,PTK2,VAV2,GNAI3,MAPK1,GNB5,CXCL12,CAP1,GNG13,EFNB3
VPA	P10-21	Epithelial Adherens Junction Signaling	3.07	0.149		VAV2,TUBB3,NOTCH3,LMO7,ACTN2,TUBB2A,AFDN,TCF7L1,TUBB,APC,TUBB2B,CTNNA2,MAGI1,TUBA1A,TUBB6,BAIAP2,MYH3,TUBA1C,TUBB4A,NOTCH1,ACVR1C,ACTN1
Saline	P10-21		1.57	0.108		VAV2,TUBB3,ACTN2,TUBB2A,TUBA4A,CTNNA1,TUBB,TUBB2B,CTNNA2,TUBA1A,MAGI1,TUBB6,BAIAP2,TUBB4A,TUBA1C,ACVR1C
VPA	P10-21	ERK/MAPK Signaling	1.47	0.108	-0.426	ITGB1,MYCN,PPP1R14C,RAF1,RAC2,PTK2B,MAPK1,PPP1CB,PPP1R11,MKNK2,PPP1R14B,DUSP2,PTK2,MYC,PAK3,PPP1R7,KL,ETS2,PPP2R3A,MKNK1,PPP2R2C,MAP2K1
Saline	P10-21		1.45	0.0985	0.894	MYCN,PPP1R14C,PTK2B,MAPK1,CRKL,PPP1R14A,PPP1R11,PPP1R14B,DUSP2,PRKCG,MYC,PTK2,PLA2G6,H3F3A/H3F3B,DUSP1,ETS2,PPP2R3A,PRKAR1B,PPP2R2C,MAP2K1
VPA	P10-21	Estrogen-mediated S-phase Entry	2.96	0.292	-2.646	MYC,CCNA2,CCND1,CDK1,CDK2,SKP2,CDC25A
Saline	P10-21		1.79	0.208	-2.236	MYC,CDK4,CDK1,CDK2,SKP2
VPA	P10-21	Fatty Acid Activation	2.8	0.316		ACSL3,SLC27A5,SLC27A2,ACSL6,ACSL4,SLC27A3
Saline	P10-21		1.53	0.211		ACSL3,SLC27A5,SLC27A2,ACSL6
VPA	P10-21	G-Protein Coupled Receptor Signaling	4.04	0.137		ADRA2B,RAF1,GPR17,CAMK4,HTR4,MAPK1,PTK2B,NFKBIE,PDE1A,HRH3,CAMK2A,DRD1,ADCY5,KL,PLCB1,SMPDL3B,RGS14,ADCY8,MAP2K1,HCAR2,HTR6,RGS2,NPY1R,MC4R,PDE1C,GNAI3,GABBR2,PLCB4,LPAR1,RASGRP1,PDE1B,S1PR1,PDE8B,HTR1F,ADRA2C,ADCY10,ADORA2A,CAMK2G
Saline	P10-21		2.19	0.104		CAMK4,MAPK1,PTK2B,CHRM4,PDPK1,PDE1A,HRH3,AVPR1A,CAMK2A,ADCY5,HTR7,PRKAR1B,PLCB1,ADCY8,MAP2K1,ADRB2,PDE2A,RGS4,PRKCG,GNAI3,ADCY9,PLCB4,TULP2,LPAR1,DUSP1,RASGRP1,PDE1B,S1PR1,CALCR
VPA	P10-21	GABA Receptor Signaling	1.84	0.151		SLC6A11,GABBR2,GABRR2,GPR37,GABRA4,UBQLN1,ADCY5,GAD1,ADCY8,GABRD,ADCY10

Saline	P10-21		2.2	0.151		SLC6A11,NSF,ADCY9,GPR37,GABRA4,ADCY5,AP2B1,ADCY8,GABRD,GABRA1,AP2A2
VPA	P10-21	GADD45 Signaling	1.37	0.211		CCND1,CDK1,CDK2,CCNB1
Saline	P10-21		1.53	0.211		CDK4,CDK1,CDK2,CCNB1
VPA	P10-21	Gap Junction Signaling	4.04	0.155		RAF1,MAPK1,PRKG2,TUBB,PRKCZ,TUBB2B,PLCE1,SP1,DRD1,ADCY5,KL,PPP3R1,PLCB1,TUBA1C,TUBB4A,ADCY8,MAP2K1,PPP3CA,TUBB3,TUBB2A,ITPR1,GNAI3,PLCB4,TUBA1A,LPAR1,TUBB6,SGSM3,ADCY10
Saline	P10-21		4.06	0.144		TUBB3,MAPK1,PDIA3,TUBB2A,TUBA4A,ITPR1,TUBB,PRKCZ,TUBB2B,PRKCG,ADCY9,GNAI3,PLCB4,TUBA1A,LPAR1,TUBB6,ADCY5,PPP3R1,SGSM3,PRKAR1B,PLCB1,TUBA1C,TUBB4A,ADCY8,MAP2K1,PPP3CA
VPA	P10-21	Germ Cell-Sertoli Cell Junction Signaling	3.47	0.148		RAC2,MAPK1,AFDN,TUBB,TUBB2B,LIMK1,PTK2,MAP3K10,CTNNA2,RHOG,KL,TUBA1C,TUBB4A,MAP2K1,ACTN1,ITGB1,MAP3K9,TUBB3,ACTN2,TUBB2A,MAPK8,GSN,TUBA1A,RND3,TUBB6,PAK3
Saline	P10-21		2.05	0.114		TUBB3,MAPK1,ACTN2,TUBB2A,TUBA4A,CTNNA1,PDPK1,TUBB,GSN,TUBB2B,PTK2,CTNNA2,RHOG,TUBA1A,TUBB6,RND3,MAPK10,TUBA1C,TUBB4A,MAP2K1
VPA	P10-21	Glutamate Receptor Signaling	5.1	0.25	0	GRIN2A,CAMK4,SLC17A6,GRIN2D,GRIA2,SLC1A3,GRIP1,GRIN3A,GRINA,GRIK5,PICK1,SLC1A2,GLUL,HOMER1,GRIK1,GRIA3
Saline	P10-21		3.77	0.203	-0.378	SLC1A6,GRIN2A,CAMK4,GRIN2D,SLC1A3,GRIP1,GRINA,GRIK4,GLUL,SLC1A2,HOMER1,GRIK1,GRIA3
VPA	P10-21	Glycolysis I	2.11	0.195		PKLR,PGAM1,PKM,Tpi1 (includes others),PFKM,ALDOC,FBP2,NAD+
Saline	P10-21		3.71	0.244		PGK1,GPI,PKLR,PGAM1,PKM,ALDOA,PFKL,Tpi1 (includes others),PFKM,ALDOC
VPA	P10-21	GNRH Signaling	2.46	0.141	0.229	RAF1,MAP3K9,MAPK1,EGR1,MAPK8,ITPR1,PRKCZ,PTK2,MAP3K10,GNAI3,PLCB4,CAMK2A,PAK3,ADCY5,PLCB1,ADCY10,ADCY8,MAP2K1,CAMK2G
Saline	P10-21		1.91	0.119	2.5	MAPK1,EGR1,ITPR1,PRKCZ,PRKCG,PTK2,ADCY9,GNAI3,PLCB4,CAMK2A,ADCY5,PRKAR1B,MAPK10,PLCB1,ADCY8,MAP2K1
VPA	P10-21	Gα12/13 Signaling	2.25	0.137	0.243	VAV2,RAF1,PTK2B,MAPK1,F2R,NFKBIE,MAPK8,CDH19,ROCK2,PTK2,ROCK1,TEC,LPAR1,LPAR2,KL,CDH8,MAP2K1,CDH13
Saline	P10-21		1.4	0.107	1.604	PTK2,ROCK2,VAV2,F2RL2,CDH9,LPAR2,LPAR1,PTK2B,MAPK1,CDH3,MEF2D,MAPK10,MAP2K1,CDH19
VPA	P10-21	Gαq Signaling	1.76	0.119	0.943	RAF1,RGS2,CAMK4,PTK2B,MAPK1,NFKBIE,CSK,GNB5,ITPR1,PRKCZ,ROCK2,ROCK1,PLCB4,RHOG,RND3,KL,PPP3R1,PLCB1,MAP2K1,PPP3CA
Saline	P10-21		2.27	0.119	1.886	CAMK4,PTK2B,MAPK1,GNB5,GNG13,RGS4,ITPR1,AVPR1A,PRKCZ,PRKCG,ROCK2,PLCB4,RHOG,RND3,PPP3R1,CALCR,PLCB1,ARHGEF25,MAP2K1,PPP3CA
VPA	P10-21	Gαs Signaling	1.83	0.133	1.604	HTR6,RGS2,ADD2,MAPK1,HTR4,GNB5,MC4R,DRD1,ADCY5,ADD1,ADCY8,ADCY10,ADORA2A,MAP2K1,HCAR2
Saline	P10-21		1.89	0.124	0.905	ADD2,MAPK1,RYR2,GNB5,GNG13,CNGA1,ADCY9,ADCY5,HTR7,ADD1,PRKAR1B,ADCY8,MAP2K1,ADRB2
VPA	P10-21	Huntington's Disease Signaling	1.97	0.113	-1.964	MAPK1,GNB5,PRKCZ,MAP3K10,ARFIP2,IGF1,SP1,KL,HDAC11,PLCB1,NAPA,CAPN5,ATP5J,CAPN6,CASP3,MAPK8,APAF1,ITPR1,HIP1,SNAP25,BCL2L1,PLCB4,PSME1,CACNA1B,PENK,STX16,CAPN2,CASP7
Saline	P10-21		2.07	0.105	-1	POLR2D,MAPK1,PACSIN1,GNB5,PDPK1,GNG13,AP2A2,PRKCZ,NSF,IGF1,PLCB1,NAPA,CAPN6,CASP3,PSME2,APAF1,BAX,ITPR1,SNAP25,RPH3A,PRKCG,BCL2L1,PLCB4,PSME1,STX16,CASP7
VPA	P10-21	Leukocyte Extravasation Signaling	3.1	0.134	0.426	RAC2,MAPK1,PTK2B,MMP14,Wasl,MMP15,AFDN,PRKCZ,ROCK2,PTK2,CTNNA2,KL,TIMP1,ARHGAP12,CLDN9,ACTN1,VAV2,ITGB1,CLDN10,ACTN2,MAPK8,THY1,SELPLG,TEC,ROCK1,GNAI3,JAM3,RASGRP1,ARHGAP35

Saline	P10-21		1.44	0.0972	2.183	VAV2,CLDN10,PTK2B,MAPK1,Wasl,CRKL,ACTN2,CTNNA1,CXCL12,THY1,PRKCZ,SELPLG,PRKCG,PTK2,ROCK2,GNAI3,CTNNA2,TIMP1,RASGRP1,MAPK10,CLDN3
VPA	P10-21	LPS/IL-1 Mediated Inhibition of RXR Function	1.95	0.115	0.816	ECSIT,NDST3,CYP3A7,SLC27A2,CPT1B,SLC27A5,ALDH1A1,ACSL4,FABP5,FABP7,HS3ST1,HMGCS1,IL1RAP,TNFRSF11B,GSTA3,GSTA2,ABCB1,ACSL3,ACSL6,MAPK8,Sult1d1,IL33,CHST1,IL1RN,ALDH3B1,SLC27A3
Saline	P10-21		1.49	0.0973	0.447	ECSIT,GSTA3,CYP3A7,ABCB1,NDST3,GSTA2,ACSL3,SLC27A2,ACSL6,Sult1d1,CHST1,SLC27A5,ALDH1A1,SCARB1,ALDH1L2,IL1RN,FABP7,ALDH3B1,FMO1,FABP5,CPT1C,TNFRSF11B
VPA	P10-21	Melatonin Signaling	1.68	0.143	0.632	GNAI3,RAF1,PLCB4,CAMK4,PLCE1,CAMK2A,MAPK1,PLCB1,MAP2K1,PRKCZ,CAMK2G
Saline	P10-21		2.02	0.143	1.508	GNAI3,PLCB4,CAMK4,CAMK2A,MAPK1,PDIA3,PRKAR1B,PLCB1,MAP2K1,PRKCZ,PRKCG
VPA	P10-21	Mitochondrial L-carnitine Shuttle Pathway	3.35	0.333		ACSL3,SLC27A5,SLC27A2,CPT1B,ACSL6,ACSL4,SLC27A3
Saline	P10-21		2.04	0.238		ACSL3,SLC27A5,SLC27A2,ACSL6,CPT1C
VPA	P10-21	Mitotic Roles of Polo-Like Kinase	7.74	0.303	-2	FZR1,KIF23,CDC25C,ESPL1,CDC20,PLK3,PTTG1,PRC1,CCNB2,ANAPC1,CDK1,CCNB1,PPP2R3A,PLK2,PKMYT1,FBXO5,PPP2R2C,CDC16,KIF11,CDC25A
Saline	P10-21		5.54	0.242	-1.941	KIF23,CDC25C,CDC20,PLK3,WEE1,PTTG1,PRC1,CCNB2,PLK1,CDK1,CCNB1,PPP2R3A,PLK2,FBXO5,PPP2R2C,KIF11
VPA	P10-21	Neuropathic Pain Signaling In Dorsal Horn Neurons	2.36	0.144	1.213	GRIN2A,CAMK4,MAPK1,GRIN2D,GRIA2,ITPR1,PRKCZ,GRINA,GRIN3A,PLCB4,CAMK2A,PLCE1,GPR37,KL,PLCB1,CAMK2G,GRIA3
Saline	P10-21		2.09	0.127	2.84	GRIN2A,CAMK4,MAPK1,PDIA3,GRIN2D,ITPR1,PRKCZ,GRINA,PRKCG,PLCB4,CAMK2A,GPR37,PRKAR1B,PLCB1,GRIA3
VPA	P10-21	nNOS Signaling in Neurons	4.94	0.269	1.667	CAPN5,CAPN6,GRIN2A,CAMK4,GRIN2D,RASD1,PRKCZ,GRINA,PFKM,GRIN3A,CAMK2A,PPP3R1,CAPN2,PPP3CA
Saline	P10-21		4.08	0.231	2.121	CAPN6,GRIN2A,CAMK4,CAMK2A,GRIN2D,PPP3R1,RASD1,PRKCZ,PPP3CA,GRINA,PFKM,PRKCG
VPA	P10-21	Parkinson's Signaling	2.38	0.312		CASP3,MAPK1,GPR37,MAPK8,SNCAIP
Saline	P10-21		2.6	0.312		SEPT5,CASP3,MAPK1,GPR37,SNCAIP
VPA	P10-21	phagosome maturation	3.33	0.152		CTSK,TUBB3,ATP6V1D,VPS41,PRDX5,TUBB2A,DYNLT1,TUBB,SNAP25,PRDX6,TUBB2B,DYNC1H1,TUBA1A,Dync1i2,TUBB6,CTSS,Atp6ap1l,STX16,TUBA1C,TUBB4A,ATP6V1G2,NAPA,ATP6V0E1
Saline	P10-21		4.5	0.159		CTSK,VPS18,TUBB3,VPS41,PRDX1,TUBB2A,TUBA4A,DYNLT1,TUBB,ATP6V1A,SNAP25,PRDX6,TUBB2B,NSF,CTSA,TUBA1A,Dync1i2,TUBB6,CTSS,STX16,TUBA1C,TUBB4A,NAPA,ATP6V1B2
VPA	P10-21	PI3K Signaling in B Lymphocytes	2.18	0.135		VAV2,CD81,RAF1,CAMK4,MAPK1,NFKBIE,ITPR1,FCGR2B,PRKCZ,PLCB4,CAMK2A,PLCE1,PPP3R1,SH2B2,PLCB1,MAP2K1,PPP3CA,CAMK2G
Saline	P10-21		1.97	0.12		VAV2,CD81,CAMK4,MAPK1,PDIA3,PDPK1,ATF6,ITPR1,PRKCZ,PLCB4,CAMK2A,PPP3R1,SH2B2,PLCB1,MAP2K1,PPP3CA
VPA	P10-21	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	1.81	0.116	0.626	MAP3K9,PPP1R14C,MAPK1,NFKBIE,MAPK8,PPP1CB,PPP1R11,PPP1R14B,PRKCZ,MAP3K10,ALB,LYZ,RHOG,RND3,PPP1R7,KL,PPP2R3A,PPP2R2C,MAP2K1,CLU,TNFRSF11B,RBP4,APOD
Saline	P10-21		1.53	0.101	2.683	PPP1R14C,MAPK1,PPP1R14A,ARG2,PPP1R11,IFNGR1,PPP1R14B,PRKCZ,PRKCG,ALB,LYZ,RHOG,RND3,PPP2R3A,MAPK10,PPP2R2C,MAP2K1,TNFRSF11B,RBP4,APOD
VPA	P10-21	Protein Kinase A Signaling	6.25	0.142	3.286	RAF1,MAPK1,GNB5,PPP1R14B,PTK2,ROCK2,CAMK2A,PHKB,DUSP3,PLCE1,PPP1R7,ADCY5,PPP3R1,PLCB1,SMPDL3B,CDC16,CDC25A,PPP1R14C,ADD2,PPP1R1B,PTCH1,PPP1R11,ITPR1,PDE1C,PYGM,PDE1B,CREM,H1f0,ADCY10,EBI3,CAMK2G,AKAP12,CAMK4,AKAP8,PTK2B,NFKBIE,PPP1CB,PDE1A,ANAPC1,PRKCZ,DUSP2,FLNA,PTPRZ1,ADCY8,MAP2K1,PPP3CA,AKAP5,CDC25C,Cdkn3,TCF7L1,ROCK1,GNAI3,PLCB4,CDC14B,ADD1,PDE8B,Ptprt

Saline	P10-21		6.37	0.132	3.727	MAPK1,GNB5,GNG13,PPP1R14B,PTK2,ROCK2,DUSP3,CAMK2A,PTPRO,ADCY5,PPP3R1,PLCB1,PPP1R14C,PDE2A,ADD2,PPP1R14A,PPP1R11,ITPR1,CNGA1,ADCY9,TULP2,PYGM,H3F3A/H3F3B,DUSP1,CREM,PDE1B,H1f0,EBI3,AKAP12,CAMK4,AKAP8,PTK2B,PDIA3,UBASH3B,PDE1A,PRKCZ,DUSP2,AKAP11,PRKAR1B,PTPRZ1,ADCY8,MAP2K1,PTPRN,PPP3CA,AKAP5,CDC25C,RYR2,Cdkn3,PRKCG,GNAI3,PLCB4,ADD1,PTGS2
VPA	P10-21	PTEN Signaling	1.59	0.124	0.258	MAST2,ITGB1,RAF1,RAC2,MAPK1,CASP3,PREX2,CCND1,PRKCZ,PTK2,BCL2L1,MAGI1,PDGFRA,MAP2K1,MAGI3
Saline	P10-21		1.34	0.107	-0.832	PTK2,SYNJ2,MAST2,BCL2L1,MAGI1,SYNJ1,CASP3,MAPK1,PDGFRA,PDPK1,PREX2,MAP2K1,PRKCZ
VPA	P10-21	Regulation of Cellular Mechanics by Calpain Protease	3.15	0.207	0.632	ITGB1,PTK2,CAPN5,CCNA2,CAPN6,MAPK1,ACTN2,CAPN2,CCND1,CDK1,ACTN1,CDK2
Saline	P10-21		1.52	0.138	1.342	PTK2,CAPN6,MAPK1,ACTN2,CDK4,CNGA1,CDK1,CDK2
VPA	P10-21	Regulation of eIF4 and p70S6K Signaling	1.67	0.118	0	ITGB1,RAF1,EIF4EBP2,MAPK1,RPS18,RPS29,PRKCZ,RPS4Y1,EIF4G2,PPP2R3A,KL,PAIP1,MKNK1,RPS27L,EIF4A1,PPP2R2C,RPS2,MAP2K1,RPSA
Saline	P10-21		5.93	0.174	1.633	MAPK1,RPS27,RPS18,PDPK1,PRKCZ,RPS28,RPS7,RPS20,PAIP1,EIF3A,PPP2R2C,RPS2,RPS12,RPS5,MAP2K1,RPS24,RPS8,RPS10,RPS29,RPS15,RPS4Y1,RPS16,RPS26,PPP2R3A,EIF4A1,RPS27L,RPS15A,RPSA
VPA	P10-21	Remodeling of Epithelial Adherens Junctions	3.56	0.206		TUBB3,RALA,MAPRE1,ACTN2,TUBB2A,TUBB,APC,TUBB2B,CTNNA2,TUBA1A,TUBB6,TUBB4A,TUBA1C,ACTN1
Saline	P10-21		3.49	0.191		CTNNA2,TUBB3,TUBA1A,TUBB6,MAPRE1,ACTN2,TUBB2A,TUBA4A,CTNNA1,TUBA1C,TUBB4A,TUBB,TUBB2B
VPA	P10-21	Role of CHK Proteins in Cell Cycle Checkpoint Control	1.39	0.145	0	CDC25C,PPP2R3A,PPP2R2C,CDK1,CDK2,CDC25A,RFC3,RAD1
Saline	P10-21		1.64	0.145	0.378	CDC25C,PPP2R3A,PPP2R2C,PLK1,CDK1,CDK2,CHEK1,RFC3
VPA	P10-21	Role of NFAT in Cardiac Hypertrophy	2.37	0.126	0	AKAP5,RAF1,CAMK4,MAPK1,SLC8A3,MAPK8,GNB5,ITPR1,PRKCZ,GNAI3,PLCB4,PLCE1,CAMK2A,IGF1,KL,ADCY5,PPP3R1,HDAC11,PLCB1,ADCY10,SLC8A1,ADCY8,MAP2K1,PPP3CA,CAMK2G
Saline	P10-21		2.37	0.116	2.837	AKAP5,CAMK4,MAPK1,PDIA3,GNB5,GNG13,ITPR1,PRKCZ,PRKCG,ADCY9,GNAI3,PLCB4,CAMK2A,IGF1,ADCY5,PPP3R1,MEF2D,MAPK10,PRKAR1B,PLCB1,ADCY8,MAP2K1,PPP3CA
VPA	P10-21	Semaphorin Signaling in Neurons	4.83	0.264		DPYSL2,ITGB1,MAPK1,DPYSL3,DPYSL5,LIMK1,ROCK2,PTK2,ROCK1,CRMP1,RHOG,RND3,PAK3,NRP1
Saline	P10-21		2.23	0.17		ROCK2,PTK2,CRMP1,SEMA4D,RHOG,RND3,MAPK1,DPYSL3,DPYSL5
VPA	P10-21	Sertoli Cell-Sertoli Cell Junction Signaling	3.1	0.14		SPTBN2,ITGB1,MAP3K9,RAF1,CLDN10,DLG1,TUBB3,MAPK1,ACTN2,TUBB2A,MAPK8,AFDN,PRKG2,TUBB,TUBB2B,MAP3K10,CTNNA2,TUBA1A,TUBB6,JAM3,TUBA1C,TUBB4A,CLDN9,ADCY10,MAP2K1,ACTN1
Saline	P10-21		1.81	0.108		SPTBN2,DLG1,CLDN10,TUBB3,MAPK1,ACTN2,TUBB2A,CTNNA1,TUBA4A,TUBB,TUBB2B,CTNNA2,TUBA1A,TUBB6,PRKAR1B,MAPK10,TUBA1C,TUBB4A,MAP2K1,CLDN3
VPA	P10-21	Signaling by Rho Family GTPases	3.03	0.129	-0.557	RAF1,SEPT9,MAPK1,PTK2B,SEPT3,ARHGEF7,GNB5,PIP4K2B,PRKCZ,LIMK1,ROCK2,STMN1,PTK2,MAP3K10,RHOG,ARFIP2,KL,BAIAP2,ARHGEF3,CDH13,MAP2K1,ITGB1,MAP3K9,MAPK8,CDH19,ROCK1,GNAI3,RND3,PAK3,CDH8,ARHGEF9,PIP4K2C

Saline	P10-21		1.32	0.0924	3.441	SEPT5,PTK2B,MAPK1,GNB5,GNG13,SEPT11,PRKCZ,CDH19,STMN1,ROCK2,PTK2,GNAI3,PIP5K1A,CDH9,RHOG,RND3,CDH3,BAIAP2,MAPK10,ARHGEF3,ARHGEF9,MAP2K1,PIP4K2C
VPA	P10-21	Sperm Motility	1.37	0.114	2.324	PLA2G16,CACNA1G,CAMK4,PTK2B,PRKG2,ITPR1,PDE1A,PRKCZ,PDE1C,PTK2,PLCB4,PLCE1,PDE1B,PLCB1,ADCY10,PAFAH1B3
Saline	P10-21		2.1	0.121	3.153	PLA2G16,PDE2A,CAMK4,PTK2B,PDIA3,ITPR1,PDE1A,CNGA1,PRKCZ,PRKCG,PTK2,PLA2G6,PLCB4,PDE1B,PRKAR1B,PLCB1,PAFAH1B3
VPA	P10-21	Sphingosine-1-phosphate Signaling	2.43	0.143	0	PTK2B,MAPK1,CASP3,PDGFA,PTK2,GNAI3,PLCB4,RHOG,PLCE1,RND3,ADCY5,KL,PDGFRA,S1PR1,PLCB1,ADCY10,ADCY8,CASP7
Saline	P10-21		2.19	0.127	1	PTK2B,MAPK1,CASP3,PDIA3,PTK2,GNAI3,ADCY9,PLCB4,RHOG,RND3,ADCY5,PDGFRA,S1PR1,PLCB1,ADCY8,CASP7
VPA	P10-21	Synaptic Long Term Depression	1.54	0.116	0.471	PLA2G16,RAF1,MAPK1,GRIA2,PPP1R17,PRKG2,ITPR1,PRKCZ,GNAI3,PLCB4,PLCE1,IGF1,PPP2R3A,PLCB1,PPP2R2C,MAP2K1,GRIA3,PAFAH1B3
Saline	P10-21		1.69	0.11	1.698	PLA2G16,MAPK1,PDIA3,RYR2,ITPR1,PRKCZ,PRKCG,PLA2G6,GNAI3,PLCB4,IGF1,PPP2R3A,PLCB1,PPP2R2C,MAP2K1,GRIA3,PAFAH1B3
VPA	P10-21	Synaptic Long Term Potentiation	5.44	0.195	0.816	RAF1,GRIN2A,CAMK4,MAPK1,GRIN2D,PPP1CB,PPP1R14B,PRKCZ,GRINA,CAMK2A,PLCE1,PPP1R7,PPP3R1,PLCB1,ADCY8,MAP2K1,PPP3CA,PPP1R14C,GRIA2,PPP1R11,ITPR1,GRIN3A,PLCB4,GRIA3,CAMK2G
Saline	P10-21		4.72	0.172	2.4	PPP1R14C,GRIN2A,CAMK4,MAPK1,PDIA3,GRIN2D,PPP1R14A,PPP1R11,ITPR1,PPP1R14B,PRKCZ,PRKCG,GRINA,PLCB4,CAMK2A,PPP3R1,PRKAR1B,PLCB1,ADCY8,MAP2K1,PPP3CA,GRIA3
VPA	P10-21	Thrombin Signaling	2.38	0.124	0.2	RAF1,CAMK4,MAPK1,F2R,GNB5,PPP1CB,PRKCZ,ROCK2,PTK2,RHOG,PLCE1,CAMK2A,KL,ADCY5,PLCB1,ARHGEF3,ADCY8,MAP2K1,ITPR1,ROCK1,GNAI3,PLCB4,RND3,ADCY10,ARHGEF9,CAMK2G
Saline	P10-21		2.41	0.115	2.558	F2RL2,CAMK4,MAPK1,PDIA3,GNB5,GNG13,PDPK1,ITPR1,PRKCZ,PRKCG,ROCK2,PTK2,GNAI3,ADCY9,PLCB4,RHOG,CAMK2A,RND3,ADCY5,PLCB1,ARHGEF3,ADCY8,ARHGEF9,MAP2K1
VPA	P10-21	α-Adrenergic Signaling	2.44	0.155	1.069	RAF1,CAMK4,MAPK1,SLC8A3,GNB5,ITPR1,PRKCZ,GNAI3,PYGM,PHKB,ADCY5,ADCY10,SLC8A1,ADCY8,MAP2K1
Saline	P10-21		2.47	0.144	1.732	GNAI3,ADCY9,CAMK4,PYGM,MAPK1,ADCY5,GNB5,PRKAR1B,GNG13,ITPR1,ADCY8,MAP2K1,PRKCZ,PRKCG

Table S2.1B.

Group	*Comparison*	*Ingenuity Canonical Pathways*	*-log(p-value)*	*Ratio*	*z-score*	*Molecules*
Saline	P10-21	3-phosphoinositide Biosynthesis	2.07	0.111		CDC25C,PPFIBP2,PPP1R14A,ERBB3,PPP4R1,PPM1K,PPP1R14B,DUSP14,DUSP2,SET,PIP5K1A,ATP1A1,SYNJ1,DUSP1,PPP2R3A,PTPRO,PDGFRA,PTPRN,THTPA,PIP4K2C,PPP3CA,NUDT1
Saline	P10-21	3-phosphoinositide Degradation	2.32	0.123		CDC25C,PPFIBP2,PPP1R14A,PPP4R1,PPM1K,PPP1R14B,DUSP14,DUSP2,SYNJ2,SET,ATP1A1,SYNJ1,DUSP1,PPP2R3A,PTPRO,PTPRN,THTPA,PPP3CA,NUDT1
Saline	P10-21	ATM Signaling	1.51	0.125	1.414	CDC25C,SUV39H1,MAPK10,CCNB2,HIST1H4J,CBX5,CDK1,CDK2,CHEK1,CCNB1
Saline	P10-21	Calcium-induced T Lymphocyte Apoptosis	1.48	0.129	2.333	CAMK4,PPP3R1,MEF2D,NR4A1,HLA-DOB,ITPR1,PRKCZ,PPP3CA,PRKCG
Saline	P10-21	CCR5 Signaling in Macrophages	1.34	0.122		GNAI3,CAMK4,MAPK1,PTK2B,GNB5,MAPK10,GNG13,PRKCZ,PRKCG
Saline	P10-21	Corticotropin Releasing Hormone Signaling	1.99	0.124	1.941	CAMK4,MAPK1,UCN3,ITPR1,PRKCZ,PRKCG,GNAI3,ADCY9,ADCY5,MEF2D,PRKAR1B,NR4A1,PTGS2,ADCY8,MAP2K1
Saline	P10-21	D-myo-inositol (1,4,5,6)-Tetrakisphosphate Biosynthesis	2.55	0.131		CDC25C,PPFIBP2,PPP1R14A,PPP4R1,PPM1K,PPP1R14B,DUSP14,DUSP2,SET,ATP1A1,SYNJ1,DUSP1,PPP2R3A,PTPRO,PTPRN,PPP3CA,THTPA,NUDT1
Saline	P10-21	D-myo-inositol (3,4,5,6)-tetrakisphosphate Biosynthesis	2.55	0.131		CDC25C,PPFIBP2,PPP1R14A,PPP4R1,PPM1K,PPP1R14B,DUSP14,DUSP2,SET,ATP1A1,SYNJ1,DUSP1,PPP2R3A,PTPRO,PTPRN,PPP3CA,THTPA,NUDT1
Saline	P10-21	EIF2 Signaling	13.4	0.228	-3.545	RPL24,RPL11,MAPK1,RPS27,Rpl36a,RPS18,RPL39,PDPK1,RPL7,RPS7,RPS28,RPL27A,RPL14,RPL35,RPS20,RPL18A,PAIP1,EIF3A,RPS2,RPS12,RPL36,RPS5,MAP2K1,RPL31,RPL18,RPS24,RPS8,RPS10,RPL12,RPS29,RPL10A,RPL15,RPL27,RPS4Y1,RPS15,RPS16,RPS26,RPS27L,EIF4A1,RPL10,RPS15A,RPL6,RPL41,RPSA,RPLP0
Saline	P10-21	Fatty Acid α-oxidation	1.72	0.2		ALDH1A1,ALDH1L2,ALDH3B1,PTGS2,ALOXE3
Saline	P10-21	GPCR-Mediated Integration of Enteroendocrine Signaling Exemplified by an L Cell	1.9	0.138		GNAI3,ADCY9,PLCB4,GIPR,PDIA3,ADCY5,PRKAR1B,PLCB1,ITPR1,ADCY8,ADRB2
Saline	P10-21	GPCR-Mediated Nutrient Sensing in Enteroendocrine Cells	1.86	0.13		GNAI3,ADCY9,PLCB4,PDIA3,ADCY5,PRKAR1B,PLCB1,GNG13,ITPR1,ADCY8,PRKCZ,PRKCG
Saline	P10-21	Hypusine Biosynthesis	1.45	0.4		DHPS,DOHH
Saline	P10-21	mTOR Signaling	7.55	0.176	1.069	MAPK1,PRKAB2,RPS27,RPS18,PDPK1,FKBP1A,PRKCZ,RPS7,RPS28,RHOG,RPS20,EIF3A,PPP2R2C,RPS2,RPS12,RPS5,RPS24,RHEB,RPS8,RPS10,RPS29,PRKCG,ATG13,DGKZ,RPS6KA6,RPS4Y1,RPS15,RPS16,RND3,PPP2R3A,RPS26,RPS27L,EIF4A1,PRR5,RPS15A,RPSA
Saline	P10-21	Netrin Signaling	1.69	0.159		PPP3R1,ABLIM3,RYR2,UNC5D,PRKAR1B,ABLIM2,PPP3CA
Saline	P10-21	Nur77 Signaling in T Lymphocytes	1.44	0.133		CAMK4,CASP3,PPP3R1,MEF2D,APAF1,NR4A1,HLA-DOB,PPP3CA
Saline	P10-21	P2Y Purigenic Receptor Signaling Pathway	1.42	0.106	1.941	MAPK1,PDIA3,GNB5,GNG13,PRKCZ,PRKCG,MYC,GNAI3,ADCY9,PLCB4,ADCY5,PRKAR1B,PLCB1,ADCY8,MAP2K1
Saline	P10-21	Protein Ubiquitination Pathway	1.59	0.0965		USP24,CRYAB,USP45,CDC20,DNAJB4,USP20,USP2,USP8,USP42,DNAJC16,HSPB6,HSPA4L,DNAJC9,DNAJC27,HSPH1,PSME2,SKP2,USP31,PSME1,USP22,USP29,BAP1,USP34,DNAJB5,UBE2C
Saline	P10-21	RAR Activation	2.75	0.122		DHRS3,MAPK1,AKR1C3,CYP26A1,SMARCE1,PDPK1,RBP1,CRABP1,PRKCZ,SMARCD3,PRKCG,ADCY9,LRAT,TNIP1,ALDH1A1,DUSP1,ADCY5,MAPK10,PRKAR1B,GTF2H5,RDH12,ADCY8,MAP2K1,RBP4
Saline	P10-21	Retinoate Biosynthesis I	1.5	0.158		DHRS3,Akr1b10,ALDH1A1,AKR1C3,RDH12,RBP1

Saline	P10-21	Role of MAPK Signaling in the Pathogenesis of Influenza	1.31	0.12	PLA2G16,PLA2G6,CASP3,MAPK1,MAPK10,BAX,PTGS2,MAP2K1,PAFAH1B3
Saline	P10-21	Superpathway of Inositol Phosphate Compounds	2.3	0.108	PPFIBP2,PPM1K,PPP1R14B,DUSP2,PLCH1,SET,ATP1A1,PTPRO,PDGFRA,PLCB1,PTPRN,PPP3CA,CDC25C,PPP1R14A,ERBB3,ITPKA,PPP4R1,DUSP14,SYNJ2,PLCB4,PIP5K1A,SYNJ1,DUSP1,PPP2R3A,PIP4K2C,THTPA,NUDT1
Saline	P10-21	The Visual Cycle	2.46	0.219	DHRS3,LRAT,Akr1b10,AKR1C3,RDH12,RBP1,RPE65
Saline	P10-21	Tight Junction Signaling	1.41	0.102	CLDN10,F2RL2,HSF1,CDK4,MPP5,CTNNA1,SNAP25,PRKCZ,NSF,PPP2R3A,PRKAR1B,STX16,PPP2R2C,STX4,NAPA,CLDN3,TNFRSF11B
Saline	P10-21	Ubiquinol-10 Biosynthesis (Eukaryotic)	1.35	0.161	CYP7B1,MICAL2,CYP26A1,BCKDHA,BCKDHB

Table S2.1C.

Group	*Comparison*	*Ingenuity Canonical Pathways*	*-log(p-value)*	*Ratio*	*z-score*	*Molecules*
VPA	P10-21	Actin Cytoskeleton Signaling	3.24	0.134	-1.826	RAC2,RAF1,F2R,MAPK1,PDGFA,ARHGEF7,PIP4K2B,PPP1CB,LIMK1,PTK2,ROCK2,KL,FLNA,BAIAP2,TMSB10/TMSB4X,MAP2K1,ACTN1,VAV2,ITGB1,TIAM1,CSK,ACTN2,FGD1,GSN,APC,ROCK1,PAK3,APC2,MYH3,ARHGAP35,PIP4K2C
VPA	P10-21	Agrin Interactions at Neuromuscular Junction	1.57	0.143	-1.414	ITGB1,PTK2,RAC2,PKLR,MAPK1,PAK3,ARHGEF7,MAPK8,UTRN,AGRN
VPA	P10-21	AMPK Signaling	1.65	0.112	1.291	ADRA2B,CHRNA4,MAPK1,SLC2A1,CPT1B,CHRNB1,SMARCE1,CCND1,PPM1G,SMARCD3,NAD+,PFKM,CCNA2,KL,PPM1B,PPP2R3A,FASN,PRKAA2,ACACA,PPP2R2C,ADRA2C,HMGCR,CHRNA3
VPA	P10-21	Apoptosis Signaling	3.28	0.18	-2.5	CAPN5,RAF1,CAPN6,MAPK1,CASP3,NFKBIE,BIRC6,MAPK8,APAF1,BAK1,CDK1,ROCK1,BCL2L1,CAPN2,MAP2K1,CASP7
VPA	P10-21	B Cell Receptor Signaling	1.76	0.116	0.218	VAV2,GAB2,RAF1,RAC2,MAP3K9,CAMK4,PTK2B,MAPK1,NFKBIE,EGR1,CSK,MAPK8,FCGR2B,PTK2,BCL2L1,MAP3K10,CAMK2A,KL,PPP3R1,MAP2K1,PPP3CA,CAMK2G
VPA	P10-21	CCR3 Signaling in Eosinophils	1.5	0.119		RAF1,CAMK4,MAPK1,GNB5,PPP1CB,ITPR1,PRKCZ,LIMK1,ROCK2,ROCK1,GNAI3,PLCB4,PAK3,KL,PLCB1,MAP2K1
VPA	P10-21	CD27 Signaling in Lymphocytes	1.52	0.154	0.378	BCL2L1,MAP3K9,MAP3K10,CASP3,NFKBIE,MAPK8,APAF1,MAP2K1
VPA	P10-21	Circadian Rhythm Signaling	1.44	0.171		GRIN2A,NR1D1,GRIN2D,BHLHE40,GRINA,GRIN3A
VPA	P10-21	D-myo-inositol (1,4,5)-Trisphosphate Biosynthesis	1.34	0.162		PLCB4,PLCE1,PIP4K2B,PLCB1,PLCH1,PIP4K2C
VPA	P10-21	Ephrin Receptor Signaling	2.4	0.13	-0.471	ITGB1,RAC2,RAF1,GRIN2A,NGEF,MAPK1,PDGFA,Wasl,GRIN2D,SH2D3C,GNB5,LIMK1,GRINA,GRIN3A,ROCK1,PTK2,ROCK2,GNAI3,PAK3,EPHA5,EPHB3,EFNB3,MAP2K1
VPA	P10-21	Fatty Acid β-oxidation I	2.39	0.2		ACSL3,SLC27A5,SLC27A2,ACSL6,ACSL4,SLC27A3,ACAA2,ECI1,NAD+
VPA	P10-21	Glioblastoma Multiforme Signaling	1.34	0.11	-1.414	RAF1,MAPK1,PDGFA,ITPR1,CCND1,APC,MYC,PLCB4,PLCE1,RHOG,RND3,IGF1,KL,PDGFRA,WNT4,PLCB1,MAP2K1,CDK2
VPA	P10-21	Glioma Signaling	1.37	0.119	-0.832	RAF1,CAMK4,MAPK1,PDGFA,SUV39H1,CCND1,PRKCZ,CAMK2A,IGF1,KL,TGFA,PDGFRA,MAP2K1,CAMK2G
VPA	P10-21	Gluconeogenesis I	1.36	0.152		PGAM1,ME3,ME1,MDH1,ALDOC,FBP2,NAD+
VPA	P10-21	GM-CSF Signaling	1.43	0.135	0	BCL2L1,RAF1,CAMK2A,MAPK1,KL,PPP3R1,CCND1,MAP2K1,PPP3CA,CAMK2G
VPA	P10-21	Gαi Signaling	2.93	0.154	0.471	ADRA2B,RAF1,GPR17,RALA,MAPK1,NPY1R,GNB5,HRH3,GABBR2,GNAI3,LPAR1,ADCY5,S1PR1,RGS14,ADRA2C,HTR1F,ADCY10,ADCY8,HCAR2
VPA	P10-21	HIPPO signaling	1.68	0.138	-0.447	DLG1,PPP1R14C,TEAD1,PPP1R7,PPP2R3A,PPP1CB,PPP1R11,PPP2R2C,PPP1R14B,LATS1,PRKCZ,SKP2
VPA	P10-21	Integrin Signaling	1.83	0.114	-0.626	ITGB1,CAPN5,RAC2,RAF1,CAPN6,RALA,MAPK1,Wasl,ACTN2,ARHGEF7,MAPK8,PPP1CB,GSN,ROCK1,PTK2,RHOG,RND3,PAK3,ARF3,KL,CAPN2,TSPAN6,MAP2K1,Arf2,ACTN1
VPA	P10-21	LXR/RXR Activation	1.4	0.117	0.302	SCD,HPX,VTN,IL33,ALB,LYZ,IL1RN,FASN,ACACA,HMGCR,IL1RAP,CLU,APOD,RBP4,TNFRSF11B
VPA	P10-21	Mevalonate Pathway I	1.4	0.185		MVD,IDI1,ACAT1,HMGCR,HMGCS1
VPA	P10-21	Mitochondrial Dysfunction	1.55	0.112		ATP5J,COX17,CASP3,NDUFS7,PRDX5,CPT1B,XDH,MAPK8,LRRK2,UQCR11,GPX7,NDUFA1,NAD+,VPS9D1,NDUFB9,COX6B2,NDUFA6,ATPAF2,CYB5A,ACO1,NDUFA8
VPA	P10-21	Molecular Mechanisms of Cancer	3.43	0.12		GAB2,RAF1,RAC2,RALA,MAPK1,ARHGEF7,NFKBIE,SUV39H1,CCND1,PRKCZ,PTK2,MYC,CTNNA2,CAMK2A,RHOG,ADCY5,KL,WNT4,PLCB1,ARHGEF3,ADCY8,MAP2K1,CDC25A,ITGB1,CDC25C,STK36,CASP3,PTCH1,APAF1,MAPK8,AURKA,BAK1,APC,BCL2L1,GNAI3,PLCB4,RND3,PAK3,RASGRP1,ADCY10,ARHGEF9,GLI1,NOTCH1,CASP7,CDK2,CAMK2G
VPA	P10-21	Oleate Biosynthesis II (Animals)	1.37	0.211		SCD,FADS2,Ptprt,CYB5A
VPA	P10-21	PAK Signaling	2.58	0.155	-2	ITGB1,RAF1,PTK2B,MAPK1,CASP3,PDGFA,ARHGEF7,MAPK8,LIMK1,PTK2,PAK3,KL,PDGFRA,EPHB3,DSCAM,MAP2K1

VPA	P10-21	Pancreatic Adenocarcinoma Signaling	1.61	0.125	-1.732	RAF1,RALA,MAPK1,SUV39H1,MAPK8,HBEGF,CCND1,BIRC5,RAD51,BCL2L1,KL,TGFA,MAP2K1,NOTCH1,CDK2
VPA	P10-21	Phospholipase C Signaling	1.33	0.102	0	RAF1,RALA,CAMK4,MAPK1,ARHGEF7,GNB5,PPP1CB,FCGR2B,PRKCZ,RHOG,PLCE1,ADCY5,PPP3R1,HDAC11,PLCB1,ARHGEF3,ADCY8,MAP2K1,PPP3CA,ITGB1,ITPR1,PLCB4,RND3,ADCY10,ARHGEF9
VPA	P10-21	PPARα/RXRα Activation	1.41	0.109	-1.134	RAF1,GPD1,MAPK1,NFKBIE,CPT1B,MAPK8,MED12,NR2F1,PLCB4,CHD5,PLCE1,ADCY5,FASN,PRKAA2,PLCB1,ADCY10,ADCY8,IL1RAP,MAP2K1,ACVR1C
VPA	P10-21	Pyridoxal 5'-phosphate Salvage Pathway	2.68	0.176		PDXK,MAP3K9,MAPK1,MAPK8,MAPK6,TTK,CDK1,LIMK1,PRKX,PAK3,PRKAA2,MAP2K1,CDK2
VPA	P10-21	Rac Signaling	1.94	0.133	0.258	ITGB1,RAF1,TIAM1,PTK2B,MAPK1,MAPK8,PIP4K2B,PRKCZ,LIMK1,PTK2,ARFIP2,PAK3,KL,BAIAP2,MAP2K1,PIP4K2C
VPA	P10-21	RAN Signaling	1.45	0.222		CSE1L,TNPO1,RANBP1,KPNA2
VPA	P10-21	RANK Signaling in Osteoclasts	1.52	0.127	0.577	MAP3K10,MAP3K9,RAF1,CAMK4,PTK2B,MAPK1,KL,PPP3R1,NFKBIE,MAPK8,GSN,MAP2K1,PPP3CA
VPA	P10-21	Regulation of Actin-based Motility by Rho	1.83	0.14	-1.155	ITGB1,ROCK1,RAC2,RHOG,RND3,PAK3,Wasl,BAIAP2,PIP4K2B,PPP1CB,GSN,PIP4K2C,LIMK1
VPA	P10-21	RhoA Signaling	2.89	0.153	0.471	NGEF,PTK2B,SEPT9,SEPT3,RTKN,PPP1CB,PIP4K2B,LIMK1,ROCK2,PTK2,ROCK1,IGF1,LPAR1,LPAR2,RND3,BAIAP2,ARHGAP12,ARHGAP35,PIP4K2C
VPA	P10-21	RhoGDI Signaling	1.76	0.117	0.943	ITGB1,ARHGDIG,ARHGEF7,GNB5,PIP4K2B,GRIP1,LIMK1,CDH19,ROCK1,ROCK2,GNAI3,RHOG,RND3,PAK3,CDH8,ARHGAP12,ARHGAP35,ARHGEF3,ARHGEF9,CDH13,PIP4K2C
VPA	P10-21	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	1.54	0.102		RAF1,SFRP2,CAMK4,MAPK1,PDGFA,NFKBIE,CCND1,PRKCZ,ROCK2,MYC,PLCE1,CAMK2A,WIF1,KL,PPP3R1,TRAF4,PLCB1,WNT4,MAP2K1,IL1RAP,PPP3CA,FCGR3A/FCGR3B,TNFRSF11B,DAAM1,TCF7L1,APC,IL33,ROCK1,PLCB4,IL1RN,APC2,CAMK2G
VPA	P10-21	Salvage Pathways of Pyrimidine Ribonucleotides	1.6	0.127		MAP3K9,MAPK1,UPP1,MAPK8,MAPK6,TTK,CDK1,LIMK1,NME3,PRKX,PAK3,PRKAA2,MAP2K1,CDK2
VPA	P10-21	Small Cell Lung Cancer Signaling	1.34	0.126		PTK2,MYC,BCL2L1,KL,NFKBIE,SUV39H1,TRAF4,APAF1,CCND1,CDK2,SKP2
VPA	P10-21	Sonic Hedgehog Signaling	2.27	0.226	-1.342	STK36,GLIS2,PTCH1,HHIP,GLI1,CDK1,CCNB1
VPA	P10-21	Stearate Biosynthesis I (Animals)	2.67	0.204		ACSL3,SLC27A5,DHCR24,SLC27A2,FASN,ACSL6,ACSL4,SLC27A3,ELOVL1,ACOT7
VPA	P10-21	γ-linolenate Biosynthesis II (Animals)	3.76	0.333		ACSL3,SLC27A5,SLC27A2,ACSL6,FADS2,ACSL4,SLC27A3,CYB5A

Table S2.2. Canonical RNA Sequencing Pathways differing between Saline and VPA amygdala at (A) P10 and (B) P21.

Ratio represents the number of genes altered between treatments within the total number of genes in that pathway, and the p-value is from a right-tailed Fisher exact test of this ratio. The z-score represents the predicted change in gene regulation of that pathway.

A.

Age	*Comparison*	*Ingenuity Canonical Pathways*	*-log(p-value)*	*Ratio*	*z-score*	*Molecules*
P10	VPA/Saline	Reelin Signaling in Neurons	3.57	0.0978		MAP3K9,APOE,MAP3K10,MAP2K7,MAP3K11,MAPT,HCK,MAPK8IP1,ITGAL
P10	VPA/Saline	Notch Signaling	2.77	0.132	2	NOTCH2,FURIN,NOTCH3,HES5,NOTCH1
P10	VPA/Saline	SAPK/JNK Signaling	2.53	0.0762	1.134	MAP3K9,MAP3K10,MAP2K7,MAP3K11,NFATC3,DUSP10,MAPK8IP1,MAP4K4
P10	VPA/Saline	Signaling by Rho Family GTPases	2.28	0.0522	0.832	MAP3K9,MAP2K7,MAP3K11,PTK2B,CDC42EP5,PIP4K2B,DES,CDH19,LIMK1,MAP3K10,WIPF1, BAIAP2,FNBP1
P10	VPA/Saline	LXR/RXR Activation	2.02	0.0625	0	APOE,IL1A,FASN,APOA5,ABCG1,NCOR2,RXRB,IL1RAP
P10	VPA/Saline	B Cell Receptor Signaling	1.9	0.0526	1.897	MAP3K9,MAP3K10,MAP2K7,MAP3K11,APBB1IP,PTK2B,NFATC3,ABL1,INPPL1,CAMK2G
P10	VPA/Saline	tRNA Splicing	1.74	0.0909		PDE3B,PDE4A,PDE8B,NAD+
P10	VPA/Saline	Regulation of Actin-based Motility by Rho	1.69	0.0645	1.633	WIPF1,PFN1,BAIAP2,PIP4K2B,FNBP1,LIMK1
P10	VPA/Saline	PPAR Signaling	1.69	0.0645	-1.633	IL1A,PDGFRA,NCOR2,INSR,IL1RAP,MAP4K4
P10	VPA/Saline	Rac Signaling	1.68	0.0583	1.134	MCF2L,MAP2K7,MAP3K11,PTK2B,BAIAP2,PIP4K2B,LIMK1
P10	VPA/Saline	IL-15 Production	1.63	0.111		TWF1,MAP3K11,PTK2B
P10	VPA/Saline	Protein Kinase A Signaling	1.61	0.0398	-0.535	PTK2B,PTPN23,NFATC3,PDIA3,PTCH1,PDE4A,DUSP15,ANAPC7,ANAPC13,PTPRF,PDE3B,FLNC ,DUSP10,PDE8B,CDC16,CAMK2G
P10	VPA/Saline	Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	1.57	0.0606	0.816	PLA2G6,NCF1,PTK2B,GPLD1,HCK,TLN1
P10	VPA/Saline	STAT3 Pathway	1.56	0.0676	2.236	MAP3K9,MAP3K10,MAP3K11,PDGFRA,INSR
P10	VPA/Saline	G-Protein Coupled Receptor Signaling	1.56	0.0432		ADRA2B,GPR17,PTK2B,PDE3B,NPY1R,GRM1,PDE4A,PDE8B,RAPGEF3,RPS6KA1,CRHR1,CAM K2G
P10	VPA/Saline	CD27 Signaling in Lymphocytes	1.5	0.0769	2	MAP3K9,MAP3K10,MAP2K7,MAP3K11
P10	VPA/Saline	Semaphorin Signaling in Neurons	1.48	0.0755		SEMA4D,DPYSL5,FNBP1,LIMK1
P10	VPA/Saline	TR/RXR Activation	1.47	0.0571		UCP2,PDE3B,FASN,APOA5,NCOR2,RXRB
P10	VPA/Saline	Axonal Guidance Signaling	1.46	0.0374		MMP21,PAPPA,PFN1,RGS3,SLIT1,NFATC3,PDIA3,SEMA5A,PTCH1,ABL1,VEGFB,DPYSL5,LIMK 1,WIPF1,SEMA6D,SEMA4D,BAIAP2
P10	VPA/Saline	cAMP-mediated signaling	1.45	0.0444	1.265	ADRA2B,GPR17,PDE3B,NPY1R,PDE4A,PDE8B,RAPGEF3,RPS6KA1,CRHR1,CAMK2G
P10	VPA/Saline	Galactose Degradation I (Leloir Pathway)	1.4	0.143		GALT,GALM
P10	VPA/Saline	Cdc42 Signaling	1.38	0.0473	-0.447	WIPF1,MAP3K11,LLGL1,CDC42EP5,BAIAP2,EXOC5,HLA-DOB,LIMK1
P10	VPA/Saline	NAD biosynthesis II (from tryptophan)	1.37	0.0882		AFMID,ABL1,NAD+
P10	VPA/Saline	Ethanol Degradation IV	1.31	0.0833		ALDH1A1,CYGB,NAD+

B.

Age	*Comparison*	*Ingenuity Canonical Pathways*	*-log(p-value)*	*Ratio*	*z-score*	*Molecules*
P21	VPA/Saline	Cholecystokinin/Gastrin-mediated Signaling	4.04	0.0849		RND2,ROCK2,IL1RN,MAPK8,SST,PRKCH,CCK,PTGS2,CCKBR
P21	VPA/Saline	Axonal Guidance Signaling	3.87	0.0441		EPHA7,SLIT1,FZD3,NFATC3,CXCR4,WNT9A,ARHGEF15,SEMA5A,GNA14,ADAMTS2,HERC2,ROCK2,TUBA8,ABLIM3,EPHA5,FIGF,PRKCH,SEMA3C,RTN4R,MYL3
P21	VPA/Saline	Calcium Signaling	3.4	0.0591	-1	HDAC9,GRIN2A,CHRNA4,MYH8,NFATC3,TRPV6,RYR2,MYH3,Tpm1,TRPC3,MYL3
P21	VPA/Saline	Protein Kinase A Signaling	3.18	0.0423	0.775	TCF4,PTPRG,NFATC3,DUSP6,RYR2,PTPN18,PDE1C,ROCK2,PTPN4,PPP1R3D,PTPRJ,TGFB3,PRKCH,PTGS2,CDC16,NFKBIB,MYL3
P21	VPA/Saline	Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	3.04	0.0504		TCF4,MMP3,DKK3,NFATC3,IL1RN,FZD3,WNT9A,MAPK8,CALCR,IL1R1,NFKBIB,XIAP
P21	VPA/Saline	Molecular Mechanisms of Cancer	2.98	0.0419		RND2,TCF4,FZD3,ARHGEF15,WNT9A,MAPK8,GNA14,XIAP,MYC,BCL2L1,CDH1,NLK,APH1A,TGFB3,PRKCH,NFKBIB
P21	VPA/Saline	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	2.91	0.0444		ROCK2,MYC,TCF4,NLK,MMP3,IL1RN,DKK3,FZD3,NFATC3,WNT9A,FIGF,PRKCH,IL1R1,NFKBIB
P21	VPA/Saline	Colorectal Cancer Metastasis Signaling	2.82	0.0476	0	RND2,MYC,BCL2L1,CDH1,TCF4,MMP3,FZD3,WNT9A,MAPK8,TGFB3,FIGF,PTGS2
P21	VPA/Saline	GPCR-Mediated Integration of Enteroendocrine Signaling Exemplified by an L Cell	2.59	0.075		ADRB1,SST,CCK,GNA14,VIP,ADCYAP1
P21	VPA/Saline	Induction of Apoptosis by HIV1	2.43	0.0833		BCL2L1,CXCR4,MAPK8,NFKBIB,XIAP
P21	VPA/Saline	Amyotrophic Lateral Sclerosis Signaling	2.32	0.0583		PRPH,BCL2L1,GRIN2A,NEFL,FIGF,SOD1,XIAP
P21	VPA/Saline	Polyamine Regulation in Colon Cancer	2.16	0.13		MYC,PSMA8,TCF4
P21	VPA/Saline	ILK Signaling	2.12	0.0455	-1.414	RND2,MYC,CDH1,MYH8,MAPK8,MYH3,FIGF,PTGS2,MYL3
P21	VPA/Saline	Gα12/13 Signaling	2.11	0.0534	-0.816	ROCK2,CDH1,F2RL2,CDH8,MAPK8,NFKBIB,MYL3
P21	VPA/Saline	IL-8 Signaling	2.08	0.0448	-0.378	RND2,ROCK2,BCL2L1,CDH1,MAPK8,FIGF,PRKCH,PTGS2,NFKBIB
P21	VPA/Saline	Gαq Signaling	2.05	0.0476	1.134	RND2,ROCK2,NFATC3,CALCR,PRKCH,GNA14,NFKBIB,AVPR1A
P21	VPA/Saline	Wnt/β-catenin Signaling	2.02	0.0471	1.134	MYC,CDH1,TCF4,NLK,DKK3,FZD3,WNT9A,TGFB3
P21	VPA/Saline	Signaling by Rho Family GTPases	1.94	0.0402	-2.333	RND2,ROCK2,MAP3K9,CDH1,ARHGEF15,CDH8,MAPK8,GNA14,SEPT11,MYL3
P21	VPA/Saline	Ephrin Receptor Signaling	1.92	0.0452	-2.236	ROCK2,EPHA7,GRIN2A,CXCR4,ARHGEF15,EPHA5,FIGF,GNA14
P21	VPA/Saline	Embryonic Stem Cell Differentiation into Cardiac Lineages	1.92	0.2		T,MESP1
P21	VPA/Saline	CD27 Signaling in Lymphocytes	1.91	0.0769		BCL2L1,MAP3K9,MAPK8,NFKBIB
P21	VPA/Saline	TNFR2 Signaling	1.88	0.103		MAPK8,NFKBIB,XIAP
P21	VPA/Saline	NRF2-mediated Oxidative Stress Response	1.72	0.0415		ERP29,MGST2,DNAJC6,MAPK8,DNAJA3,PRKCH,SOD1,MGST3
P21	VPA/Saline	Circadian Rhythm Signaling	1.66	0.0857		GRIN2A,VIP,ADCYAP1
P21	VPA/Saline	G-Protein Coupled Receptor Signaling	1.64	0.036		GRM2,ADRB1,DUSP6,CNR1,CALCR,GNA14,RGS12,NFKBIB,AVPR1A,PDE1C

P21	VPA/Saline	PCP pathway	1.63	0.0635	-1	ROCK2,FZD3,WNT9A,MAPK8
P21	VPA/Saline	Glutamate Receptor Signaling	1.61	0.0625		GRM2,GRIN2A,SLC1A6,GLS
P21	VPA/Saline	Tight Junction Signaling	1.58	0.0419		MPDZ,F2RL2,YKT6,MYH8,MYH3,TGFB3,MYL3
P21	VPA/Saline	April Mediated Signaling	1.56	0.0789		NFATC3,MAPK8,NFKBIB
P21	VPA/Saline	Huntington's Disease Signaling	1.55	0.0364	0	PSMA8,BCL2L1,HDAC9,YKT6,GLS,MAPK8,PRKCH,GNA14,STX1A
P21	VPA/Saline	CXCR4 Signaling	1.51	0.0405	-0.816	RND2,ROCK2,CXCR4,MAPK8,PRKCH,GNA14,MYL3
P21	VPA/Saline	B Cell Activating Factor Signaling	1.5	0.075		NFATC3,MAPK8,NFKBIB
P21	VPA/Saline	Hepatic Cholestasis	1.5	0.0402		CYP27A1,IL1RN,MAPK8,TGFB3,PRKCH,IL1R1,NFKBIB
P21	VPA/Saline	Thyroid Cancer Signaling	1.48	0.0732		MYC,CDH1,TCF4
P21	VPA/Saline	TR/RXR Activation	1.45	0.0476		ENO1,ADRB1,AKR1C3,ME1,THRB
P21	VPA/Saline	IL-10 Signaling	1.45	0.0556		IL1RN,MAPK8,IL1R1,NFKBIB
P21	VPA/Saline	RhoGDI Signaling	1.44	0.0391	1.633	RND2,ROCK2,CDH1,ARHGEF15,CDH8,GNA14,MYL3
P21	VPA/Saline	Mouse Embryonic Stem Cell Pluripotency	1.44	0.0472	0.447	MYC,TCF4,T,FZD3,XIAP
P21	VPA/Saline	GABA Receptor Signaling	1.43	0.0548		GAD2,GABRG1,KCNQ3,AP2S1
P21	VPA/Saline	Pyridoxal 5'-phosphate Salvage Pathway	1.41	0.0541		MAP3K9,MAPK8,PLK1,PRKCH
P21	VPA/Saline	MIF Regulation of Innate Immunity	1.4	0.0682		MAPK8,PTGS2,NFKBIB
P21	VPA/Saline	Netrin Signaling	1.4	0.0682		NFATC3,ABLIM3,RYR2
P21	VPA/Saline	Aryl Hydrocarbon Receptor Signaling	1.39	0.0411		MYC,MGST2,MAPK8,TGFB3,NFIB,MGST3
P21	VPA/Saline	Salvage Pathways of Pyrimidine Ribonucleotides	1.38	0.0455		MAP3K9,MAPK8,PLK1,PRKCH,UCK2
P21	VPA/Saline	LPS/IL-1 Mediated Inhibition of RXR Function	1.37	0.0354		MGST2,SLC27A2,IL1RN,MAPK8,CPT1C,NDST4,IL1R1,MGST3
P21	VPA/Saline	Epithelial Adherens Junction Signaling	1.36	0.0405		CDH1,TCF4,TUBA8,MYH8,MYH3,MYL3
P21	VPA/Saline	Hepatic Fibrosis / Hepatic Stellate Cell Activation	1.36	0.0374		MYH8,IGFBP3,MYH3,TGFB3,FIGF,IL1R1,MYL3
P21	VPA/Saline	Gluconeogenesis I	1.35	0.0652		ENO1,ME1,FBP2
P21	VPA/Saline	Regulation of the Epithelial-Mesenchymal Transition Pathway	1.34	0.037		CDH1,TCF4,FZD3,APH1A,WNT9A,TGFB3,JAG1
P21	VPA/Saline	Agranulocyte Adhesion and Diapedesis	1.33	0.0368		MMP3,MYH8,CXCR4,IL1RN,MYH3,IL1R1,MYL3
P21	VPA/Saline	Mitochondrial L-carnitine Shuttle Pathway	1.3	0.0952		SLC27A2,CPT1C

Table S2.3. Canonical Proteomic Pathways differing between Saline and VPA amygdala at P21.

Ratio represents the number of genes altered between treatments within the total number of genes in that pathway, and the p-value is from a right-tailed Fisher exact test of this ratio. The z-score represents the predicted change in gene regulation of that pathway.

Age	*Comparison*	*Ingenuity Canonical Pathways*	*-log(p-value)*	*Ratio*	*z-score*	*Molecules*
P21	VPA/Saline	Actin Cytoskeleton Signaling	5.06	.0345	-0.378	FLNA,PIP5K1C,ARPC2,MRAS,RDX,TMSB10/TMSB4X,ARPC4,NCKAP1
P21	VPA/Saline	Actin Nucleation by ARP-WASP Complex	2.71	.0536		ARPC2,MRAS,ARPC4
P21	VPA/Saline	Adenine and Adenosine Salvage I	1.41	.111		PNP
P21	VPA/Saline	Agrin Interactions at Neuromuscular Junction	1.42	.0286		LAMC1,MRAS
P21	VPA/Saline	Amyotrophic Lateral Sclerosis Signaling	2.70	.0333		GRIN1,CAT,GLUL,SLC1A2
P21	VPA/Saline	Asparagine Degradation I	1.66	.200		ASRGL1
P21	VPA/Saline	Calcium Signaling	1.31	.0161		GRIN1,RYR2,RAP1A
P21	VPA/Saline	Clathrin-mediated Endocytosis Signaling	3.58	.0300		AP2A1,PIP5K1C,ARPC2,AP2B1,AP2A2,ARPC4
P21	VPA/Saline	CTLA4 Signaling in Cytotoxic T Lymphocytes	2.02	.0303		AP2A1,AP2B1,AP2A2
P21	VPA/Saline	Endometrial Cancer Signaling	1.46	.0303		MRAS,CTNNB1
P21	VPA/Saline	Ephrin B Signaling	1.36	.0267		MRAS,CTNNB1
P21	VPA/Saline	Ephrin Receptor Signaling	2.95	.0282	-1.342	GRIN1,ARPC2,MRAS,RAP1A,ARPC4
P21	VPA/Saline	Epithelial Adherens Junction Signaling	4.30	.0405		CDH2,ARPC2,MRAS,CTNNB1,RAP1A,ARPC4
P21	VPA/Saline	fMLP Signaling in Neutrophils	1.72	.0234		ARPC2,MRAS,ARPC4
P21	VPA/Saline	GABA Receptor Signaling	4.75	.0685		AP2A1,AP2B1,MRAS,GABBR1,AP2A2
P21	VPA/Saline	Germ Cell-Sertoli Cell Junction Signaling	1.37	.0170		CDH2,MRAS,CTNNB1
P21	VPA/Saline	Glutamate Receptor Signaling	2.55	.0469		GRIN1,GLUL,SLC1A2
P21	VPA/Saline	Glutamine Biosynthesis I	1.52	.0143		GLUL
P21	VPA/Saline	Glutathione Biosynthesis	1.32	.0909		GSS
P21	VPA/Saline	Glutathione Redox Reactions II	1.46	.125		GLRX
P21	VPA/Saline	Glycolysis I	1.85	.0488		PFKL,Tpi1 (includes others)
P21	VPA/Saline	Guanine and Guanosine Salvage I	1.41	.111		PNP
P21	VPA/Saline	Gα12/13 Signaling	1.69	.0229		CDH2,MRAS,CTNNB1
P21	VPA/Saline	Gαi Signaling	1.77	.0244		MRAS,GABBR1,RAP1A
P21	VPA/Saline	Gαs Signaling	1.86	.0265		RYR2,MRAS,RAP1A
P21	VPA/Saline	HIF1α Signaling	1.82	.0256		MRAS,TCEB1,Ldha/RGD1562690

P21	VPA/Saline	Integrin Signaling	1.79	.0182	-1	ARPC2,MRAS,RAP1A,ARPC4
P21	VPA/Saline	Lipid Antigen Presentation by CD1	3.70	.115		AP2A1,AP2B1,AP2A2
P21	VPA/Saline	Molecular Mechanisms of Cancer	1.57	.0131		RABIF,PA2G4,MRAS,CTNNB1,RAP1A
P21	VPA/Saline	Neuroprotective Role of THOP1 in Alzheimer's Disease	2.96	.0652		PDYN,AGT,APP
P21	VPA/Saline	NRF2-mediated Oxidative Stress Response	1.98	.0207		CAT,MRAS,CLPP,GSTP1
P21	VPA/Saline	Ovarian Cancer Signaling	1.57	.0205		PA2G4,MRAS,CTNNB1
P21	VPA/Saline	Prostate Cancer Signaling	3.03	.0408		PA2G4,MRAS,CTNNB1,GSTP1
P21	VPA/Saline	Protein Kinase A Signaling	1.49	.0124	1	FLNA,RYR2,CTNNB1,RAP1A,SIRPA
P21	VPA/Saline	Protein Ubiquitination Pathway	1.56	.0154		PSMB3,UBE2M,TCEB1,UBE3A
P21	VPA/Saline	Pyruvate Fermentation to Lactate	1.36	.100		Ldha/RGD1562690
P21	VPA/Saline	Rac Signaling	3.72	.0417	-1.342	PIP5K1C,ARPC2,MRAS,ARPC4,NCKAP1
P21	VPA/Saline	Regulation of Actin-based Motility by Rho	2.09	.0323		PIP5K1C,ARPC2,ARPC4
P21	VPA/Saline	Remodeling of Epithelial Adherens Junctions	2.47	.0441		ARPC2,CTNNB1,ARPC4
P21	VPA/Saline	Renal Cell Carcinoma Signaling	2.23	.0361		MRAS,TCEB1,RAP1A
P21	VPA/Saline	RhoA Signaling	2.65	.0323	0	PIP5K1C,ARPC2,RDX,ARPC4
P21	VPA/Saline	RhoGDI Signaling	4.84	.0391	1.134	CDH2,PIP5K1C,ARPC2,MRAS,RDX,ARPC4,ARHGDIB
P21	VPA/Saline	Signaling by Rho Family GTPases	3.08	.0241	-0.816	CDH2,PIP5K1C,ARPC2,MRAS,RDX,ARPC4
P21	VPA/Saline	Sulfate Activation for Sulfonation	1.46	.125		PAPSS1
P21	VPA/Saline	Superoxide Radicals Degradation	1.36	.100		CAT
P21	VPA/Saline	Synaptic Long Term Potentiation	1.72	.0234		GRIN1,MRAS,RAP1A
P21	VPA/Saline	Thyroid Cancer Signaling	1.85	.0488		MRAS,CTNNB1
P21	VPA/Saline	Virus Entry via Endocytic Pathways	3.93	.0463		AP2A1,FLNA,AP2B1,MRAS,AP2A2
P21	VPA/Saline	Xanthine and Xanthosine Salvage	1.46	.125		PNP

Table S2.4. Diseases and Functions RNA Sequencing Categories differing from P10-21 in (A) both VPA and Saline amygdala, (B) exclusively in Saline amygdala or (C) exclusively in VPA amygdala both VPA and Saline amygdala.

The z-score represents the predicted change in gene regulation of that pathway, and the p-value is from a right-tailed Fisher exact test of the ratio of number of genes altered between treatments within the total number of genes in that pathway.

A.

Group	*Comparison*	*Diseases or Functions Annotation*	*p-Value*	*Predicted Activation State*	*Activation z-score*	*# Molecules*
Saline	P10-21	cell cycle progression	3.03E-11	Decreased	-2.119	138
VPA	P10-21		1.56E-13	Decreased	-3.542	158
Saline	P10-21	hepatocellular carcinoma	0.000000818	Decreased	-2.131	104
VPA	P10-21		0.000000227	Decreased	-2.48	116
Saline	P10-21	liver carcinoma	3.51E-09	Decreased	-2.131	501
VPA	P10-21		2.39E-11	Decreased	-2.268	569
Saline	P10-21	neurological signs	4.15E-27	Increased	2.18	149
VPA	P10-21		4.89E-29	Increased	2.573	164

B.

Group	*Comparison*	*Diseases or Functions Annotation*	*p-Value*	*Predicted Activation State*	*Activation z-score*	*# Molecules*
Saline	P10-21	cellular homeostasis	9.57E-08	Increased	3.372	197
Saline	P10-21	transport of inorganic cation	0.00000422	Increased	2.062	51
Saline	P10-21	transport of metal ion	0.0000015	Increased	2.062	49
Saline	P10-21	transport of molecule	1.38E-15	Increased	2.137	237
Saline	P10-21	size of body	0.000019	Increased	2.577	112
Saline	P10-21	ataxia	0.00000114	Decreased	-2.451	45
Saline	P10-21	generalized seizures	0.00000132	Decreased	-2.341	20
Saline	P10-21	Movement Disorders	5.88E-31	Decreased	-3.056	220
Saline	P10-21	seizure disorder	1.33E-20	Decreased	-3.516	108
Saline	P10-21	seizures	1.16E-21	Decreased	-3.44	96
Saline	P10-21	tremor	0.00000218	Decreased	-2.928	28
Saline	P10-21	skin lesion	0.000000467	Decreased	-2.232	531
Saline	P10-21	skin tumor	0.000000228	Decreased	-2.571	528

Saline	P10-21	cell death of cancer cells	5.37E-08	Increased	3.363	64
Saline	P10-21	cell death of osteosarcoma cells	5.12E-09	Increased	4.914	29
Saline	P10-21	cell death of tumor cells	0.000000113	Increased	3.01	72
Saline	P10-21	necrosis of tumor	5.94E-08	Increased	3.01	74
Saline	P10-21	metabolism of membrane lipid derivative	3.5E-11	Increased	2.101	70
Saline	P10-21	synthesis of lipid	0.000000211	Increased	2.625	99
Saline	P10-21	glucose metabolism disorder	0.00000831	Decreased	-2.024	166
Saline	P10-21	sensation	0.00000113	Increased	2.224	51

C.

Group	*Comparison*	*Diseases or Functions Annotation*	*p-Value*	*Predicted Activation State*	*Activation z-score*	*# Molecules*
VPA	P10-21	morbidity or mortality	1.73E-23	Increased	4.529	403
VPA	P10-21	neonatal death	0.0000104	Increased	3.378	69
VPA	P10-21	organismal death	2.27E-24	Increased	4.336	402
VPA	P10-21	cytokinesis	0.000000418	Decreased	-2.052	34
VPA	P10-21	mitosis	2.7E-10	Decreased	-2.782	80
VPA	P10-21	branching of cells	6.88E-13	Decreased	-2.263	83
VPA	P10-21	formation of plasma membrane projections	3.08E-21	Decreased	-2.058	138
VPA	P10-21	generation of cells	1.69E-19	Decreased	-2.001	332
VPA	P10-21	proliferation of cells	1.21E-26	Decreased	-2.222	519
VPA	P10-21	sprouting	1.43E-12	Decreased	-2.341	85
VPA	P10-21	cell movement	3.46E-17	Decreased	-3.031	311
VPA	P10-21	cell movement of neurons	9.12E-11	Decreased	-3.285	55
VPA	P10-21	invasion of tumor cell lines	0.00000247	Decreased	-2.178	75
VPA	P10-21	migration of cells	1.78E-17	Decreased	-2.882	288
VPA	P10-21	congenital anomaly of central nervous system	9.96E-08	Increased	3.484	59
VPA	P10-21	congenital encephalopathy	9.96E-08	Increased	3.484	59
VPA	P10-21	congenital malformation of brain	0.000000178	Increased	3.484	58
VPA	P10-21	death of embryo	0.00000125	Increased	2.177	31
VPA	P10-21	midline defect	2.17E-08	Increased	3.434	53
VPA	P10-21	neural tube defect	0.00000011	Increased	3.235	33
VPA	P10-21	dyskinesia	9.02E-28	Increased	2.194	154
VPA	P10-21	neuromuscular disease	1.92E-27	Increased	2.22	199
VPA	P10-21	astrocytoma	0.000000683	Decreased	-2.2	116

VPA	P10-21	carcinoma in lung	1.47E-10	Decreased	-2.027	250
VPA	P10-21	cell proliferation of tumor cell lines	2.27E-09	Decreased	-3.027	93
VPA	P10-21	cell transformation	0.00000343	Decreased	-2.325	71
VPA	P10-21	epithelial cancer	4.21E-43	Decreased	-3.174	1355
VPA	P10-21	genital tract cancer	1.85E-15	Decreased	-2.207	623
VPA	P10-21	glioblastoma cancer	0.00000235	Decreased	-2	63
VPA	P10-21	glioma cancer	0.00000121	Decreased	-2.186	65
VPA	P10-21	gliomatosis	2.12E-08	Decreased	-2.556	140
VPA	P10-21	growth of carcinoma	0.0000027	Decreased	-2.265	29
VPA	P10-21	growth of malignant tumor	2.18E-08	Decreased	-2.035	77
VPA	P10-21	malignant neoplasm of male genital organ	1.92E-09	Decreased	-2.207	212
VPA	P10-21	malignant solid tumor	2.77E-41	Decreased	-2.006	1413
VPA	P10-21	mammary tumor	2.74E-19	Decreased	-2.105	361
VPA	P10-21	neoplasia of epithelial tissue	1.63E-44	Decreased	-3.091	1363
VPA	P10-21	pelvic cancer	4.59E-15	Decreased	-2.207	655
VPA	P10-21	prostate cancer	1.14E-08	Decreased	-2.207	202
VPA	P10-21	skin cancer	9.03E-08	Decreased	-2.535	583
VPA	P10-21	tumor in nervous system	3.01E-08	Decreased	-2.114	169
VPA	P10-21	tumorigenesis of tissue	9.1E-45	Decreased	-3.131	1377
VPA	P10-21	renal lesion	3.46E-10	Increased	2.182	198
VPA	P10-21	concentration of lipid	3.97E-08	Decreased	-2.258	126
VPA	P10-21	axonogenesis	2.58E-09	Decreased	-2.055	49
VPA	P10-21	branching of neurons	6.05E-14	Decreased	-2.045	72
VPA	P10-21	development of neurons	4.44E-24	Decreased	-3.1	176
VPA	P10-21	migration of neurons	9.8E-10	Decreased	-3.228	52
VPA	P10-21	neuritogenesis	3.07E-21	Decreased	-2.148	135
VPA	P10-21	synaptic transmission of cells	1.59E-09	Increased	2.816	34
VPA	P10-21	synaptic transmission of pyramidal neurons	6.49E-08	Increased	2.16	12

Table S2.5. Diseases and Functions RNA Sequencing Categories differing between Saline and VPA amygdala at (A) P10 and (B) P21. The z-score represents the predicted change in gene regulation of that pathway, and the p-value is from a right-tailed Fisher exact test of the ratio of number of genes altered between treatments within the total number of genes in that pathway.

A.

Age	*Comparion*	*Diseases or Functions Annotation*	*p-Value*	*Predicted Activation State*	*Activation z-score*	*# Molecules*
P10	VPA/Saline	morbidity or mortality	5.93E-12	Decreased	-7.245	142
P10	VPA/Saline	organismal death	9.34E-12	Decreased	-7.268	140
P10	VPA/Saline	differentiation of cells	5.15E-14	Increased	3.329	137
P10	VPA/Saline	generation of cells	6.07E-12	Increased	2.764	123
P10	VPA/Saline	microtubule dynamics	2.4E-09	Increased	2.645	75
P10	VPA/Saline	organization of cytoskeleton	2.75E-09	Increased	2.703	85
P10	VPA/Saline	formation of cellular protrusions	3.48E-09	Increased	3.196	63
P10	VPA/Saline	proliferation of cells	7.67E-09	Increased	2.065	165
P10	VPA/Saline	formation of plasma membrane projections	1.23E-08	Increased	2.796	47
P10	VPA/Saline	quantity of cells	0.00000493	Increased	4.374	91
P10	VPA/Saline	sprouting	0.00000601	Increased	2.505	30
P10	VPA/Saline	endocytosis	0.00000665	Increased	2.041	32
P10	VPA/Saline	cell survival	0.0000119	Increased	3.438	58
P10	VPA/Saline	branching of cells	0.000047	Increased	2.939	27
P10	VPA/Saline	differentiation of central nervous system cells	0.0000681	Increased	2.121	12
P10	VPA/Saline	cell viability	0.000154	Increased	3.153	49
P10	VPA/Saline	differentiation of embryonic tissue	0.000749	Increased	2.169	17
P10	VPA/Saline	quantity of adipose tissue	0.000756	Increased	2.249	18
P10	VPA/Saline	proliferation of blood cells	0.00106	Increased	2.187	44
P10	VPA/Saline	colony formation of cells	0.00115	Increased	2.777	22
P10	VPA/Saline	development of body trunk	5.35E-09	Increased	2.409	78
P10	VPA/Saline	mass of organism	0.00000185	Increased	2.635	27
P10	VPA/Saline	size of body	0.0000136	Increased	4.247	51
P10	VPA/Saline	formation of muscle	0.000112	Increased	2	26
P10	VPA/Saline	phosphorylation of protein	0.000101	Increased	2.154	39
P10	VPA/Saline	organization of cytoplasm	3.48E-09	Increased	2.703	91

P10	VPA/Saline	cell movement	2.33E-12	Increased	2.878	120
P10	VPA/Saline	migration of cells	9.5E-11	Increased	2.916	107
P10	VPA/Saline	transport of molecule	0.000047	Increased	2.977	80
P10	VPA/Saline	urination disorder	0.000884	Decreased	-2.028	19
P10	VPA/Saline	Growth Failure	0.00000628	Decreased	-4.626	40
P10	VPA/Saline	Hypoplasia	0.000399	Decreased	-4.139	30
P10	VPA/Saline	hypoplasia of organ	0.000456	Decreased	-3.862	27
P10	VPA/Saline	dysgenesis	0.000727	Decreased	-4.14	31
P10	VPA/Saline	tremor	0.0000311	Decreased	-3.274	14
P10	VPA/Saline	Movement Disorders	0.000151	Decreased	-4.372	58
P10	VPA/Saline	hyperactive behavior	0.000142	Decreased	-2.902	13
P10	VPA/Saline	cancer	1.75E-16	Increased	2.981	464
P10	VPA/Saline	malignant solid tumor	1.27E-15	Increased	2.129	458
P10	VPA/Saline	tumorigenesis of tissue	7.3E-15	Increased	2.019	442
P10	VPA/Saline	transformation of fibroblast cell lines	0.000301	Increased	2.813	18
P10	VPA/Saline	cell transformation	0.000779	Increased	2.162	26
P10	VPA/Saline	development of neurons	2.26E-09	Increased	3.019	59
P10	VPA/Saline	neuritogenesis	3.46E-08	Increased	2.796	45
P10	VPA/Saline	axonogenesis	0.00000643	Increased	2.578	20
P10	VPA/Saline	differentiation of neuroglia	0.000229	Increased	2.057	14
P10	VPA/Saline	quantity of neurons	0.000369	Increased	2.766	26

B.

Age	*Comparison*	*Diseases or Functions Annotation*	*p-Value*	*Predicted Activation State*	*Activation z-score*	*# Molecules*
P21	VPA/Saline	neuronal cell death	3.21E-08	Decreased	-2.095	41
P21	VPA/Saline	cell death of sympathetic neuron	0.00138	Decreased	-2.217	5
P21	VPA/Saline	morbidity or mortality	0.00000278	Increased	5.105	96
P21	VPA/Saline	organismal death	0.00000279	Increased	5.186	95
P21	VPA/Saline	condensation of cells	0.000173	Decreased	-2	4
P21	VPA/Saline	mineralization of cells	0.0014	Decreased	-2	6
P21	VPA/Saline	size of body	0.000751	Decreased	-3.028	36
P21	VPA/Saline	ion homeostasis of cells	0.000244	Decreased	-2.591	27
P21	VPA/Saline	flux of inorganic cation	0.00126	Decreased	-2.246	15
P21	VPA/Saline	secretion of molecule	0.00000129	Decreased	-2.012	32
P21	VPA/Saline	Growth Failure	0.000997	Increased	2.383	27
P21	VPA/Saline	Infarction	0.00015	Decreased	-2.449	20
P21	VPA/Saline	acute coronary syndrome	0.000379	Decreased	-2	15
P21	VPA/Saline	infarction of heart	0.00147	Decreased	-2.236	13
P21	VPA/Saline	Gastrointestinal Tract Cancer and Tumors	4.24E-16	Increased	2.472	291
P21	VPA/Saline	malignant neoplasm of large intestine	7.06E-15	Increased	2	278
P21	VPA/Saline	colorectal cancer	8.2E-11	Increased	2	204
P21	VPA/Saline	Bleeding	0.000781	Increased	2.211	21
P21	VPA/Saline	memory	0.000636	Increased	2.398	15

Table S2.6. Diseases and Functions Proteomic Categories differing between Saline and VPA amygdala at P21.

The z-score represents the predicted change in gene regulation of that pathway, and the p-value is from a right-tailed Fisher exact test of the ratio of number of genes altered between treatments within the total number of genes in that pathway.

Age	*Comparison*	*Diseases or Functions Annotation*	*p-Value*	*Predicted Activation State*	*Activation z-score*	*# Molecules*
P21	VPA/Saline	formation of cellular protrusions	0.000106	Decreased	-2.453	16
P21	VPA/Saline	migration of fibrosarcoma cell lines	3.36E-08	Decreased	-2.4	6
P21	VPA/Saline	proliferation of cells	0.000898	Decreased	-2.239	35
P21	VPA/Saline	neuritogenesis	0.0000494	Decreased	-2.581	13
P21	VPA/Saline	development of neurons	0.000108	Decreased	-2.282	15

Chapter 3

Increased Stress Vulnerability in Rats Exposed to Valproic Acid Prenatally

Abstract

Incidence of autism spectrum disorder (ASD) is estimated at 1 out of 68 children and of these 40% will also be diagnosed with an anxiety disorder. Therefore, we investigated stress susceptibility in the valproic acid (VPA) model of autism. Rats were exposed *in utero* to VPA or saline and assigned to acute (AS) or chronic (CS) unpredictable shock stress (USS) or non-shock control. Two levels of stress were used as AS and CS evoke distinct responses that could be differentially affected by VPA. Juvenile male and female, VPA and saline, and USS and control rats, were compared in measures of anxiety-like (self-grooming and exploration) and social (preference and novelty) behaviors. We determined that both AS and CS reduced social preference in VPA animals but only VPA+CS animals increased grooming behavior. Adolescent VPA animals did not show a reduction in social behaviors. Our results support the hypothesis that elevated rates of anxiety disorders in individuals with autism may be the result of a greater innate vulnerability to stress.

1. Introduction

The diagnosis of Autism Spectrum Disorder (ASD), has increased dramatically in the last 70 years, with the most recent prevalence estimates in the US as high as 1 in 68 children (Asperger, 1944; Fombonne, 2009; Kanner, 1943; U.S. Department of Health and Human Services, 2014). Notably, anxiety disorders in individuals with ASD are twice as common as in the general population (van Steensel et al., 2011; Vasa and Mazurek, 2015; White et al., 2009). Measures of anxiety correlate with the severity of ASD symptoms, and individuals with ASD and their parents report that symptoms of anxiety have a greater impact on quality of life than those of ASD (Hallett et al., 2013; Ogawa et al., 2016; Ozsivadjian et al., 2012; Wood and Gadow, 2010). In addition, stress is a known precipitating factor for anxiety disorders, particularly chronic stressors that place demands on individuals which can outstrip their adaptive capacity (McEwen, 2004; McEwen et al., 2012; Schmidt et al., 2000). Yet little preclinical research has examined stress susceptibility in models of ASD (Benno et al., 2009; Schneider et al., 2008). The question remains, does ASD increase the vulnerability to stress induced anxiety disorders?

Examining the interaction between stress, anxiety and ASD is difficult in clinical studies because, while the correlation is strong, the direction of cause and effect is unknown. One possibility is that hardships from living with autism are greater than those experienced by typically developing individuals, and this additional stress triggers anxiety disorders (Wood and Gadow, 2010). Alternatively, though anxiety and autism may share a common origin, e.g. a teratogen or genetic inheritance, they are otherwise independent, co-occurring disorders (Kerns and Kendall, 2012). Thus, an individual with autism may have increased vulnerability to anxiety disorders, such that an equivalent level of stress may serve to trigger an anxiety disorder in them, unlike in a typically developing individual who is not otherwise predisposed to anxiousness (Nugent et al., 2011; Schmidt et al., 2000).

To examine stress susceptibility and ASD we turned to a widely used animal model with high face and construct validity: prenatal exposure to valproic acid (VPA, Ardinger et al., 1988; Binkerd et al., 1988; Bromley et al., 2013; Chomiak et al., 2013; Jäger-Roman et al., 1986; Markram et al., 2008; Schneider et al., 2007; Schneider and Przewłocki, 2005; Zaccaria et al., 2010). VPA is the only environmental insult known to increase ASD incidence so substantially (Bromley et al., 2013; Christensen et al., 2013; Ornoy et al., 2015). In rodents, dams dosed with VPA during a sensitive gestational phase recapitulate much of the ASD phenotype, giving birth to pups that are less social and display more repetitive and anxiety-like behaviors (Chomiak et al., 2013; Kim et al., 2011; Markram et al., 2008; Rodier et al., 1997; Schneider and Przewłocki, 2005). Research by our group and others has linked prenatal VPA exposure to disruption of growth and neural development pathways in social brain regions, such as the amygdala (Banerjee et al., 2016; Barrett et al., 2017; Bringas et al., 2013).

Here, we used the prenatal VPA exposure model of ASD to examine the impact of stress on anxiety-like behaviors and social interaction in exposed versus non-exposed rats. Significantly, using a combination of acute and chronic stressors, we explored synergistic effects of stress and prenatal VPA exposure. We report that VPA exposure makes juvenile male rats more vulnerable to chronic stress, increases the display of anxiety-like behaviors, and reduces time spent socializing compared to vehicle or non-stressed controls.

2. Methods

2.1 Animals

Time mated pregnant Sprague-Dawley dams (Charles River, Wilmington, MA) were received at embryonic (E) day 5 or 6 and maintained on a 12:12H light dark cycle with food and water *ad libitum*. Dams were randomly assigned to receive either 500 mg/kg VPA (Sigma-Aldrich, St. Louis, Missouri) in 0.9% saline, or saline alone, delivered to dams daily from E11 to E13 by gavage. At postnatal (P) day 21 pups were weaned and group housed with same sex siblings. All protocols

strictly conformed to the Guidelines for the Care and Use of Laboratory Animals by the National Institute of Health as well as Emory University's Institutional Animal Care and Use Committee.

2.2 Stress paradigm

To determine the effects of acute stress (AS) versus chronic stress (CS) on behavior we employed an unpredictable shock stress (USS) paradigm. Animals were placed in a shock chamber (20 x 20 x 24 cm, San Diego Instruments, San Diego, CA) with a stainless-steel grid floor. Stressed animals received two 8-min blocks of random shock presentations (8 shocks, 1s, 0.5 mA, scrambled foot-shocks with an inter-trial interval of 20-100s) separated by an 8-minute period without shock presentations. Non-shocked controls were placed in the same chamber and then removed after 25 minutes. Chambers were cleaned and disinfected after every animal.

AS: A total of 81 animals from 18 different litters (n = 9 - 10 animals per group) were used for behavioral testing between P31-33. Animals were randomly assigned to undergo a single USS or non-shock control session, followed by a 30-minute habituation to the testing room, and then behavioral testing.

CS: Animals were randomly assigned to undergo 4 days of USS or to non-shock control sessions. Behavioral testing was carried out after an incubation period of 6-8 days. To investigate the effects of CS at different developmental stages, animals underwent CS at juvenile (P21-25; 64 animals from 11 litters; n = 7 - 8 animals per group) or adolescent (P40-44; 97 animals from 31 litters; n = 9 - 14 animals per group) developmental stages (Tseng et al., 2009). Behavioral testing took place at P31-33 for juveniles and between P50-52 for adolescent groups.

2.3 Behavioral testing

Open Field and Social Behavior. Between P31-33 for juvenile CS and AS animals, and between P50-52 for the adolescent CS cohort, social and anxiety-like behaviors were assessed in a variant

of Crawley's three-chamber test (Moy et al., 2004). Behavioral tests consisted of open field (OF), social preference (SP), and social novelty (SN) in a 27.3 x 90.2 x 91.1 cm arena under red light.

Animals were habituated in the empty arena for 5 minutes which also served as a test of exploratory behavior in the OF. A novel, same-age, conspecific probe animal in a wire cage was then placed in one corner of the arena, and an empty cage in the opposite corner. Probe animals were randomly selected, non-drug exposed, unstressed animals that had not previously been used in behavioral studies. The proportion of time spent with the social stimulus versus alone over a 5-minute period was defined as average SP. A second novel, same-age, conspecific probe animal was then placed in the previously empty cage and the proportion of time spent with the new versus the old probe animal over 5 minutes was used as the measure of the test animal's preference for SN. If the animal spent more than 50% of their interaction time in the social zone in SP or with the new stimulus animal in SN they were designated as preferring socializing or novelty, respectively. Time spent exploring or in proximity (<20 cm) to the probe animal or empty cage was measured by automated software (TopScan, Cleversys, Reston, VA).

A trained observer, blind to group membership, manually logged duration of grooming behavior while reviewing recorded tests.

2.4 Statistical analysis

Statistics were analyzed in SPSS v24 (IBM, Chicago, IL) and Prism (GraphPad, La Jolla, CA) with an alpha level of 0.05. Grooming durations were analyzed in a mixed ANOVA with test arena as the within-subjects factor and sex, drug, and treatment as between-subject factors. Exploration time in the center of the OF and indices of SP and SN were analyzed in a three-way ANOVA with sex, drug, and treatment as between-subject effects. Because of an *a priori* assumption that they would respond differently, males and females were then analyzed separately in 2 x 2 ANOVAs (drug x treatment). If the assumption of sphericity was violated in the mixed ANOVA, either the Greenhouse-Geisser (if $\varepsilon < .75$) or the Huynh-Feldt (if $\varepsilon > .75$) correction was used to adjust the

degrees of freedom, ensuring the test would not be too permissive. Data was transformed when the Shapiro-Wilk test indicated that there were deviations from normality or when Levene's Test of Equality of Variance showed groups violated the assumption of homogeneity of variance. To test whether a significantly different proportion of animals displayed a social preference we used Fisher's exact test comparing either non-shock control or USS prenatal VPA with saline exposed animals. When an interaction of either condition (drug or treatment) was detected by the ANOVA, we performed tests of main effects with a Holm-Bonferroni correction for multiple comparisons, separately for males and females; adjusted p values are reported.

3. Results

3.1 Acute Stress

The combination of AS and VPA had little effect on measures of anxiety-like behavior but did have compounding effects on social behavior.

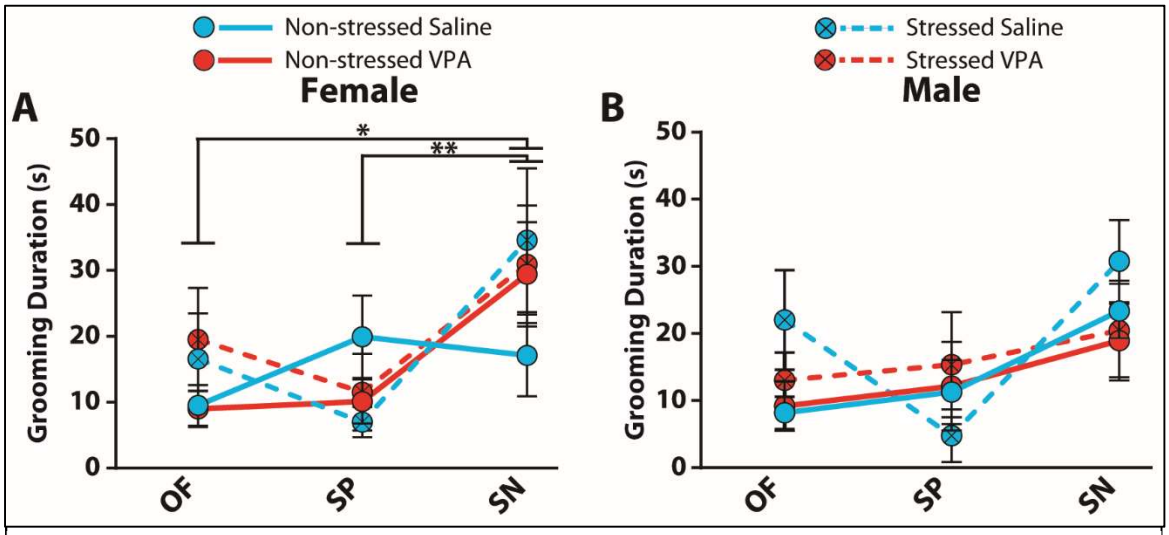

Figure 3.1. Grooming increased in the social novelty arena but not by acute stress. Females (**A**) displayed increased grooming in the SN arena compared to OF or SP, but there were no between group differences. Grooming in males (**B**) did not differ significantly. (n = 8 – 10 for each group *p<.05, **p<.01)

3.1.1 Grooming

Since self-grooming behavior in rodents is increased after a stressful experience, and is associated with activation of sub-cortical networks that control the endocrine responses to stress, we examined

this behavior in our animals (Füzesi et al., 2016). Differences in grooming behavior were not observed between groups in any arena, but a significant increase in grooming was detected over time in females. Overall grooming in females (**Fig. 3.1A**) was higher in the social novelty arena than in either the open field (adjusted p=.016) or the social preference (adjusted p=.007) arena. Grooming behavior was affected by the test arena in both females (**Fig. 3.1A**, mixed ANOVA $F_{1.943,67.998}$=10.077, p<.001), and males (**Fig. 3.1B**, mixed ANOVA $F_{2,66}$=4.920, p=.01), however no other significant interactions were detected in either sex. This suggests that the testing increased anxiety-like behavior and, in females, this was most apparent by the last test of the session. Male grooming was also increased in the SN arena, but after correction this trend was not significant (adjusted p=.131).

As Mauchly's Test of Sphericity showed that the assumption of sphericity had been violated in females (χ^2= 6.328, p=.042) the Huynh-Feldt correction was applied to degrees of freedom in the within-subject tests (ε=.971); there was no violation in males (p=.295). We found no effect of sex in any test arena (p=.693). While there were significant deviations from normality in some of the cells of the ANOVA, square root transformation violated Levene's test of equality of variance for some cells, therefore statistical tests were done on untransformed data. Results were identical using transformed or untransformed data. Post-hoc p-values were adjusted after correction for 9 comparisons, including within subject (OF vs. SP, OF vs. SN, SP vs. SN) and between subject (non-stressed saline vs. non-stressed VPA, acute stress + saline vs. acute stress + VPA), by the Holm-Bonferroni method.

3.1.2 Open Field

No main or interaction effects were detected in the OF in females or males.

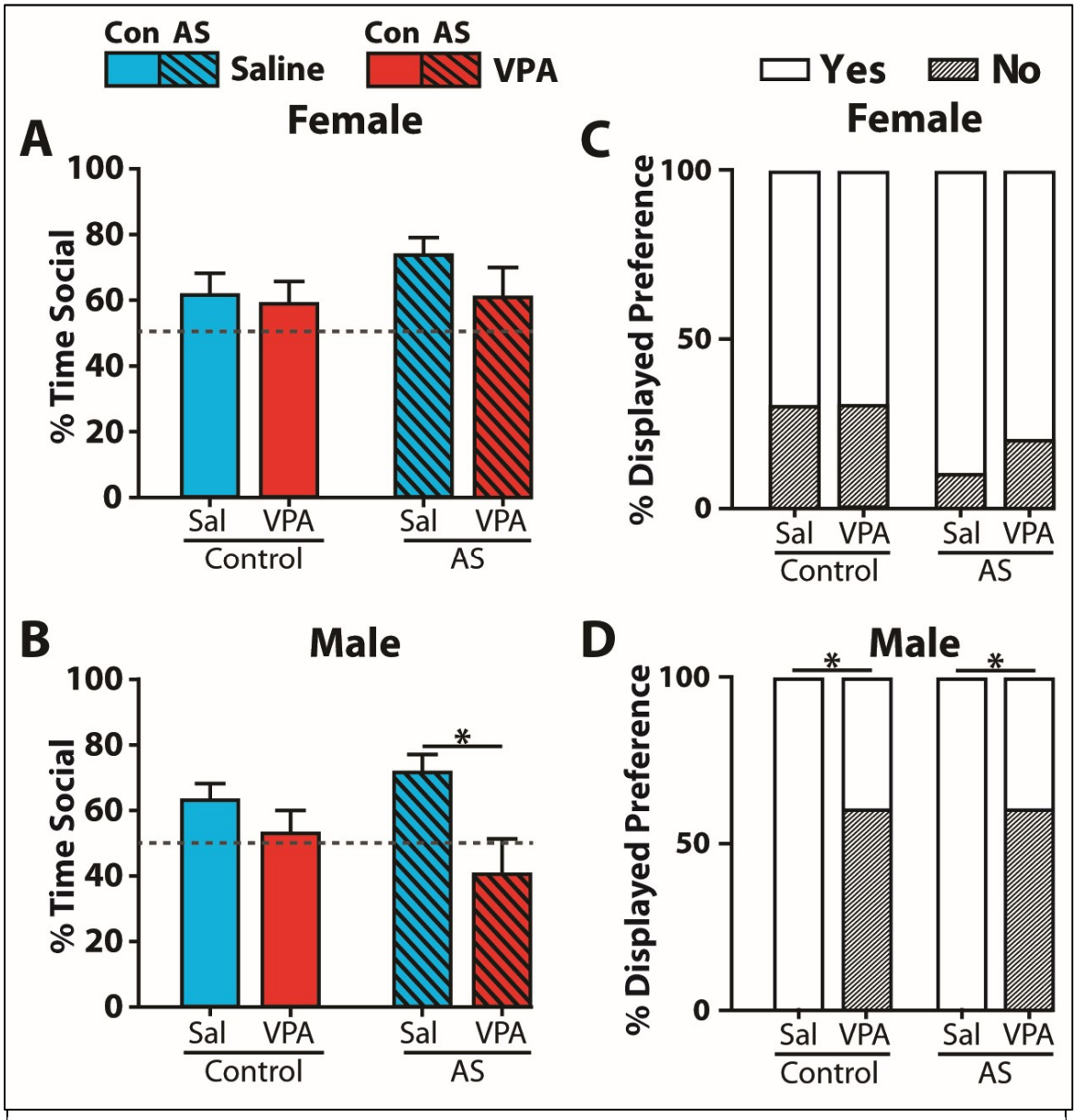

Figure 3.2. Social preference decreased in male VPA rats after acute stress. (**A**) Average preference for social interaction was unchanged in females but (**B**) significantly reduced in VPA+AS males. (**C**) Displayed preference was high in all groups of females but (**D**) was greatly reduced in male VPA exposed rats. Error bars represent the standard error of the mean for that group (n = 8 – 10 for each group *p<.05.

3.1.3 Social Interaction

We have previously shown social deficits in rats prenatally exposed to VPA throughout development (Barrett et al., 2017). Here we extended these observations to juvenile rats after acute

stress. VPA exposed animals had reduced social preference compared to saline, primarily in males which experienced AS (**Fig. 3.2B, D**). While non-shocked males did not differ, there was a significant decrease in average SP in VPA+AS compared to saline+AS (**Fig 3.2B**, main effect: $F_{1,36}$=7.084, adjusted p=.022).

There was no main or interaction effect of sex (p=.147) on average SP. There were no significant main or interactions effects in females (**Fig. 3.2A**). As variances were unequal in males (Levene's p=.004) a reflected square root transformation was applied to the data before statistical analysis. A significant effect of drug was detected in males (**Fig. 3.2B**, 2-way ANOVA $F_{1,36}$ = 5.924, p=.020), but no effect or interaction with treatment. Main effect p values adjusted for 2 comparisons: saline vs. VPA and saline+AS vs. VPA+AS.

Of the 40 female rats in the AS cohort, 31 displayed a preference (>50% time spent with social stimulus animal) irrespective of treatment or drug (**Fig. 3.2C**). There were no statistically significant associations between drug and preference as determined by Fisher's exact test. Though 20/20 saline exposed males displayed a preference, this was true of only 8/20 VPA exposed males and was not affected by AS (**Fig. 3.2D**). Fisher's exact test showed that in males there was a significant association between drug and preference for both non-shock and USS groups (p=.011 adjusted).

In SN after AS there was no effect of sex and no differences in preference for novelty in either males or females in any condition.

3.2 Juvenile Chronic Stress

With chronic stress, as with acute, synergistic effects with VPA were seen, but they were of greater magnitude, included measures of anxiety-like behavior, and were sex specific.

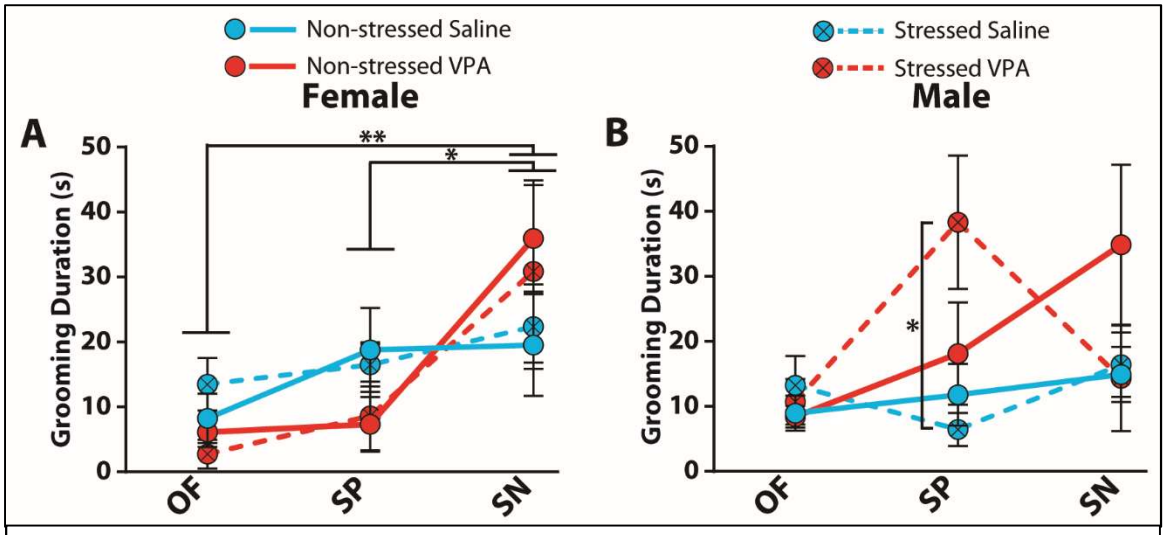

Figure 3.3. Enhanced grooming in juvenile VPA males after chronic stress. (**A**) There were no differences between groups of female rats in the SP or SN arena but there was a non-specific increase in grooming in the SN compared to the OF or SP arena. (**B**) Male grooming was unchanged in the OF but increased in VPA+CS in the SP arena. (n = 7 – 8 for each group, *p<.05, **p<.01).

3.2.1 Grooming

Increased grooming behavior was seen in the chronically stressed, VPA exposed rats, but this increase depended on both the test arena and sex. In females, there was significantly more grooming in the social novelty arena than in the open field (p=.003 adjusted) or social preference (p=.019 adjusted) arena (**Fig. 3.3A**) similar to what was seen in the acute stress cohort (**Fig. 3.1A**). An effect of arena (mixed ANOVA: $F_{1.442,38.934}$=12.988, p <.001) and an interaction of arena x drug (mixed ANOVA: $F_{1.442,38.934}$=4.380, p =.030) was detected. No differences were seen between female groups, in any arena.

Chronic stress in juvenile, male, VPA exposed rats (**Fig. 3.3B**) led to greatly increased grooming in the social preference arena compared to saline+CS (main effect: $F_{1,26}$=9.792, p=.039 adjusted). There was a significant effect of arena (mixed ANOVA: $F_{2,52}$=3.429, p=.040) and an interaction of arena x drug x treatment (mixed ANOVA: $F_{2,52}$=3.802, p=.029). There was a trend towards

increased grooming by non-shocked, VPA males in the SN arena, but after correction for multiple comparisons this was not significant (main effect: $F_{1,26}$=4.654, p=.242 adjusted).

Mauchly's Test of Sphericity revealed the assumption of sphericity was violated in females (χ^2=11.875, p=.002, Greenhouse-Geisser corrected ε=.721) but not males (χ^2=3.749, p=.153). Before separating sexes for analysis, we saw that there was a significant interaction of arena x sex x drug ($F_{1.934,102.527}$=3.114, p = .050). As in the acute stress test, post-hoc p-values were adjusted for 9 comparisons.

3.2.2 Open Field

There was no indication that VPA and/or CS reduced exploratory behavior in the OF.

3.2.3 Social Interaction

Synergistic effects of VPA exposure and chronic stress were apparent in the test of social preference as well as a moderating effect of sex. There was a trend of VPA+CS females (**Fig. 3.4A**) interacting more than saline+CS (main effect: $F_{1,28}$=4.940, p=.068 adjusted). Conversely, VPA+CS males (**Fig. 3.4B**) interacted significantly less than saline+CS (main effect: $F_{1,25}$=8.863, p=.013 adjusted). A

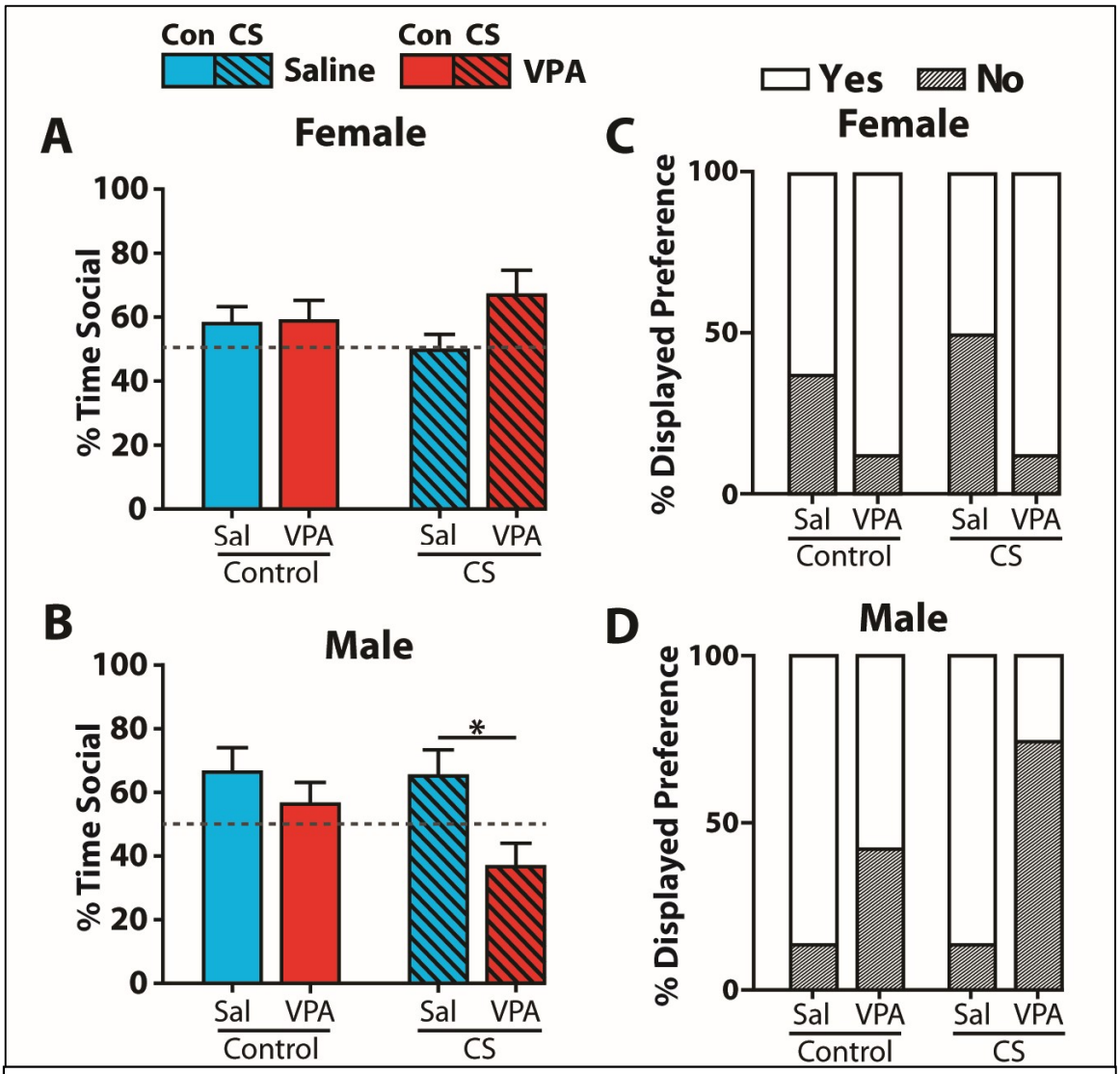

Figure 3.4. Social preference in juvenile male rats significantly reduced by chronic stress. (**A**) Average interaction times were elevated, although not significantly, in VPA+CS exposed females but (**B**) substantially reduced in males. (**C**) The proportion of VPA females that displayed a preference was increased while (**D**) fewer male VPA rats displayed a preference, particularly after CS. Error bars represent the standard error of the mean for that group (n = 7 – 8 for each group, *p<.05).

significant sex x drug (ANOVA: $F_{1,53}$ = 10.538, p=.002) and three-way sex x drug x treatment (ANOVA: $F_{1,53}$= 4.007 p=.050) interactions were detected in SP.

Out of 16 juvenile females prenatally exposed to VPA, 14 displayed a preference compared to 9/16 saline exposed, irrespective of treatment (**Fig. 3.4C**). Fisher's exact test showed no significant differences in either non-shock control or USS groups. Only 2/14 saline males failed to display a preference compared to 3/7 prenatal VPA exposed, non-shock control and 6/8 VPA+CS males (**Fig. 3.4D**). Fisher's exact test suggested that juvenile USS males had a trend towards an association between drug and preference (p=.061, adjusted) while non-shock controls did not differ (p=.4896).

With SN in CS there was a significant effect of sex ($F_{1,54}$ = 4.944, p=.030) and an interaction of sex x drug ($F_{1,54}$ = 5.010, p=.029), but after separating for analysis there were no effects or interactions in either males or females.

3.3 Adolescent Chronic Stress

The age of stress exposure and/or testing greatly affected the results seen in VPA treated rats.

Grooming behavior was not examined in the adolescent cohort.

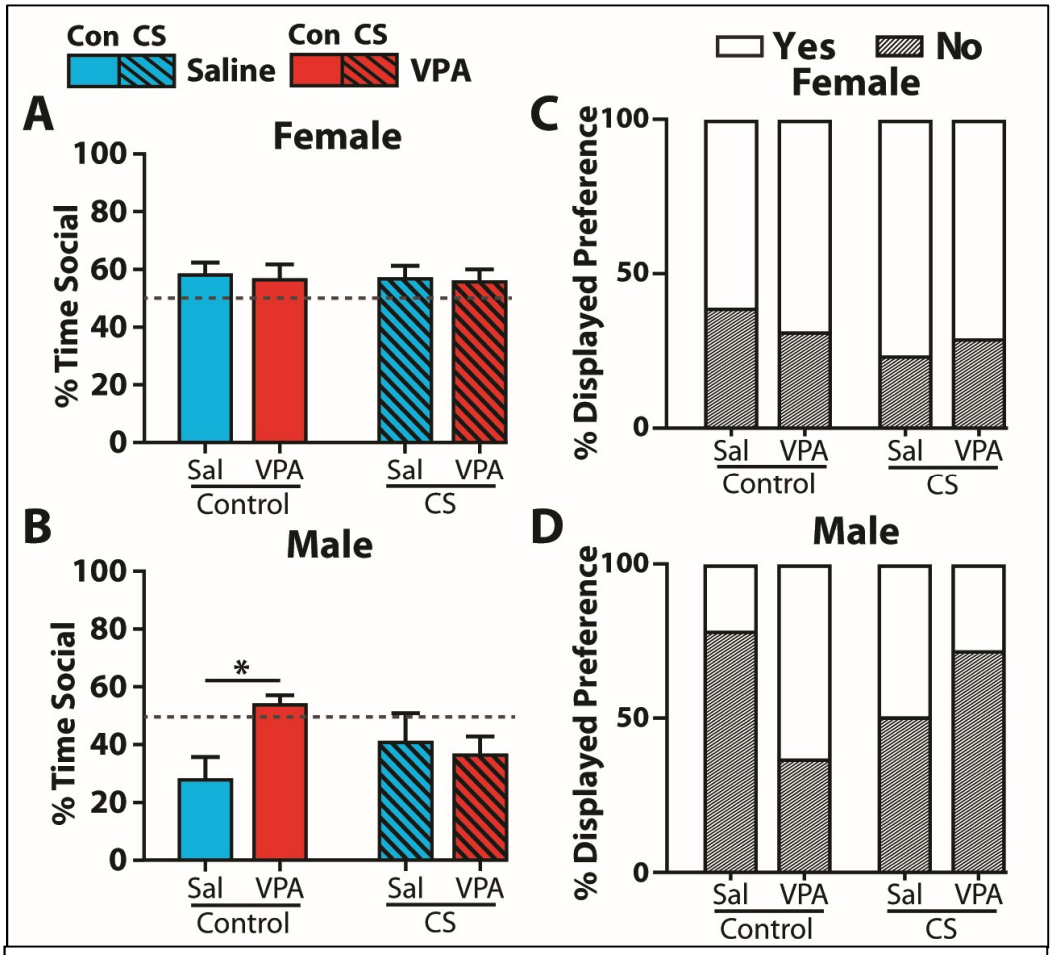

Figure 3.5. Social preference elevated in VPA exposed males after adolescent chronic stress. (**A**) SP was unaltered in the females but (**B**) was increased in non-shocked VPA males, while VPA+CS had equal preference compared to saline+CS. (**C**) The majority of females displayed a social preference regardless of group. (**D**) More adolescent, non-shocked VPA males formed a preference than saline but this was pattern reversed with CS. Error bars represent the standard error of the mean for that group (n = 9 – 14 for each group *p<.05).

3.3.1 Open Field

Females spent more time in the center than males (3-way ANOVA $F_{1,110}$= 10.304, p=.002), but no effects or interactions of drug or treatment were detected after splitting by sex.

3.3.2 Social interaction

Adolescent male VPA exposed rats displayed a greater social preference than saline controls. Across treatments females (**Fig. 3.5A**) did not differ in average social preference. In males (**Fig. 3.5B**) the non-shock control VPA displayed significantly higher SP than did the saline (main effect:

$F_{1,40}$=7.301, p=.020 adjusted). CS increased average SP in saline males while reducing VPA preference to the level of saline (p > .05).

Levene's test showed that variances were unequal in males (p=.009) but transformation failed to correct this. Since ANOVA is relatively robust with regards to unequal variance we proceeded with the analysis (Weerahandi, 1995). A three-way interaction of sex x drug x treatment (ANOVA: $F_{1,89}$= 4.494, p=.037) was detected in SP.

Of the 53 females in the adolescent CS cohort, 37 showed a preference for social interaction across all conditions (**Fig. 3.5C**). Males displayed preference was similar to average SP (**Fig. 3.5D**), with 7/9 control saline failing to show a preference, which increased to 5/10 in animals that experienced CS. In prenatal VPA exposed males, 7/11 non-shocked controls displayed a preference, compared to only 4/14 of those that experienced CS. No associations were significant according to Fisher's exact test.

In the adolescent CS cohort there were no effects or interactions of sex, drug, or treatment on SN.

4. Discussion

We have shown that prenatal VPA exposure and stress interact to potentiate ASD-like behaviors, including both anxiety-like and social deficits. This supports the hypothesis that the increase in anxiety disorders seen in individuals with autism is the result of greater vulnerability to stress. We have also demonstrated that both chronic and acute stress can alter behavior, but only chronic stress does so in a sex-dependent manner, primarily intensifying the male rats' ASD-like behaviors (**Fig. 3.6A-B**). The age of stress exposure is also important, and the critical period for the interaction of USS and VPA is early in postnatal development, before PND 40.

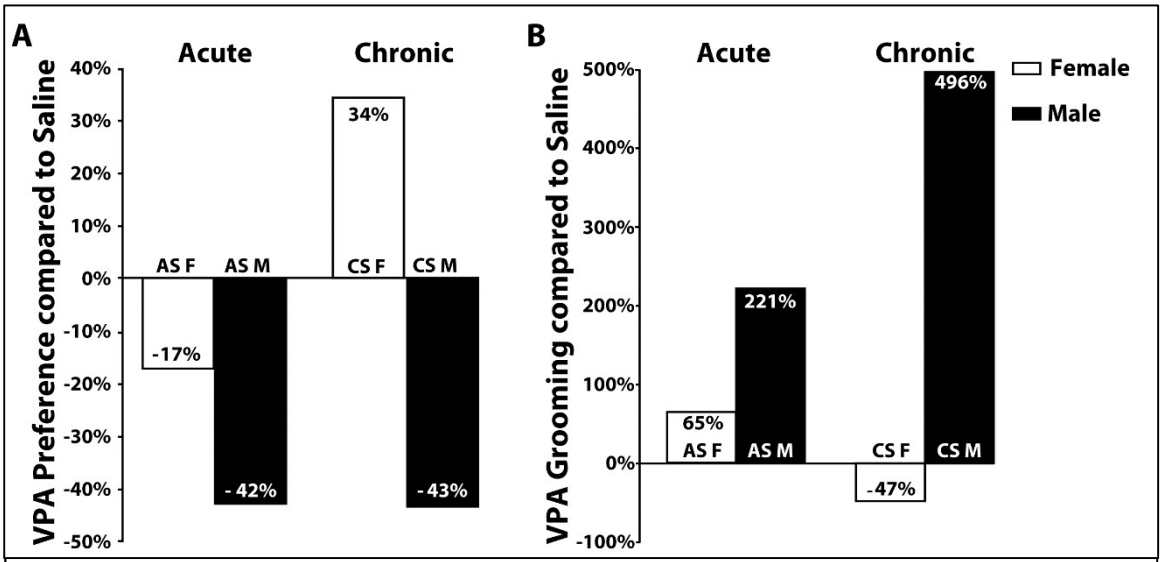

Figure 3.6. USS intensifies autism-like behavior in VPA compared to USS saline. (**A**) Both acute and chronically stressed juvenile VPA males exhibit a similar decrease in social preference, but only CS VPA females have an increased preference. (**B**) Male VPA grooming is substantially enhanced in the social preference arena.

Significantly, anxiety-like and social behavior alterations were dependent not only on VPA exposure but on the sex, age of animals, and severity of stress experienced. Chronic stress increased grooming in the social preference arena in VPA exposed males, relative to saline+CS males (**Fig. 3.3B, 3.6B**). This was not observed in female CS (**Fig. 3.3A, 3.6B**) and neither the male or female acutely stressed rats were significantly different from saline (**Fig. 3.1A-B, 3.6B**). Both acute and juvenile, chronically stressed male VPA rats had reduced preference for social interaction, but female and adolescent VPA+CS rats did not. Our finding that juvenile male VPA+CS rats interact less and females more (**Fig. 3.4A-B, 3.6A**) is reflective of the sex bias in autism; male children are diagnosed with ASD at an approximately 4.5-fold higher rate than females (U.S. Department of Health and Human Services, 2014; Werling and Geschwind, 2013a, 2013b). This is also consistent with the sex bias towards males in FVS-linked autism (Bromley et al., 2013; Christensen et al., 2013). There are many possible sources of this bias including underdiagnoses in girls, but our findings suggest there is also additional vulnerability in males (Beery and Zucker, 2011; Giarelli et al., 2010; Lai et al., 2013; Zuloaga et al., 2014). Our model's vulnerability to stress is evident in measures of anxiety-like and social behavior, and has implications for future clinical studies.

4.1 Anxiety

The increase in grooming during exposure to social stimuli reflects increased stress susceptibility in the juvenile, VPA exposed, chronically stressed males (**Fig. 3.3B, 3.6B**). Heightened grooming was not seen in acute stress animals (**Fig. 3.1A-B, 3.6B)**, which suggests that this behavior is not as labile as the preference for social interaction. This is unlike what was observed after AS by Füzesi et al., (2016) but their mice only exhibited increased grooming in their home cages, when placed in a novel environment, such as our behavior testing arena, grooming was decreased.

One interesting observation was that grooming behavior was substantially increased in the juvenile VPA+CS males, but only in the social preference arena (**Fig. 3.4B, 3.6B**). This may indicate that self-grooming successfully reduced their anxiety, since grooming was not increased during the social novelty phase of testing. The non-shocked male VPA, as well as the juvenile CS females and female AS rats (**Fig. 3.1A, 3.3A-B**), all displayed heightened grooming in this final phase of testing. This suggests a nonspecific increase in anxiety-like behavior resulting from extended separation from their home cage, and that the tendency to express this behavior was elevated in VPA+CS males before testing began.

We considered excessive grooming in our animals (**Fig. 3.1A-B, 3.3A-B**) a sign of anxiety, but it may more properly model repetitive behavior (Benno et al., 2009; Carter et al., 2011; Kalueff and Tuohimaa, 2005; Servadio et al., 2015; Wurzman et al., 2015). Whether grooming represents anxiety or repetitive behavior could be a distinction without a difference; repetitive behaviors in children with autism are enhanced by stress and anxiety, and may serve as a physical outlet for emotions that are difficult or impossible for them to express verbally (Bitsika et al., 2015; Ozsivadjian etal., 2012).

4.2 Social Behavior

Average social preference was reduced in the male VPA+AS rats (**Fig. 3.2B**) but it was only in the CS animals that sex played a moderating role in the response to stress (**Fig. 3.4A-B**). In the juvenile

CS cohort, compared to their saline counterparts, female VPA+CS rats interacted more (though this comparison was not significant after Bonferroni correction), and males significantly less (**Fig. 3.6A**). It is notable that, despite the difficulty of detecting interaction effects with small group sizes, a full three-way interaction of sex x drug x treatment was observed (Tordjman et al., 2014). A three-way interaction was also seen in the adolescent CS cohort (**Fig. 3.5A-B**), although the effects of sex, VPA and USS were very different than in juveniles.

Dichotomizing preference emphasized the social deficits of the VPA model, but only in males. In the acutely stressed cohort, most females (**Fig. 3.2C**) displayed a preference regardless of condition, while VPA males (**Fig. 3.2D**) were significantly less likely to show a preference and this was not affected by stress. Juvenile, chronically stressed, female rats (**Fig. 3.4C**) were prosocial; males exposed to both VPA and CS (**Fig. 3.4D**) were less so. In the adolescent CS cohort (**Fig. 3.5C-D**) this differential response to stress was not evident; non-shock and USS animals no longer differed significantly from each other. Overall these results are analogous to what was seen analyzing average social preference.

This is the first study to examine the interaction of sex and chronic or acute stress on these behaviors in an animal model of autism. The behavioral alterations Schaafsma et al. (2017) observed were most pronounced in *Cntnap2* knock-out, stressed, male mice, but their environmental stress was delivered via maternal immune activation, not directly to the test subjects. Benno et al. (2009) saw an enhanced response to acute stress in the inbred BTBR mouse model of ASD, with elevated corticosterone levels and less exploratory behavior in the elevated plus maze but they only examined male mice, did not see changes in grooming, and did not examine social behavior. While Schneider et al. (2008) included both sexes and AS in his study of behavioral and immunological differences in VPA rats, the only outcome measure after stress was plasma corticosterone levels, not behavior.

Significant differences were seen in comparisons between males and females, VPA and saline and USS and control, across both social and anxiety-like behaviors. Future studies will examine how the underlying neurobiology differs between these groups and how that correlates with their altered behaviors, improving our understanding of ASD and expediting the discovery of more effective therapies and treatments for co-occurring anxiety.

4.3 Human Implications

Our model of autism uses VPA, a histone deacetylase inhibitor, to disrupt the expression of a wide number of genes at a critical stage of brain development, equivalent to the first trimester in humans, when the neural tube closes (Eikel et al., 2006; Kim et al., 2011; Roullet et al., 2013). The use of this agent to create autism-like behaviors must be reconciled with the finding that autism has very high heritability (Hallmayer et al., 2011; Tordjman et al., 2007). But because this model is not the product of a single gene mutation it is in some ways more representative of the many individual's whose autism is not associated with any known monogenic cause. Kosmicki et al. (2017) reported that autism is associated with higher rates of de novo protein truncating mutations, and that more severe symptoms of autism and intellectual disability are associated with the highest rates of mutations. Whether this increase in mutations in ASD is also associated with increased anxiety is not yet known.

Stress can be either beneficial (eustress) or harmful (distress) and what is positive and motivating for one individual can be debilitating for another; the effect of stress is dependent on the interpretation of the experience (Le Fevre et al., 2006; McEwen et al., 2012; Mineka and Oehlberg, 2008; Selye, 1955). Responses to stress differ in terms of the demand they place on the homeostasis of the organism, acute stressors are more likely to elicit an adaptive response, while chronic stressors are more disruptive (Monroe, 2008). One explanation for the increased rate of anxiety disorders in individuals with autism is that unpredictable social encounters and omnipresent

noxious stimuli (changing schedules, red cars, labels on clothes, etc.) results in more distress in their lives and fuel for their anxiety (Wood and Gadow, 2010).

Alternatively, though an individual with ASD may have galvanizing experiences unlike those of a typically developing individual, the total amount of distress they experience could be the same but their diathesis greater (Kerns and Kendall, 2012; Mineka and Oehlberg, 2008; Schmidt et al., 2000). Separating these two hypotheses in humans is difficult and if we are to find therapies and treatments for those with co-occurring anxiety disorders and autism, an understanding of the underlying neurobiology is necessary. For that reason, we decided to go beyond measuring baseline anxiety to explore stress susceptibility in the prenatal VPA exposure model of autism (Bambini-Junior et al., 2011; Bromley et al., 2013; Favre et al., 2013; Mabunga et al., 2015; Markram et al., 2008; Roullet et al., 2013). Our results support the innate vulnerability hypothesis, as the same magnitude of USS served to alter behavior in VPA exposed, but not saline control rats.

This research is important because while the social deficits of autism are a hallmark of the disorder, for individuals with autism and their families dealing with the social dimension is often easier than the anxiety (Corbett et al., 2009; Ozsivadjian et al., 2012). There is substantial evidence for overlap of autism and anxiety disorders, and the challenge of separating the effects of both is only beginning to be addressed (Hallett et al., 2013; Kerns and Kendall, 2012; Ogawa et al., 2016; Spiker et al., 2011; Wood and Gadow, 2010). Through developing this important but underrecognized element of autism in a pre-clinical setting we can speed the discovery of new insights and treatments to reduce the burden of anxiety in individuals with autism.

As initially proposed in **Chapter 1**, and further explored in **Chapter 2**, though the expression of autism is unique to every individual, there are common biological pathways that are likely disrupted across many, if not most, of those with the disorder. In this chapter, we explored anxiety-like and social behaviors in the VPA model of autism, two domains that are highly associated with both autism and the amygdala. In **Chapter 2** we found that by the time of weaning the growth pattern

of the amygdala has been significantly altered. Here we confirmed that this has predisposed the male, juvenile, VPA exposed rat to more extreme and disruptive patterns of behavior after stress. Given the heterogeneity of ASD these finding cannot be presumed to apply to every individual with autism but they nevertheless provide a foundation for the final chapter of this dissertation: Conclusion and Future Directions in the Study of Anxiety and Autism.

Chapter 4

Conclusion and Future Directions in the Study of Anxiety and Autism

In the preceding chapters we have explored how the disruption of developmental pathways from prenatal VPA exposure models autism spectrum disorder, alters the amygdala, and predisposes anxiety-like behavior. In **Chapter 2** we compared early life behavior and, with next generation sequencing, a broad swath of genes active in postnatal amygdala development in both typically developing and VPA exposed rats. In **Chapter 3** we saw how this disruption set the stage for additional dysfunction as a result of stress exposure and how the nature of these impairments was dependent on characteristics like sex, age, and whether the stress was acute or chronic. Future research should explore in detail how environmental experience can modify the neurobiology of the amygdala and whether it is possible to intervene and prevent the synergistic effects of stress and VPA exposure. New molecular tools could be used to pinpoint alterations in circuitry and disrupted molecular pathways at a cellular level. Detailed examination of other behaviors and biomarkers associated with autism could serve to differentiate the population and permit precision medicine. Ultimately, this work has the potential to lead to new and improved therapeutic interventions that might help the millions of individuals with autism and their families find optimal outcomes.

Troublingly, ASD is an immense and expanding problem that extends beyond the directly affected individuals to the whole of society. The economic impact in the US alone for the cost of services, productivity lost, and other factors related to ASD is estimated at $250 billion per year (Buescher et al., 2014). This analysis does not include non-monetary costs like the lifelong psychological burden of coping with a disabling disorder. In addition, the tools available for mitigating autism's impact are inadequate to the task; it is estimated that 50% of individuals with ASD who receive treatment experience no benefit from that intervention (Stahmer et al., 2010). Much of the difficulty lies with diagnoses, not in establishing whether or not some individual falls on the spectrum, but what that placement signifies from a research perspective. In many ways the label of ASD is

"ultimately a convenient fiction from the biological perspective" (Müller and Amaral, 2017) critical for securing support and resources for the client, but too imprecise for rigorous inquiry.

Several initiatives are aimed at improving the current situation. One, as described in **Chapter 1**, is the development of RDoC to better define functional units of analysis that are disrupted in ASD. A corollary to the RDoC initiative is establishing a more accurate baseline of what constitutes 'normal'. To that end the NIH is beginning a program, All of Us, that aims to enroll 1 million individuals for a detailed analysis of individual genetics, environments and outcomes with the explicit goal of advancing precision medicine (Precision Medicine Initiative (PMI) Working Group, 2015). Another is the Autism Sequencing Consortium which seeks to leverage next generation sequencing, similar to the technology we used in **Chapter 2**, in cohorts of tens of thousands to uncover more about the genetic inheritance and expression that instantiates ASD (Buxbaum et al., 2012). This is necessary to address the heterogeneity of ASD, as only very large sample sizes are adequately powered to identify subgroups, though advances in data science may also help in this process (Carmona et al., 2015; Lavrac, 2013). But an alternative approach that can be applied to more fundamental questions of how the social brain does or does not function is available: animal models of ASD. These can provide mechanistic insight, test potential therapies, and allow a degree of resolution that would be extraordinarily difficult to match if research were restricted to human subjects.

One of the more interesting implications of the RNA and protein sequencing work done in **Chapter 2** is that the effects of VPA on gene expression in the amygdala are simultaneously stochastic and ordered. Stochastic in the sense that the exact genes altered by VPA by may differ from one rat to another, but at the same time these alterations are consistently impacting a select number of pathways: first an increase in nervous system development and cellular growth, followed by a decrease in the same (**Fig. 2.5A**). The behavioral outcome is consistent with a collective liability as well, as seen in **Chapter 3**. The effects of VPA exposure appear to diminish over time, as

adaptive strategies are learned perhaps, but a stressful experience reveals them again (**Fig. 3.6A-B**) particularly when that stress is chronic. This is a pattern that is reflective of what is observed clinically, as ASD becomes apparent early in life, with consistent behavioral, communication, and socialization deficits. Following an intervention, especially while the child is young, some recovery is possible, but vulnerability remains.

In **Chapter 2** we saw that VPA exposed pups exhibited both substantial communication deficits (**Fig 2.1F-K**), as well as alterations in social behavior (**Fig. 2.1P,Q**), and that these changes in behavior occurred in parallel to changes in amygdala molecular pathways (**Fig. 2.2, 2.3, 2.4**). The two timepoints chosen, P10 and P21, are good starting points to trace the alteration of these pathways, but future studies should sample more deeply, including into embryonic stages, as did a recent effort to sequence a transcriptomic atlas of the non-human primate developing brain (Bakken et al., 2016). An improved understanding of altered embryogenesis as a result of VPA exposure would be an excellent initial step, and the next could be linking these changes to the phenotypic diversity and associated biomarkers of the model.

In our studies we have typically separated animals that were to be used for biochemistry from those to be used for behavior. This has the advantage of ensuring that the gene expression differences we observed would be an accurate reflection of the developmental alterations over time without added confounds. The downside is that we were unable to correlate individual behaviors with gene expression or other biomarkers. One exception is the work reported in **Chapter 3**. Within a half hour of concluding testing in the social arena, animals were sacrificed and tissue collected from several brain regions, including the amygdala. This could be used for RNAseq or proteomics, to extend the results from **Chapter 2**. In addition, a regression analysis could be performed to see which genetic pathways are implicated as mediators of the social deficits and anxiety-like behavior that we observed.

Using the gene expression of the amygdala to predict whether a social preference would be formed, or anxiety-like behavior exhibited, would be an impressive advancement in the study of ASD, but new technologies are making investigations with an even higher resolution possible. Sakurai et al. (2016) developed a technique for capturing activated neuronal ensembles (CANE) that allowed them to pinpoint a group of neurons in the hypothalamus responsible for social-fear behavior, enabling optogenetic modulation of behavior, as well as transsynaptic pathway tracing. CANE could be used to investigate social preference formation and expression, and to see how such a network is disrupted in form and function by VPA. It would also allow the investigation of the electrophysiological characteristics of individual cells in the network and for single cell RNAseq to reveal what makes those cells distinct (Wills et al., 2013).

In addition to changes in gene expression, another biomarker that could be fruitful to investigate, and that has been associated with ASD for many years, is peripheral hyperserotonemia. Other groups have reported this result after prenatal VPA exposure, though this is not universal (Lim et al., 2017; Narita et al., 2002). Our preliminary attempts to measure 5HT in blood serum (collected at the same time as brain tissue after behavior in the experiments of **Chapter 3**) seemed promising, but because of technical issues we were unable to accurately analyze these samples. It remains to be seen if hyperserotonemia has predictive utility for behavior or response to treatment.

A related issue that should be considered in the use of animal models generally, and with VPA specifically, is standardization between laboratories, a long-standing concern (Crabbe et al., 1999). Discordant findings have been reported in previous studies showing deficits apparent only in some measures of sociability, such as reduced pins or play behavior or even enhanced social behaviors (Cohen et al., 2013; Markram et al., 2008; Schneider and Przewłocki, 2005; Štefánik et al., 2015). Sources of variability such as the age of the test and probe animals, or inter-litter variation likely play a role (Lazic & Essioux, 2013 but see Giovanoli & Meyer, 2013). In addition, if the amount of incidental stress that animals experience differs between investigators, it would follow that social

preference would be altered as well. Though temporary arousal from acute stress did not significantly increase grooming behavior, there was an impact on average social preference in males (**Fig. 3.2B 3.6A-B**), illustrating that it is relatively easy to affect these behaviors. Moreover, without exogenous stress juvenile male VPA and Saline differed little in percent of time spent in social proximity (**Fig. 3.2B, 3.4B**) and indeed, average preference was increased relative to saline in older animals (**Fig. 3.5B**). However, dividing animals by whether they display a preference revealed stronger effects of prenatal drug exposure and reduced differences attributable to unpredictable shock stress in the juvenile animals (**Fig. 3.2C-D, 3.4C-D**). A final consideration is group size, if non-shocked animals from both acute and juvenile chronic stress cohorts are combined, then a small, nearly significant decrease is seen in the average social preference of male VPA rats (Saline 65.4%, VPA 54.5%, $F_{1,67}$=3.89, p=.053), underlining the importance of appropriately powered experimental design.

One of the most consistent findings in the demographics of ASD is a male sex bias of about 4.5:1 (Christensen et al., 2016). Conversely, the reverse is seen in rates of internalizing disorders, with diagnoses of anxiety and depression twice as common in females (Lewinsohn et al., 1998). Why then did we not see more evidence of anxiety symptoms in our female rats, as was reported in a small clinical population of high functioning children and adolescents with ASD (Solomon et al., 2012)? One possibility is that the dose of VPA was insufficient to induce an autism-like phenotype. This may be due to increased liability in the male brain, or to protective effects in the female (Baron-Cohen et al., 2011). Theoretically, a higher dose of VPA, or a longer exposure period, could engender autism- and anxiety-like symptoms in female rats, but increased fetal toxicity would likely make this approach untenable (Khera, 1992). As an alternative, we could adopt the approach of Yochum et al. (2010) who combined a transgenic mouse with postnatal VPA exposure and found that females had elevated hippocampal apoptosis compared to both wildtype and saline exposed mice. Considering that women with autism typically have more severe symptoms, and more of their

male relatives have increased symptoms of autism than vice versa, a greater genetic liability than men with ASD is likely (Jacquemont et al., 2014; Szatmari et al., 2012). Therefore, a combination of transgenic predisposition and environmental trigger, in the form of a normally sub-threshold VPA exposure for instance, might be the most valid model of ASD in females.

It is also possible that autism-like symptoms would be apparent with careful behavioral analyses of female VPA exposed rats in a group housing setting, similar to what was seen in an ethological study of 'camouflaged' play behavior in elementary school girls with ASD (Dean et al., 2016). It is important to continue, as the NIH has directed, including both male and female subjects in animal research (Clayton and Collins, 2014). Not only would it be impossible to investigate the roots of ASD's sex bias otherwise, but there are many concerns unique to women with ASD that should be examined (Beggiato et al., 2017; Rynkiewicz et al., 2016; Watkins et al., 2014).

The overall question of how the amygdala is affected by, and/or contributing to, the phenotype of autism, especially what role it plays in the predisposition to anxiety disorders, is only beginning to be addressed. In children, some portion of amygdala volume variability is due to comorbid anxiety (Juranek et al., 2006); it may be that the apparent regression in amygdala volume in adults with ASD is related to this (e.g. increased glucocorticoids leading to more pruning and apoptosis) but the actual etiology is unknown. With the prenatal VPA exposure model this is a question that can be answered. We have shown in **Chapter 2** that from P10 to P21 there is a shift from overactivation of growth pathways to inhibition of them in the VPA amygdala. The result of this divergence, as seen in **Chapter 3**, is a rat that is sensitive to stress and predisposed to anxiety-like behavior. With insight into how stress exposure has changed the neurobiology of the VPA amygdala it could be possible to identify new, druggable, therapeutic targets. By leveraging VPA with appropriately powered clinical studies, the promise of precision medicine can be realized and perhaps, the burden of autism alleviated.

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