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The Role Of The Nmda Receptor In Shaping Cortical Activity During Development

Jacob Michael Kayle Lister

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Currently, it is estimated that neuropsychiatric disorders will affect 20-25% of humans in their lifetime. These disorders are a major cause of mortality, suffering, and economic cost to society. Within this broad class, neurodevelopmental disorders (NDDs), including intellectual disability, autism spectrum disorder, and schizophrenia, are estimated to affect 2-5% percent of the world population. Devastatingly, we lack fundamental treatments for NDDs, which have proved some of the most imposing disorders to understand scientifically. The challenge is twofold: first, NDDs affect the most complex aspects of human cognition; second, pathogenesis begins early in neural circuit development, but we lack predictive biomarkers before overt behavioral deficits are apparent. Although we have identified many genes associated with these disorders, how underlying genetic disruptions lead to pathological neural network development and function remains unclear. The overarching framework of this dissertation is that all NPDs are disorders of distributed neural networks, and pathophysiology must be understood at this level to effectively intervene clinically.

The cerebral cortex is necessary for complex human capacities, and cortical dysfunction is hypothesized to be central to the pathophysiology of NDDs. NMDA glutamate receptors (NMDARs) are important for the development of local circuit features in the cortex, for normal neurocognitive function, and are strongly implicated in NDDs. However, the role of NMDARs in the development of the large-scale cortical network dynamics that underly higher cognition has
not been well examined. Understanding the role of NMDARs at this network level is critical because large-scale “functional connectivity” patterns are thought to be hallmarks of normal cortical function, are hypothesized to be disrupted in NDDs, and may be detectable in humans using non-invasive neuroimaging or electrophysiology.

In the studies presented in this dissertation, I (in collaboration and with the support of my colleagues) tested the role of the NMDAR in shaping large-scale cortical network organization using in vivo widefield imaging of whole cortex spontaneous activity in developing mice. I found that NMDAR function in the lineage that includes cortical excitatory neurons and glia, specifically, was critical for the elaboration of normal cortical activity patterns and dynamic network organization. In the first set of experiments, NMDARs were deleted in glutamatergic excitatory neurons (Emx1-cre\(^{+/\text{WT}}\)/Grin1\(^{f/f}\); referred to as EX-NMDAR KO mice) or GABAergic inhibitory neurons (Nkx2.1\(^{+/\text{WT}}\)/Grin1\(^{f/f}\); referred to as IN-NMDAR KO mice). The developing cortex normally exhibits a diverse range of spatio-temporal patterns, reflecting the emergence of functionally associated sub-networks. In EX-NMDAR KO mice, normal patterns of spontaneous activity were severely disrupted and reduced to a nearly one-dimensional dynamic space dominated by large, cortex-wide events. Interestingly, in IN-NMDAR KO mice, the structure and complexity of spontaneous activity was largely normal.

In the next set of experiments, I tested the role of extrinsic thalamic neurotransmission on cortical activity during development. Deleting the vesicular glutamate transporter from thalamic neurons while leaving cortical NMDARs intact (Sert-\text{Cre}\(^{+/\text{WT}}\), vglut1\(^{-/-}\), vglut2\(^{f/f}\); referred to as TH-VG KO mice) led to a shift in cortical activity patterns towards large domains of activity, reminiscent of patterns observed in EX-NMDAR KO mice. This manipulation also reduced the dimensionality of cortical activity, though not as severally as in EX-NMDAR KO mice. In a final set of experiments, I tested cortical activity in three established mouse models of mono-genetic
causes of NDDs in humans: the FMR1-KO mouse based on Fragile X Syndrome, the CNTNAP2-KO mouse, and the TS2-neo mouse based on Timothy Syndrome. In all three of these mouse models, I found that large-scale cortical activity patterns were largely normal, but there was a statistically significant shift towards reduced cortex-wide synchrony and increased dimensionality of spontaneous activity, which may be consistent with the dysconnectivity hypothesis of autism.

In a final set of experiments, we tested our hypothesis, based on past literature and our results in EX-NMDAR KO and TH-VG KO mice, that the disruptions in cortical activity was predominantly due to the developmental loss of activity-dependent wiring of circuits. To test the developmental versus acute role of NMDAR function in shaping cortical activity, I blocked NMDAR pharmacologically in wild-type mice. I found that acute NMDAR blockade shifted cortical activity to a restricted dynamic space similar to that observed in EX-NMDAR KO mice and more extreme than that observed in TH-VG KO mice. These results strongly reinforce the critical role of NMDAR in shaping cortical activity during development, and suggest that a substantial component of that may be through NMDAR’s role in synaptic transmission and moment to moment cortex-wide circuit function.

Overall, these results provide critical insight into the role of NMDARs and the glutamatergic system in cortical network functional organization during development. Specifically, they highlight the essential role of NMDARs in excitatory neurons on the functional connectivity and dynamic repertoire of the cortical network during development. These results make novel contribution to our understanding of how NMDARs may contribute to the pathophysiology of NDDs. Specifically, they contribute powerful new insight into to a critical
mechanistic question about the cell-specific role of NMDARs in the pathophysiology of schizophrenia and the mechanisms of NMDAR antagonists, which have transformed psychiatry recently due to their rapid-acting anti-depressant and anti-suicidal properties. Furthermore, they identify a patterns of large-scale network dysfunction that might be detectable in humans using noninvasive functional imaging or electrophysiology.
The Role of the NMDA Receptor in Shaping Cortical Activity during Development

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Presented to the Faculty of the Graduate School
Of
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Doctor of Philosophy

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Thesis Overview

The experiments presented in this dissertation are motivated by the challenge of treating neuropsychiatric developmental disorders (NDDs). The guiding framework is that neuropsychiatric disorders generally, and NDDs, specifically are diseases driven by dysfunction of distributed neural networks. Therefore, in order to effectively intervene and improve outcomes for people suffering from such disorders, we must understand the pathogenesis at the level of neural circuit organization and function. The experimental approach in these studies uses molecular genetic tools that have been established in the mouse model system to 1) manipulate genes implicated in human neuropsychiatric disorders and 2) readout neural activity directly to deepen our understanding of normal and abnormal neural network development. First, we use targeted genetic deletion of genes or cellular processes implicated in neurodevelopmental disorders. Second, we use genetically encoded calcium indicators to read-out neural activity \textit{in vivo} with high spatial and temporal resolution. Using these approaches, we demonstrate that disrupting critical components of neural transmission and synaptic plasticity implicated in NDDs drastically alters spontaneous neural network dynamics across the cerebral cortex during critical periods of development.

Chapter 1 introduces the relevant background for this study. First, I discuss NDDs in terms of clinical presentation, phenotype, genetics, and circuit level dysfunction. Second, I discuss normal development and function of the nervous system with a focus on the cerebral cortex, the region most responsible for human cognitive capacities and a central site of pathology in neuropsychiatric disorders. Third, I discuss the role of the NMDA receptor, a fundamental
Chapter 2 discusses the experimental and analytic methods used in this study. The main experimental elements are targeted genetic deletions, fluorescent calcium indicators for widefield imaging, electrophysiology for recording cortical local field potential, and pharmacology for blocking NMDAR mediated neural transmission. The main analytic elements are the principles of time-series correlation and functional connectivity, linear and non-linear dimensionality reduction approaches, and analysis of electrophysiology.

Chapter 3 presents the core study of my thesis which tests the role of the NMDA receptor in the large scale cortical functional organization during critical periods of post-natal development. I use targeted deletion to test NMDAR function in the two major cell types of the cerebral cortex – the excitatory neurons that release the neurotransmitter Glutamate and the inhibitory neurons that release the neurotransmitter GABA. I find that NMDAR deletion in the excitatory population, specifically, causes a major disruption of spontaneous activity: normal patterns and functional networks fail to emerge and cortical activity shifts to a reduced dynamic repertoire dominated by large scale cortical events.

Chapter 4 discusses a complimentary study that leaves cortical NMDAR intact and tests the role of thalamic glutamatergic neurotransmission in the development of normal patterns of cortical activity. Here, the vesicular glutamate transporter (both VG1 and VG2) is deleted in the primary
input region to the cortex – the thalamus. Blocking thalamic glutamatergic neurotransmission disrupts cortical activity patterns along a similar though less extreme abnormal trajectory as NMDAR knockout mice. In TH-VG KO mice, spontaneous cortical activity shifts towards large, cortex wide events and reduced dimensionality, but retains a more normal dynamic structure than does cortical deletion of the NMDA receptor.

Chapter 5 discusses a final set of studies in three other mouse models based on mono-genetic causes of human NDDs: the Fmr1 KO mouse model based on Fragile X syndrome, the TS2-neo mouse model based on Timothy Syndrome, and the Cntnap2 KO mouse based on a novel genetic cause of intellectual disability (ID), autism spectrum disorder (ASD), and attention deficit hyperactivity disorder (ADHD) identified in an affected family. Although, cortical spontaneous activity patterns and network organization is grossly normal in these mice, there are trends towards an reduced cortex wide synchrony and increased dimensionality, which may be consistent with the dysconnectivity hypothesis of ASD.

Chapter 6 discusses the general implications of this work and proposed next steps for understanding principals of cortical development and pathophysiology of neurodevelopmental disorders. I propose next steps to further elaborate the mechanisms of NMDA function cortical development and discuss pharmacologic experiments that shed further light on NMDARs role in shaping cortical activity during these time periods. These results strongly reinforce the critical role of NMDAR in shaping cortical activity during development, but suggest that a substantial component of that may be through NMDAR role in synaptic transmission and moment to
moment cortex-wide circuit function. I discuss the implication of all these studies for our understanding of NDDs and neuropsychiatric disorders, broadly. Finally, I discuss how these studies and the framework of this research relates to some of the most exciting areas of psychiatric diagnoses and treatment, specifically neural network-based assessment and circuit targeted neuro-modulatory approaches.
Chapter 1: Introduction

Neurodevelopmental disorders

Neuro-developmental disorders (NDDs), including intellectual disability (ID), autism spectrum disorder (ASD), and schizophrenia (SZ), are estimated to affect 2-5% percent of the world population (Fombonne et al. 2009; Maulik et al. 2011; Whiteford et al. 2013; Chong et al. 2016; Chiarotti et al. 2020). Devastatingly, we lack fundamental treatments for these disorders, and they have proved some of the most imposing disorders to understand scientifically. The challenge posed is twofold: first, NDDs affect the most complex aspects of human cognition; second, we lack definitive biomarkers and diagnosis is based on behavior, but pathogenesis likely begins early in neural circuit development before overt behavioral deficits are apparent. Although we have identified many genes associated with these disorders, how underlying genetic disruptions lead to pathological neural network development remains unclear (Hamdan et al. 2011; Iossifov et al. 2014; Ronemus et al. 2014; Fitzgerald et al. 2015; Geschwind et al. 2015; Sanders et al. 2015; Avramopoulos et al. 2018; Li et al. 2018). It is critical to understand when and how neural circuits become disrupted in order to guide interventions that can improve neurodevelopmental outcomes.

The current concepts of “neurodevelopmental” and “neuropsychiatric” disorders have arisen over the course of the 20th century as the frameworks of modern psychology and psychiatry have evolved. From a clinical standpoint, the framework for defining this family of disorders is articulated in The Diagnostic and Statistical Manual on Mental Disorders (DSMV), which guides diagnosis in psychiatry and medicine. The challenge of treating the following (distinct but related) neurodevelopmental disorders were the primary motivators for the work in this thesis:
intellectual disability (ID), autism spectrum disorders (ASD), and schizophrenia (SZ). While these disorders can have distinctive phenotypic features and developmental time-courses, my organizational framework is that they are all disorders of cognition caused by abnormal cortical development and function that manifests at the level of neural network activity patterns and organization.

“Intellectual disability” was known as “mental retardation” both clinically and in the press until DSM V (2013), with the name change reflecting the appreciation of the stigma associated with the earlier terminology. Intellectual disability (intellectual developmental disorder) is characterized by deficits in general mental abilities, such as reasoning, problem solving, planning, abstract thinking, judgment, academic learning, and learning from experience. The deficits result in impairments of adaptive functioning, such that the individual fails to meet standards of personal independence and social responsibility in one or more aspects of daily life, including communication, social participation, academic or occupational functioning, and personal independence at home or in community settings (DSM V). There are three criteria that must be met. “1) Deficits in intellectual functions, such as reasoning, problem solving, planning, abstract thinking, judgment, academic learning, and learning from experience, confirmed by both clinical assessment and individualized, standardized intelligence testing. 2) Deficits in adaptive functioning that result in failure to meet developmental and sociocultural standards for personal independence and social responsibility. Without ongoing support, the adaptive deficits limit functioning in one or more activities of daily life, such as communication, social participation, and independent living, across multiple environments, such as home, school, work, and community. 3) Onset of intellectual and adaptive deficits during the developmental period” (DSM V). There
are no universal biomarkers associated with ID, and diagnosis is based on clinical evaluation of individual functioning.

The term “autism”, which literally means “without speech”, was first used in its modern sense by Leo Kanner (Kanner L 1943), to describe a population of patients who often had not developed active speech and exhibited almost no social interaction. He described them as displaying "autistic aloneness" and "insistence on sameness". The modern conception of ASD retains the recognition of these aspects through two diagnostic categories: “1) Persistent deficits in social communication and social interaction across multiple contexts, as manifested by deficits in social-emotional reciprocity, deficits in nonverbal communicative behaviors used for social interaction, deficits in developing, maintaining, and understanding relationships, and 2) restricted, repetitive patterns of behavior, interests, or activities manifested in stereotyped or repetitive motor movements, use of objects, or speech, insistence on sameness, inflexible adherence to routines, or ritualized patterns of verbal or nonverbal behavior, highly restricted, fixated interests that are abnormal in intensity or focus, hyper- or hypo-reactivity to sensory input or unusual interest in sensory aspects of the environment” (DSM V).

During the 20th century, we have achieved important success in the areas of ID and ASD through several main routes. First, we have identified important treatable causes of developmental disorders: toxins, including Lead (Pb), mercury (Hg), and arsenic (As) (McDermott et al. 2011) and inborn disorders of metabolism (Saudubray et al. 2018). Second, through greater public awareness, early clinical diagnosis at one to two years of age, and intense early behavioral interventions, we have massively improved the trajectory of those with ASD, such that a large number develop language and can participate in society to varying degrees (Reichow et al. 2012).
Nonetheless, numerous children never achieve the capacity to live independently and a great many never enjoy the full enjoyment of a social existence such as romantic partnership (see the recent Netflix show, “Love on the spectrum,” for a contemporary popular portrayal of this aspect). Although genetic causes can be identified for some cases of both ID and ASD, a large portion of both remain idiopathic. Equally important, clinically definable features of ID and ASD do not emerge until 1-2 years of age, but pathological neurodevelopment begins long before during early post-natal and perhaps if prenatal periods. Thus, alternative approaches to predict and assess neurodevelopment are needed.

An alternative approach to using genetics to predict and define neurodevelopment is using readouts of neural activity. Pioneering work in this area has shown promise. As early as three months of age, EEG data has a strong correlation with autism diagnostic scores at three years of age (Bosl et al., 2018). EEG abnormalities in preterm infants within the first post-natal month predict developmental delay (Hayashi-Kurahashi et al., 2012). EEG data also shows that infants at high familial risk for autism have reduced frontal power at three months across frequency bands, and this was associated with poorer expressive language at twelve months (Levin et al., 2017). An EEG study found that hyper-connectivity in infants, especially over frontal and central areas, is associated with later ASD diagnosis (Orekhova et al., 2014). An additional promising biomarker has been the use of eye-tracking, with failure of infants to appropriately track the eyes of adults predictive of later autism diagnoses (Jones and Klin 2013).

Although less relevant from the standpoint of early prediction and intervention, work in adults with ASDs has led to one prominent theory that autism can be characterized as a “developmental disconnection syndrome,” as there is a preponderance of evidence towards
increased connectivity within local areas but reduced connectivity between distributed areas. (Geshwind and Levitt 2007). Studies have found a complex pattern of increases and decreases in functional connectivity observed in both task related and spontaneous activity (Geshwind and Levitt 2007; Bednarz et al. 2018). A disturbance of connection in higher order social regions is among the most well replicated finding (Philip et al. 2012). But the major challenge remains identifying predictive biomarkers during early development when the possibility for altering neurodevelopmental trajectory is greatest.

In DSM V, the “Schizophrenic disorders” are a separate classification from the “Neurodevelopmental disorders”, though many clinicians and researchers view SZ as a neurodevelopmental disorder (Weinberger DR 1987, Lewis and Levitt 2002) and genetic risk genes for ID, ASD, SZ (as well as other neuropsychiatric disorders) are highly overlapping (Ronemus et al 2014; Fitzgerald et al 2015). SZ is defined by abnormalities in one or more of the following five domains: “1) delusions, 2) hallucinations, 3) disorganized thinking (speech), 4) grossly disorganized or abnormal motor behavior (including catatonia), and 5) negative symptoms” (DSM V). While anti-psychotic medications and social intervention can help people live with less severe “positive symptoms”, we still lack fundamental disease altering treatments for the disorder and the majority of patients never recover from first episode psychosis sufficiently to lead independent lives (Chong et al 2016). Importantly, we have no effective treatments for the cognitive symptoms, which are the most important predictor of recovery (Tripathi et al. 2018). While the definitive symptoms typically appear in the late adolescent period, there are multiple streams of evidence that disrupted neurodevelopmental processes starting during the pre-natal period can influence pathophysiology. Prenatal infectious exposures
and maternal starvation both increase risk for schizophrenia (Brown et al. 2010). Children born into immigrant populations in urban environments have increased rates and adolescent cannabis use also increases risk (van Os et al. 2010). Additionally, motor coordination may be impaired during early childhood (Walker and Lewine 1990), and deficits in cognition are also evident during childhood in many cases (Carrion et al. 2016).

A critical tool to understand how this complex process goes wrong is the use of animal models to understand brain development and function. Animal models allow for experimental access and control of neural processes in ways not possible in humans. In particular, by disrupting genes and molecules linked to human NDDs, we can gain unique access into how these components function in normal development and mechanisms by which they drive abnormal development and function in ways that manifest as NDDs. A number of validated mouse models have been generated and the approach offers enormous promise, as the mouse combines the most advanced molecular genetic toolbox in a mammalian model system. Some mice based on human disease include NMDA KO mice as models of schizophrenia (Mohn et al. 1999; Belforte et al. 2010; reviewed in Lee and Zhou 2019), FMR1 KO based on fragile x syndrome (Bakker et al. 1994), and CNTNAP2 KO mice based on a gene identified that causes ID, ASD, ADHD, and epilepsy in humans with high penetrance (Peñagarikano et al. 2011). Many studies in these mice have demonstrated behavioral disruptions reminiscent of these illnesses, offering validity to the relevance of these models. Furthermore, alterations in neural activity, synaptic plasticity, and circuit connectivity offering insight into possible mechanisms and treatment targets.
The Cerebral Cortex

The cerebral cortex is the most highly evolved region in mammalian evolution and is critical for higher order complex processing that integrates the sensory, motor, and cognitive domains (Rakic R 2002). The cortex mediates complex sensory, motor, and cognitive functions through precise wiring of local circuits that subserve specific functions and appropriate coordination of neural activity across distributed networks (Harris and Mrsic-Flogel 2013). It has a three-dimensional organization, with the surface thought of as the “x-y” plane and that is organized into regions and areas based on the types information that is processed – e.g. visual, somatosensory, and motor. Perpendicular to the surface of the brain can be thought of as the “z-dimension” and is formed by a six layered ‘microcolumn’, which is thought of as the functional unit of the neocortex (Mountcastle V 1995). All regions of the neocortex have characteristic input and output layers: with inputs from the primary sensory thalamic nuclei synapsing primarily in layer IV, outputs to sub-cortical and cortical regions from layer V, and cortical-cortical connection from layer II/III. There are ‘primary’ sensory areas which receive direct input via the thalamus from that sensory periphery and then feed this information onward to higher order regions which integrate information from different modalities which is believed to allow the emergence of complex and higher order representation, thoughts, and action. This organization combines high specialization with high integration, which is believed to underlie the remarkable diversity, flexibility, adaptability of human cognition (Mountcastle V 1995).

The recognition of the importance and organization of the cerebral cortex began in the 1860s – 1880s through a combination of tragic accidents and focused studies. Famous patients such Phineas Gage showed that damage to small portions of the cortex, in his case the left frontal
lobes, could lead to profound changes in personality and behavior. Studies based on electrical stimulation by David Ferrier and Victor Horsely in the 1800s and independent investigations by Charles Sherrington, Harvey Cushing, and Wilder Penfield, demonstrated that different regions of the cortex were associated with distinct functions (Silverstain et al. 2015). For instance, stimulation of the somatosensory cortex could cause tingling in distinct areas of the body, stimulation of motor cortex could drive brief movements, and stimulation in visual cortex could cause visual phenomena. Koblinian Broadman distinguished areas of the neocortex based on cytoarchitectural features and divided them into 52 regions, which have continual organizational value to this day (Broadman K 1909, Zilles et al. 2018).

Studies by Vernon Mountcastle in the 1950s helped reveal that the cortex was organized as “columns” with vertical patches of neurons that were functionally connected with similar sensory response properties (Mountcastle VB 1957). Single unit recording studies in cat visual cortex by Hubel and Wiesel in the 1970s demonstrated cells that responded to varying features of a visual stimulus, including the location in the visual field (retinotopy), orientation, and direction of static and moving experimentally presented patterns. They went on to demonstrate that visual cortex is organized into maps, such as those for retinotopy or ocular dominance, and that development of these maps is activity dependent during critical periods in development (Hubel and Wiesel 1970s, Shatz and Stryker 1988). From a developmental standpoint, work by Pasko Rakic and colleagues beginning in the 1960s and 1970s helped explained the developmental origins of the cortex, showing that cortical progenitors migrate along radial glia and to settle as neurons and glia in an inside out fashion, generating the layered cortex and functional microcolumns previously observed. (Rakic P 1972, 1988, 2009).
The cortex is not only important for processing sensory input and generating motor output. Lesion studies in the 1930s by Carlyle Jacobsen in 1937 demonstrated the unique role of the prefrontal cortex in keeping information online during tasks, or “working memory” (Jacobsen CF 1935, 1936, Arnsten AFT 2013). Monkeys with lesions to the dorsolateral prefrontal cortex (dLPFC) region could solve complex spatial problems if the information was present in the environment but had no capacity to remember the location of a piece of food for even a few seconds if it was hidden under a cup. Beginning in the 1970s, Fuster and Patricia Goldman-Rakic elucidated the cellular basis of working memory, by identifying distinct classes of cells in the dLPFC that persisted in firing during the delay period of the task. Work by Goldman-Rakic located spatial working memory in the caudal two-thirds of the principal sulcus, hinting to parallel organization in processing streams in PFC between spatial and visual features just as in upstream visual areas (Goldman and Rosvold 1970, Goldman et al. 1971). In the 1970s, Fuster made the first recordings of cells in dLPFC while monkeys performed a spatial working memory task (Fuster JM and Alexander GE, 1971). Multiple cell types were observed during these tasks: cells that responded to the sensory cue (Cue cells), cells that responded in anticipation of and during the motor response (Response cells), and, most interestingly, cells that maintained persistent firing across the delay period (Delay cells). Goldman-Rakic built on this work by developing the visually guided saccade task and showed that delay cell firing was spatially tuned: i.e. a given cell will persist in firing for a specific retinotopic location and this retinotopy is stable over trials and days. Furthermore, the cell’s firing was inhibited when a cue opposite to its preferred orientation is being held in mind, suggesting an inhibitory component to the circuit basis of working memory.
Across species, excitatory neurons that release the neurotransmitter, glutamate, are generally thought to constitute 70-80% of all neocortical neurons and make both local and long range connections (DeFelipe and Fariñas 1992; Markram et al. 2004, Rakic P 2002; Petersen and Crochet 2013). Inhibitory neurons that release the neurotransmitter, GABA, make up 20-30% of neocortical neurons but are critically important for excitatory/inhibitory balance, information processing, and flexible behavior (Le Magueresse and Monyer 2013). Importantly, however, there are both species- and region-specific differences that may be important for distinctive regional functions (e.g. working memory functions mediated by layer II/III in prefrontal regions) and differences between species (e.g. primates versus rodents) that may be important for the substantially increased cognitive capacities in humans and primates. For instance, a recent study showed that the ratio of interneuron classes in the primate cortex shifted from sensory to executive regions, which may contribute to the unique working memory functions mediated by frontal areas (Torres-Gomez et al. 2020). From a functional perspective, this study found a decrease in the ratio of narrow spiking (putative parvalbumin positive and calbindin positive interneurons) to broad spiking (putative calretinin positive interneurons or pyramidal neurons) from medial temporal cortex (MT) to medial superior temporal cortex (MST) to lateral prefrontal cortex (PFC). Labeling revealed that the proportion of PV in cortical layers II/III decreased and the proportion of CR interneurons (putatively analogous to VIP interneurons in mice) increased from MT/MST to PFC. Moreover, based on both physiological recordings and immunostaining, they found the proportion of pyramidal cells to be closer to 50% in layer II/III in all three areas. The authors note that this finding does not necessarily contradict previous studies but rather reflects an increase in the proportion of interneurons in superficial layers.
In mice, GABAergic inhibitory neurons derived from the medial ganglionic eminence are characterized as the parvalbumin-expressing interneurons (PV) and somatostatin-expressing interneurons (SOM). PV cells constitute ~40% of all cortical interneurons and ~30% of neurons in layer II/III, are fast-spiking and target the somata and axon initial segments of pyramidal neurons to exert powerful inhibition and control their output. SOM cells constitute ~30% of all cortical interneurons and ~20% in layer II/III, and target the dendritic compartments of pyramidal neurons to control their input. GABAergic inhibitory neurons derived from the caudal ganglionic eminence express 5HT3AR and constitute ~30% of all interneurons in the cortex and ~50% in layer II/III. Within this class are vaso-intestinal peptide-expressing interneurons (VIP), which constitute ~2% of interneurons and reside mainly in layer II/III. VIP interneurons target SOM interneurons for disinhibition and receive long-range neuro-modulatory input and have been strongly implicated in state dependent transitions and could thus be posited to exert powerful control of an entire circuit (Petersen and Crochet 2013; Wamsley and Fishell 2017).

Studies in layer II/III of rodent somatosensory cortex and visual cortex argue that circuits operate with a “sparse code”: despite a dense barrage of similar excitatory input to excitatory cells in a functional column, an individual cell fires sparse action potentials, leading to a wide distribution of firing rates with a long tail, with the majority of neurons firing sparsely and a minority of neurons firing frequently (Jadhav et al. 2009; Petersen and Crochet 2013, Harris and Mrsic-Flogel 2013; Buzsaki and Mizuseki 2014). This distribution is reflected in the difference between the mean to the median firing rate, with one study finding a mean firing rate of 1.7 Hz but a median of 0.2 Hz across neurons in adult layer II/III barrel cortex (Petersen and Crochet 2013). Based on studies in adult cortex, we know that this property is enforced by strong
inhibition (Haider et al. 2013) and this organization may allow for functional sub-networks of strongly connected cells (Ko et al. 2013). Developmentally, this property emerges over the first 1-2 weeks in the rodents (Golshani et al. 2009). In the first week, cortical circuits firing synchronous bursts punctuated by periods of silence. Starting around post-natal day 11-12 (P11-12), an activity independent switch occurs in which the cortex switch from discontinuous bursts of synchronous activity to continuous activity that shifts between more synchronized and desynchronized periods. This precise wiring requires finely tuned anatomical connectivity between excitatory and inhibitory neurons and appropriate functional dynamics, (Colonnese et al. 2010; Colonnese and Khazipov 2010).

Neural activity and activity dependent plasticity play an important role in the refinement of fundamental circuit features in the cortex, even before extensive sensory and behavioral engagement with the environment is apparent (Goodman and Shatz 1993; Katz and Shatz 1994). During ~P6-12 in mice, both excitatory and inhibitory progenitors have entered the cortex and are sending out diffuse projections, that are highly plastic over the next week (Cruz-Martin and Portera-Caillau 2014). During this period, there is a wave of apoptosis as numbers of excitatory and inhibitory cells are established, a process that is neural activity-dependent (Bandler et al. 2017; Wamsley and Fishell 2017). By P11-13 in mouse, a major developmental switch occurs at the levels of gene expression, synapses, circuits, and behavior (Fertuzinhos et al. 2014). These switches include the expression of parvalbumin (PV) in PV+ interneurons (Xu et al. 2008; Bandler et al. 2017), the synaptic strengthening and switch from NR2B to NR2A expression (Williams et al. 1993), the switch in electrophysiological patterns from the immature bursting period driven by “spindle bursts” to the mature period characterized by ongoing internally maintained
spontaneous activity (Colonnese et al. 2010; Colonnese and Khazipov 2010). At the behavioral level, mice open their eyes and begin to explore their environment. Of particular note, these immature cortical activity patterns known are conserved across mammalian evolution. They are the predominant activity pattern in rodent cortex until ~P10-11 and until ~birth in humans, and they appear to play a critical role in establishing circuits before extensive engagement with the external environment (Colonnese et al. 2010; Colonnese and Khazipov 2010).

Neural activity at individual synapses and amongst circuits is the critical foundation of dynamic neural systems. But in order to get from cells and circuits to perceptions, emotions, thoughts, and behaviors, we have to understand how circuits and components distributed through the nervous system interact and coordinate. Large scale functional connectivity patterns are thought to be hallmark of normal cortical function and reflect the underlying functional capacity of complex nervous systems to operate flexibly and adaptively (Mohajarani et al. 2013; Ma et al 2016; Vanni et al. 2017). It is widely believed that these functional networks are disrupted in neuropsychiatric disorders (Geshwind D. 2007; Menon V. 2011; Bednarz and Kana 2018). In humans, use of network dynamics recorded using fMRI or EEG to study and track the development of the nervous system during pre and early post-natal period of brain development, prior to extensive overt behavior, has shown exciting early promise at predicting neurological and behavioral outcomes (Khan et al. 2008; Hayashi-Kurahashi et al. 2012; Haartsen et al. 2019; Pillay et al. 2020).

In mice, previous work recording large-scale brain activity using fluorescent sensors of calcium (Chen et al. 2013; Dana et al. 2014; Madisen et al. 2015) have revealed structured dynamic patterns in adult mouse cortex, with functionally associated brain regions activating and
deactivating together during spontaneous activity (Lu et al. 2012; Mohajarani et al. 2013; Ma et al. 2016; Vanni et al. 2017). Furthermore, these functional networks are evident starting early in post-natal development in the mouse (Khazipov and Luhman 2006; Colonnese and Khazipov 2012; Ackman et al. 2015, Barson, Hamodi et al. 2019). A fundamental outstanding question is to what extent these functional networks are dependent on molecules and processes implicated in human NDDs and to what extent they are dependent on activity dependent developmental processes. Understanding the answer to this question will help us interpret data in humans and guide what level or type of analysis might best be used to discern abnormal network development and predict later disease.

The NMDA Glutamate Receptor

The NMDA receptor (NMDAR) for the neurotransmitter glutamate is a central molecule in normal and abnormal brain development and function. One of the fundamental frameworks of modern neuroscience is that the cellular basis of learning and memory derives from alterations in the strength of neuronal connections as a function of their activity. Before its neurochemical and molecular components were identified, this concept was articulated famously by Donald Hebb in his 1949 book, *The Organization of Behavior*, which laid out the framework for synaptic plasticity, encapsulated by the famous passage: “Let us assume that the persistence or repetition of a reverberatory activity (or "trace") tends to induce lasting cellular changes that add to its stability... When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased” (Hebb D 1949).
In the 1960s and 1970s, studies utilizing the hippocampus identified the process of long-term potentiation (LTP), the experimentally inducible change in synaptic strength, as the mechanism of plasticity and physiological marker of cellular memory. These experiments established that LTP consists of two components: the first consisting of a persistent increase in synaptic strength and the second consisting of an increase in the excitability of the neuron (granule cells of the hippocampus) (Bliss et al., 1973; Bliss and Collingridge 1993). In 1983, Gary Lynch and colleagues found that LTP in CA1 pyramidal cells could be blocked by the intracellular injection of the Ca2+ chelator EGTA, which demonstrated that post-synaptic cell involvement was essential for the induction of LTP (Lynch et al., 1983). Complimenting this finding, Collingridge and colleagues found that the induction but not the expression of LTP was blocked by APV, the antagonist of the NMDAR. Studies in the 1950s had identified glutamate as a major excitatory neurotransmitter and defined two types of receptors, NMDA and non-NMDA based on response to pharmacological agonists (Curtis et al. 1960). Studies went on to demonstrate that unique properties of the NMDAR, including its permeability to calcium and Mg2+ block that requires depolarization to make NMDAR permeable to Ca2+, allow it to serve as the theoretic “Coincident detector” of neural activity and specific cellular mediator of input specificity, cooperativity, and associativity in the hippocampal circuits.

The NMDAR is strongly linked to NDDs genetically, with mutations in the genes coding for NMDAR sub-units tied to a spectrum of NDDs including ID, ASD, SZ, and epilepsy (Watkins JC 1972; Watkins and Evans 1981; XiangWei et al. 2008; Hamdan et al. 2011; Tarabeux et al. 2011; Lee et al. 2015; Lemke et al. 2016). Mutations affecting the obligate NR1 subunit, specifically, causes ID with complete penetrance, and can also cause ASD and epilepsy in many affected
individuals (Lemke et al. 2016). Additionally, in a study of individuals with NDDs, ~7% of those with ASD and ~8% of those with SZ had a mutation in an NMDAR receptor gene (Yu et al. 2018). The high penetrance in NDDs makes NMDAR mutations an ideal general model for NDD pathogenesis. The high prevalence in NDDs makes understanding NMDAR dysfunction directly relevant for a large class of patients.

NMDAR has also been strongly linked to human psychiatric disease pharmacologically. NMDAR antagonists, including phencyclidine and ketamine, were observed in the 1960s and 1970s to induce a psychotic state in humans similar to schizophrenia. Thus, they have subsequently been used in both humans and model organisms as the gold standard to model psychosis. Additionally, it is now appreciated that autoantibodies against the NMDAR lead to a syndrome consisting of psychosis, seizures, memory deficits (as well as central hypoventilation and loss of consciousness if untreated) (Vitaliani et al 2005). The now well described NMDA autoimmune encephalitis and is often associated with a teratoma. Thankfully, even when it is not associated with a tumor, it can often be treated by intravenous immunoglobulin and plasmapheresis (Titulaer et al. 2013, Cellucci et al. 2020). However, the disorder offers further evidence for the role of the NMDAR in neuropsychiatric disease.

Studies in model organisms demonstrate that NMDAR is necessary for normal cortical synaptic plasticity in vitro in the form of both long-term potentiation and long-term depression (Crair and Malenka 1995; Malenka and Feldman 2004; Feldman DE 2012), for the development of refined sensory maps (Cline et al. 1987; Kleinschmidt et al. 1987; Li et al 1994; Iwasato et al. 1997, 2000), and for normal cognitive function (Mohn et al. 1999; Duncan et al. 2004; Milenkovic et al. 2014; Mielnik et al. 2020; Finlay et al. 2015; Lee and Zhou 2019). In mice, whole body
knockout of the obligate NR1 subunit or the NR2B subunit is perinatally lethal (Forrest et al. 1994; Li et al. 1994; Kutsuwada et al. 1996), whereas NR1 and NR2B knock-down mice grow to adulthood but have severe cognitive and behavioral impairments (Mohn et al. 1999; Duncan et al. 2004; Halene et al. 2009; Milenkovic et al. 2014). Targeted NMDAR KO of other channel subunits, in both excitatory and inhibitory neurons, and across cortical and sub-cortical regions leads to a range of physiological, cognitive, and behavioral deficits (McHugh et al., 1996; Tsien et al., 1996; Sprengel et al. 1998; Brigman et al. 2008; Kehrre et al. 2008; Belforte et al. 2010; Badanich et al. 2011; von Engelhardt et al. 2011; Carlen et al. 2012; Rompala et al. 2013; Finlay et al. 2015; Tatard-Leitman et al. 2015; Salmi et al. 2019). Delineating the region and cell specific roles of NMDAR function is therefore an essential task to understanding distinct pathogenesis of NDDs.

Mutations in genes coding for all subunits of the NMDAR have been linked to NDDs and neuro-psychiatric disease broadly, including attention deficit disorder (Kim et al. 2018), manic depressive disorder (Fountoulakis KN 2012), substance abuse disorder (Chen et al. 2018) in addition to autism, schizophrenia and epilepsy spectrum illnesses. It is thought that sub-unit, temporal timing, cell specific contribution, and nature of the mutation all contribute to the varied abnormal trajectories that can result from NMDAR mutations (Endele et al., 2010). Given that genetic insults to NMDARs are linked to cognitive disorders, important next questions for the field to answer are when and how NMDAR are impairment manifests at the global level? To what extent is disfunction cortical or sub-cortical in origin? What cell types are most important? Why can similar mutations in humans lead down different temporally and phenotypically distinct trajectories as in autism, schizophrenia, epilepsy, or attention deficit disorder (ADHD)?
In vivo imaging of whole cortex activity during development

While results from my studies cannot address directly or answer fully all of the questions just raised, I hope that my results do provide several novel and relevant insights. The goals of our studies were as follows: 1) Test how proposed pathological mechanisms of neurodevelopmental disorders impact development of cortical network. 2) Determine extent to which developmental patterns of neural activity are dependent on developmental processes. 3) Identify signatures of abnormal network function that may reflect convergent mechanisms and biomarkers relevant for both further studies of mechanisms and for translational potential targets in humans. We assessed cortex development using widefield imaging of spontaneous activity because we reasoned that this level of analysis is ideal to link defects in individual genes to mammalian behavior across development and across spatial scales. We were guided by approaches established in humans for assessing large scale, system wide neural dynamics, such as functional magnetic imaging (fMRI), but was able to capitalize on the substantial advantages afforded by calcium imaging: cell-restricted expression of the fluorescent indicator and high spatial and temporal resolution.
Chapter 2: Methods

Experimental Preparation

Animal subjects

All mice used in this study were cared for following the Yale Institutional Animal Care and Use Committee (IACUC) and US Department of Health and the Human Services guidelines. Mice were housed on a 12-h light/dark cycle with food and water available *ad libitum*. To record whole cortex activity *in vivo*, we expressed the genetically encoded calcium indicator GCaMP6 under a pan-neuronal promoter (Chen et al. 2013) in excitatory specific NMDAR knockout mice (EX KO mice) or inhibitory specific NMDAR knockout mice (IN KO mice) and their littermate controls (CTL mice). All mice used were generated using lines available from the Jackson Laboratory (Bar Harbor, ME). Emx1-Cre mice (B6.129S2-Emx1<sup>tm1(cre)Kri</sup>/J, JAX stock number 005628) (Gorski et al. 2002) were purchased from the Jackson Laboratory. Nkx2.1-Cre mice (C57BL/6J-Tg(Nkx2-1-cre)2Sand/J, JAX stock number 008661) (Xu et al. 2008) were obtained from Dr. Jessica Cardin. NR1-flox mice (B6.129S4-Grin1<sup>tm2Stl</sup>/J, JAX stock number 005246) (Tsien et al. 1996) were obtained from Dr. Takuji Iwasato. Snap25-GCaMP6s mice (B6.Cg-Snap25<sup>tm3.1Hz</sup>/J, JAX stock number 025111) (Chen et al. 2013) were obtained from Dr. Hongkui Zeng.

**WT Mice:** All experimental mice lines in these studies were bred with Snap25-GCaMP6s mice, which express GCaMP6-s (G6s) under the pan-neuronal Snap25 promoter in a heterozygous fashion. In the NMDAR study, EX KO, IN KO, and CTL mice were either Snap25-G6s<sup>+/-</sup> or Snap25-G6s<sup>-/-</sup>. In mice that were Snap25-G6s<sup>-/-</sup>, we achieved pan-neuronal G6s expression using viral mediated expression delivered via transverse sinus injections (see below).
**EX KO mice:** To generate NMDAR knockout in cortical glutamatergic excitatory neurons, we crossed Emx1-Cre mice (which express Cre recombinase in excitatory neurons and glia of the neocortex, hippocampus, and olfactory bulb) to Grin1\(^{fl/fl}\) mice (Grin1 codes for the obligate NR1 sub-unit of the NMDAR). Offspring heterozygous for Emx1-Cre and homozygous for Grin1-flox (Emx1-cre\(^{+/wt}\),Grin1\(^{fl/fl}\) mice) are excitatory neuron specific NMDAR knockout mice and are referred to as **EX KO mice** (see *Introduction, Chapter 3, and Discussion* for reference to previous studies using similar mouse lines).

**IN KO mice:** To generate NMDAR knockout in cortical GABAergic inhibitory neurons, we crossed Nkx2.1-Cre mice (which express Cre recombinase in neurons derived from the medial ganglionic eminence including parvalbumin positive and somatostatin positive GABAergic interneurons) to Grin1\(^{fl/fl}\) mice. Offspring heterozygous for Nkx2.1-Cre and homozygous for Grin1-flox (Nkx2.1-Cre\(^{+/wt}\), Grin1\(^{fl/fl}\) mice) are inhibitory neuron specific NMDAR knockout mice and are referred to as **IN KO mice** (see *Introduction and Discussion* sections for reference to previous studies using similar mouse lines).

**CTL mice:** Littermates with all other possible genotypes were used as controls and are referred to as **CTL mice**. These included 1) Emx1-Cre\(^{+/wt}\), Grin1\(^{fl/wt}\) mice; 2) Nkx2.1-Cre\(^{+/wt}\), Grin1\(^{fl/wt}\) mice; 3) Cre\(^{-}\), Grin1\(^{fl/fl}\) mice; 4) Cre\(^{-}\), Grin1\(^{fl/wt}\) mice.

**TH-VG KO mice:** We maintained and bred Sert-cre\(^{+/−}\) (B6.129(Cg)-Slc6a4tm1(cre)Xz/J, SERT-cre, Jackson stock number 014554, Zhuang et al 2005) Vglut1\(^{+/−}\); Vglut2\(^{fl/+}\), Sert-
Cre/−;Vglut1/−;Vglut2f/−, and Vglut1+/−;Vglut2f/− mice on a mixed C57B/6J and CD1 background and used Vglut1−/−;Vglut2f/− mice as littermate controls for TH-VG KO (Sert-Cre+/−;vglut1−/−;vglut2f/f, and Sert-Cre+/−;Vglut1−/−;Vglut2f/f). Vglut1−/− (Wojcik et al. 2004) and TH-VG KO mice (Li et al. 2013) have previously been described. See Chapter 4 for further discussion of previous studies using similar mouse lines.

**FMR1 KO mice:** B6.129P2-Fmr1tm1Cgr/J (Fmr1 KO, Jackson stock number 003025, Bakker et al. 1994) mice were bred with Snap-25-G6 mice and are referred to as FMR1 KO mice. See Chapter 5 for further discussion of previous studies using similar mouse lines.

**CNTNAP2 KO mice:** B6.129(Cg)-Cntnap2tm1Pele/J (Cntnap2 Ko mice, Jackson stock number 017482, Poliak et al. 2003) were bred with Snap-25-G6 mice and are referred to as CNTNAP2 KO mice. See Chapter 5 for further discussion of previous studies using similar mouse lines.

**TS mice:** B6.Cg-Cacna1ctm2ltl/J (TS2-neo, G406R mutation mice, Jackson stock number 019547) mice were bred with Snap-25-G6 mice and are referred to as TS mice (Bader et al. 2011, Bett et al. 2012). See Chapter 5 for further discussion of previous studies using similar mouse lines.

**WT mice:** For MK 801 pharmacological experiments, we used Snap-25-GCaMP6s mice which we refer to as WT. Mice in these experiments did not receive sinus injections.
We achieved viral mediated expression of pan-neuronal G6s via transverse sinus injections at P1-2 as previously described (Barson, Hamodi et al. 2020; Hamodi et al. 2020). In brief, mice were anesthetized using hypothermia for ~2 minutes prior to surgery and then kept cold for a further 10-12 minutes during the procedure. Pulled glass micropipettes (1B150F-4; World Precision Instruments) were loaded with pAAV.Syn.GCaMP6s.WPRE.SV40 (Viral prep #100843-AAV9, Addgene, Watertown, MA) (Chen et al 2013), and then 1-2uL was pressure-injected into each transverse sinus. Mice were then placed on a temperature-controlled heat pad immediately after surgery and returned to their mothers following return of spontaneous movements.

For widefield imaging experiments, mice aged P6-13 were surgically prepared using approaches described previously (Ackman et al. 2014; Barson, Hamodi et al. 2020). In brief, we performed a 20-30 minute surgery in which the scalp and connective tissue were cleared and a layer of clear dental cement was applied to provide the field of view for widefield imaging of whole cortex spontaneous activity. Two screws were placed just anterior and posterior to the cortex over the olfactory bulb and cerebellum, respectively, to allow head-fixation of the animal during imaging. Meloxicam (0.3 mg/kg) was delivered before the procedure and for at least three days following the procedure. Mice were acutely anesthetized during surgery with 1.5-2.5% isoflurane delivered with continuous 100% oxygen. General anesthesia was augmented with local anesthetic of 1.0%lidocaine/0.5%marcane. Following initial recover from anesthesia, mice were returned to their home cage for further recovery. Most imaging sessions were performed on subsequent
days, but if imaging was performed on the same day, mice were allowed to recover at least twice the time spent under anesthesia plus an additional 1 hour (typically 3-4 hours recovery).

For electrophysiology experiments, we performed craniotomies under the same general anesthesia protocols as described above. To do so, we used a combination of scalpel and dental drill to make openings through the bone while leaving the dura intact. One ~1x1mm craniotomy was made over the somatosensory cortex to record local field potentials (LFP) and a second ~1x1mm craniotomy was made over the cerebellum for placement of a reference electrode. Protective coverings of 2% agarose (Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline were applied over both craniotomies and kept hydrated throughout the experiment.

**Widefield Calcium Imaging (All figures)**

**Data acquisition**

Widefield calcium imaging was performed *in vivo* on head-fixed, unanesthetized mice using a Zeiss AxioZoom V16 microscope with a 1X macro-objective (Carl Zieiss Inc., White Plains, NY) (**Figure 2.1**). Illumination was provided by an LED source (X-Cite XLED1; Alghanim Electronics, Shuwaikh, Kuwait) with blue light (470 nm, Chroma ET470/20x; Chroma Technology Corp., Bellows Falls, VT) for GCaMP imaging and was collected by a sCMOS camera (pco.edge 4.2; PCO AG, Kelheim, Germany). Imaging was performed at 10 frames per second with a 512x500 pixel imaging field with a ~20x20 um/pixel resolution and collected using CamWare V3.17 software (PCO AG, Kelheim, Germany).
**Pre-processing of widefield imaging data**

Raw TIFF movies were pre-processed with the following steps: 1) A region of interest (ROI) mask around the cortex was manually applied in ImageJ. 2) Movies were spatially down-sampled using average-pooling with a 2x2 filter. 3) To remove slow drifts in baseline fluorescence, a temporal top-hat filter using a 300-frame filter object was applied. 4) Intensity was normalized to generate a ΔF/F₀ for each pixel: \[ \Delta F/F₀ = (F_t - F₀)/F₀ \] where \( F_t \) = the top-hat filtered trace for each pixel and \( F₀ \) = the top-hat filtered mean fluorescence for each pixel. 5) In most mice, we addressed potential for signal artifact using dual wavelength imaging in which blue wavelength light optimized for GCaMP6 emission is interleaved with ultraviolet (UV) wavelength light that elicits predominantly GCaMP independent fluorescence. The UV elicited fluorescence is presumed to reflect non-specific signal including z-axis motion artifact and hemodynamic artifact. The UV signal was regressed out from the blue light signal in order to generate the ΔF/F₀.

**Fraction of cortical pixels active (Figures 3.1a, 3.1b, 6.2)**

To calculate the fraction of cortical pixels active (FA) at a given frame, we binarized the ΔF/F₀ matrix by counting a pixel as either active or not active using a z-score based threshold such that pixels with intensity less than threshold were set to 0 (“inactive”) and greater than threshold were set to 1 (“active”). The FA was then calculated for each frame as: \( \text{FA} = \frac{\# \text{ active cortical pixels}}{\# \text{ total cortical pixels}} \). A threshold of z-score = 0 (mean) was used in Figure 3.1a and Figure 6.1, and a threshold of z-score = 1 was used in Figure 3.1b.
**Functional connectivity / Seed based correlations (Figures 3.2a, 3.2b, 4.1, 5.1, 6.3)**

To determine functional connectivity profiles of spontaneous cortical activity, we placed 1000 seeds evenly spanning the entire field-of-view across the cortex (500/hemisphere). Then, for each seed pixel, we used the built-in MATLAB function ‘corr’ to calculate the Pearson’s correlation coefficient (CC) between that seed pixel’s fluorescence trace and the fluorescence traces of all cortical pixels ipsilateral and contralateral to the seed. To quantify the strength of cortex-wide correlation, we took the mean ipsilateral and contralateral CCs for all seed pixels, yielding a single mean cortex-wide correlation value for that mouse. For partial correlation analysis (Figure 3.2b), we used the built-in MATLAB function ‘partialcorr’ to calculate the correlation following the multiple linear regression of the mean activity trace for all cortical pixels.

**Principal component analysis (Figures 3.3, 6.4)**

We performed principal component analysis (PCA) on the raw ΔF/\(F_0\) matrix using singular value decomposition (built-in MATLAB function ‘svd’). To quantify the total variance explained for each principal component, we calculated the eigenvalue for each PC by taking the square of the singular values (output ‘s’ from the svd function). For a given PC, \(PC_a\), and a given singular value, \(S_a\): the eigenvalue, \(\lambda_a = (S_a)^2\). For each mouse, we calculated the % Variance Explained for each PC: \(\% \text{ Variance Explained for } PC_a = \frac{\lambda_a}{\sum \lambda_i} \). We also calculated the dimensionality of the spontaneous activity via the Participation Ratio (PR): \(PR = \left(\sum \lambda_i\right)^2 / \sum \lambda_i^2\) (Gao et al. 2017; Recanatesi et al. 2019). We statistically compared the % Variance Explained by the first PC and the PR for each mouse.
Diffusion Mapping (Figures 3.4, 6.5)

We performed a diffusion map analysis (Coifman et al. 2005) using a custom MATLAB GUI (https://github.com/CairLab/GUI_dimReduction) in which frames were projected to and visualized in two-dimensional space based on the fluorescent intensity of the cortical pixels. In brief, a Gaussian kernel was used to project data points to a weighted graph (each data point is a vector whose entries are the $\Delta F/F_0$ values of pixels in a given frame). The analysis modeled the data similarity by conducting random walks on the constructed Gaussian-weighted graph and computing eigenfunctions of the corresponding Markov matrix. The eigenfunctions were used to compute diffusion coordinates and distances, which quantified similarities of the frames in terms of their patterns of fluorescence. We first performed diffusion map analysis on all mice without specifying the parameter, $\sigma$, which determines diffusion speeds on the Markov matrix. We then determined a common $\sigma$ value, $\sigma=8$, that was intermediate between the $\sigma$ range of values for CTL mice and EX KO mice. To visualize the diffusion map and quantify the mean pairwise distance between datapoints (frames), we performed a second diffusion map analysis for all mice using $\sigma=8$.

Electrophysiological recordings (Figures 3.5A, 3.5B)

Data Acquisition

LFP signals were collected using silicon probes (NeuroNexus Technologies, Ann Arbor, MI) with either 16 recording sites on 1 shank (100-μm vertical separation between recording sites; 1-2-MΩ impedance) or with 4 recording sites on each of 4 shanks (100-μm vertical separation between recording sites; 125-μm horizontal spacing between shanks; 1-2-MΩ impedance). An
insulated silver wire (0.25-mm diameter; Medwire, Mt. Vernon, NY) placed above the cerebellum served as a reference electrode. Signals were pre-amplified 10x (MPA8I preamplifiers; Multi Channel Systems MCS GmbH, Reutlingen, Germany) before being amplified 200x and band-pass filtered at 0.3-5000 Hz (Model 3500; A-M Systems, Inc., Carlsborg, WA). The amplified and filtered signals were sampled at 20 kHz using a digital interface (Power 1401 mk 2; Cambridge Electronic Design, Cambridge, UK). LFP data were collected from n=4 CTL mice and n=5 EX KO mice. In n=3 CTL mice and n=2 EX KO mice, LFP data was recorded simultaneously with widefield calcium imaging. The timing and absolute amplitude of LFP activity were similar across channels spanning multiple cortical layers in both CTL and EX KO mice and thus a single, artifact-free superficial channel was used for the analyses described in the Results Section.

Data Analysis

Data were analyzed using Spike2 (Cambridge Electronic Design, Cambridge, England) and MATLAB software. To calculate a dispersion index (DI) of signal strength across time, we rectified the selected LFP trace and then calculated a ratio of the variance, σ, to the mean, μ: DI = σ/μ. To compare electrophysiology and calcium events, we identified large electrophysiological events based on a threshold = mean + 2 standard deviations. Of all identified large events, we used only those that followed the previous large events by >2 seconds in order to calculate a spike-triggered average of the calcium frames surrounding the event.
MK 801 Injections (Figure 6.2 – Figure 6.5)

Following baseline spontaneous recordings, the NMDAR antagonist, MK-801 (Tocris, Bristol, United Kingdom) at 0.5 mg/kg, 1.0 mg/kg, or 3.0 mg/kg was injected IP at P8-9 or P13-15 (Coan et al. 1987, Huettner et al. 1988). Data were collected immediately following injection and for a period of 2 hours after. Analysis was restricted to 30 – 60 minutes post injection, consistent with peak response period from previous studies (Miyamoto et al. 2000), although effects on dynamics typically persisted for several hours.

Statistical analysis

Following calculations using custom MATLAB scripts, all significance testing was performed using Graphpad Prism software (GraphPad Software, San Diego, CA). One-way analysis of the variance (ANOVA) followed by Tukey’s HSD post-hoc test was used to test statistical significance between the three groups (e.g. CTL, EX KO, and IN KO) at a single age range of P7-9 or P11-17. Student’s T-test was used to compare within each group between the two ages (e.g. CTL P7-9 vs CTL P11-17). Stars indicate significance $p<0.05$.

Data and Code Availability

Datasets were analyzed using custom scripts written in MATLAB (The MathWorks, Inc., Natick, MA) and freely available at https://github.com/CrairLab. The datasets used in the current study and more detailed steps to perform statistical analysis are available upon request.
Figure 2.1: Schematic of widefield imaging
**Figure 2.1: Schematic of widefield imaging.** Left: Schematic of widefield imaging of spontaneous cortical activity in unanesthetized, head-fixed neonatal mice. Mice expressing pan-neuronal GCaMP6s were imaged during P7 to P17. Right: Field of view and example of normalized (DF/F) fluorescence in a P8 littermate control mouse (CTL).
Chapter 3: The Role of the NMDA Receptor in Shaping Cortical Activity during Development

Adapted from the following manuscript in revision at Cerebral Cortex:

The development of normal spontaneous neural activity patterns in the mouse cortex depends on the expression of the NMDA type glutamate receptor in excitatory neurons

Jacob Lister, Ethan J. Mohns, Yixiang Wang, Xinxin Ge, Seneca Oxendine, Ali S. Hamodi, Daniel Barson, Yueyi Zhang, N. Dalton Fitzgerald, Sofia Fertuzinhos, James Ackman, Michael C. Crair
Introduction

Numerous sources of evidence point to the cerebral cortex as a critical region to target to understand pathogenesis of NDDs, since it is necessary for complex human capacities (Mountcastle et al 1995; Goldman-Rakic PS 1995; Fuster JM 2001; Rakic P 2002; Arnsten AFT 2013), dysfunction in this region is thought to be central to the pathology of neurocognitive disorders (Casanova and Casanova 2019), and NDMAR is one of the fundamental mediators of activity dependent development in this region (Crair and Malenka 1995; Iwasato et al. 1997, 2001; Malenka and Feldman 2004; Feldman 2012).

Cortical circuits are composed of excitatory and inhibitory neurons, and the precise connectivity between these populations is critical for cortical function and implicated in the pathophysiology of NDDs (Sohal and Rubenstein 2019). There is evidence that NMDAR function in both excitatory and inhibitory neurons is important for the development of cellular and local circuit features in the cortex, for neurocognitive function, and for NDD pathophysiology; however, the cell-type specific role of the NMDAR in the development of cortical networks involved in higher cognition has not been well examined. Excitatory (glutamatergic) neurons account for ~75-90% of cortical neurons and are the driving force of the central nervous system (Marin and Rubenstein 2003; Le Magueresse and Monyer 2013). Previous work using mice with the obligate NR1 subunit of the NMDAR knocked out from cortical excitatory neurons shows that this receptor is essential for the development of somatotopic organization of whisker representation in the primary somatosensory “barrel” cortex (S1) (Iwasato et al. 2000; Datwani et al. 2002; Lo et al. 2013; Mizuno et al. 2014). Inhibitory (GABAergic) neurons account for ~10-25% of cortical neurons only but are known to be important in maintaining overall
excitatory/inhibitory balance (Froemke RC 2015; Tatti et al. 2017) and trafficking of information (Puzerey and Galan 2014; Mongillo et al. 2018). Previous work in mice with NR1 knocked out in inhibitory cells reveals disruptions of interneuron migration, diversification and integration into the cortical circuit (Bortone and Polleux 2009; Matta et al. 2013; De Marco Garcia et al. 2011, 2015; Akgul and McBain 2016; Chittajallu et al. 2017; Mayer et al. 2018; Priya et al. 2018; Cornford et al. 2019; Hanson et al. 2019; Mahadevan et al. 2020). Despite the evidence for NMDARs role in cellular and local circuit formation in the cortex, NMDARs role in shaping large-scale cortical network dynamics in vivo has not been examined.

Here, we sought to characterize the role of NMDAR in the development of cortical functional organization from a system-wide, dynamic perspective using wide-field imaging of spontaneous cortical activity in unanesthetized neonatal mice. Our goal was not to “model” a specific disorder explicitly, but rather to ask a fundamental question about the principles that govern the development of cortical circuitry. Characterizing development of higher order cortical circuits from the perspective of large-scale dynamics is fundamentally important because functional connectivity patterns are thought to be hallmarks of normal cortical function and may be disrupted in neuropsychiatric disorders (Geschwind D. 2007; Menon V. 2011; Mohajerani et al. 2013; Ma et al 2016; Vanni et al. 2017; Bednarz and Kana 2018). To determine the role of the NMDAR in shaping the development of cortical network dynamics, we recorded spontaneous neural activity using widefield calcium imaging and electrophysiological recordings of cortical local field potentials in unanesthetized NMDAR KO mice during a critical time window for circuit formation. We tested the developmental function of the NMDAR in the two major neuronal populations in the cortex by expressing the genetically encoded calcium indicator GCaMP6 under
a pan-neuronal Snap-25 promoter (Madisen et al. 2015) in excitatory specific NMDAR knockout mice (referred to as EX KO mice) or inhibitory specific NMDAR knockout mice (referred to as IN KO mice). We focused on the time period from the end of the first post-natal week to the beginning of the third: a critical time window in cortical development when fundamental features of circuit connectivity are first established, the cortex shifts from an immature bursting period to its mature electrophysiological properties, and mice begin to explore their environment [Khazipov et al 2004; Hanganu et al. 2006; Golshani et al. 2009; Colonnese et al 2010; Colonnese 2014; Luhmann and Khazipov 2018; Gribizis et al. 2019]. Importantly, an analogous transition occurs in humans around the time of birth [Milh et al 2007; Chipaux et al 2013].

Despite evidence for the importance of NMDAR function in both these populations to normal cortical circuit development and neurocognitive function (Belforte et al 2010; Rompala et al 2013), we were surprised to see vastly different impacts on cortical activity in our cell-specific KO mice. In EX KO mice, we observed an extreme phenotype: the entire cortical network operated in a functionally hyper-connected and hyper-synchronous mode as early as post-natal day 7 (P7), with spontaneous activity locked in a single recurrent motif characterized by bilateral bursts of activity involving the majority of the observable cortex. In IN KO mice, on the other hand, large-scale cortical activity during this period in development was not different from normal based on our measures. We characterized cortical activity in several ways including a summary measure of whole cortex dynamics, analysis of pairwise correlations across the cortex (analogous to “functional connectivity” analysis of resting state fMRI in humans), and principal component analysis, showing that complexity of global activity in EX KO mice is substantially reduced with a dominant global activity pattern accounting for 85-95% of the total moment to
moment variance. We went on to visualize the distinctive dynamics in EX KO mice using diffusion mapping, a non-linear dimensionality reduction technique, which shows how the dynamical structure shifts from a highly varied but tightly clustered network space in control (CTL) mice to an extended manifold that appears like a one-dimensional line between two extreme network states in EX KO mice. Finally, using electrophysiological recordings of cortical local field potential (LFP), we identify unusual large amplitude spiking events in EX KO mice that are closely associated with the aberrant cortex-wide calcium fluorescence.

Taken together, these results demonstrate that the large-scale structure of spontaneous activity in the mouse cortex depends on the function of NMDAR in cortical excitatory neurons. In the absence of NMDAR, the cortex does not behave like many independent units, but rather like a single connected sheet locked into one dominant dynamic trajectory. This extreme network state may offer a biomarker in humans for both identifying and tracking response to intervention at an early stage of network development, prior to behavioral manifestation, when these responses have the greatest opportunity to alter developmental trajectory for children. As NMDAR is widely implicated in human disease, the trajectory of cortical network development observed in this study can serve as a prediction and point of comparison for NDDs, generally.
Results

Spatiotemporal properties of whole cortex spontaneous activity in neonatal mice are severely disrupted following NMDAR knockout in cortical excitatory neurons

We used widefield, mesoscopic calcium imaging to examine the spontaneous activity in neonatal control mice (CTL mice), cortical excitatory neuron NMDAR knockout mice (EX KO mice), and cortical inhibitory neuron NMDAR knockout mice (IN KO mice) (see Methods). We focused our recordings on the end of the first post-natal week to the beginning of the third. During this critical time window in cortical development, fundamental features of circuit connectivity are first established, the cortex shifts from an immature bursting period to its mature electrophysiological properties, and mice begin to explore their environment (Khazipov et al 2004; Hanganu et al. 2006; Golshani et al. 2009; Colonnese et al 2010; Colonnese MT 2014; Luhmann and Khazipov 2018; Gribizis et al. 2019).

The large-scale, real-time calcium dynamics look strikingly abnormal in EX KO mice in comparison to CTL and IN KO mice. Examples that highlight the distinct spatiotemporal dynamics of spontaneous cortical activity at P8-9 and P13-14 are shown in Figure 3.1A(a). Activity in CTL mice spontaneously elaborates a range of spatial patterns, reflecting neural activity in distinct cortical regions that evolves across time. In contrast to CTL mice, EX KO mice demonstrate spontaneous activity characterized by large, synchronous cortex-wide events. Interestingly, the dynamical pattern of spontaneous activity in IN KO mice is similar to the pattern of activity observed in CTL mice, perhaps surprisingly considering evidence supporting an important role of NMDA function in cortical interneuron development (Belforte et al 2010; Rompala et al 2013).
To appreciate the overall dynamics of neural activity across the cortex, we measured the mean $\Delta F/F_0$ across the whole cortex and the fraction of cortical pixels active (FA) across time (Figure 3.1A(a), see Methods). In CTL and IN KO mice, the mean $\Delta F/F_0$ and FA fluctuate, with small changes reflecting evolving spatiotemporal dynamics, but rarely is the cortex completely active (with high mean $\Delta F/F_0$ and FA) or completely silent (with low mean $\Delta F/F_0$ and FA). On the other hand, in EX KO mice the whole cortex turns on and off together (alternating epochs of high and low mean $\Delta F/F_0$ and FA). Histograms summarizing these differences across the recording sessions show that CTL and IN KO mice display a continuous distribution across the entire range of FA, whereas the EX KO mice display a distribution shifted to the poles (Figure 3.1A(b) and Figure 3.1B(a)). To quantify this striking difference in the distribution of cortical dynamics between groups, we calculated the mean proportion of time that less than 0.1 of cortical pixels were active (FA<0.1), 0.1-0.9 pixels were active (0.1<=FA>=0.9), and greater than 0.9 pixels were active (FA>0.9). (Figure 3.1A(c), group Mean±Standard Error of the Mean (SEM) are shown for FA<0.1, 0.1<=FA>=0.9, FA>0.9: P7-9 CTL=0.12±0.01, 0.84±0.02, 0.03±0.01, EX KO=0.53±0.03, 0.26±0.04, 0.21±0.01, IN KO=0.18±0.01, 0.79±0.02, 0.03±0.01, $p<0.05$; P11-17 CTL=0.13±0.01, 0.81±0.02, 0.06±0.01, EX KO=0.43±0.02, 0.29±0.02, 0.28±0.01, IN KO=0.13±0.01, 0.81±0.02, 0.06±0.01, $p<0.05$; see also Figure 3.1B(b)). These differences indicate that knockout of NMDAR in excitatory neurons (EX KO mice) drastically alters the normal spatiotemporal statistics of spontaneous activity, with the cortex alternating between all-on and all-off epochs.
Figure 3.1A: Whole cortex spontaneous activity dynamics in NMDAR KO mice during development

A. Characteristic spatial patterns and temporal dynamics

![Images of spatial patterns and temporal dynamics for P8-9, P13-14, P11-17 stages with CTL, EX-NMDAR KO, IN-NMDAR KO conditions]

B. Fraction of cortical pixels active (FA)

![Graphs showing FA across P7-9, P11-17 stages with CTL, EX KO, IN KO conditions]

C. Proportion of time spent in different FA ranges (FA < 0.1, FA 0.1 - 0.9, FA > 0.9)

![Scatter plots showing proportion of time across CTL, EX KO, IN KO conditions for P7-9, P11-17 stages]
Figure 3.1A. Whole cortex spontaneous activity in NMDAR KO mice during development.

(A) NMDAR was knocked out in either Glutamatergic (EX KO) or GABAergic (IN KO) cortical neurons. Characteristic image sequences and temporal dynamics are shown for individual CTL, EX KO, IN KO mice at P8-9 and P13-14. For each mouse, the top row shows a characteristic 5 second image sequence. The middle row shows a trace of mean $\Delta F/F_0$ across the cortex for the 200 second (2000 frame) period from which these examples sequences are drawn (as indicated by the dashed lines). The bottom row shows a trace of the fraction of cortical pixels active above their mean fluorescence (FA) for the same 200 second period. (B) For quantification, we grouped P7-9 (CTL: n=7 mice and 202,361 frames/5.62 hours; EX KO: n=5 mice and 184,119 frames/5.11 hours; IN KO: n=3 mice and 77,393 frames/2.42 hours) and P11-17 (CTL: n=11 mice and 295,538 frames/8.21 hours; EX KO: n=6 mice and 233,628 frames/6.49 hours; IN KO: n=4 mice and 87,066 frames/2.42 hours). The graphs are histograms that show the proportion of total time versus FA (range between FA=0 and FA=1 is divided into 20 bins) for all mice in that group. (C) The graphs show mean proportion of time for all mice when FA > 0.1 (top graph), FA = 0.10 - 0.90 (middle graph), and FA > 0.90 (bottom graph). Star indicates $p$-value less than 0.05 following one-way ANOVA with Tukey’s multiple comparisons test.
Figure 3.1B:

A. FA (Threshold = z-score 1)

B.
**Figure 3.1B.** Same analysis as figure 1 but with FA threshold = z-score 1. For FA > 0.1, p7-9: EX KO vs CTL \( p < 0.05 \), EX KO vs IN KO \( p < 0.05 \); p11-17: EX KO vs CTL \( p < 0.05 \), EX KO vs IN KO \( p < 0.05 \). For FA = 0.10 - 0.90, p7-9: EX KO vs CTL \( p < 0.05 \), EX KO vs IN KO \( p < 0.05 \); P11-17: EX KO vs CTL \( p < 0.05 \), EX KO vs IN KO \( p < 0.05 \). For FA > 0.90 p7-9: EX KO vs CTL \( p < 0.05 \), EX KO vs IN KO \( p < 0.05 \), p11-17: EX KO vs CTL \( p < 0.05 \), EX KO vs IN KO \( p < 0.05 \).
Disruption of normal spontaneous activity in EX KO mice is reflected in abnormal functional connectivity maps

We next sought to characterize the effect of deleting NMDARs on regional topography by examining functional correlations in different areas of the cortex. Previous studies in both mice and humans show that functionally related regions display similar time courses of spontaneous activity (Fransson et al. 2007; Fair et al. 2009; Lu et al. 2012; Mohajerani et al. 2013, Ackman et al. 2014). We computed correlation maps, which show the strength of the Pearson’s correlation coefficient across the cortex relative to a seed pixel (see Methods), to examine the relationship between neural activity in a seed region of interest and neural activity in other cortical regions in CTL, EX KO, and IN KO mice (characteristic examples shown in Figure 3.2A (a)). We quantified the mean correlation coefficient (CC) for all seeds and observed significant differences between EX KO and IN KO or CTL groups as shown in Figure 3.2A (b) (CC Ipsilateral to seed, CC Contralateral to seed, Mean±SEM: P7-9 CTL=0.36±0.02, 0.26±0.04, EX KO=0.90±0.03, 0.89±0.04, IN KO=0.39±0.04, 0.32±0.05, p<0.05; P11-17 CTL=0.53±0.02, 0.45±0.02, EX KO=0.89±0.01, 0.89±0.01, IN KO=0.45±0.06, 0.39±0.07, p<0.05).

In Figure 3.2A (a), we highlight differences in cortical functional connectivity using seeds in three well-separated networks in the developing brain: the frontal/motor, somatosensory, and visual cortices (Ackman et al. 2014; Barson, Hamodi et al. 2020). In CTL mice, there are distinct and discrete functional networks of regions with correlated spontaneous activity, depending on the location of the seed. The frontal/motor seed is highly correlated (CC range=0.6:0.9) with the surrounding frontal motor region in both the ipsilateral and contralateral cortex, but weakly correlated (CC range=0.0:0.4) with the majority of the cortex posterior to these regions. The
somatosensory seed region is similarly highly correlated with the surrounding somatosensory area, with the ipsilateral motor area, and with the contralateral somatosensory and associated motor area. Thus, correlations in spontaneous activity reflect functional association rather than mere spatial distance. Also note that at P7-8 the right visual cortex is highly correlated with ipsilateral visual cortex but not the contralateral visual cortex or the anterior cortex. This correlation pattern reflects the developmental stage of the mouse, which is blind at this age, with activity in visual cortex primarily driven by retinal waves that occur independently within each eye (Ackman et al. 2012). By P13-14, approximately the time of eye opening, correlation with contralateral visual cortex has increased but visual cortex is still not strongly correlated with anterior regions of the cortex. In general, functional connectivity within IN KO mice is similar to CTL mice, with distinct functional regions easily identifiable, as would be expected from the greater similarity between the spatiotemporal properties of their spontaneous activity.

In contrast to CTL and IN KO mice, in EX KO mice the structure of these networks is severely degraded. Activity in the right somatosensory region or in the right visual region is highly correlated with activity across the entire cortex, and large portions of the cortex are nearly perfectly correlated (CC range=0.8:1). Each seed is almost fully bilaterally correlated within its area (CC range>0.9), while slightly less correlated with other areas (CC range=0.7:0.9). Notably, in EX KO mice at P8-9, retinal waves are observable in the cortex (Ackman et al. 2012; Burbridge et al. 2014; Gribizis et al. 2019), which can occur either with or without concurrent whole cortex activations. The distinct behavior of retinal waves from the predominant whole cortex activity is reflected in the slightly lower correlation between primary visual areas and the rest of the cortex in EX mice at P8-9 (CC range=0.5:0.6). The unilateral nature of spontaneous retinal waves is
reflected in the subtle difference in correlation between the ipsilateral visual area (CC range=0.9:1) and the contralateral visual area (CC range=0.6:0.8) in EX KO mice at this younger age. By P13-14, when mice are opening their eyes and spontaneous retinal waves disappear, the visual hemispheres are nearly completely bilaterally correlated (CC range=0.9:1) in EX KO mice. Nonetheless, it is still possible to pick out subtle topographical differences between frontal/motor, somatosensory, and visual areas.

To investigate the subtle differences in topology of the maps that are difficult to distinguish from the dominant activity patterns in EX KO mice, we performed a partial correlation analysis on the same data following a regression of global mean activity (see Methods, Figure 3.2B); note that regressing out globally shared activity shifts low correlations to negative values. In CTL and IN KO mice, this regression reinforces the areas of strength while maintaining the same predominant topology. Notably, in EX KO mice, the global regression abolishes the anterior posterior correlations, revealing the remaining activity in this region to be highly anti-correlated. This regression unsurprisingly abolishes the difference in mean correlation strength between the groups (Figure 3.2B (b) Left Graph); however, driven by these highly anti-correlated anterior and posterior poles, activity in EX KO mice is still more highly synchronous than in CTL and IN KO mice, as reflected in the mean of absolute value of the CC (Figure 3.2B (b) Right Graph, Absolute Value CC Ipsilateral to Seed, Absolute Value CC Contralateral to Seed, Mean±SEM: P7-9: CTL=0.25±0.01, 0.19±0.01, EX KO=0.44±0.02, 0.38±0.03, IN KO=0.25±0.02, 0.20±0.01, p<.05; P11-17: CTL=0.34±0.01, 0.28±0.01, EX KO=0.47±0.01, 0.44±0.01), IN KO=0.36±0.02, 0.31±0.04; CTL vs EX KO p<.05. All other p=n.s.). These data suggest that the complexity of spontaneous
activity that is independent of the dominant pattern is also significantly reduced in EX KO mice compared to the CTL and IN KO mice.
Figure 3.2A: Functional connectivity maps of spontaneous cortical activity

A. Characteristic seed based correlation maps of spontaneous cortical activity

B. Mean correlations across cortex
Figure 3.2A. Functional connectivity maps of spontaneous cortical activity.

(A) Characteristic seed-based correlation maps from 10 minute recordings of individual mice. Example seeds in the Frontal/Motor Cortex (top row), Somatosensory Cortex (middle row), and Visual Cortex (bottom row) at P8-9 are shown on the left and at P13-14 on the right. The colorbar represents the strength of correlation (CC) between the seed location (marked by the black dot) and the rest of the cortex. These correlation coefficients range between -1 and 1, with 1 indicating completely correlated activity and -1 indicating completely anti-correlated (note the graphs are displayed between CC=0 (Blue) and CC=1 (Red)). (B) For summary quantification, 1000 seeds are placed evenly across the cortex and the mean CC between that seed and the ipsilateral and contralateral cortex is calculated. Graphs show the mean CC for all seeds for individual animals and the group means for Ipsilateral CC and Contralateral CC. Star indicates p-value less than 0.05 following one-way ANOVA with Tukey’s multiple comparisons test or Student’s T-test.
Figure 3.2B

A. Characteristic seed based partial correlation maps following global mean regression

P8-9

Frontal/Motor Seed

Somatosensory

Visual Seed

B. Mean partial correlations across cortex for all seeds

Correlation Coefficient

Correlation Coefficient (Absolute Value)
3.2B. Functional connectivity maps of spontaneous cortical activity following global regression of mean activity. (A) Characteristic seed based partial correlation maps using the same seeds and records from figure 2. Colorbar now represents the strength of correlation between -1 and 1, with the scales now changed from -1 to 1 to reflect the shift to negative correlations introduced by the regression of the globally shared activity. (B) Top panel shows mean CC and bottom panel show mean absolute value of the CC. Star indicates P value less than .05 following one-way ANOVA with Tukey’s multiple comparisons test. For absolute value of R-value in cortex ipsilateral to seeds, p7-9: EX KO vs CTL $p<0.05$, EX KO vs IN KO $p<0.05$; p11-17: EX KO vs CTL $p<0.05$, EX KO vs IN KO $p<0.05$. For cortex contralateral to seeds, p7-9: EX KO vs CTL $p<0.05$, EX KO vs IN KO $p<0.05$; P11-17: EX KO vs CTL $p<0.05$, EX KO vs IN KO $p<0.05$. 
**Linear and non-linear dimensionality reduction techniques reveal reduced complexity of spontaneous activity in EX KO mice**

To further demonstrate the dominance of widespread, bilaterally correlated activity and the concomitant reduction in overall dynamical complexity of spontaneous activity in EX KO mice, we performed principle component analysis (PCA) on the ΔF/F₀ movies (see Methods) (Figure 3.3). PCA is an unbiased, linear dimensionality reduction technique that allows identification of dimensions (principal components (PCs)) that best explain the observed variance in the data. PCs of spontaneous activity can reveal recurring motifs in the data, which can be ranked by how well they explain the variance in that activity. We show examples of the spatial structure of these motifs and the variance explained by each PC in Figure 3.3 (a). PCA analysis of cortical spontaneous activity revealed that the majority of the variance of spontaneous activity, even in CTL and IN KO mice at both ages, can be explained by a small number of PCs; however, consistent with previous analyses, activity in EX KO mice is pushed to an extreme in which activity is nearly one-dimensional. Figure 3.3 (b) quantifies the percent of variance explained by the first five PCs for all mice (Figure 3.3 (b), leftmost graph, PC1 % Variance Explained, Mean±SEM: P7-9: CTL=64.00%±2.69%, EX KO=93.75%±1.35%, IN KO=64.32%±1.00%, p<0.05; P11-17: CTL=73.72%±2.73%, EX KO=94.42%±0.49%, and IN KO=75.86%±0.35%; p<0.05). Figure 3.3 (c) quantifies dimensionality of the activity through the Participation Ratio (see Methods, Figure 3.3 (c) Participation Ratio, Mean±SEM: P7-9 CTL=6.75±0.57, EX KO=1.46±0.10, and IN KO=5.82±0.89, p<0.05; P11-17: CTL=5.96±0.86, EX KO=1.41±0.07, and IN KO=7.93±2.12, p<0.05). Thus, PCA analysis quantitatively reinforces the impression that cortical spontaneous
activity in EX KO is dominated by cortex-wide spontaneous activity fluctuations, unlike in CTL and IN KO mice.
Figure 3.3: Principal component analysis of spontaneous cortical activity

A. Characteristic principal components (PCs) and % variance explained

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<th>PC2</th>
<th>PC3</th>
<th>PC4</th>
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<td>00.61</td>
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<tr>
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<td>24.22</td>
<td>06.40</td>
<td>03.61</td>
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<td>02.49</td>
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<td>05.55</td>
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<td>02.62</td>
</tr>
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B. Percent Variance Explained

C. Dimensionality
Figure 3.3. Principal component analysis of spontaneous cortical activity. (A) Characteristic examples of individual mice at P8-9 and P13-14 showing spatial motifs and percentage of variance explained for the first five principal components. (B) Summary for all mice, with the graphs showing the percentage of variance explained by each of the first five principal components. Star indicates $p$-value less than 0.05 one-way ANOVA with Tukey’s multiple comparisons test. (C) Dimensionality as calculated from the Participation Ratio.
To further visualize the dynamic structure of the data, we used diffusion mapping, a non-linear dimensionality reduction approach that allows visualization of the underlying manifold of a high dimensional dataset by taking into account local geometry of the data (Coifman et al. 2005) (Figure 3.4, See Methods). Original frames of fluorescent values are modelled as vertices in a weighted graph; then, a gaussian kernel is used to take a random walk through the data to determine a probability of diffusing from one point to another. This non-linear diffusion probability is embedded into Euclidean space with greater distance reflecting lower probability of diffusing from one point to the next.

The examples show that activity in CTL mice encompasses a tight cluster that appears as a cloud centered around a mean, (note axes on the order of $10^{-3}$-$10^{-6}$ a.u. in the P8-9 example and $10^{-3}$-$10^{-4}$ a.u. in the P13-14 example). On the other hand, in EX KO mice, the manifold of evolving activity patterns appears one dimensional – forming a single line extended over a long distance in this space (note axes on the order of $10^{-2}$-$10^{-3}$ a.u. in the P8-9 example and $10^{-3}$-$10^{-4}$ a.u. in the P13-14 example) that reflects the oscillation between extremes of minimal and maximal whole network activity (Figure 3.4 (b), pairwise distance, Mean±SEM: P7-9: CTL=$2.42x10^{-4}$±$8.13x10^{-05}$, EX KO=$7.90x10^{-3}$±$2.59x10^{-03}$, $p<0.05$; P11-17: CTL=$3.46x10^{-4}$±$1.46x10^{-04}$, EX KO=$6.69x10^{-3}$±$1.85x10^{-03}$, $p<0.05$). Once again, this analysis reveals the intrinsic manifold and dominant dynamics underlying the brain activity patterns in EX KO mice that vacillate between extremes of quiescence and large-scale correlated activity across the cortex.
Figure 3.4: Diffusion map of spontaneous cortical activity

A. Characteristic diffusion map

B. Mean pairwise distance between frames
**Figure 3.4. Diffusion map of spontaneous cortical activity**

**(A)** Characteristic diffusion maps of 10 minute/6000 frame movies in CTL and EX KO at P8-9 and P13-14. Each datapoint represents a single frame plotted in diffusion space (x-axis is Diffusion Map 1 and y-axis is Diffusion Map 2), with distance (arbitrary units) reflecting strength of transition probability between frames. Diffusion map parameter sigma=8, with the color from blue to yellow reflecting time from frame 1-6000. **(B)** Summary for all mice showing the mean pairwise distance in diffusion space. P7-9 and P11-17. Star indicates p-value less than 0.05 following Student’s t-test.
Whole cortex calcium events are reflected in the cortical local field potential in EX KO mice

To investigate the electrophysiological signatures of these correlated events in EX KO mice, we used sixteen channel silicon electrodes to record local field potentials (LFPs) from CTL and EX KO mice through the depth of the cortex (Figure 3.5A and Figure 3.5B). LFP dynamics and the associated widefield calcium activity are shown in Figure 3.5A (a), which displays examples of raw traces for a P17 CTL and P16 EX KO mouse. CTL mice display a typical continuous LFP pattern that shifts between periods of high and low amplitude relative to the mean (Timofeev et al. 2020). EX KO on the other hand, display a relatively flat LFP, punctuated by large amplitude bursts. This difference is captured in the dispersion index (DI) (see Methods), with the ratio of SD/Mean below 1 for all CTL mice and near or above 1 for all EX KO mice (Figure 3.5A (c), DI Mean±SEM: CTL=0.81±0.03, EX KO=1.12±0.08; p<0.05).

In EX KO mice, the electrophysiological read-out of a local portion of cortex reveals a pattern of activity similar to the whole cortex dynamics. To determine the association between dynamics at the electrophysiological and whole cortex level, we compared the LFP to simultaneously recorded whole cortex fluorescence (Figure 3.5A (a) and 3.5A (b)). LFP fluctuations in the CTL mice do not bear an obvious relationship to global calcium dynamics. However, in EX KO mice, spikes in the LFP are closely associated with the global cortical dynamics, often with the initial LFP spikes preceding an increase in global activity and multiple LFP spikes underlying a single widefield calcium event. To quantify this relationship, we calculated the average cortex-wide fluorescence in a three second window around each identified spike in the LFP (Figure 3.5A (c)). Subtle dynamics can be observed in the CTL mice, with weak consistent activity across the cortex both before and after the spikes, while in EX KO mice there is relative
silence preceding the spikes followed by activation across the entire cortex that persists for several seconds (note the same analysis for one other CTL and one other EX KO mouse in Figure 3.5B). These recordings reveal that the large cortex-wide events observed in EX KO mice are closely associated with large amplitude electrophysiological events.
Figure 3.5A: Spontaneous local field potential and associated whole cortex activity

A. Characteristic local field potential (LFP) dynamics and concurrent whole cortex activity

B. Whole cortex activity associated with LFP spikes (spike triggered average)

C. Dispersion Index

Dispersion Index

SD/MEAN

CTL (P17)  
EX-NMDAR KO (P16)

DF/F

Average Rectified LFP spike

Average spike-triggered whole cortex activity
Figure 3.5A. Spontaneous local field potential and associated whole cortex activity.

(A) Characteristic examples at P8-9 and P13-14. Top row: 20 seconds of local field potential (LFP). Middle row: 5 seconds of LFP. Bottom row: 5 seconds of concurrent whole cortex activity. (B) Whole cortex activity associated with LFP spikes in the same mice from 5A. Top row: Average rectified LFP spike. Bottom row: Associated whole cortex activity triggered on detected LFP spikes. (C) Summary of dispersion index (DI) of rectified LFP power for n=4 CTL mice and n=5 EX KO. Star indicates p-value less than 0.05 following Student’s t-test.
A. Characteristic local field potential (LFP) dynamics and concurrent whole cortex activity

CTL (P15)  
EX-NMDAR KO (P18)

B. Whole cortex activity associated with LFP spikes (spike triggered average)
3.5B. Spontaneous local field potential and associated whole cortex activity. Same analysis as Figure 3.5a showing data from different CTL and EX KO mice.
Activity events show characteristic trajectories in EX-NMDAR KO mice

We have added a figure which shows examples of sequences leading to ‘whole cortex events’ and where they originate from. Activity generally starts in a smaller area first and then propagates to the whole cortex. There are several main initiation points. Most common are association regions (frontal, parietal, and retro-spenal). Activity in primary sensory regions (somatosensory, visual, auditory) can also initiate activity in some cases, especially in P7-9 mice. This new analysis and Figure 3.6 shows that activity can be initiated in both association and primary sensory regions, but activity then flows through one or several of the association regions before encompassing the whole cortex, in a restricted and consistent propagation stream. The average activation sequence reflects which regions are the origins most often.
Figure 3.6: Characteristic sources and sequences of cortical activity events

A. Characteristic sequences of cortical activity events

B. Average sequence of cortical activity events
Figure 3.6: Characteristic Sources and Sequences of Cortical Activity Events. (A) Four characteristic examples of activity sequences in the same mouse at P8 and at P13. Time 0.0 seconds is the first frame with FA > 0 after a period of at least 1 seconds of network silence (10 frames of FA = 0). (B) Average cortical activity before and after all identified transitions from >=1 second/10 frames of FA=0 across a 10 minute recording session (P8 n=64 events; P13 n= 79 events).
Discussion

In this study, we combined a targeted genetic approach with widefield calcium imaging and electrophysiology in vivo to assess the impact of the NMDA receptor on the development of spontaneous activity patterns in the mouse cortex. Despite substantial evidence for the importance of NMDAR function in both excitatory and inhibitory neuronal populations, our results demonstrate drastically distinct impacts on cortical spontaneous activity depending on the cellular population that loses NMDAR function. Whereas NMDAR loss in the inhibitory neurons results in little, if any, impact on the basic statistics and structure of whole cortex spontaneous activity observable with widefield imaging, NMDAR function in excitatory neurons proves necessary for the elaboration of large-scale network connectivity: in EX KO mice, normal spatiotemporal dynamics are dramatically shifted to a restricted, low complexity mode as early as P7. Using global cortical dynamics, pairwise correlations between cortical pixels, dimensionality reduction techniques, and electrophysiology, we comprehensively characterize the functional organization of spontaneous cortical activity and demonstrate that activity is locked into a low dimensional dynamic state in EX KO mice.

To better understand these aberrant activity patterns, we look to the known impact of the NMDAR deletion in cortical excitatory neurons on cortical circuits. EX KO mice have disrupted anatomical connectivity in the thalamic-cortical circuit that transmits somatosensory information from the whiskers to the cortex (Iwasato et al., 2000; Datwani et al., 2002; Lo et al., 2013; Zhang et al. 2013; Mizuno et al., 2014). In normal development, neuronal projections are initially guided to their target area by pre-programmed molecular factors, and neurons initially sprout extensive axonal and dendritic projections. Then, neural activity and activity dependent processes refine
these diffuse projections into precise connections characteristic of the complex and highly skewed pattern of wiring found in mature circuits (Goodman and Shatz 1993; Katz and Shatz 1994; Hofer et al. 2011; Ko et al. 2011, 2013; Li and Crair 2011; Erzurumlu and Gaspar 2012; Harris and Mrsic-Flogel 2013; Buzsáki and Mizuseki 2014; Cossell et al. 2015; Okun et al. 2015; Han et al., 2018; Kim et al. 2018). However, in EX KO mice, axons from somatosensory thalamus conveying information from individual whiskers innervate layer IV of S1, but layer IV excitatory neurons (stellate cells) fail to form the characteristic barrel pattern by orienting their axons towards thalamic projections from a single whisker. Furthermore, in EX KO mice, S1 neurons elaborate extensive dendritic trees with increased spine density that radiate in all directions and cover large territories, cortical discrimination of individual whisker deflections in S1 is degraded, and cortical-cortical projections are aberrantly diffuse (Iwasato et al., 2000; Datwani et al., 2002; Lo et al., 2013; Zhang et al. 2013; Mizuno et al., 2014; Zhou et al. 2021).

We propose that the failure to elaborate the normal network structure and the dominance of whole cortex-wide events in spontaneous activity in EX KO mice is the physiological reflection of this unrefined anatomy. Moreover, our widefield imaging results demonstrate that failed circuit refinement is not limited to the anatomical organization of S1 as previous studies have shown, but rather is manifest in the functional organization of the whole cortex. We offer a model of these distinct anatomical connectivity patterns in Figure 3.7. In this cartoon model of cortical excitatory circuit connectivity, we depict three excitatory neurons (represented by black dots) in three regions (Left Somatosensory Cortex, Right Somatosensory Cortex, and Right Frontal/Motor Cortex) and show the connectivity pattern between them (represented by blue lines in CTL mice and red lines in EX KO mice).
Normal NMDAR function during development:
Refined local and long range connections and distinct functional networks

Loss of NMDAR function during development:
Unrefined local and long range connectivity and low dimensional network space dominated by whole cortex activity events
Figure 3.7. Model of NMDAR dependent cortical network connectivity underlying spontaneous activity patterns. (A) In controls, neural activity patterns drive circuit refinement via NMDA mediated plasticity, resulting in precise local and long-range connections, distinct pathways of information and distinct functional sub-networks. (B) Following loss of NMDA receptor function in excitatory neurons, connections fail to refine and neurons remain diffusely interconnected both in terms of local and long-range connections. Any increase in activity in the system, locally, will entrain the whole cortical network, resulting in the dominant whole cortex activity patterns observed.
In both cases, long-range axon targeting is normal, as it is guided by molecular factors that are independent of NMDAR function. In CTL mice, early developmental neural activity patterns drive circuit refinement via NMDA mediated plasticity, resulting in precise local and long-range connections that allow for distinct pathways of information and distinct functional sub-networks. With loss of NMDA receptor function in excitatory neurons that occurs in EX KO mice, connections fail to refine under the guidance of patterned neural activity, resulting in excitatory neurons that have diffusely interconnected local and long-range connections. We propose that the consequence of this diffuse, unrefined connectivity is that any local increase in activity in the system will strongly drive the whole cortical network, resulting in the restricted dominant whole cortex activity pattern observed.

The mechanism responsible for this failed circuit refinement is likely NMDAR’s canonical role in synaptic plasticity as a “coincident detector” of pre-synaptic glutamate release and post-synaptic depolarization. Importantly, NMDARs play an important role in cortical circuits to both strengthen synapses through long term potentiation (LTP) and to weaken synapses through long term depression (LTD) (Crair and Malenka 1995; Malenka and Bear 2004; Watkins and Jane 2006; Feldman DE 2012). We suggest that the unrefined and hyper-connected cortical circuitry in EX KO mice highlights the critical role of cortical excitatory NMDAR in weakening of synaptic connections during development, likely through an NMDAR dependent LTD mechanism. However, it is also possible that non-canonical pathways might be involved in the abnormal anatomical and functional connectivity. For instance, an interaction between EPHRIN-B/EPHB signaling and NMDARs has been implicated in some aspects of colossal development (Zhou et al. 2021). EPHRIN-B/EPHB are axon guidance molecules, so this observation suggests an interesting
new aspect linking “activity independent” and “activity dependent” aspects of circuit formation through NMDAR dependent mediation (Li and Crair 2011; Erzurumlu and Gaspar 2012). Another non-canonical NMDAR mechanism that might contribute to the observed phenotype is suggested by the developmental downregulation of gap junctions (electrical synapsis) observed in hypothalamic circuits, which is dependent on NMDAR function (Arumugam et al. 2005). A similar persistence of gap junctions in an unrefined and hyperconnected cortical excitatory neuronal network might underlie some of the large-scale correlated activity across the cortex.

Considering the importance of NMDAR function in inhibitory cells for cortical circuit development and cognitive behaviors, it is notable that NMDA knockout in inhibitory cells did not cause major disruptions to large-scale activity patterns during this time period (Bortone and Polleux 2009; Belforte et al. 2010; De Marco Garcia et al. 2011, 2015; Matta et al. 2013; Akgul and McBain 2016; Chittajallu et al. 2017; Mayer et al. 2018; Priya et al. 2018; Cornford et al. 2019; Hanson et al. 2019; Mahadevan et al. 2020). It is important to note that the absence of gross disruptions in IN KO mice does not mean that inhibitory neuronal function is not important for the normal development of global functional connectivity, as it is possible that other NMDAR independent pathways may be utilized by inhibitory neurons during this developmental period. Moreover, our results do not imply that NMDAR function in inhibitory neurons is unimportant for all aspects of neural development or cognition. NMDAR function in inhibitory neurons may be important for local functional connectivity, which is not detectable through widefield imaging. Additionally, our study focused on developmental periods before extensive behavior, so it is plausible that the absence of NMDA function in inhibitory cells may impact global activity patterns in the setting of cognitive and behavioral demands that emerge later in development.
Considering the role of inhibitory cells in gating of information and attention, it may be that task engagement is needed to elicit differences in patterns of functional connectivity. Finally, it is also important to note that there are some trends towards increased bilateral synchrony in IN KO compared to CTL mice that may become functionally important at later stages of development. Nonetheless, the difference between the effects of NMDA knockout in the two cell types is striking and implies that NMDA loss in excitatory cells is sufficient to comprehensively disrupt cortical development.

While the effects seen in EX Ko mice is likely mediated predominantly by neuronal loss of NMDAR, Emx1 is not only expressed in excitatory neurons. It is also radial glia, Cajal-Retzius cells, astrocytes, and oligodendrocytes of the cortex, hippocampus and olfactory bulb that originate from the Emx1-expressing lineage (Gorski et al. 2002). Therefore, NMDAR roles in these other cells may also contribute to the phenotype in EX KO mice. In astrocytes, NMDARs have been demonstrated and show to be capable of expressing all NMDAR sub-units and have the potential to contribute to ionic flux and changes in calcium concentration through both ionic and metabotropic mechanisms. Expression level of NMDAR in astrocytes is often low, but is upregulated in activated astrocytes by tissue stress. In activated astrocytes, there is evidence for signaling through NMDARs contributing to both neuronal protection and to deregulation of astrocytic proteins critical for their functions. Calcium signaling in cortical astrocytes has been shown to exhibit both spontaneous and sensory evoked activity (Stobart et al. 2018), but there has been only a single study that has demonstrated evidence for NMDARs in astrocytes contributing to synaptic events (Letelier et al. 2016). IN ex vivo hippocampal slices and in vitro dissociated hippocampal cultures, they demonstrated an involvement of astrocytes in hetero-
synaptic presynaptic plasticity which exerts an inhibitory tone on excitatory synapses in CA1. Blocking this plasticity and NMDAR tone in astrocytes promoted homogenization of convergent presynaptic inputs. The authors suggest that this astrocyte-dependent cellular mechanism may enhance the heterogeneity of presynaptic strengths of convergent connection, which may help boost the computational power of dendrites.

In humans, mutations in genes coding for all subunits of the NMDAR have been linked to NDDs and neuro-psychiatric disease broadly, including intellectual disability (ID), autism spectrum disorder (ASD), and schizophrenia (SZ), as well as attention deficit disorder, manic depressive disorder, and substance use disorder (XiangWei et al. 2008; Hamdan et al. 2011; Tarabeux et al. 2011; Fountoulakis KN 2012; Lee et al. 2015; Lemke et al. 2016; Chen et al 2018). It is thought that sub-unit, temporal timing, cell specific contribution, and the nature of the mutation all contribute to the variability in abnormal developmental trajectories that can result from NMDAR mutations (Endele et al., 2010). Most relevant for comparison to our current study, previous work characterizing patients with mutations in the obligate NR1 sub-unit, specifically, found almost exclusively dominant negative missense mutations that led to a severe neurological phenotype, characterized by intellectual disability (ID) in 100% of individuals with mutations, epilepsy in 65%, and autism spectrum disorder (ASD) in 22% (Lemke et al. 2016; Platzer et al. 2019).

The extreme disruption of normal cortical activity patterns in EX KO mice is consistent with an extreme phenotype observed in humans with NR1 mutations, and our results may help disambiguate whether certain aspects of the phenotype are dependent on cortical NMDAR function. Most importantly, we would suggest that the reduction in the dimensionality of
spontaneous activity patterns in EX KO mice is consistent with the complete penetrance of ID
associated with NR1 mutations. Additionally, these low dimensional spontaneous dynamics
characterized by recurring whole cortex events are reminiscent of the tendency towards
repetitive motor behaviors associated with ASD.

Finally, while spontaneous activity is highly correlated across the cortex in EX-NMDAR KO
mice, there is no behavioral evidence of seizures and there is no electrophysiological evidence of
ictal activity, as characterized by either periodic spike and wave discharges (with consistent
frequencies and inter-spike intervals, often around ~3 HZ) or a long envelope of increasing LFP
spike rate and amplitude with high frequency oscillations (usually ~60-120 HZ) (Krestel et al.
2004; Le Van Quyen 2006; Panayiotopoulos CP 2008; Fisher et al. 2015). Clearly, LFPs in EX KO
mice are different from normal LFP patterns, as they are characterized by isolated or short
sequences of high amplitude events that are associated with whole cortex events observable
with widefield calcium imaging (Figure 5). These events are self-limiting, occur with varying inter-
spike intervals and rates (~0.3 – 4.0 HZ), with several distinct waveforms, and an absence of
associated high frequency activity (Figure 5). Such dynamics are in contrast to the “recruitment
rhythm” of a seizure, in which each LFP spike seems to enlist more neurons for subsequent spikes.

The LFP patterns observed in EX KO are reminiscent of “interictal spikes”, which are
commonly observed in both human epileptic patients and animal models (Rodin et al 2009). In
mice they have been defined as having a duration <200 ms and a large amplitude often >2x
background activity (Erbayat-Altay et al 2007). Two recent studies observed similar events while
simultaneously recorded LFP and widefield calcium imaging and warrant comparison to our
current study. Rossi et al 2017 induced focal seizures in visual cortex with picrotoxin. They
observed both 1) prolonged seizure activity ("ictal events") which had oscillatory frequency of 6-11 HZ, lasted many seconds and spread spatially across the cortex and 2) brief interictal events which were short in duration and remained localized as standing waves.

A potential concern is raised by Steinmetz et al 2017, which reported epileptiform events that resembled interictal spikes in several transgenic mouse models expressing both cre recombinase (including Emx1-cre) and GCaMP (Ai93 and Ai94). In Steinmetz et al they observe some generalized seizures in subset of mice, but do not observe spike-wave discharges characteristic of ictal activity in rodent models of epilepsy that occur at higher rates of 7-9 HZ (Fisher et al, 2014). The events in EX KO recordings thus have similar characteristics to these "interictal spikes" in terms of their brief duration variable and high amplitude. Importantly, they are distinct from ictal activity as they are self-limiting, with a slow and variable occurrence rate (~0.3 – 4.0 HZ), without associated high frequency oscillatory activity. The experiments of Steinmetz et al do appropriately raise concern that GCaMP6 expression in EX-NMDAR KO mice is responsible for these aberrant patterns, but importantly, these whole cortex calcium events occurred only EX-NMDAR KO mice or in WT mice after MK-801 injection. Widefield activity was completely normal in N=5 Emx1-cre+, Nr1fl/wt, Snap-25G6+ mice that also received viral injections of AAV9-syn-G6. Electrophysiology was recorded in one such mouse (CTL P15 in Figure 3.5B) with no interictal-like events observed. We thus feel confident that the whole cortex activity seen in EX-NMDAR KO mice and after MK-801 administration are the result of NMDAR loss within the cortical circuit. These results have potential relevance to humans with NR1 mutations, as 65% have epileptiform activity (Lemke et al. 2016; Platzer et al. 2019).
Rather than being “epileptic”, we interpret these dynamics in EX KO mice as reflecting a cortical network with unrefined local and long-range connectivity but with grossly integrated functional connections between excitatory and inhibitory neurons. This network connectivity would allow brief high amplitude events involving the excitatory population across the cortex, but the excitatory discharge would quickly drive the inhibitory population to suppress the events through feedback inhibition. We speculate that the restricted cortical dynamics in EX KO mice may reflect the neurobiological signature of cognitive dysfunction caused by NR1 loss of function in humans. As there is no behavioral or electrophysiological evidence of epilepsy in either EX KO mice or IN KO mice, this aspect of the human syndrome may be more likely explained by NR1 function in sub-cortical regions.

Our results are also relevant for human neurodevelopment and NDDs not directly driven by NMDAR mutations. The in vivo recording techniques used in our study offer a readout that capitalizes on neuron-restricted expression and high spatial and temporal resolution. However, these techniques and the physiological patterns they reveal may be translatable to humans using non-invasive approaches for assessing large scale, system wide neural dynamics, such as functional magnetic imaging (fMRI) or electroencephalogram (EEG). Work in humans using fMRI and EEG has revealed the existence of brain functional networks in pre and early post-natal periods (Fransson et al. 2007; Fair et al. 2009; Thomason et al. 2014; van den Heuvel et al. 2015). During this period in humans, there are not overt behaviors to reveal underlying pathology, and so identifying biomarkers or signposts of normal and abnormal development is critical for identifying and intervening during this critical window (Geshwind D 2007; Bednarz and Kana 2018). Using these tools to track development has already shown promise in predicting future
neuro-cognitive deficits (Hayashi-Kurahashi et al. 2012; Haartsen et al. 2019; Pillay et al. 2020). Our results suggest that a range of mutations or disturbances that impact NMDAR pathways, specifically, or activity dependent processes, generally, might drive network dynamics towards a regime that could be measurable in large-scale functional connectivity or electrophysiological waveform patterns.

In summary, decades of work in human genetics has identified many mutations that can lead to cognitive disability and NDDs (Fombonne et al. 2009; Maulik et al. 2013; Iossifov et al. 2014; Ronemus et al. 2014; Fitzgerald et al. 2015; Sanders et al. 2015). The canonical view is that genetic insults lead to abnormal development and function of neural networks that then manifest as cognitive disability later in life. These genes are predominantly associated with neuronal development and function, including axonal growth, synaptic function and plasticity. Our results demonstrate how eliminating this single receptor in a critical neuronal population can fundamentally disrupt the development of long-range cortical network functional connectivity. Our results also have implications for understanding a fundamental principal of cortical circuit development: in the absence of NMDAR, the cortex will not behave like a set of independent units but rather like a unified sheet locked into a single dominant activity mode. This extreme network state may offer a biomarker in humans to diagnose and to track response to intervention at an early stage of network development prior to behavioral manifestation, when these responses have the greatest opportunity to alter developmental trajectory for children. Not only does understanding the function of the NMDAR have direct relevance for people with NMDAR mutations, the early disruption of cortical network development observed in this study can serve as a point of comparison for the neurocognitive disorders more broadly. Such an identifiable
circuit level phenotype could be a particularly useful signpost in cases in which a single genetic cause cannot be identified.
Chapter 4: The Role of Thalamic Neurotransmission on Shaping Cortical Activity during Development

Results from this chapter are part of a manuscript in preparation for submission:

The molecular and functional properties of somatosensory cortical circuits are dictated by early sensory experience, rather than spontaneous cortical activity

Ethan Mohns, Jacob Lister, Sofia Fertuzinhos, N. Dalton Fitzgerald, Aude Sabino Martinez, Ali Hamodi, Daniel Barson, Michael Crair
**Introduction**

In this study, we tested the role of glutamatergic neurotransmission from thalamo-cortical projections into the cortex by deleting the vesicular glutamate transporter. Previous work in these mice showed that blocking thalamic neuro-transmission prevented formation of barrel columns in the cortex (Li et al. 2013). In this study, we expressed snap-25 GCaMP6s in these mice (TH-VG KO) and recorded spontaneous cortical activity. At P6-7, TH VG KO display localized activity with similar spatial structure to controls; however, activity events were shorter duration, which likely reflects fluorescence in thalamocortical axons that is not transmitted to the cortex to induce thalamocortical spindle oscillations. At P13-15, once endogenous cortical activity normally begins, TH-VG KO display disrupted cortical activity patterns with increased areas of activation and increased bilateral synchrony. These effects were similar though not as extreme as those seen in EX-NMDAR KO mice. These results implicate bottom-up thalamic input as an important source for refining cortical-cortical connectivity.

Studies in EX-NMDAR KO mice demonstrate that cortical network functional organization is exquisitely sensitive to NMDAR function within cortical cells. We hypothesized that this is largely due to NMDAR’s role as a mediator of synaptic plasticity, allowing cortical neurons to alter the strength of their synapses as a function of the input. From the standpoint of cortical development, activity from the thalamus and the sensory periphery guides the refinement of cortical circuits, including thalamocortical and intracortical neuronal connectivity and the distribution and spacing of cortical columns, especially during critical periods of development, presumably through activity-dependent mechanisms (Hensch, 2004). For instance, whisker removal or monocular deprivation during an early “critical period” shifts the anatomical and
functional properties of neurons in the cortex to favor the remaining nondeprived whiskers or eye. In human development, insults that affect neural activity including hypoxic events and maternal drug consumption can lead to neurocognitive disfunction. We therefore sought to test the role of thalamic activity on development of cortical spontaneous activity.

To do so, we used TH-VG KO mice (see Methods) (Li et al. 2013). These mice have normal NMDAR function, but do not release glutamate from the thalamus. Previous work using these mice demonstrated that glutamate release from thalamocortical neurons was essential for cortical barrel column development. Additionally, the differentiation of neurons and the elaboration of superficial layers in the cortex were also disrupted upon removal of excitatory input from the thalamus. These experiments help define limits on the role of intrinsic factors in cortical development and establish a role for extrinsic, presumably activity-dependent factors on cortical columnar, laminar, and neuronal morphological development.
Results

We performed widefield imaging acutely at P13-15. Figure 4.1 shows functional connectivity maps based on seed-based correlations and gives the mean correlation between all seeds and the rest of the cortex. Figure 4.2 shows principal component analysis of spontaneous activity (see Methods). At P13-15, TH-VG KO mice exhibit cortical activity that is shifted to large bilaterally synchronous events, reminiscent of EX-NMDAR KO mice, as evidenced by increased size of cortical events relative to controls and functional connectivity maps that reflect strong bi-lateral synchrony. Notably, the deficit is less extreme in TH-VG KO than in EX-NMDAR KO mice. In TH-VG KO mice, while activity appeared strikingly bi-laterally synchronous, TH-VG KO mice also exhibited a larger range of spatiotemporal patterns and few periods where both anterior and posterior cortical areas are activated together. These differences are evident in the FC maps, which show increased correlation with the contralateral region compared with controls but not between anterior and posterior regions Figure 4.1 (CC Ipsilateral to seed, CC Contralateral to seed, Mean: P13-15 CTL=0.55, 0.50, TH-VG KO=0.64, 0.62, EX-KO=0.89, 0.88, \( p<0.05 \)). These differences are also reflected in the PCA analysis, which shows lower dimensionality that CTL mice but still significantly different from EX-NMDAR KO mice Figure 4.2 (PC1 % Variance Explained, Mean: P13-15 CTL=50%, TH-VG KO=62%, EX-KO=86%, \( p<0.05 \); PR, Mean: P13-15 CTL=3.65, TH-VG KO=2.44, EX-KO=1.32, \( p<0.05 \)).
Figure 4.1: Functional connectivity maps of spontaneous activity TH-VG KO mice

A. Characteristic seed based correlation maps of spontaneous cortical activity

<table>
<thead>
<tr>
<th>Seed Type</th>
<th>WT</th>
<th>TH-VG KO</th>
<th>EX-NMDAR KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal/Motor</td>
<td>![Image](frontal_motor WT)</td>
<td>![Image](frontal_motor TH-VG KO)</td>
<td>![Image](frontal_motor EX-NMDAR KO)</td>
</tr>
<tr>
<td>Somatosensory</td>
<td>![Image](somatosensory WT)</td>
<td>![Image](somatosensory TH-VG KO)</td>
<td>![Image](somatosensory EX-NMDAR KO)</td>
</tr>
<tr>
<td>Visual</td>
<td>![Image](visual WT)</td>
<td>![Image](visual TH-VG KO)</td>
<td>![Image](visual EX-NMDAR KO)</td>
</tr>
</tbody>
</table>

B. Mean correlations across cortex

Correlation Coefficient (CC)

<table>
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<tr>
<th>Ipsi</th>
<th>Contra</th>
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**Figure 4.1. Functional connectivity maps of spontaneous cortical activity in TH-VG KO mice.** (A) Characteristic seed-based correlation maps in CTL, EX-NMDA KO, and TH-VG KO mice. Colorbar represents the strength of correlation (CC) between the seed location (marked by the black dot) and the rest of the cortex. These correlation coefficients range between -1 and 1, with 1 indicating completely correlated activation and -1 indicating completely anti-correlated (note the graphs are displayed between CC=0 (Blue) and CC=1 (Red)). Examples from 10 minute recordings of individual mice are shown for seeds in the right somatosensory cortex (top row), and left visual cortex (bottom row). Examples at P13-14 on the right. (B) For summary quantification, 50 seeds are placed randomly across the cortex and the mean CC between that seed and the ipsilateral and contralateral cortex is calculated. Graphs show the mean CC and SEM for the group means for all seeds. P13-16 N=9 CTL, N=6 EX NMDA KO, N=4 TH VG KO Star indicates p value less than .05 following one-way ANOVA with Tukey’s multiple comparisons test. For P13-15: TH VG KO vs CTL p<0.05, EX KO vs CTL p<0.05, TH VG KO vs CTL p<0.05.
Figure 4.2: Principal component analysis of spontaneous cortical activity in TH-VG KO mice

A. Characteristic principal components (PCs) and % variance explained

B. Percent Variance Explained

C. Dimensionality
Figure 4.2. Principal component analysis of spontaneous activity in TH-VG KO mice. A)

Characteristic examples of individual mice at P13-15 showing spatial motifs and percentage of variance explained for the first five principal components. (B) Summary for all mice, with the graphs showing the percentage of variance explained by the first five principal components. Star indicates $p$-value less than 0.05 one-way ANOVA with Tukey’s multiple comparisons test. (C) Dimensionality as calculated from the Participation Ratio.
Discussion

Using mice in which cortical NMDAR function is normal but upstream thalamic glutamate is blocked, we demonstrate the whole cortex spontaneous activity is disrupted along a similar, though less extreme, trajectory as EX-NMDAR KO mice. Importantly, the cortex does not become intrinsically active until ~P10-12 and its activity is predominantly driven by sub-cortical thalamic inputs which are known to be necessary to refine cortical circuits. Thus, these results support the interpretation that disrupting activity dependent process of cortical circuit development prevents the emergence of normal patterns of spontaneous activity, shifting the dynamics towards large, bi-laterally synchronous activity events. These results suggests that such network patterns are signatures of a convergent pathological process during development – disrupted activity dependent plasticity – which may be disrupted as a result of a range of environmental or genetic factors in development. 1) Inhibiting the intrinsic ability of cortical excitatory neurons to respond and undergo neuroplastic changes through NMDAR signaling or 2) removing the sub-cortical glutamatergic inputs that guide cortical development during critical periods can both drive a similar common pathway reflecting failure of refined circuit connectivity.

It is notable that this manipulation has a less extreme effect on cortical activity as EX-NMDAR KO. A potentially implication of these results is that thalamic activity is particularly responsible for promoting asymmetry between analogous cortical regions (such as left and right somatosensory regions), but less critical for refining cortical-cortical connections between distinct cortical regions (such as visual and somatosensory). Our EX-NMDAR KO results would argue that connectivity between areas – such as between somatosensory and visual regions – is also activity dependent, but perhaps relies more critically on intra-cortical activity.
However, we should be cautious in over-interpreting this aspect of the results as the TH-VG KO likely reduces but does not completely eliminate thalamic glutamate release. Indeed, in Li et al. 2013 reported that Sert-Cre expression is much weaker in the dLGN and MGN in comparison to the somatosensory thalamus and Vglut2 mRNA and VGLUT2 protein levels were only modestly decreased in the dLGN (68.9% of control mRNA levels) and MGN (48.4% of control mRNA levels) of ThVGdKO mice at P12. In contrast, Vglut2 mRNA in the VB was only 13.5% of control levels and VGLUT2 protein levels was 20% of control levels at P4. Unlike in the barrel cortex, they did not observe lamination defects the visual or auditory cortex. Importantly, despite no lamination defects in these other regions, TH-VG KO mice clearly display abnormal cortical activity in visual regions. However, we cannot rule out that cortical activity might be further disturbed if a more complete blockade of glutamatergic transmission were achieved.
Chapter 5: Cortical Activity during Development in other Mouse Models of Neurodevelopmental Disorders

Introduction

The experiments showing the profound impact of NMDAR disruption on cortical development offers a point of comparison with other putative disease-causing genes in humans. In the final section, we discuss preliminary studies testing three other mono-genetic causes of neurodevelopmental disorders with validated mouse models: the FMR1 KO Mouse based on Fragile X Syndrome, the CNTNAP2 Mouse, and the CACA1 Mouse Model of Timothy syndrome.

Fragile X Syndrome and the FMR1 KO Mouse Model

Fragile X Syndrome (FXS) is the most common known monogenic cause of ASD/ID (Hagerman RJ et al. 2017). It is caused by the functional silencing of the mRNA binding protein, fragile X mental retardation protein 1 (FMR1). Although caused by a single gene, FMR1 is dynamically regulated with unique developmental, tissue, and cell-type expression and numerous known functions, as it can target up to 4% of mRNAs in the mammalian genome (Hagerman RJ et al. 2017). The consequences of the loss of function of this gene are highly variable in humans (Hagerman RJ et al. 2017); thus, it presents similar challenges to understanding heterogeneity of the many genetic insults that can lead to ASD/ID but has the experimental advantage of being caused by a loss of function in a single gene, which might be used to unlock mechanisms of pathogenesis that describe final common pathways of dysfunction across ASD/ID.
Since the FMR1 KO mouse was introduced in 1994 (Dutch-Belgian Fragile X Consortium 1994), an enormous research effort has elaborated pathophysiology at the cellular level *in vitro*, with strong evidence for disruption in excitatory and inhibitory neurotransmission and synaptic plasticity deficits in the neocortex and associated brain regions (Kazdoba et al. 2016a; Contractor et al 2015). In terms of the study of cortical network activity, groups have reported disruptions in cortical circuitry *in vitro* (Gibson et al. 2008; Bureau et al. 2008; Testa-Silva et al. 2012; La Fata 2014) and *in vivo* (Hays et al. 2011; Goncalves et al. 2013; He et al. 2017). *In vivo*, there have been several published reports of spontaneous neural activity in the neocortex in unanaesthetized FMR1 KO mice. A study using multi-electrode LFP recordings in primary somatosensory cortex in adult FMR1 KO mice found longer durations of spontaneously occurring “up states”, which suggested a signature of “hyper-excitability” (Hays et al 2011). Another group used two-photon calcium imaging and electrophysiology (whole-cell patch clamp and multi-electrode LFP) to record spontaneous neuronal ensemble activity in at 1 week, 2 week, and 3-4 weeks in primary somatosensory cortex (Gonçalves et al 2013). They found developmental and state specific differences: during slow wave sleep at P12-14, unanesthetized FMR1 KO mice exhibited higher average firing rates and elevated network synchrony measured as a higher Pearson correlation coefficient recorded using 2-photon imaging with a pan cellular fluorescent indicator (Oregon Green BAPTA). The overall evidence from studies on FMR1 KO mice suggests that “hyper-excitability” at the synaptic, circuit, and behavioral level is at the core of the pathophysiology of the illness. (Contractor et al. 2015).

Of note, these studies have been carried out exclusively in local circuits using local field potential recordings or 2-photon imaging of local cell populations. There have been no mesoscale
recordings reported and so we do not know the impact of FMR1 deletion on the functional connectivity of the cortical network. There is a widespread hypothesis that there is reduced connectivity in large-scale networks in ASD (Geshwind and Levitt 2007), with long-range connections, with connectivity to higher order neocortical regions suggested to be weaker. In humans, there have only been a few studies looking at FXS specifically (Bednarz and Kana 2018; Hall et al. 2013; van der Molen 2014; Heard et al. 2014; Bruno et al. 2017). Using resting state EEG in males with FXS, Van der Molen et al. 2014 found decreased in global functional connectivity in specific frequency bands (alpha and beta) and increased connectivity in long range (fronto-posterior) and short range (frontal-frontal and posterior-posterior) clusters (Hall et al. 2013). Additionally, Heard et al. 2014 reported abnormal EEG findings with slowing of background rhythm and epileptiform discharges in a small group of children with FXS whose parents reported behaviors resembling seizures (Van der Molen et al. 2014).

The work on FMR1 KO mice has led to many novel treatment approaches and over twenty trials in humans, all of which have been unsuccessful (Kazdoba et al. 2016a, 2016b; Erickson et al. 2017). We hypothesize that we must test FMR1 KO mice at the level of cortical functional connectivity to effectively link between mouse models and humans.

**CNTNAP2 Mouse Model**

Contactin-associated protein-like 2 (CASPR2) is encoded by *CNTNAP2* and clusters voltage-gated potassium channels (Kv1.1) at the nodes of Ranvier. A homozygous mutation of *CNTNAP2* identified in Old Order Amish children causes cortical dysplasia, focal epilepsy, relative macrocephaly, and diminished deep-tendon reflexes. Intractable focal seizures begin in
early childhood, followed by language regression, hyperactivity, impulsive and aggressive behavior, and mental retardation that developed in all children with this mutation. Temporal-lobe specimens showed evidence of abnormalities of neuronal migration and structure, widespread astrogliosis, and reduced expression of CASPR2. Cntnap2−/− mice exhibit epileptic seizures and abnormal electroencephalogram (EEG) pattern, neuronal migration abnormalities, reduced numbers of interneurons, and reduced cortical neuronal synchrony (Peñagarikano et al. 2011). Furthermore, they display hyperactivity and deficits in core ASD behavioral domains. Specifically, they show stereotypic motor movements and behavioral inflexibility, as evidenced by impaired reversal learning on the Morris Water Maze task and the spontaneous alternation T maze test. Additionally, they exhibited motor stereotypies as evidenced by increased grooming. Studies in CNTNAP2 showed a decrease in synchrony between cell pairs using two-photon calcium imaging in layer II/III of somatosensory cortex in young adult mice (2-4 months of age). Notably that difference was subtle (difference ~0.1 mean correlation) (Penagarikano et al 2011). Notably, a later study also showed reduced local and long-range connectivity in pre-frontal and midline connectivity hubs using resting state FMRI performed under anesthesia (Liska et al 2018).

Timothy syndrome and the TS2-neo Mouse Model

Timothy syndrome is a disorder characterized by multiorgan dysfunction including lethal arrhythmias, webbing of fingers and toes, congenital heart disease, immune deficiency, intermittent hypoglycemia, cognitive abnormalities, and autism. Timothy syndrome results from the de novo Ca(V)1.2 missense mutation G406R (Splawski et al. 2004). Ca(V)1.2, the cardiac L-
type calcium channel, is important for excitation and contraction of the heart, as well as in all affected tissues. The G406R mutation produces maintained inward Ca(2+) currents by causing nearly complete loss of voltage-dependent channel inactivation. This likely induces intracellular Ca(2+) overload in multiple cell types. In the heart, prolonged Ca(2+) current delays cardiomyocyte repolarization and increases risk of arrhythmia, the ultimate cause of death in this disorder. Along with normal general health, activity, and anxiety level, TS2-neo mice showed markedly restricted, repetitive, and perseverative behavior, altered social behavior, altered ultrasonic vocalization, and enhanced tone-cued and contextual memory following fear conditioning. (Bader et al. 2011)
Results

We have collected a preliminary data set of whole cortex mesoscale imaging in FMR1 KO mice, TS-neo mice, and CNTNAP2 KO mice and littermate controls expressing GCamp6 under a pan neuronal (snap-25) promoter at two weeks (P12-16), N=4 (FMR1 KO), N=2(TS-neo) and N=2(CNTNAP2). Figure 5.1 shows characteristic seed-based maps in FMR1 KO, TS, and CNTNAP2 mice. These examples suggest that large scale topography in these mice is largely normal, but the cortex-wide synchrony is decreased compared to CTL mice. Figure 5.1 (CC Ipsilateral to seed, CC Contralateral to seed, Mean: P13-15 CTL=0.55, 0.50, FMR1 KO=0.38, 0.34, CNTNAP2 KO=0.43, 0.38, TS2-neo=0.40, 0.37, EX-KO=0.89, 0.88, p<0.05). Figure 5.2 shows principal component analysis in NDD model mice, which also shows that the first PC explains less of the total variance and the participation ratio reflected the dimensionality is increased. Figure 5.2 (PC1 % Variance Explained, Mean: P13-15 CTL=50%, FMR1 KO=37%, CNTNAP2 KO=40%, TS2-neo=37%, EX-KO=86%, p<0.05; PR, Mean: P13-15 CTL=3.65, FMR1 KO=6.50, CNTNAP2 KO=5.62, TS2-neo=6.78, EX-KO=1.32, p<0.05).
Figure 5.1: Functional connectivity maps of spontaneous cortical activity in NDD model mice

A. Characteristic seed based correlation maps of spontaneous cortical activity

<table>
<thead>
<tr>
<th>Seeded Region</th>
<th>CTL</th>
<th>FMR1 KO</th>
<th>CNTNAP2 KO</th>
<th>TS2-neo</th>
<th>EX-NMDAR KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal/Motor Seed</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
</tr>
<tr>
<td>Somatosensory</td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
</tr>
<tr>
<td>Visual Seed</td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
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</tbody>
</table>

B. Mean correlations across cortex

[Graph showing mean correlations across cortex]
Figure 5.1. Functional connectivity maps of spontaneous cortical activity in mouse models of NDDS. (A) Characteristic seed-based correlation maps from 10 minute recordings of individual mice. Example seeds in the Frontal/Motor Cortex (top row), Somatosensory Cortex (middle row), and Visual Cortex (bottom row) at P8-9 are shown on the left and at P13-14 on the right. The colorbar represents the strength of correlation (CC) between the seed location (marked by the black dot) and the rest of the cortex. These correlation coefficients range between -1 and 1, with 1 indicating completely correlated activity and -1 indicating completely anti-correlated (note the graphs are displayed between CC=0 (Blue) and CC=1 (Red)). (B) For summary quantification, 1000 seeds are placed evenly across the cortex and the mean CC between that seed and the ipsilateral and contralateral cortex is calculated. Graphs show the mean CC for all seeds for individual animals and the group means for Ipsilateral CC and Contralateral CC. Star indicates p-value less than 0.05 following one-way ANOVA with Tukey’s multiple comparisons test or Student’s T-test.
Figure 5.2: Principal component analysis of spontaneous cortical activity in NDD model mice

A. Characteristic principal components (PCs) and % variance explained

B. Percent Variance Explained

C. Dimensionality
Figure 5.2. Principal component analysis of spontaneous activity in mouse models of NDDs. A) Characteristic examples of individual mice at P13-15 showing spatial motifs and percentage of variance explained for the first five principal components. (B) Summary for all mice, with the graphs showing the percentage of variance explained by the first five principal components. Star indicates p-value less than 0.05 one-way ANOVA with Tukey’s multiple comparisons test. (C) Dimensionality as calculated from the Participation Ratio.
Chapter 6: Conclusions, Implications, and Future Directions

Summary

In summary, these results show 1) that excitatory specific knockout of NMDAR drives cortical activity to an extreme state in which normal activity is almost completely degraded, 2) blocking thalamic neurotransmission to the cortex leads to disrupted cortical activity by P13 along a similar trajectory as EX NMDAR KO but to a less extreme degree, and 3) several other mechanistically valid models based on mono-genetic causes of NDDs show cortical activity patterns that are grossly normal and with reduced cortex-wide synchrony when viewed through widefield mesoscopic imaging. Taken together, these results demonstrate an essential role for NMDAR in cortical development and we have hypothesized that this role is largely due to its capacity to mediate patterned neural activity through the thalamus in addition to potentially intracortical sources. These studies lead to a number of future questions and implications for disease in humans that will be discussed below.

Activity dependent mechanisms during cortical development

As discussed above, we think that the combined evidence in EX-NMDA KO and TH-VG KO mice from our own studies and previous studies argues in favor of the notion that the normal complexity and organization of whole cortex spontaneous dynamics is a reflection of activity dependent refinement of cortical circuits (See Figure 3.7). In multiple cortical and sub-cortical circuits, the development of long-range connectivity begins first with neurons sending out axonal projections to the proper target area guided by axon guidance molecules (e.g. VPM thalamic projections to somatosensory cortex). Neurons initially make synaptic connections with a large
number of neighbors before refining into precise targets through activity dependent processes. Based on previous work, in both EX-NMDAR KO and TH-VG KO mice thalamic axons still target the cortex and create a gross whisker map, but the somatosensory cortex fails to form organized barrel maps of whisker representation (Iwasato et al. 2000, Li et al. 2013). There are normal cell numbers, but the dendritic distributions of layer IV cells are different. In EX NMDAR KO mice, there is an increase in dendritic density relative to controls (~20%, see above, Datwani et al. 2002), whereas in the TH-VG KO mice there is a greater than 3-fold reduction in dendritic density relative to controls. Furthermore, in EX KO mice, layer IV cortical cells failed to show orientation bias of their dendrites towards single thalamocortical projection clusters (63% in control vs 17% EX NMDAR KO, Datwani et al. 2002), showed an increase in dendritic density (20% greater spine density on secondary dendrites in EX NMDAR KO compared to Control, Datwani et al. 2002), and had increased input from multiple whiskers rather than a single whisker (Lo et al 2015). A mechanism for this connectivity was suggest by a study which showed that NMDAR was responsible for the stabilization of individual dendrites by increased motility of dendrites both inside and outside the TCA cluster (Mizuno et al. 2013). Functionally, this diffuse and unrefined anatomical connectivity was reflected by spatially diffuse voltage sensitive dye (VSD) responses in barrel cortex to deflections of single whiskers (half max area = 1.86mm² in EX KO mice vs .71mm² in control, Lo et al 2013). Also observed in this functional study, layer IV neurons in EX NMDA KO mice showed reduced temporal summation response to tonic deflection of whisker (5.6 spikes in control versus 2.3 in EX NMDAR KO, Lo et al. 2013), presumably due to loss of the long channel kinetics of NMDARs. Overall, this work suggests that neural activity but not NMDAR is required for the maintenance of synapses, and NMDAR’s specific contribution is the
instantiation of Hebbian plasticity – the strengthening and weakening of connections as a function of pre and post synaptic spiking.

Outstanding questions and proposed experiments to determine mechanisms of NMDAR function in cortical development

Experiment 1: Test role of arousal in mediating whole cortex, synchronous activity

The dynamic state of the mature cortex is linked closely to the arousal state of the animal, with increased arousal and attention associated with a “de-synchronized” LFP and decreased arousal and sleep associated with larger amplitudes oscillations or “slow waves”. The immature cortex, on the other hand, is characterized by a flat LFP punctuated by “spindle bursts”, which are driven primarily by the periphery (e.g. muscle twitches and retinal waves). As shown in Figure 6.5, in an EX NMDA KO mouse at P16, large activity events are clearly independent of movement and persist through periods of sleep, as reflected in the low amplitude of the electromyogram (EMG) and videographic observation. However, the question remains how changes in arousal either associated with or independent of movement affect global cortical activity.

We propose first to perform a careful analysis of data already collected to determine the relationship between large amplitude events and 1) physiological proxies for arousal – heart and breathing rate - and 2) movement and movement transitions. While the results of such analysis will be informative, the question will remain open how individual neuromodulators would distinctly affect activity. Increased neuromodulator tone tends to desynchronize the EEG while leaving overall firing rate intact (Harris and Thiele 2011). In the prefrontal cortex, low levels of
neuromodulators are necessary for circuit functioning, with optimal levels of dopamine further refining working memory representations, whereas at higher levels working memory function is degraded and behavioral control shifts to sub-cortical circuitry (Arnsten et al. 2012). Thus, we might interpret neuromodulatory influences in the mature cortex as primarily shifting the local and network level operational state. Observations from our work show prevalence of high amplitude LFP and whole cortex events during quiet resting or sleep period, with periods of sustained movement (and presumed increases in neuromodulatory tone) restricting activity to midline and somatosensory trunk regions, albeit still in a bilaterally synchronous fashion. (see Figure 6.5). Before P11-12 and the onset of slow waves, cortical state is thought to be independent of arousal state (e.g. Colonnese et al 2010). However, large amplitude events are clearly evident during this period (see Chapter 3). We would propose to closely characterize the relationship between changes in arousal and large amplitude events. Moreover, we would propose at both ages to test the impact on spontaneous activity following local application of pharmacological agents.
Figure 6.1: Relationship between movement, LFP, and widefield cortical activity in EX-NMDAR KO mice
Figure 6.1: Relationship between movement, LFP, and widefield cortical activity in EX-NMDAR KO mice. Simultaneously recorded electromyograms, local field potential, and whole cortex Ca2+ activity. Top section shows 40 second period. The bottom section shows 20 seconds of that period in conjunction with whole cortex activity.
**Experiment 2: Characterize response to peripheral driven inputs**

Peripheral driven events are evident in EX NMDAR KO mice. Retinal waves are visible alongside and distinct from large activity events through P11-12 in EX NMDAR KO mice. Using data already collect, we would aim to characterize their properties to determine whether they change across development. Stage 2 waves appear grossly normal in EX NMDAR KO mice, but a previous study from our lab demonstrated that the cortex does not automatically respond to peripherally driven waves after P10 (Gribizis et al. 2019). Considering the abnormal cortical dynamics and activity patterns, it would be informative to quantify how stage 3 waves are transformed in the cortex. Similarly, following whisker deflections, discrete whisker responses in the contralateral somatosensory region can be evoked in EX NMDAR KO mice, suggesting these events are distinct from large scale cortical events. Both these sets of observations help to reinforce that the dominant large events are not merely the result of sensory inputs from the thalamus.

**Experiment 3: Determine cellular and local circuit basis of disrupted activity patterns**

Whole cortex calcium dynamics and LFP recordings suggest a hyper-synchronous local circuit with a large percentage of participation by cells in the circuit. Alternatively, these dynamics could be driven primarily from a hyper-connected and frequently firing sub-network across the cortex. 2-photon imaging of local circuits would be ideal to determine the underlying cellular dynamics because it allows the observation of a large number of neurons, including infrequently firing neurons. Using 1P/2P multiscale imaging (Barson, Hamodi et al. 2019), we would directly quantify the relationship between the numbers of cells participating in the local event and the largescale fluorescence. These approaches would further allow us to answer the question of whether there
are cells the fire independently or whether smaller sub-networks exist that are drowned out by large amplitude, synchronous events. This experiment could also be used to compare EX NMDAR KO mice with WT 1 mg/kg MK-801 to determine whether similar large scale activity patterns are driven by similar dynamics at the local circuit level.

**Experiment 4: Determine long range anatomical connectivity underlying disrupted activity patterns**

A recent study (Zhou et al 2020) examined callosal projections between somatosensory regions in EX NMDAR KO mice. They observed that differences in projection patterns could be observed as early as P6, were severe by P14, and persisted through P30 (i.e. through the ~30 day lifespan of the mice). Most prominently, whereas in normal mice, callosal projections terminated at the S1/S2 border, in EX KO mice projections were strong to all layers and projected diffusely throughout S1. They measured this difference first by the fluorescence intensity, which was restricted to the lateral extent in control mice but evenly distributed in EX NMDAR KO mice. They also measured the fluorescence density across S1, which was greater in EX NMDAR Ko mice compared to controls by more than a factor of 2.

These results provide strong evidence for diffuse unrefined connectivity between homologous cortical regions, providing a clear anatomical substrate for the nearly perfect bilateral correlations of spontaneous activity in EX KO mice. However, equally striking in our whole cortex recordings are the high correlations between anterior cortex (e.g. motor and somatosensory areas) and posterior cortex (e.g. visual areas). We therefore propose to test whether anatomical connectivity can also help explain this physiological pattern. To test this
hypothesis, we would perform viral tracing experiments, and carefully quantify the distribution in unrelated regions (e.g. injection in somatosensory regions and test in visual). For example, we could inject AAV-CAG-GFP-flex (Plasmid#59331) or AAV-TD-Tomato-flex (Addgene, 28306-AAV1) into S1 at P0. At P14, we would sacrifice the mouse, make coronal sections, and image across the cortex. Our expectation would be significantly more fluorescence in regions such as V1 or retrosplenial cortex in EX NMDAR KO mice compared to controls.

To test the capacity for initiation of large events in different regions throughout the cortex, we would drive individual regions optogenetically using channelrhodosin-2. For example, we could inject AAV-CAG-ChR2-tdTOMATO (#SL100897, SignaGen Laboratories) in either V1, S1, or M2 and test whether whole cortex events can be elicited. In particular, we would test whether this ability exists 1) at P7 when callosal projections begin to differentiate and when whole cortex events are clearly present, but intrinsically generated cortical slow waves have not yet begun and 2) at P14, when callosal projections are diffuse in EX NMDAR KO mice and slow waves have begun during normal development.

**Experiment 5: Test developmental versus acute NMDAR role on cortical activity**

A fundamental question our study leaves open is the acute versus developmental role of NMDAR function in the disrupted cortical activity that we observe. This question has direct relevance on human disease from the standpoint of understanding when pathogenesis begins and how early in development it might be necessary to detect and intervene in order to reverse developmental trajectory. This question can be viewed from two angles, both of which are potential experimentally testable. First, to what extent does
the observed cortical activity reflect failures of earlier developmental refinement processes. Second, to what extent are normal patterns (and ultimately circuit function) restorable at later ages.

To distinguish excitatory specific NMDAR function in the developmental versus acute setting, future experiments could use a tamoxifen inducible Emx1-cre (Emx1-CreERT2, Jackson 027784, Kessaris et al. 2006) to exert temporal control over NMDAR deletion. There are several key windows that would be relevant to test: 1) delivery of tamoxifen starting at P0 to determine whether pre-natal NMDAR function is important for our observed phenotype, 2) delivery starting ~P3-4 to test the dependence on NMDAR function before onset of intrinsic cortical activity, which begins around P10-12 in mice (see above) 3) delivery starting around P11-12 to determine the acute role of the loss after initial circuit balance has been established.

Because this approach would require long-term future studies, we aimed to gain initial insight into the question by testing whether acute pharmacological blockade of the NMDAR could recapitulate cortical dynamics seen in EX NMDAR KO mice (Figures 6.2 – 6.5). Although this approach sacrifices cellular and regional specificity available through cell-specific deletions, it is a tractable way to gain initial insight into this question. We performed this experiment using the NMDAR antagonist MK 801 (Coan et al. 1987, Huettner et al. 1987), which we applied acutely to wild type (Snap-25 G6) mice at P7-9 and P13-15. We initially tested several doses at P7-9, including 0.5 mg/kg, 1/0 mg/kg, and 3.0 mg/kg. These reflected a range of doses in adult mice that lead to a range of responses from hyperactivity and cognitive deficits in some tasks at lower doses, to reduced activity and severe cognitive deficits at intermediate doses, and nearly complete reduced activity and general anesthesia at higher doses (Miyamoto et al. 2000). At this early post-natal timepoint, 0.5 mg/kg MK 801 led to inconsistent behavioral and cortical dynamic responses (n=4 mice, data not shown). At 3.0 mg/kg, mice were universally anesthetized, and
cortical activity was drastically reduced, with periods on the order of many seconds with no activity, punctuated by brief burst of activity (N=4 mice, data not shown). However, at 3.0 mg/kg, although the mice universally regained wakefulness after 4-6 hours, none regained full neurobehavioral capacities. Specifically, they flailed their limbs, but did not regain the ability to move their body in a coordinated fashion, including walking, turning over, or feeding. This permanent behavioral damage may be consistent with known lesions caused by high dose exposure to MK-801, known as Olney’s lesions (Olney and Labruyere 1989). However, at 1 mg/kg, P7-9 mice were also anesthetized, but were able to reawaken and regain full behavioral function and normal cortical dynamics. Therefore, we focused our analysis on 1 mg/kg (See Figures 6.2 – 6.5).

Injection of MK-801 1.0 mg/kg shifted dynamics towards larger areas of activity as measured by Fraction of Cortical Pixels Active (Figure 6.2 (c), group Mean±Standard Error of the Mean (SEM) are shown for FA<0.1, 0.1<=FA<=0.9, FA>0.9: P7-9 WT=0.21±0.02, 0.69±0.04, 0.06±0.01, WT 1 mg/kg MK-801=0.55±0.02, 0.31±0.03, 0.12±0.01, EX KO=0.53±0.03, 0.26±0.04, 0.21±0.01; P11-17 WT=0.20±0.01, 0.70±0.01, 0.10±0.01, WT 1 mg/kg MK-801=0.43±0.01, 0.35±0.01, 0.22±0.01, EX KO=0.43±0.02, 0.29±0.02, 0.28±0.01, p<0.05), increases in cortex wide and bilateral synchrony (Figure 6.3 (b) (CC Ipsilateral to seed, CC Contralateral to seed, Mean±SEM: P7-9 WT=0.45±0.03, 0.35±0.03, WT 1 mg/kg MK-801=0.68±0.02, 0.65±0.02; EX KO=0.90±0.03, 0.89±0.04, P11-17 WT=0.57±0.01, 0.52±0.01, WT 1 mg/kg MK-801=0.45±0.06, 0.39±0.07, EX KO=0.89±0.01, 0.89±0.01), and reduction in dimensionality as reflected in the increased variance explained by the first principal component and the reduced participation ratio (Figure 6.4 (b), leftmost graph, PC1 % Variance Explained, Mean±SEM: P7-9: WT 40.18±2.41%,
WT 1 mg/kg MK-801=71.83%±2.67%, EX KO=93.75%±1.35%; P11-17: WT=52.76%±1.35%, WT 1 mg/kg MK-801=77.64%±0.99%; EX KO=94.42%±0.49%, and p<0.05; Figure 6.4 (c) Participation Ratio, Mean±SEM: P7-9 WT =5.42±0.43, WT 1 mg/kg MK-801=2.12±0.19, EX KO=1.46±0.10, and, p<0.05; P11-17: WT=3.43±1.64, WT 1 mg/kg MK-801=1.64±0.04 EX KO=1.32±0.07, and, p<0.05).

The differences in these dynamics can be visualized by the diffusion maps shown in Figure 6.4. This shift to larger domains of activity and more restricted dynamic motifs moved the activity patterns towards those seen in EX KO mice. This shift was intermediate at P7-9 and was even closer at P13-15. We quantified these differences by comparing WT, WT 1 mg/kg MK-801, and EX KO mice. For every measure in Figure 6.1 – 6.3, WT were significantly different from both WT 1 mg/kg and EX KO mice.

Our initial hypothesis was that observed activity patterns in EX KO mice were predominantly the result of developmental effects on circuit connectivity and synaptic transmission. To our surprise, we find that acute NMDAR antagonism causes major shifts in cortical activity that substantially recapitulates what we see in EX KO mice, especially at P13-15. These results suggest that the capacity of the cortex to elaborate a wide range of spontaneous activity patterns relies on the acute physiological function of NMDAR in cortical excitatory neurons. Loss of this function results in a dynamic network space that is highly restricted in mice who have developed normally to this point.

A second and equally relevant question is to what extent are normal patterns (and ultimately circuit function) restorable at later ages. One approach to test the capacity rescue could be through viral vector mediated re-expression of NR1 in excitatory cells. Another approach would involve using whole body knockdown. A recent study using a global NMDA receptor knockdown and rescue found that several behaviors could be partially – though not fully rescued – with re-expression of NMDA receptors in adulthood (Lee et al. 2019). They did so by a targeted
insertion of a *loxP* site to the *neo* cassette (that causes the reduced NR1 expression), allowing it to be excised following Cre recombination to restore the wild-type locus. This study raises the hopeful possibility that some potential for recover may persist in the system. Our own work suggests that the structure of motifs as well as the patterns of connectivity beyond the dominant mode bear resemblance to normal structure. Perhaps the gross patterns of connections independent of NMDAR function would provide a scaffold for improved function if NMDA receptor is restored. However, based on the incomplete recovery in this study and human evidence in NDDs, certain capacities cannot reach full potential if normal developmental windows are missed (Fombonne E. 2009). We could test and establish the effects on cortical activity in NMDAR KD mice, and then, test re-expression. In either case, key age points to test would be 1) at birth, 2) around P3-4, 3) after P11-12 for similar reasons as above.
Figure 6.2: Whole cortex spontaneous activity dynamics after MK-801 administration

A. Characteristic spatial patterns and temporal dynamics

B. Fraction of cortical pixels active (FA)

C. Proportion Time
Figure 6.2 Whole cortex spontaneous activity dynamics after MK-801 administration. (A) Wild type (snap-25 G6) mice were recorded at baseline (0 mg/kg MK-801) and then after delivery of MK 801 1mg/kg. Characteristic image sequences and temporal dynamics are shown at P8-9 and P13-14 in WT, WT 1 mg/kg MK-801, and EX KO mice. For both ages, example 5 second image sequences are shown. The first trace shows mean $\Delta F/F_0$ across the cortex for a 200 second (2000 frame) period from which these examples sequences are drawn. The second trace shows the fraction of cortical pixels active above their mean fluorescence (calculated individually for each pixel) for the same 200 second period (FA). (B) For quantification, we grouped P7-9 (WT: N=7 mice; WT 1 mg/kg MK 801: N=4 mice; EX KO: N=5 mice) and P11-17 (WT 0 mg/kg MK 801: N=4 mice; WT 1 mg/kg MK 801: N=4 mice; EX KO: N=6 mice). The histograms show proportion of time for the range of FA for all mice. (C) The graphs show average proportion of time for all mice for when FA > 0.1 (top graph), FA = 0.10 - 0.90 (middle graph), and FA > 0.90 (bottom graph). Star indicates $p$ value less than 0.05 following one-way ANOVA with Tukey’s multiple comparisons test. For FA < 0.1 P7-9: WT vs WT 1 mg/kg MK 801 $p$<0.05, WT 1 mg/kg MK 801 vs EX KO $p$=n.s.; P11-17: WT vs WT 1 mg/kg MK 801 $p$<0.05, WT 1 mg/kg MK 801 vs EX KO $p$=n.s. For FA > 0.90, P7-9: WT vs WT 1 mg/kg MK 801 $p$<0.05, WT 1 mg/kg MK 801 vs EX KO $p$<0.05; P11-17: WT vs WT 1 mg/kg MK 801 $p$<0.05, WT 1 mg/kg MK 801 vs EX KO $p$<0.05.
Figure 6.3: Functional connectivity maps of spontaneous activity after MK-801 administration

A. Characteristic seed based correlation maps of spontaneous cortical activity

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<thead>
<tr>
<th></th>
<th>P8-9</th>
<th>P13-14</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>WT 1 mg/kg-MK 801</td>
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B. Mean correlations across cortex
**Figure 6.3. Functional connectivity maps of spontaneous cortical activity.** (A) Characteristic seed based correlation maps. Colorbar represents the strength of correlation (CC) between the seed location (marked by the black dot) and the rest of the cortex. These correlation coefficients range between -1 and 1, with 1 indicating completely correlated activation and -1 indicating completely anti-correlated (note the graphs are displayed between CC=0 (Blue) and CC=1 (Red)). Examples from 10 minute recordings of individual mice are shown for seeds in the frontal/motor cortex (top row), somatosensory cortex (middle row), and visual cortex (bottom row). Examples at p8-9 are shown on the left and at p13-14 on the right. (B) For summary quantification, 1000 seeds are placed evenly across the cortex and the mean CC between that seed and the ipsilateral and contralateral cortex is calculated. Graphs show the mean CC for all seeds for individual animals and the group means for ipsilateral, contralateral, and the difference between ipsilateral and contralateral. Star indicates $p$ value less than .05 following one-way ANOVA with Tukey’s multiple comparisons test. For cortex ipsilateral to seeds, P7-9: WT vs WT 1 mg/kg MK 801 $p<0.05$, WT 1 mg/kg MK 801 vs EX KO $p<0.05$; P11-17: WT vs WT 1 mg/kg MK 801 $p<0.05$, WT 1 mg/kg MK 801 vs EX KO $p<0.05$. For cortex contralateral to seeds, P7-9: WT vs WT 1 mg/kg MK 801 $p<0.05$, WT 1 mg/kg MK 801 vs EX KO $p<0.05$; P11-17: WT vs WT 1 mg/kg MK 801 $p<0.05$, WT 1 mg/kg MK 801 vs EX KO $p<0.05$. 
Figure 6.4: Principal component analysis of spontaneous cortical activity after MK-801 administration

A. Characteristic principal components (PCs) and % variance explained

<table>
<thead>
<tr>
<th></th>
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<th>PC 2</th>
<th>PC 3</th>
<th>PC 4</th>
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<tr>
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<td>14.66</td>
<td>03.30</td>
<td>01.87</td>
<td>00.94</td>
</tr>
<tr>
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<td>04.70</td>
<td>00.91</td>
<td>00.61</td>
<td>00.55</td>
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B. Percent Variance Explained

C. Dimensionality
Figure 6.4. **Principal component analysis of spontaneous cortical activity.** (A) Characteristic examples of individual mice at p8-9 and p13-14 showing spatial motifs and percentage of variance explained for the first five principal components. (B) Summary for all mice, with the left graph showing the percentage of variance explained by the first principal component and the right graph showing the dimensionality as reflected by the participation ratio. P7-9 and P11-17. Star indicates $p$ value less than 0.05 one-way ANOVA with Tukey’s multiple comparisons test. For variance explained by the first PC: P7-9: WT vs WT 1 mg/kg MK 801 $p<0.05$, WT 1 mg/kg MK 801 vs EX KO $p<0.05$; P11-17: WT vs WT 1 mg/kg MK 801 $p<0.05$, WT 1 mg/kg MK 801 vs EX KO $p<0.05$. For dimensionality, P7-9: WT vs WT 1 mg/kg MK 801 $p<0.05$, WT 1 mg/kg MK 801 vs EX KO $p<0.05$; P11-17: WT vs WT 1 mg/kg MK 801 $p<0.05$, WT 1 mg/kg MK 801 vs EX KO $p<0.05$. 
Figure 6.5: Diffusion map of spontaneous cortical activity after MK-801 administration

A. Characteristic diffusion map
Figure 6.5. Diffusion map of spontaneous cortical activity (A) Characteristic diffusion maps of 20 minute/12000 frame movies in WT and WT 1 mg/kg MK 801 at P8-9 and P13-14. The axes represents distance in diffusion space, which reflects similarities in terms of transition probability between data points, with parameter sigma=8 (arbitrary units). Each dot represents a single frame and the color reflects time from frame 1-12000 (blue to yellow). Diffusion map parameters sigma = 8.
**Experiment 8:**

For a complete pathophysiological and mechanistic understanding of neurodevelopmental and neuropsychiatric disorders, we must link 1) fundamental insults to 2) neural circuit development and function to 3) behavioral function. We have demonstrated that disrupting cortical NMDAR function or thalamic neurotransmission drives clear abnormalities in cortical activity patterns during spontaneous activity in neonatal periods, establishing a clear connection between fundamental insults (step 1) and neural circuits during development (step 2). They have established a clear signature of abnormal dynamic network coordination, and lay solid groundwork to connect neural circuit function (step 2) to behavioral function (step 3). A number of recent studies have used widefield imaging of the cortex in adult mice performing perceptual decision making tasks have shown widespread activation across the cortex, with region specific dynamics that are critical for different aspects of task performance (Guo et al. 2014, Goard et al. 2016; Wekselblatt et al. 2016; Allen et al. 2017; Kyriakatos et al. 2016; Makino et al. 2017, Salkov et al. 2019). These studies provide strong evidence that whole cortex dynamics are a meaningful scale to understand neural dynamics during task performance.

A large body of work on mice with NMDA receptor mutations show a range of behavioral abnormalities including impairments in cognition, social behavior, hyper-locomotion [Mohn et al. 1999; Duncan et al. 2004; Milenkovic et al. 2014; Mielnik et al. 2014; Finlay et al. 2015; Lee and Zhou 2019; Lee et al. 2019; Mielnik et al. 2019]. However, studies examining neural dynamics either locally or globally during task learning or performance are lacking. The potential space here is large and will demand the work of many researchers to fully elaborate the relationship between abnormal neural dynamics and behavioral function. Notably, platforms have recently
been introduced to perform whole cortex calcium imaging in freely moving mice (Rynes et al. 2020), which greatly expands the space of tasks possible, in particular opening the space to examine naturalistic behavior and learning environments.

From the standpoint of our model, the potential for behavioral tasks is limited because EX-NMDAR KO mice only live to P30-40. However, the natural behaviors of the mice are suggestive of core features of intellectual disability and autism. Specifically, based on our observations, EX-NMDAR KO mice can display extended periods of asocial interactions where they exhibit periods of both minimal movement and perseverative grooming-like paw movements. We have also observed EX-NMDAR KO mice exhibiting periods of hyper-active, purposeless running in which they even seem to run into the walls of their cages. When the top of the cage is lifted, normal mice become active, moving to different areas of the cage and standing to sniff. EX-NMDAR KO mice exhibit almost no obvious exploratory or investigative behavior. At other times, EX-NMDAR KO mice can be observed huddling with their littermates in a manner that is more suggestive of normal behavior. However, even during these periods, when their littermates are huddled quietly and presumably sleeping, EX NMDAR KO mice do not stay still, often continually climbing and digging at their littermates.

Therefore, the natural behavior of these mice offers a platform that does not require training to link neural activity to their abnormal behavior. Our own studies have demonstrated that both normal and EX-NMDAR KO mice are readily accepted by their parents following a preparation for chronic widefield imaging as early as P6. Therefore, there is no reason not to believe that a preparation for freely moving imaging or electrophysiology would not be feasible in a neonatal or juvenile mouse from the standpoint of parental nurturing. However, the weight
of the device and the effect on feeding would be potential concerns. The difficulty of nursing with a permanent device could be overcome through experimenter feeding. However, the weight poses a considerable technical challenge because EX-NMDAR KO mice rarely grow beyond 6-8 grams. A recent device for freely moving mesoscopic imaging weighs > 3 grams (Rynes et al. 2020) and an example device for 2-photon cellular resolution imaging weighs 2.15 grams (Zong et al. 2017). The most promising approach may be to use freely moving electrophysiology, especially as our recordings demonstrate that there are clear local electrophysiological signatures of whole cortex activity in EX NMDAR KO mice. An example of a device that allow recording of up to 100 neurons with Neuropixels probes (and recycling of probes) weighs ~2 grams (Juavinett et al. 2019).

However, if the technical challenge could be overcome, then the potential to record neural activity even during these basic naturalistic activities could provide a rich space to understand behavioral challenges experiences by these mice. For instance, let us consider having simultaneous recordings from a littermate CTL and EX-NMDAR KO mouse during the behavioral sequence of huddling and then sleeping at around P16-18. First of all, normal dynamics in freely moving mice in naturalistic environments has not examined, and so the relationship between whole cortex dynamics and 1) active huddling behavior, 2) transition to stable positions, and 3) transition to sleep would first need to be defined. This characterization of normal dynamics would provide novel insight into the relationship between neural dynamics and behavior during development. The next major question would be the relationship of activity patterns, in particular the large cortex wide events, to the range of abnormal behaviors observed in EX NMDAR KO mice. Two primary questions would be: 1) do whole cortex burst appear associated with
repetitive behavioral movements or are they suppressed during active movement as suggested by spontaneous head fixed recordings (Figure 6.1), and 2) are the onset or offset of large bursts associated with or predictive of either the initiation or cessation of particular behavioral patterns.

However, as mentioned, the physical development and lifespan of EX-NMDAR KO mice limits their potential use in behavioral and combined imaging/behavioral experiments. An alternative approach would be to use an NMDAR whole body knockdown (KD) in which NMDAR is expressed at only 5-10% of normal levels. These mice live to adulthood and display deficits characteristic of intellectual disability and autism spectrum disorders including increased repetitive behaviors and motor stereotypy, deficits in social and sexual interactions, self-injury, altered ultra-sonic vocalizations, reduced pre-pulse inhibition (Mohn et al. 1999, Gandel et al. 2012). Furthermore, Milenkovic et al. 2014 defined a developmental trajectory for core cognitive functions including social behavior (sociability assay, Moy et al 2004), working memory (using the Y-maze assay, Mandillo et al 2008), executive function (using the puzzle box assay, Ben Abdallah et al. 2011), locomotor activity and stereotypy (using digital activity monitors to measure distance travelled, stereotypy number and time) at 3, 6, and 12 weeks of age. Interestingly, they identified distinct developmental trajectories for each of these aspects of behavioral function. In terms of locomotor function, hyperlocomotion was most substantial in juvenile mice and plateaued in adult mice, while stereotypy progressively worsened with age. Impairments in working memory and sociability were sexually dimorphic, with deficits first detected in peri-adolescent males but only detected in adult females. Executive function was most impaired in peri-adolescent mice of either sex. Furthermore, while juvenile mutant mice had some ability to problem solve in the puzzle box test, the same mice lost this ability when tested 4 weeks
later. These experiments demonstrate the robust differences in a range of relevant behaviors for ID and ASD in NMDAR KD mice during both juvenile and adult periods. The improved health of these NMDAR KD mice compared to EX NMDAR KO mice argues that available tools for imaging or electrophysiology in freely moving mice could be used to study the neural underpinnings of their behavioral abnormalities.

A further exciting potential of using NMDAR KD mice is the recent unpublished report in which they generated a reversible NMDAR KD mouse (See above, Mielnik et al. 2019). Using their reversible cre-based approach, they demonstrated full recovery in startle response, and partial recovery in hyperlocomotion, sociability (three chamber test), and executive functioning (puzzle box). The use of these mice would allow experiments to not only directly link neural dynamics and behavior, but also to test the relationship between restored behavioral function and underlying dynamics.

**Experiment 8: Establish NMDAR mice as model to optimize in utero gene therapy approaches**

For NDDs in humans, in cases when a genetic cause can be identified during prenatal periods (for instance, in cases of an already affected sibling or in the future if more widespread whole exome screening becomes available), gene therapy offers curative potential. However, there are many technical and conceptual challenges that remain, including route of delivery and timing (Massaro et al. Nature 2018). The NMDAR offers an ideal target to test methodology of gene therapy related to neurodevelopment. The loss of function of this single gene causes a major, easily observable disruption in activity that would allow easy testing of the intervention at early
developmental points. Additionally, there are robust cognitive deficits in mice (see above) that also offer relevant downstream readouts.

Translation to humans

Establishing models of NMDAR dysfunction in primates

The genetic toolbox of the mouse provides enormous experimental advantages as highlighted by the current study and the enormous body of work referenced. However, directly translating from mouse to humans is problematic and has failed on multiple occasions (e.g. Hyman SE 2014). In particular, the mouse cortex is substantially smaller and lacks many associational and pre-frontal regions and region-specific layers (e.g. deep layer III in primate pre-frontal cortex) that underly the much richer cognitive capacities of humans, such as language or higher order executive functioning. However, recent advances (e.g. using CRISPR-Cas9 or TALEN mediated gene editing) have begun to demonstrate the potential for generating transgenic non-human primates, including macaques (Sasaki et al. 2009, Liu et al 2014). These approaches have been used to test other genes implicated in neurodevelopmental disorders. For instance, Liu et al 2016 demonstrated germline transmission and autism-like behaviors (including decreased sociability) in transgenic monkeys overexpressing MeCP2, the mutation associated with Rett syndrome. The large body of evidence implicating the NMDAR receptor in neurodevelopmental disorders and cortical development would argue that this would be a valuable target for transgenic primates. Doing so would open up a range of powerful experimental possibilities, including defining neural activity patterns during development (as we have done in the current study) and testing primate
social and cognitive behaviors. Such models would have substantially increased relevance for
human disease, especially of complex cognitive functioning.

**Implications for pathogenesis of NDDs**

In humans, mutations in genes coding for all subunits of the NMDAR have been linked to NDDs
and neuro-psychiatric disease broadly, including intellectual disability, autism spectrum disorder,
and schizophrenia, as well as attention deficit disorder, manic depressive disorder, and substance
abuse disorder (XiangWei et al. 2008, Hamdan et al. 2011, Tarabeux et al. 2011, Lee et al. 2015,
Lemke et al. 2016, Kim et al. 2018, Fountoulakis KN 2012, Chen et al 2018). It is thought that sub-
unit, temporal timing, cell specific contribution, and the nature of the mutation all contribute to
the varied abnormal trajectories that can result from NMDAR mutations (Endele et al., 2010).
Studies characterizing patients with mutations in the obligate NR1 sub-unit, specifically, found
almost exclusively dominant negative missense mutations that led to a severe neurological
phenotype, characterized by intellectual disability (ID) in 100% of individuals with mutations,
epilepsy in 65%, and autism spectrum disorder (ASD) in 22% (Lemke et al. 2016; Platzer and
Lemke 2019). We acknowledge the limitation of using mice to model more complex and distinctly
human aspects of cognition, as mice lack many of the higher order cortical regions that subserve
these functions. Nonetheless, the extreme disruption of cortical activity in EX NMDAR KO mice is
consistent with an extreme phenotype, and our results can help disambiguate whether certain
aspects of the phenotype are dependent on cortical NMDAR function.

Most importantly, we would suggest that the major reduction in complexity and normal
activity patterns are consistent with the complete penetrance of ID associated with NR1
mutations. Interestingly, the restricted, low dimensional space of spontaneous activity in EX NMDAR KO mice is reminiscent of the repetitive motor behaviors associated with ASD. However, the extreme whole cortex synchrony in EX NMDAR KO mice is not fully consistent with the “developmental disconnection syndrome” model of autism spectrum disorder (Geschwind and Levitt 2007), which posits that a decrease in connectivity between associational and frontal regions (e.g. dorsolateral prefrontal and anterior cingulate regions, Mundy P 2003) underly the deficits in executive functionally and behavioral flexibility seen in the disorder. Work in humans based on NR1 mutations as well as the work in model systems showing the extreme effect of NMDAR deletions on cortical circuitry and cognitive function, would suggest that EX NMDAR KO mice are best considered a model of disrupted cognition rather than ASD, per se. In humans with ASD who have otherwise intact IQs, this pattern of reduced functional connectivity between certain areas in conjunction with increased connectivity within areas may account for the restricted interests and difficulties with shifting attention without the overall loss of cognitive capacities. On the other hand, severe cognitive deficits, seen either with or without a concurrent ASD diagnoses, may be reflected in more a unitary brain wide neural pattern blocking global function.

Interestingly, neither EX NMDAR KO nor IN NMDAR KO mice displays behavioral evidence of seizures. LFPs in EX KO mice are notable for individual high amplitude spikes but are clearly distinct from spike-wave patterns and rhythmic spiking with high frequency oscillations typically seen in epilepsy (Krestel et al. 2004, Venzi et al. 2014, Fisher et al. 2015, Kadiyala et al. 2016). It is perhaps surprising that loss of glutamatergic NMDA receptors in glutamatergic excitatory cells would drive high amplitude events, as increased excitatory currents through NMDA receptors are
often thought to be associated with seizures (Sibarov et al. 2017). However, this may be explained by the previous work showing increased AMPA mediated currents in layer IV cortical neurons in EX NMDAR KO mice (Lo et al. 2013). Importantly, however, the LFP in EX NMDAR KO mice does not display persistent rhythmic spiking that builds in amplitude and intensity. We suggest that the lack of NMDAR results in brief high amplitude population spikes, but that a grossly functional inhibitory network is sufficient to immediately suppress the network-wide excitatory discharge.

Our experiments may also help shed light on why similar genetic risk exists for both early presenting developmental disorders like ID and ASD versus later presenting developmental disorders like schizophrenia (SCZ). An important hypothesis of SCZ is that it results from a wave of excessive pruning in late adolescence (Feinberg I 1982, Zipursky et al. 1992), which is reflected in a decrease in spine density, especially in layer II/III of frontal cortical regions (Goldman-Rakic and Selemon 1997, Glantz and Lewis 2000, Selemon LD 2001, Kolluri et al. 2005). The network disruptions in EX NMDAR KO mice suggest a distinct pathological process of failed pruning earlier in development rather than excessive pruning later in development. We speculate that these differences may offer insight into differences between ID/ASD on the one hand and SCZ on the other. In the former case, neural circuits never achieve appropriate refinement in early periods, and the deficits in circuit functioning are further exacerbated over time, especially without intervention and support. On the other hand, in SCZ, although initial insults may prime the system (e.g. pre-natal maternal illness) and certain deficits in working memory or executive functioning may be observable in childhood, many aspects of normal development and normal neural
circuitry can be achieved until the full pathological events later in adolescence lead to the full manifestation of the disorder.

Our MK-801 results may also offer insight into to mechanisms of SCZ. There have been two prominent theories of schizophrenia, that have at times been expressed as a debate. First is the dopamine hypothesis, based on the evidence that anti-psychotics are dopamine (D2 receptor) antagonists, suggesting that an excess of dopamine drives symptoms. Second is the glutamate hypothesis, based on the evidence that NMDAR blockers like phencyclidine and ketamine cause a psychotic response in normal humans that is often indistinguishable from SZ. The presumed mechanism by which blockade of a receptor for glutamate, an excitatory neurotransmitter, led to psychosis, a presumed hyperexcitable network state, was that at psychotomimetic doses, NMDAR receptors on inhibitory interneurons would be preferentially active. This concept was supported by studies in mice using acute application of the NMDAR blocker ketamine has found a preferential action on NMDAR currents in inhibitory cells, which help maintain excitatory drive onto inhibitory cells, reducing inhibitory cell firing and an increase in excitatory cell firing. Furthermore, mice with NMDAR KO in inhibitory cells demonstrate behavioral deficits thought to be proxies for SZ (Mohn et al 1999; Duncan et al. 2004; Belforte et al. 2010; Rompala et al. 2013). However, our experiments showing a disruption of cortical dynamics by acute MK-801 that largely recapitulates the dynamics of EX NMDAR KO mice suggests that the systemic effects of NMDAR antagonists may be more dependent on excitatory rather than inhibitory NMDAR.

Such an interpretation would be more consistent with the evidence from primate work, which also casts doubt on the hypothesis that NMDAR function in inhibitory neurons is
responsible for the psychotomimetic effects. First of all, while NMDAR receptors are expressed highly on interneurons in juvenile monkey, they are expressed at low levels in adult monkeys (Gonzalez-Burgos and Lewis 2012, Hoftman et al 2017). Moreover, work by Patricia Godman-Rakic, Amy Arnsten and colleagues has demonstrated that recurrent excitatory network activity underlying working memory in prefrontal cortical regions is uniquely dependent on NR2B receptors in deep layer III neurons (in addition to neuromodulators norepinephrine, dopamine, and acetylcholine) (Wang et al. 2013, Arnsten et al. 2015, Yang et al 2013, Wang et al. 2007). As deficits in working memory are a core feature of schizophrenia, it is much more likely that blockade of these receptors underlies the effects of NMDAR receptor blockers (or at least the cognitive aspects) and dysfunction of these cells in SCZ, which have been shown in post-mortem studies to have reduced dendritic spines (Glantz and Lewis 2000; Kolluri et al. 2005).

Summary

Decades of work in human genetics has identified many genetic insults that can lead to cognitive disability and NDDs (Fombonne et al. 2009; Maulik et al. 2013; Iossifov et al. 2014; Ronemus et al. 2014; Sanders et al. 2015; Geshwind and 2015, Fitzgerald et al. 2015). The canonical view is that genetic insults lead to abnormal function of neural networks that manifest as cognitive disability later in life. These genes are predominantly associated with neuronal development and function – including axonal growth, synaptic function and plasticity. Our work shows the fundamental role of a single receptor and signaling pathway – the NMDA receptor – in establishing long-range cortical network “functional” connectivity. We have demonstrated, using EX NMDAR KO mice, TH-VG KO mice, and MK-801 application in WT mice, that mutations and
activity dependent processes related to NDDs can cause gross disruptions in neural activity dynamics.

Understanding the function of the NMDAR has direct relevance for a substantial class of patients. The findings of our work utilizing in vivo recording of neural activity can also have broader implications for human investigative and diagnostic approaches. The trajectory of cortical network development observed in this study can serve as a prediction and point of comparison for the neurocognitive disorders more broadly. Such an identifiable circuit level phenotype could be a particularly useful signpost in cases in which a single genetic cause cannot be identified.

Finally, our results have implications for understanding a fundamental principal of cortical circuit dynamics during development, generally: in the absence of NMDAR, the cortex will not behave like many isolated units but rather like a single connected sheet locked into a single dominant trajectory. This extreme network state may offer a biomarker in humans for both identifying and tracking response to intervention at an early stage of network development – prior to behavioral manifestation – when these responses have the greatest opportunity to alter developmental trajectory for children. Our work should further motivate increased efforts to use non-invasive tools to identify abnormal signatures and trajectories of human brain development. A long-term future sequence for identifying and treating NDDs might be: 1) pre-natal screening of brain activity (analogous to the use of ultrasound to assay the pre-natal heart), 2) identification of abnormal patterns motivating genetic sequencing, 3) identification of putative causative genes, 4) gene therapy mediated restoration of abnormal function.
Epilogue: The Future of Psychiatry

To conclude this dissertation, I would like to look forward and reflect on some of the broader trends in psychiatry. The motivation for my research has been to engage in science that may contribute to our understanding of normal and pathogenic cortical development and function. But perhaps an even more fundamental motivation has been to develop knowledge and tools to contribute as an effective physician-scientist for patients suffering from mental illness. Psychiatry is currently undergoing a state of transformation, driven by several factors that relate scientifically to work in this thesis. I hope that the experimental, analytic and conceptual approaches I have developed through this training have prepare me to contribute to these broader goals.

I would like to highlight three aspects in particular: first, the use of brain imaging for early detection and prediction of neurodevelopmental disorders; second, the combination of brain imaging and neuromodulation for precision, circuit driven medicine; and third, the recognition and mainstream acceptance of a group of powerful rapid acting medications for treatments of serious psychiatric and medical disorders. At the heart of these major developments is the model of neuropsychiatric diseases as disorders of distributed neural networks, with a prominent role for the NMDAR as the target for the rapid-acting and profoundly powerful medicine, ketamine.

Neural network characterization and prediction of neurodevelopmental disorders

The explicit focus of this dissertation has been elaborating the pathogenesis of neurodevelopmental disorders (NDDs), which present the distinct challenge of affecting neural development long before behavioral biomarkers are apparent. Identifying biomarkers or
signposts of normal and abnormal development is thus essential for identifying and intervening during early stages of pathogenesis (Geshwind D 2007; Bednarz and Kana 2018). Thankfully, work in humans using functional approaches has begun to show promise. Neuroimaging approaches have revealed brain networks display structural organization even in pre- and early post-natal periods (Fransson et al. 2007; Fair et al. 2009; Thomason et al. 2014; van den Heuvel et al. 2015), and there is accumulating evidence that use of these tools have the capacity to predict future neuro-cognitive deficits (Khan et al. 2008; Hayashi-Kurahashi et al. 2012; Haartsen et al. 2019; Pillay et al. 2020). Perhaps the most compelling example comes from the Infant Brain Imaging Study Network (The Infant Brain Imaging Study Network – Infant Brain Imaging Study (ibis-network.com)), which has predicted future ASD, based on both anatomical and functional measures, with incredible accuracy (Hazlett et al. 2017; Emerson et al. 2017). Using prospective neuroimaging of 6 month-old infants with high familial risk of ASD, a cross validated machine learning algorithm applied at 6 months had a positive predict value of 100%, a negative predictive value of 96%, a specificity of 100%, and a sensitivity of 81.8% for the diagnosis of ASD at 24 months (Emerson et al. 2017).

Such studies raise the possibility of having a clinical useful screening tools even earlier in post-natal development with high predictive value. Of course, it is an ongoing challenge to know how to effectively intervene, but being able to identify neonates who are likely to develop ASD is game-changing. The history of ASD already demonstrates the incredible difference early intensive early intervention can make, with intensive behavioral and symptomatic treatment starting at 2-3 years old can massively shift the developmental trajectory. Thus, even behavioral intervention during neonatal development could have massive impacts on shifting the
neurodevelopmental course. For instance, from the standpoint of eye tracking, infants who will go on to develop ASD start out by attending to eyes at 2 months (Jones and Klin 2013). But they then exhibit mean decline in eye fixation from 2 to 6 months of age, as their non-ASD counterparts continue to strengthen this fundamental source of social attention and information. Even for parents and trained therapists to be attuned to this and make sure the child is engaged and understanding may be transformative. And of course, the ability to predict and then track pathophysiological trajectory across development can also guide the development of new pharmacologic or neuromodulatory treatment approaches.

From the standpoint of schizophrenia, a similar problem exists whereby once a person has their first psychotic break, typically around 17-25 years of age, much of the underlying pathophysiology has already begun. There is evidence that this may even begin as far back as second trimester in utero (Brown and Derkits 2008). And other evidence exists that differences in motor coordination and cognitive function may be evident as far back as early childhood (Walker and Lewine 1990). Substantial neuroimaging and electrophysiological approaches have shown disruptions in major functional connections that seem to underly this illness. Studies by the large scale NAPLS-2 consortium combined a range of parameters including brain networks in prodromal populations has led to a predictive nomogram with an area under the curve of 0.79 (Carrion et al 2016; Cannon et al 2016). Work using circuit-based analysis of dysfunctional network dynamics have already been shown some initial promise, for instance targeting the auditory cortex of auditory hallucinations (Hoffman et al 2003) and restoring prefrontal cortex to cerebellum connectivity for negative symptoms (Brady et al. 2019).
Circuit based therapies and neuromodulation

As already alluded to, recording brain networks does not only have value prediction and biomarker identification, but also for guiding circuit-based interventions, including trans-magnetic stimulation (TMS), deep brain stimulation (DBS), and focused ultrasound. The application for these is furthest along in major depression (MDD), but there is every expectation that the principles and approaches can be applied to psychiatric disorders broadly. Clinically diagnosable MDD affects as many as one in five people during their lifetime and is the leading cause of disability worldwide (https://www.nimh.nih.gov/health/statistics/major-depression). Currently, standard of care is the use combination of conventional anti-depressant medications (SSRI, SNRI), which can take weeks to months to respond, and therapy, but as many as 30% do not respond to standard therapy (https://www.nimh.nih.gov/health/statistics/major-depression).

Early understanding of the functional organization of the cortex came through direct stimulation of specific areas which reveal anatomic locations for specific functions. Work over the past decades has moved us from recognizing that such an organization is applicable not only to sensory and motor functions but also to complex human emotions and cognition that are disrupted in psychiatric brain disorders. The origins of circuit modulation can be traced back to Wilder Penfield, who stimulated and mapped the cortex, demonstrating that there is a functional geography and anatomic locations critical for specific functions, including memory (Penfield W 1952). Jean Talairach demonstrated that this concept could also be applied to neuropsychiatric disorders by showing that lesions in could treat cases of severe OCD (Talairach J 1952). Mahlon Delong showed that frontal sub-cortical circuitry has a general structure (cortex, to striatum, to...
thalamus), with distinct loops designated for motor behaviors and emotions (Wichmann and Delong 2006). Tony Barker in the 1980s showed that non-invasive magnetic stimulation to the motor cortex could induce lasting increase in excitability (Barker et al. 1985).

From the standpoint of MDD, hyper-metabolism of frontal regions had been identified and Alvaro Pascual-Leon and Bob Post identified hypometabolism of LDLPFC. There was convergent clinical and neuroimaging evidence that hypoactivity of this region would be a good target (Reviewed in George et al. 2002). In 1995, the first patient received excitation to left dorsolateral prefrontal cortex for depression (Reviewed in George et al. 2002) using parameters derived from motor physiology findings (Pascual-Leon 1994). Parameters evolved over time with longer duration, higher intensities, and more pulses per day producing incrementally greater efficacy. The treatment was approved by the FDA in 2008 based on 30% remission and 50% responder rate in treatment resistant depression (O’Reardon et al 2007), which has proved relatively durable for ~50% responders (Senova et al. 2018). The treatment was approved with the following parameters: 10 HZ single pulse response, 1 session/day, 5 sessions/week, 30 sessions/course, with a total of 90,000 pulses/course and with the target area identified by ruler-based methods for target region.

A second major improvement, theta burst stimulation, derived from stimulations parameters based on physiological hippocampal rhythms which was confirmed in a non-inferiority trial (Blumberger et al. 2018). This approach treats over the same number of days, but now with 3x 50 hz pulses, every 200 ms, for 2 seconds, with an 8 second interval. (Huang Neuron 2005), thus allowing 3 minutes instead of 37 minutes and 1/5 as many pulses. Unfortunately, this approach still required many weeks of treatments. However, the benefits demonstrated by
improving stimulation parameters inspired the current iteration: the Stanford Accelerated Intelligent Neuromodulation Therapy (SAINT) approach. This approach combined “spaced learning” approaches with functional network driven targeting to compress 6 weeks of TMS treatment into 5 days with 80-90% remission in treatment resistant depression.

Their approach was based on evidence for optimal learning paradigms based on “spaced learning”, which has showed that learning intervals of ~1 hour are optimal in both rodents and humans (Kramar et al. 2012, Nettekoven et al. 2014). Moreover, they took advantage of contemporary circuit-based understanding of depression. Work in depression has continued to demonstrate that it is a disorder of functional networks. Alan Schatzberg’s group demonstrated that depressed subjects show increased connectivity between subgenual cingulate cortex and default mode network, as well as hypo-activity of the left DLPFC (Grecius et al. 2007). Work from Michael Greicius and Michael Fox using lesion-based connectivity studies, strongly reinforced the left DLPFC as a critical target for neuromodulation. Their approach was to take strokes in diverse brain regions that had caused clinical depression in previously healthy individuals, and overlay these onto functional network maps of resting state library of the human connectome project. By doing so they could identify overlapping networks amongst the lesions. In normal connectivity, Left DLPFC and subgenual cingulate (sgACC) are highly interconnected, with DLPFC exhibiting a strong inhibitory drive onto the sgACC to regulate mood. In MDD, DLPFC becomes less active and fails to inhibit sgACC. This network pathology seems to cause cognitive impairment and inwardly directed negative thoughts (Baxter et al. 1989; Drevets et al. 1997). This work strongly reinforced that TMS is effective in MDD by stimulating the DLPFC, re-activating functional inhibitory drive onto sgACC, which corrects the network imbalance and normalizes mood regulation. Negative
connectivity between DLPFC and sgACC associated with TMS efficacy, and proximity to ideal DLPFC spot correlated with treatment improvement (Avissar et al. 2017; Weigand et al. 2018).

Rapid-acting pharmacotherapies

The final transformation is the recognition (or re-recognition) of a suite of highly psychoactive compounds for rapidly altering mood and psychiatric illness: ketamine, MDMA, and psychedelics. Ketamine has had longstanding medical use as an anesthetic and psychedelics (psilocybin, LSD) were studied widely and intensely in the 1950s and 1960s, but were criminalized in the 1970s (Pollan M 2020). These medications continued to live vibrant lives in underground, recreational, and counter-cultural settings. However, each of them has been discovered or returned to mainstream psychiatry out of darkness in the past twenty years. Ketamine’s journey is a distinctly Yale story, where research in the 1990s on human subjects as a model of psychosis, led to the recognition that it had rapid mood-altering properties. MDMA’s journey can be credited substantially to Rick Doblin and the Multidisciplinary Association for Psychedelic Studies (MAPS) (https://maps.org/), which began in the 1980s to fund research to build a case for its use in therapeutic settings. Psilocybin was rejuvenated in particular by two seminal studies. One of which demonstrated its capacity to elicit a “mystical-type experience” in healthy subjects (Griffiths et al. 2006) and one which demonstrated its capacity to relieve existential distress in patients with terminal cancer (Grob et al. 2011). Ketamine is now used widely across the country, and a version of it – esketamine – has achieved FDA approval for depression (Singh et al. 2016). MDMA assisted psychotherapy and psilocybin are completing phase 3 trials, and expected to be FDA approved within the coming years.
These substances have now been studied extensively using brain imaging, which has revealed both distinctive and overlapping changes, but with a consistent theme that despite the distinct neurochemical targets of these medicines, they each appear to drive their changes in conjunction with changes in functional connectivity between cortical networks. Interestingly, ketamine has been shown to increase pre-frontal global brain connectivity, the average correlation of each voxel with all other voxels (a measure similar to cortex-wide correlation strength) (Driesen et al. 2013, Anticevic et al. 2015). In a study of 57 MDD vs 25 HC, there was widespread reduction of GBC for pre-frontal regions in MDD, and normalization of GBC post-ketamine predicted treatment response (Murrough et al. 2016, Abdallah et al. 2017).

Whereas NMDAR is the primary target of ketamine, psychedelics target a different neurotransmitter system and have been demonstrated to rely specifically on activating the Serotonin 2A receptor. Interestingly, these receptors exert an excitatory effect on neurons that express it and are especially highly expressed in layer V neurons of the association cortex. Work has particularly implicated the posterior cingulate cortex, a dense connectivity hub and integration center of the default mode network (Hagmann 2008). The DMN is engaged during self-reflection (Qin and Northoff, 2011), complex mental-imagery (hassibis and mgure, 2009), mental time travel (Buckner and Carroll 2007), theory of mind (Spreng et al. 2009), ego as emergent property of self-organized activity in DMN (Carhart-Harris and Friston 2010). Decrease in activity during active engagement in task. In MDD, DMN connectivity is disrupted, with increased functional connectivity between the MPFC and PCC connectivity associated with rumination in people with depression, which appears to lock people into a restricted trajectory (Hamilton et al. 2011). Carhart-Harris 2012 PNAS showed decreased CBF after psilocybin
particularly in PCC, thalamus, and MPFC. Psilocybin caused reduced blood flow to the ACC, which
predicted intensity of subjective effects, and caused reduced connectivity of nodes of default
mode network with hippocampus (mPFC and PCC).

Thus, both circuit-based targeting and diverse pharmacologic agents demonstrate the possibility
of shifting functional networks from patterns that maintain and reinforce mental illness to those
that allow for plasticity and healing. It is not unreasonable to predict a future for psychiatry based
on genuine circuit-based precision medicine: where most patients who present to the emergency
room with severe suicidality, depression, and perhaps even psychosis, will have their brain
dynamics immediately recorded, their network patterns mapped, and targeted pharmacologic
and neuro-modulatory treatment plans established with a high probability of substantively
reducing their symptoms. I hope and trust that the learning and training provided by this
graduate research will allow me to participate productively during this incredible period for the
treatment of mental illness.
References


11. Bader PL; Faizi M; Kim LH; Owen SF; Tadross MR; Alfa RW; Bett GC; Tsien RW; Rasmusson RL; Shamloo M. 2011. Mouse model of Timothy syndrome recapitulates triad of autistic traits. Proc Natl Acad Sci U S A 108(37):15432-7


13. Bakker CE; Verheij C; Willemsen R; Vanderhelm R; Oerlemans F; Vermey M; Bygrave A; Hoogeveen AT; Oostra BA; Reyniers E; Debourele K; Dhooge R; Cras P; Vanvelzen D; Nagels G; Martin JJ; Dedyn PP; Darby JK; Willems PJ; The Dutch-Belgium Fragile X Consortium. 1994. Fmr1 knockout mice: a model to study fragile x mental retardation. The Dutch-Belgium Fragile X Consortium Cell 78(1):23-33


64. Elston GN. Cortex, cognition and the cell: new insights into the pyramidal neuron and prefrontal function Cereb Cortex. 2003 Nov;13(11):1124-38


82. Fuster JM. The prefrontal cortex--an update: time is of the essence. Neuron. 2001 May;30(2):319-33


114. Hays SA et al. Altered neocortical rhythmic activity states in FMR1 KO mice are due to enhanced mGluR5 signaling and involve changes in excitatory circuitry. J Neurosci Oct 5;31(40):14223-34. (2011)

L, Piven J; IBIS Network; Clinical Sites; Data Coordinating Center; Image Processing Core; Statistical Analysis. Early brain development in infants at high risk for autism spectrum disorder. Nature. 2017 Feb 15;542(7641):348-351.


207. PENFIELD W. Memory mechanisms. AMA Arch Neurol Psychiatry. 1952 Feb;67(2):178-98.


210. Poliaik S; Salomon D; Elhanany H; Sabanay H; Kiernan B; Pevny L; Stewart CL; Xu X; Chiu SY; Shragger P; Furley AJ; Peles E. 2003. Juxtaparanodal clustering of Shaker-like K+ channels in myelinated axons depends on Caspr2 and TAG-1. J Cell Biol 162(6):1149-60


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221. Rynes L et al. Miniaturized head-mounted device for whole cortex mesoscale imaging in freely behaving mice. bioRxiv 2020.05.25.114892


244. Titulaer, Maarten J; McCracken, Lindsey; Gabilondo, Iñigo; Armangué, Thais; Glaser, Carol; Izuka, Takahiro; Honig, Lawrence S; Benseler, Susanne M; Kawachi, Izumi; Martinez-Hernandez, Eugenia; Aguilar, Esther; Gresa-Arribas, Núria; Ryan-Florance, Nicole; Torrents, Abiguei; Saiz, Albert; Rosenfeld, Myrna R; Balice-Gordon, Rita; Graus, Francesc; Dalmau, Josep (2013). "Treatment and prognostic factors for long-term outcome in patients with anti-NMDA receptor encephalitis: An observational cohort study". The Lancet Neurology. 12 (2): 157–65.


