Anatomical and Functional Characterization of the Ventral Hippocampus in a Rodent Model of Schizophrenia Neuropathology

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Schizophrenia is a debilitating, life-long illness with a still-unknown, complex etiology and, currently, no cure. Many studies have implicated the hippocampus and the parahippocampal region as a place of both primary pathology in the disease and as regions correlated to symptom severity. To better understand the pathophysiology of the region and potentially uncover mechanisms of the disease, the appropriate choice of an animal model is essential. The “MAM E17” model of hippocampal pathology shows anatomical, neurophysiological, and behavioral changes relevant to schizophrenia. Because of these wide-ranging disease-relevant changes, we aimed to relate anatomical to neurophysiological phenotypes in this model. We also performed experiments to assess the feasibility and validity of transferring the MAM E17 model to the mouse in order enable future studies of the genetic basis of the vulnerability or resilience to MAM. In adult offspring of rats exposed to methylazoxymethanol (MAM) at embryonic day 17 (E17), we found changes in regional hippocampal anatomy and subicular pyramidal cell morphology with homology to abnormalities reported in schizophrenia. Specifically, we found a decrease in dendritic spine density in specific regions of the dendrite of ventral subicular neurons. At the neurophysiological level, we observed abnormalities in afferent-evoked synaptic responses in the ventral subiculum. These changes were not however, accompanied by changes in in vivo spontaneous spike activity in subicular neurons. In the mouse, MAM was found to
have much less impact on brain development, as observed at the gross morphological level. However, these mice showed an increased sensitivity to some psychostimulants and a weak trend for metabolic abnormalities relevant to schizophrenia. We conclude from the rat studies that prenatal disruption of brain development by MAM at E17 in the rat, a manipulation that leads to a profile of gross anatomical and cognitive deficits relevant to schizophrenia, also leads to ‘dysconnectivity’ between the ventral subiculum and its inputs. While further work is needed to understand this, we speculate that this synaptic dysconnectivity may contribute to the cognitive deficits in this model and, further, may model an aspect of hippocampal pathophysiology in schizophrenia. A better understanding of these circuits could point to new strategies for treating this disease.
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Chapter 1
General introduction

Schizophrenia arises from gene and environment interactions

Schizophrenia is a chronic and debilitating illness with a lifetime risk of about 0.7% (Saha et al. 2005). Its early onset (Hafner et al. 1993) and impact on quality of life (Pinikahana et al. 2002) contribute to the disease’s high economic burden to society, estimated to be tens of billions of dollars annually (Wu et al. 2005). The disease has high but heterogeneous heritability, with multiple genes associated with increased risk for the disease but no risk allele being prevalent in the population. This has given rise to the “common disease – rare allele” hypothesis (McClellan et al. 2007) for genetic associations. These rare alleles likely interact with one or more of the known environmental risk factors to increase risk of the disease (McDonald & Murray 2000). Many risk factors cluster around the perinatal period, including maternal infection (Brown & Derkits 2010) or malnutrition (Susser et al. 2008) during pregnancy, and obstetric complications (Mittal et al. 2008).

Neuropathology of schizophrenia

Schizophrenia is not typically associated with significant decreases in overall brain size, however select regional brain volume changes are prevalent. Increased size of the lateral and third ventricles is the most prevalent finding (Shenton et al. 2001; McCarley et al.
Smaller volumes in the temporal lobe, and in particular the medial temporal lobe, are also among the most replicated findings in the disease (Heckers 2001). Given the heterogeneous nature of both the etiology and symptoms of the disease, well replicated findings could provide important clues about the primary pathologies of the disease. Neuroimaging studies show that volume changes in the medial temporal lobe, specifically the hippocampal formation, are present at or before first onset of the disease (Lawrie et al. 1999; Velakoulis et al. 1999; Pantelis et al. 2003) and may be predictive of psychosis. This suggests that hippocampal changes may be involved in the pathogenesis of the disease and are not secondary to an effect of treatment. Hippocampal volume loss seems to be greater in chronic schizophrenia than in first onset (Velakoulis et al. 2006) regardless of type of medication, suggesting that this phenotype of the illness is relevant to disease progression and long-term disease outcomes.

The medial temporal lobe contains the hippocampal formation, the amygdala, and the overlying parahippocampal gyrus. The hippocampal formation can be further divided into the entorhinal cortex, the dentate gyrus, the cornu ammonis (CA) fields, and the subiculum. Advances in neuroimaging techniques over the past decades have allowed for increasingly fine in vivo distinction of these structures within the medial temporal lobe. Changes in the shape of the hippocampus have been localized to CA1 and subiculum in some studies (Csernansky et al. 1998; Wang et al. 2001) and to preferentially affect the mid-to anterior hippocampal formation (Narr et al. 2004). More studies are needed to definitively determine which, if any, hippocampal subregions are preferentially impacted. However the overall consistency of medial temporal lobe findings strongly implicates this region, and specifically the hippocampal formation, in the pathology of schizophrenia.
The consistency of medial temporal lobe findings in schizophrenia is all the more notable given the relatively small size of the loss: just 4% by one meta-analysis (Nelson 1998). The small magnitude of the effect is consistent with the idea that schizophrenia is not a neurodegenerative disease; few studies find evidence for neuronal cell death or gliosis postmortem (Roberts et al. 1987). Studies investigating the pathology of the hippocampus typically examine alterations of existing structures and proteins, not neurodegenerative markers. Early postmortem evidence of changes in the hippocampal formation in schizophrenia implicated aberrant migration patterns of entorhinal cortical cells (Jakob & Beckmann 1986) and CA1 cells (Kovelman & Scheibel 1984). This early data must be viewed with caution, however, as attempts to replicate have yielded both positive (Arnold et al. 1991; Arnold et al. 1997; Conrad et al. 1991) and negative (Bernstein et al. 1998; Casanova & Rothberg 2002) findings. Some of the discrepancy likely arises from the irregular shape of the human hippocampal formation, especially in the anterior region (Dwork 1997; Casanova & Rothberg 2002; Bernstein et al. 1998), and the irregularities observed in the healthy human entorhinal cortex (Insausti et al. 1995). Neither the size of hippocampal subfields nor their cell density appeared altered between patients and controls in two detailed postmortem stereological studies (Heckers et al. 1991; Walker et al. 2002), however these results were not in line with other accounts of volume loss observed postmortem in schizophrenia (Falkai & Bogerts 1986; Jeste & Lohr 1989). Inconsistencies among postmortem anatomical studies could be largely the result of differences in methodology, case selection, and use of appropriate controls (Dwork 1997).

Schizophrenia does not have the features of a neurodegenerative disease, and as such may cause abnormalities of existing proteins and gene expression in the hippocampal
formation (Harrison 2004). A large-scale microarray study found that the hippocampus had one of the highest levels altered gene expression levels compared to a number of brain regions (Katsel et al. 2005). Furthermore, changes in protein expression were found to be greater in the anterior compared to posterior hippocampal formation (Nesvaderani et al. 2009). Many of the histochemical abnormalities observed in schizophrenia can be linked to glutamatergic transmission, including deficits of the dendritic spine-associated spinophilin (Law et al. 2004), the presynaptic protein synaptophysin (Eastwood et al. 1995), AMPA/kainite receptors (Kerwin et al. 1988; Harrison et al. 1991), and N-methyl-D-aspartate (NMDA) receptor subunit NR1 (Law & Deakin 2001; Gao et al. 2000). Consistent with these neurochemical findings is the finding of decreased dendritic spines in the subiculum of schizophrenia patients (Rosoklija et al. 2000 and Andy Dwork, personal communication). Decreased dendritic spines has been observed elsewhere in the temporal lobe, in the auditory cortex (Sweet et al. 2008), and moreover could not be attributed to the effects of antipsychotics in exposed versus control monkeys. Together these histochemical and structural findings support the neuroimaging results in pointing to the hippocampus as a site of pathology in schizophrenia.

**Pharmacological evidence of glutamatergic transmission deficits in schizophrenia**

Alterations of glutamatergic transmission can be tested pharmacologically in schizophrenia patients. Administration of the non-competitive NMDA antagonist ketamine in healthy individuals produces transient behavioral responses that mimic that positive and negative symptoms of schizophrenia (Krystal et al. 1994) and exacerbates psychotic
symptoms in schizophrenia patients (Lahti 2001; Malhotra et al. 1997). Decreased binding of MK801 to NMDA receptor site has been observed postmortem in schizophrenia brains (Beneyto et al. 2007). These studies and others suggest a role of NMDA receptor dysregulation in the symptoms of schizophrenia and perhaps an avenue for therapeutic intervention (Moghaddam 2003). These findings support the postmortem evidence of decreased NR1 subunit mRNA in the hippocampus cited above. Bolstering that line of evidence is the finding of reduced NMDA receptor binding in vivo in the hippocampus of unmedicated schizophrenia patients compared to controls (Pilowsky et al. 2006). Modulation of the NMDA receptor is a promising and active target for schizophrenia therapy (reviewed in (Goff & Coyle 2001)). Intriguingly, NMDA antagonism leads to increased glutamate efflux (Moghaddam 2003; Jentsch & Roth 1999), suggesting that outcome of putative NMDA receptor hypofunction does not necessarily mean a deficit in glutamatergic transmission.

**Functional evidence of hippocampal abnormalities in schizophrenia**

Complementing the postmortem findings in schizophrenia are in vivo measurements of hippocampal function. A number of techniques can be applied to the human brain in vivo to help elucidate changes in the functioning of the hippocampus, and three will be mentioned here in relevance to the study of schizophrenia: magnetic resonance spectroscopy (MRS) to investigate neurochemical alterations, positron emission tomography (PET), and magnetic resonance imaging (MRI), the latter two to investigate both resting and task-evoked changes in blood flow or its correlates in the disease state.
Markers of neuronal integrity. MRS studies point to decreased N-acetylaspartate (NAA), a neuronal metabolite reflecting cell viability or number, in the medial temporal lobe. The magnitude of change from control subjects has been correlated to age of onset of the disease (Fukuzako et al. 1995), and the decrease is observed in medication-naïve first-onset schizophrenia patients (Bertolino et al. 1998; Cecil et al. 1999; Fannon et al. 2003). Decreased NAA in the temporal lobe is consistently found in meta-analyses of the literature (Steen et al. 2005; Steen et al. 2006; Brugger et al. 2011). These data provide in vivo evidence for cellular changes that may underlie the reduced volume of the medial temporal lobe in schizophrenia patients.

Basal hippocampal activity. Basal activity in the hippocampus, often measured as cerebral blood flow or its correlates, is generally increased in the hippocampal formation schizophrenia, and these increases have been found to be correlated with symptom severity (Friston et al. 1992; Liddle et al. 1992; Kawasaki et al. 1996; Molina et al. 2003). Moreover, these increases are seen at first episode (Molina et al. 2003; Molina et al. 2005) and may be partially normalized by antipsychotic medication (Medoff et al. 2001). Meta-analyses of studies investigating the entirety of the temporal lobe, however, show mixed results in terms of the direction of resting functional changes in the temporal lobe (Davidson & Heinrichs 2003; Zakzanis et al. 2000). The varying results could arise partly from medication status or from differences in patient populations; one study observed that sporadic cases of schizophrenia showed temporal hypofusion of cerebral blood flow at rest while familial cases hyperperfused (Malaspina et al. 2004). Just as increasingly fine anatomical studies of the hippocampal formation have found preferential involvement of certain areas or regions of the area, functional studies have attempted to isolate specific regional changes. In a slice
taken from the mid-hippocampal formation, higher cerebral blood volume was found preferentially in CA1 in schizophrenia compared controls, and this elevation correlated with positive symptom severity (Schobel et al. 2009b). This study highlights the importance of carefully defining the area of investigation in a region as heterogeneous as the hippocampal formation. Future studies will be needed to confirm and extend these findings.

**Regional correlations in the basal state.** Recent investigations have focused on so-called resting-state networks (Raichle et al. 2001) in schizophrenia using blood-oxygen level dependent MRI and have seen decreased correlations among various brain regions (Bluhm et al. 2007; Liang et al. 2006; Lynall et al. 2010). This global loss of correlations was seen at first episode psychosis but was somewhat mitigated after six weeks of treatment with antipsychotics (Lui et al. 2010). The functional implications of these resting state correlations are poorly understood in healthy individuals, so the function impact of its degradation in schizophrenia is opaque. Changes in global correlations, however, suggest some level of dysconnectivity and, in schizophrenia, have been hypothesized to contribute to positive and negative symptoms (Stephan et al. 2009).

**Task-evoked activity.** In addition to resting-state or basal activity, hippocampal activity has also been probed in task-related condition. Memory task-related activity in the temporal lobe in schizophrenia is generally decreased compared to controls (Heckers et al. 1998; Weiss et al. 2003; Ongur et al. 2006). The results of task-related temporal lobe or hippocampal activation are consistent enough to stand up in a meta-analysis (Achim & Lepage 2005) but findings in individual studies may depend on the particular memory task used (Achim et al. 2007). Thus in task conditions there is consistent evidence of a failure to recruit the hippocampal formation or temporal lobe while in resting conditions there is
somewhat consistent evidence that the hippocampus is hyperperfused or hypermetabolic. Together this suggests that in addition to, or perhaps due to, the higher resting metabolic state, the hippocampus cannot be recruited appropriately under cognitive demand.

**Summary of hippocampal pathology in schizophrenia.** The evidence pointing to an involvement of the hippocampus and related medial temporal lobe structures in the neuropathology of schizophrenia comes from *in vivo* and *in vitro* methods. Structural pathology measured *in vivo* is observed in populations at high risk for developing schizophrenia and may predict onset of psychosis. There is some evidence of progressive volume loss over time; however the role of medication in this process is unknown. Some studies with higher resolution have suggested that the mid- to anterior hippocampus and/or the CA1 and subicular regions are preferentially affected in volume or shape, however more studies will be needed to confirm these findings. Focal changes in hippocampal volume observed *in vivo* are supported by histological investigations of cellular level alterations, though the evidence is sometime inconsistent. Abnormalities in glutamate neurotransmission are implicated by many of the histological alterations, and these have informed *in vivo* studies the behavioral and possible treatment effects of NMDA-receptor targeting. Given the involvement of NMDA receptors in at least some aspects of schizophrenia, and the counter-intuitive effect of NMDA receptor blockade, it is perhaps not surprising that functional measures such as NMR, PET, and MRI show changes in the temporal lobe in schizophrenia. These studies supply evidence of decreased neuronal viability or numbers in the medial temporal lobe as well as a hypermetabolic state at rest and hypometabolic during task conditions, perhaps a function of decreased global brain connectivity. The confluence of
evidence of hippocampal pathology in schizophrenia leads to the consideration of its organization and function in the healthy brain, the topic of the next section.

**Anatomy of the hippocampus**

Nestled in the medial temporal lobe, the human hippocampal formation is conventionally defined as comprising the entorhinal cortex, the dentate gyrus, CA3, CA1, and subiculum (Amaral & Witter 1995). The hippocampal region is often defined as consisting of the above structures but excluding the entorhinal cortex, while the hippocampus proper is solely the Cornu Ammonis regions. Regardless of convention, the term hippocampus is sometimes applied interchangeably with the above terms, so the reader must take care when interpreting studies that putatively investigated the hippocampus. Delimiting one subregion from another remains difficult with certain *in vivo* techniques, though the distinctions at the cellular level are well-appreciated (Amaral & Witter 1995). In this text, subregions will be denoted as defined above with the adjective “hippocampal” used to refer to general principles or findings from studies in which resolution of hippocampal subcircuits was not possible. In the human, the “long” axis of the hippocampus runs anterior to posterior, giving rise to distinctions between the anterior and posterior hippocampus. In the rodent, the long axis of the hippocampus takes a curved path in both the dorsoventral and mediolateral directions. Its dorsal extreme is labeled the septal pole while the ventral extreme is the temporal pole; the long axis is distinguished by the dorsal and ventral portions. The septal pole or dorsal hippocampus in the rodent corresponds to the posterior end of the human hippocampus, likewise, the temporal pole or ventral hippocampus
corresponds to the anterior end of the human (Amaral & Witter 1995). Descriptions in this
text to the hippocampus will mostly utilize the rodent terminology.

The circuit created by the largely unidirectional glutamatergic projections between
torhinal cortex, dentate gyrus, CA3, and CA1 has been extensively studied. The
projections of the entorhinal cortex, dubbed the perforant path, innervate all other parts of
the hippocampal formation. Pyramidal cells of layer II entorhinal cortex give rise to
projections to the dentate gyrus and CA3 while pyramidal cells of layer III mainly project to
CA1 and subiculum (Witter et al. 2000). The major projection target of CA1 is the
subiculum, and as such the subiculum is considered to be a major output of the hippocampal
circuit (Witter et al. 1990; O’Mara et al. 2001). However, both subiculum and CA1 send
projections to entorhinal cortex, though to different cellular layers, and complete a circuit
(Naber et al. 2001a). This circuit is repeated along the long axis of the hippocampal
formation (Andersen et al. 1971). The majority of hippocampal efferents, including
projections to entorhinal cortex, amygdala, striatum, other limbic basal forebrain regions,
and the hypothalamus, arise from CA1 and subiculum (Swanson & Cowan 1977). This
review will focus mainly on the anatomy of the subiculum.

**The tripartite organization of the subiculum**

The subiculum can be considered the “fourth synapse” of the trisynaptic circuit as
parts of CA1 send heavy projections to the subiculum (Amaral et al. 1991). The subiculum,
in turn, is the main site of origin of the fornix (Swanson & Cowan 1975). The subiculum can
be distinguished from CA1 by a rather abrupt widening of the pyramidal cell layer
accompanied by decreased packing density of the cells. Stratum radiatum and stratum lacunosum moleculare of CA1 become the inner and outer portions, respectively, of the subiculum molecular layer; the stratum oriens disappears completely. The pyramidal cells of the subiculum send their apical dendrites through the molecular layer of the subiculum while their basilar dendrites mingle within the pyramidal layer (Harris et al. 2001). Compared to CA1 cells, subicular cells show minimal axonal collateralization (Namura et al. 1994; Naber & Witter 1998). One striking feature of the subiculum is its strict topographical organization of efferents, afferents, or both in all three axes: dorsoventrally following the long axis of the hippocampus, proximodistally in relation to CA1, and laminarily within the molecular layer. Each of these topographical distinctions will be considered in turn.

**Dorsoventral organization of the subiculum.** Hippocampal area CA1 and the entorhinal cortex are the main afferents to the subiculum (Witter & Groenewegen 1990). As noted above, all areas of CA1 along the dorsoventral axis project to subiculum (Amaral et al. 1991). The entorhinal cortex can be split into a number of distinct areas (Witter et al. 1989), and its projections are organized topographically. The dorsal subiculum receives projections from more dorsal and lateral portions of medial and lateral entorhinal cortices while the ventral subiculum receives projections from the more medial portions of medial and lateral entorhinal cortices (Witter et al. 1989). While the afferents from CA1 and entorhinal cortex are well-characterized, other projections to the subiculum are incompletely characterized, perhaps due in part to their lower densities (Amaral & Witter 1995; O’Mara 2005). Both perirhinal and postrhinal cortices project to the dorsal subiculum (Naber et al. 1999; Naber et al. 2001b) but comparable findings have not been made in the ventral subiculum. Indeed retrograde studies investigating subicular afferents have focused mainly on the entorhinal
cortex (Van Groen & Lopes da Silva 1986). One study found projections to the ventral but not dorsal subiculum arising from the basolateral amygdala (French et al. 2003). Midline thalamic projections target the subiculum in a topographical pattern (Wouterlood et al. 1990). Projections from the medial septal complex reach the dorsal subiculum (Chandler & Crutcher 1983). Overall, many tracing studies do not sufficiently distinguish dorsal from ventral subicular targets (O’Mara 2005). Two major subcortical projections to the ventral subiculum are the nucleus reuniens and basolateral amygdala; both of these regions receive hypothalamic projections. Given that the subiculum targets the hypothalamus, this creates loops: hypothalamic-thalamic-subicular-hypothalamic and hypothalamic-amygdala-subicular-hypothalamic loops (Petrovich et al. 2001; Risold et al. 1997).

The subicular efferents along the dorsoventral gradient are better understood, possibly because the projections are more numerous. Projections to the entorhinal cortex, lateral septal complex, nucleus accumbens, presubiculum, and medial mammillary nucleus arise from the entire dorsoventral extent of the subiculum, however the exact region of innervations varies according to the dorsoventral site of origin (Naber & Witter 1998; Witter & Groenewegen 1990; Ishizuka 2001). Focusing on the ventral subiculum reveals additional projections to the ventromedial hypothalamic nucleus, infralimbic cortex, and amygdala (Canteras & Swanson 1992; Namura et al. 1994) (also see references within Naber & Witter 1998). The dorsal subiculum, in contrast, projects to the retrosplenial cortex and the perirhinal cortex (Naber & Witter 1998). The distinct sets of projection targets in the ventral and dorsal subiculum are taken to support a role of the dorsal subiculum in memory formation and the ventral subiculum in the regulation of stress responses (see below).
**Proximodistal organization of the subiculum.** In addition to the dorsoventral gradient of the subiculum along the septotemporal axis of the hippocampus, there is a further delineation of inputs and outputs relative to CA1. Following the nomenclature used in the hippocampus proper, the portion of a region that is relatively closer to dentate gyrus along the trisynaptic circuit is referred to as proximal while the portion within the region that is farther away is considered distal. Thus the proximal subiculum is the portion of the subiculum that is relatively closer to CA1, and hence the dentate gyrus, while distal subiculum is relatively far from CA1 and closer to presubiculum. This categorization divides the subiculum into three zones: proximal, middle, and distal subiculum, following the use of Amaral et al. 1991. This division is significant in that both the efferents and afferents to the region are spatially restricted to an extent along this dimension. CA1 efferents to the subiculum form a series of nested loops: the longest “loop” from proximal CA1 to distal subiculum, the shortest from distal CA1 to proximal subiculum, and a third loop intermediate between the other two (Amaral et al. 1991). This organization seems to hold at the level of a single CA1 cell projecting to approximately one third of the field of subiculum (Tamamaki et al. 1987). This organization is also respected by the entorhinal cortical efferents: medial entorhinal cortex projections target the more distal subiculum along the extent of the septotemporal axis while lateral entorhinal cortex projections target the proximal subiculum along the septotemporal axis (Van Groen & Lopes da Silva 1986).

**Laminar organization of the subiculum.** The final level of organization discussed here is the segregation of inputs within the molecular and pyramidal cell layers of the subiculum. Since afferents from CA1 and entorhinal cortex comprise the majority of incoming fibers to the subiculum, the laminar organization of these inputs will be
considered. Across the proximodistal and dorsoventral axes, the input region of CA1 and entorhinal cortical projections are spatially segregated. Entorhinal projections synapse on the outer portion of the subiculum molecular layer (Baks-te Bulte et al. 2005; Witter & Groenewegen 1990) while CA1 projections synapse on the basilar dendrites in the pyramidal cell layer and the proximal apical dendrites in the inner portion of the molecular layer (Amaral et al. 1991).

In sum, the tripartite organization of the subiculum along the dorsoventral and proximodistal gradient results in a separation of synaptic flow among CA1, subiculum, entorhinal cortex, and the other projections to and from subiculum. While the projections from CA1 probably fall into three broad divisions within the subiculum, other afferents to the subiculum and the structure of its efferents have not been delineated so precisely. Thus the subiculum can be divided roughly into quadrants: dorsal-proximal, dorsal-distal, ventral-proximal, and ventral-distal, each with its own pattern of inputs and outputs (Naber & Witter 1998). Within each quadrant, the afferents from CA1 synapse relatively closer to the pyramidal cell body while the afferents from entorhinal cortex synapse relatively farther away in the outer portion of the molecular layer.

Neurophysiology of the subiculum

Compared to the wealth of knowledge of the physiological properties and connectivity of CA1 pyramidal cells, the physiological characterization of subicular pyramidal cells is incomplete, especially of the ventral subiculum. Despite the differences of connectivity between the dorsal and ventral subiculum recognized by anatomists, the
electrophysiological investigations of the subiculum do not always distinguish their placements along this gradient. When the recording location is made explicit, it is often the dorsal subiculum. The rather small body of literature comprising the electrophysiological nature of the subiculum will be summarized here, with as much attention as possible being paid to dorsoventral location of the recordings.

Intracellular recordings of subicular neurons performed in vitro routinely characterize these cells as “bursting” or “regular spiking”, based on their responses to depolarizing current injection. Bursting cells respond to a one second intracellular current pulse with a group of action potentials followed by 20-30 msec without spiking before bursting again. In contrast, regular spiking cells fire single action potentials throughout the duration of the stimulus (Stewart & Wong 1993; Taube 1993). These and other papers reported varying ratios of bursting to regular spiking cells but this could be due to a gradient in the percent of bursting cells increasing along the proximal-distal gradient of CA1 (Jarsky et al. 2008; Kim & Spruston 2011a), at least in the ventral subiculum. The significance of the bursting phenotype is unclear, but it has been hypothesized to regulate hippocampal output (Jarsky et al. 2008), potentially by controlling intra-subicular circuits (Menendez de la Prida 2006).

Within the dorsal subiculum, stimulation of CA1 in a hippocampal in slice leads to a field excitatory postsynaptic potential (fEPSP) followed by a later onset inhibitory component (Finch & Babb 1980; Taube 1993). This same mixed response of the dorsal subiculum was observed after entorhinal cortical stimulation (Behr et al. 1998). In that study the investigators noted occasional convergence of entorhinal afferents and CA1 afferents on to single cells and simultaneous activation of the inputs had an additive effect. This additive
response was also seen using voltage sensitive dyes during simultaneous CA1 and entorhinal cortical stimulation (Cappaert et al. 2007). This evidence of convergence was predicted by the anatomy (Naber et al. 2001a).

Early recordings done in the subiculum of the anesthetized cat showed that lateral entorhinal cortex stimulation elicited the largest fEPSP in dorsal subiculum while medial entorhinal cortex stimulation elicited the largest fEPSP in ventral subiculum (Van Groen & Lopes da Silva 1986). Later studies performed in vivo in the rat dorsal subiculum showed mixed excitatory and inhibitory responses after CA1 stimulation and mostly, but not exclusively, inhibitory responses from entorhinal cortical stimulation (Gigg et al. 2000). The group also showed convergent inputs from CA1 and entorhinal cortex onto the same subicular cell, in line with the in vitro work.

**Functions of the dorsal and ventral subiculum and implications for disease states**

The functions of the dorsal hippocampus and subiculum are better characterized than those of their ventral counterparts. The roles of the dorsal regions in spatial memory and navigation are well-established (Moser & Moser 1998; Naber et al. 2000). Both the dorsal and ventral hippocampus and subiculum play a role in sensorimotor integration (Bast & Feldon 2003). The ventral subiculum, on the other hand, is more specifically involved in stress responses and limbic system-mediated affective, cognitive and behavioral processes. Regulation of the stress response arises from the anatomical projections to hypothalamic and limbic forebrain structures that in turn synapse on the paraventricular hypothalamic nucleus (Herman & Mueller 2006). Indeed, lesions of the ventral but not dorsal CA1/subicular region reduce anxiety in behavioral tests (Bannerman et al. 2003). The ventral subiculum
also occupies a pivotal place in the limbic system by its ability to regulate dopamine release in the ventral striatum (Legault et al. 2000; Floresco et al. 2001; Floresco & Grace 2003) due to its projections to the nucleus accumbens and subcortical regions that regulates ventral tegmental dopamine cell firing. These roles of the ventral subiculum in sensorimotor integration, stress response, and dopamine regulation have likely significance for schizophrenia (Grace 2010; Bast & Feldon 2003). Thus the ventral subiculum occupies a unique place to regulate multiple neural systems implicated in the psychopathology of schizophrenia. Understanding the possible mechanisms by which the region could drive or modulate diseases processes can be approached through the use of appropriate animal models, as discussed in the next section.

Disease-relevant alterations in the MAM 17 model of schizophrenia neuropathology

The heterogeneous nature of the symptoms and pathology of schizophrenia makes modeling the disease a challenge. One approach, based on neurodevelopmental hypotheses of the etiology of schizophrenia, has been to use neurodevelopmental manipulations in rodents to produce key brain and behavioral phenotypes observed in the disease. Ideally such a neurodevelopmental model should reproduce key neuronal and brain phenotypes observed in schizophrenia and be able to be used to discover mechanisms linking these ‘neurophenotypes’ to disease-relevant abnormalities in cognition and behavior. The MAM E17 rodent model meets key criteria as such a ‘neuro-patho-developmental model’. It shows construct validity in psychosis-relevant measures including sensorimotor gating and responsivity to psychostimulants and it shows relatively selective pattern of cognitive
deficits observed in schizophrenia. Furthermore, the gross histology and initial findings of
neuronal activity in the hippocampus in the model indicates that the neuropathology has
relevance to what is reported in schizophrenia, as detailed below.

The generation of the model was initially informed by the research showing that
perinatal factors could increase the risk of schizophrenia later in life combined with early
studies showing patterns of cellular disarray in the entorhinal cortex and hippocampus
(Jakob & Beckmann 1986; Kovelman & Scheibel 1984). Later researchers observed protein
markers in the temporal lobe consistent with developmental disturbances, leading to the
suggestion that the effects of disrupting cortical migration patterns could be more important
than the disruption mechanisms (Akbarian et al. 1993). Thus disrupting neurogenesis during
prenatal development was suggested as a way to model the observed defects with potential
relevance to schizophrenia (Talamini et al. 1999; Ghajarnia et al. 1998). One way to disrupt
neurogenesis is through the administration of the methylating agent methylazoxymethanol
acetate (MAM) (Nagata & Matsumoto 1969), temporally targeted to when peak
neurogenesis of the temporal cortex and underlying hippocampus have been reported (Bayer
& Altman 1995). Thus prenatal exposure to MAM targeted to embryonic day (E) 17 was
suggested as a way to selectively disrupt temporal corticogenesis in rats (Grace et al. 1998).
At this stage of neonatal development, neuronal proliferation and migration has peaked in
many cortical regions and is nearly complete or has paused in in most subcortical and
cerebellar regions (Bayer & Altman 1995). The offspring of dams injected with MAM on
gestational day 17, MAM E17 animals, show a variety of anatomical, neurochemical, and
physiological phenotypes relevant to schizophrenia.
Overall brain volume loss is subtle (Flagstad et al. 2004) if present at all (Fiore et al. 2004) in MAM E17 animals. Consistent reports show the presence of regionally specific volume loss (Moore & Grace 1997; Moore et al. 2006; Flagstad et al. 2004; Featherstone et al. 2006; Penschuck et al. 2006; Sanderson et al. 2011). Specifically, MAM-induced reductions in tissue area or regionally volume are consistently observed in limbic and paralimbic cortices including hippocampus, medial thalamus, and medial prefrontal cortex while area such as cerebellum and midbrain appear unaffected (Moore et al., 2006). There have also been reports in MAM-treated animals of subtle changes in cell migration patterns in the ventral hippocampus and entorhinal cortex (Flagstad et al. 2004; Gourevitch et al. 2004; Matricon et al. 2010). Finally, recent evidence suggests decreased myelination in the corpus callosum and cingulum in reduced in MAM E17 animals (Chin et al. 2010), however replications of this effect in additional cohorts of animals will be necessary to strengthen this finding. These anatomical findings are in line with reports from the schizophrenia literature showing volume reductions in the hippocampus and temporal lobe and the findings may replicate the slight neuronal migration patterns sometimes observed in schizophrenia (Harrison 2004).

Only a few studies have investigated histochemical changes in the MAM E17 model. Decreased parvalbumin expression was observed in the dorsal hippocampus but not the prefrontal cortex, while other calcium-binding proteins calretin and calbindin were not affected in either region (Penschuck et al. 2006). That study did not include subiculum, however a later study found ventral hippocampus/subiculum decreases of parvalbumin in MAM E17 animals (Lodge et al. 2009). Reduced parvalbumin immunoreactivity in hippocampal regions and the prefrontal cortex is in line with reports from schizophrenia (for
review, see (Reynolds et al. 2001)). Changes in brain-derived neurotrophic factor and nerve growth factor were investigated as potential long-term markers of altered neurodevelopment (Fiore et al. 2004). Regionally specific changes were observed, but some differences between 3-month old rats and 9-month old rats were difficult to interpret and suggest that more studies are needed.

Recordings in MAM E17 prefrontal cortical neurons uncovered changes in the membrane properties of these cells including depolarized resting membrane potential, depolarized spike firing threshold, increased input resistance, and altered pattern of spike discharge (Lavin et al. 2005; Moore et al. 2006). Cortical dopamine fails to modulate pyramidal neuron spike activity in the prefrontal cortex (Lavin et al. 2005; Goto & Grace 2006). In both the prefrontal cortex and the ventral striatum there is evidence of a disruption in the stability of up and down states in these neurons, which was interpreted as potentially affecting the ability of the neurons to function as coincidence detectors (Moore et al. 2006). In the hippocampus, there is preliminary evidence of increased spike firing of the presumed projection neurons of the ventral CA1/subiculum which could contribute to the increased population firing rate of dopaminergic cells in the ventral tegmental (VTA) area (Lodge & Grace 2007; Lodge & Grace 2008). Indeed, local ventral hippocampal or systemic application of a novel benzodiazepine decreased the population activity of VTA dopaminergic cells in MAM E17 animals (Gill et al. 2011). Electrophysiology recordings that targeted dorsal CA1 in slice recordings isolated deficits in synaptic transmission accompanied by increased CA1 neuronal excitability in the MAM E17 model (Sanderson et al. 2011).
The set of electrophysiology experiments performed in the ventral hippocampus/subiculum provides evidence that abnormal hippocampal activity may mediate increased spontaneous activity in the dopamine system in the MAM E17 model (Lodge & Grace 2011). This provides a key rationale for using this model to investigate brain changes relevant to schizophrenia. Namely, that hippocampal abnormalities observed in schizophrenia could be causally related to dopaminergic abnormalities in the disease. These initial reports in the MAM E17 model, however, left important gaps in our knowledge about the model. It remains uncertain if MAM E17 induction reliably models the hippocampal hypermetabolism seen at rest in patients and if morphological changes observed in the hippocampal region of schizophrenia patients are seen in the rodent model. Moreover, the physiological and molecular mechanisms that may link these phenotypes in the model and, possibly, in schizophrenia, are not understood.

To address these gaps in knowledge, I conducted a series of experiments to examine the following questions:

1. Does a mid-gestational induction of nucleic acid alkylation in the brain by MAM E17 lead to reductions in hippocampal size and morphological abnormalities in subicular neurons observed in the hippocampus of schizophrenia?

2. Does the MAM E17 rodent model exhibit evidence for changes in glutamatergic synaptic transmission in the ventral subiculum? Are there abnormalities in the input/output function?

3. Are the changes in synaptically evoked activity reflected in the spontaneous single unit and population firing characteristics in the intact MAM E17 brain?
4. Are there changes in resting cerebral blood volume (rCBV) in the MAM E17 hippocampus in vivo, as measured using methods similar to those that reveal increases in rCBV in schizophrenia patients?

I hypothesized that the MAM E17 model would exhibit schizophrenia-relevant structural and functional changes in the hippocampal formation that could underlie the behavioral changes seen in the model.
Chapter 2
Materials and Methods

Animals

Animal care

All procedures followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Columbia University and New York State Psychiatric Institute Institutional Animal Care and Use Committees.

MAM E17 model induction

Rats: Timed pregnant Wistar rats from Hilltop Lab Animals (www.hilltoplabs.com), Taconic (www.taconic.com), or Charles River (www.criver.com) were delivered on embryonic day (E) 15 and housed singly in cages with standard and enrichment bedding for nesting. Dams were injected i.p. with methylazoxymethanol acetate (MAM) (MRI Global, www.mriglobal.com) dissolved in normal saline or saline alone (vehicle control) on E17 (day of vaginal plug was considered E0). The dosage of MAM was 22-25 mg/kg, as noted in individual experiments. Offspring of dams injected with MAM on E17 are referred to as MAM E17 rats. Offspring of dams injected with vehicle are control rats. Litters were culled to 8 pups on postnatal day (P) 1 or 2. In most experiments, culled litters were biased toward males in order to achieve sufficient cell sizes with one sex; however, both sexes were included in experiments where possible. Offspring of injected dams were weaned between postnatal day 21 and 23. Offspring were group or paired housed with food and water ad libitum in a temperature and humidity controlled room with a 12 hour light-dark cycle.
Experiments were performed during the animals’ light cycle. The sex and age distributions were matched for MAM-E17 rats and controls within each experiment.

**Mice:** Timed pregnant female C57BL/6 mice were delivered from Jackson Laboratories (www.jax.org) on E15 and housed singly. Dams were injected i.p. with MAM (22-25 mg/kg) dissolved in normal saline or saline alone (vehicle control) on E17 (day of vaginal plug was considered E0). All other animal care procedures corresponded to those used on rats. Two sets of mice were generated for these experiments. The first set of animals comprised 6 MAM E17 mice (4 males) and 6 controls (3 males) with an average age of 99 ± 4.0 days. The second set comprised 8 MAM E17 mice (all males) and 5 controls (all males) with an average age of 120 ± 1.2 days.

**Hippocampal anatomy**

*Gross morphology*

Male rats from dams injected with vehicle or 25mg/kg MAM were used in this experiment.

Whole hippocampal volume in horizontal sections: Animals in this study were either 183 days old (control animals) or 184 days old (MAM E17). Animals were deeply anesthetized and perfused with ice cold normal saline followed by cold 4% paraformaldehyde in phosphate buffer. Brains were kept in 4% paraformaldehyde for at least 24 hours before being cryoprotected in ascending sucrose solutions. Brains were hemisected and the right half was used for this experiment; the other half was saved for another experiment. Hemibrains were sliced on a cryostat in 40 micron horizontal sections. Every third section
was thaw mounted, dehydrated, and stained with Cresyl Violet and Neutral Red (4:1) and prepared for examination under the microscope. The right hippocampal formation, comprised of the hippocampus proper, the dentate gyrus, and subiculum, was traced in StereoInvestigator (MBF Bioscience, www.mbfbiomonitor.com) using the 2.5x objective. Sections spanning approximately 8.6 to 3.1 mm ventral from were included. Tissue sets from each treatment group were matched for the start and end location. All rat stereotaxic references and measurements are given relative to Bregma, following Paxinos and Watson (Paxinos & Watson 1998). Volume projections were estimated in StereoInvestigator using Cavalieri’s principles. Volume estimates were compared using a paired T-test.

**Area of rat ventral hippocampus in horizontal sections:** The average age of animals used in this experiment was 103 ± 3.2 days; there was no difference in the distribution of ages between treatment groups. Tissue was prepared as in whole hippocampal volume study but slices were 40 microns and tissue underwent one round of coverslipping and recoverslipping before being stained with Cresyl Violet and Neutral Red. The area of the contour was calculated for each horizontal slice, beginning ventrally at the level matching the shape of the blades of the dentate gyrus at Bregma -7.8 in the atlas and continuing dorsally eight sections for a total span of 1.08 mm (for a schematic, see Figure 2.1). Contour areas were analyzed as a function of distance along the dorsoventral axis compared between gestational treatment groups using a mixed ANOVA.
**Figure 2-1**
Whole rat brain showing approximate locations of the span of slices used in hippocampal area measurements. Horizontal sampling area is shown in green; coronal sampling area is shown in blue. Note the overlap of the regions in the ventral hippocampus. Figure modified from Witter et al. 1989.

**Area of rat hippocampus in coronal sections:** The average age of animals used in this study was 83 ± 2.6 days (total of 10 animals); there was no difference in the distribution of ages between treatment groups (independent samples t-test, p = .355, n = 5). A separate set of animals were either perfused and cryoprotected as above or had brains removed fresh and kept in formalin for at least 24 hours before cryoprotection. Brains were sliced coronally in 45 micron sections. Sections were thaw mounted, stained with Cresyl Violet and Neutral Red (4:1) and prepared for examination under a microscope. The hippocampal formation containing the same regions as defined above comprised the region of interest. Brains were analyzed from the most caudal slice containing subiculum molecular layer as well as the pyramidal layer, at approximately Bregma -7.39 in the atlas, and continuing rostrally every third slice (inter-slice interval 0.135 mm) for 13 slices, to approximately Bregma -5.6 (Figure 2-1). Contour areas were analyzed as a function of distance along the rostrocaudal axis compared between gestational treatment groups using a mixed ANOVA. There was no effect of preservation method on area measurements.

**Area of mouse hippocampus in horizontal sections imaged in vivo:** The average age of the mice was 120 ± 1.2 days (n = 5); there was no difference in ages between the groups (independent samples t-test, p = .232). The second set of MAM E17 and control mice as
detailed in “Animal Care: Mice” portion of the methods were used in this experiment. Mice were imaged with a 9.4 T vertical Bruker magnet (Oxford Instruments Ltd, www.oxinst.com) in a spectrometer (AVANCE 400WB spectrometer; Bruker NMR, www.bruker.com) with a 30 mm inner diameter birdcage radiofrequency probe and a shielded gradient system (100 G/cm). Axial T2-weighted images were acquired with repetition time of 3500 msec and time to echo 32.88 msec. Eight slices were acquired with a acquisition matrix of 256 x 192; field of view was 22.0 by 16.5 mm; voxel size was 0.086 mm x 0.0086 x 0.6 mm (0.0044 mm³; in-plane resolution was 0.0074 mm². Each set of images required 16 minutes to acquire. One set of images was acquired before contrast administration and seven sets were acquired after contrast (gadodiamide, 10 mmol/kg via i.p. catheter). All sets were acquired while the animal was anesthetized to minimize head movement (1.5% v/v isoflurane gas (1 liter/min air flow) delivered through a nose cone). Breathing rate and blood oxygen saturation were measured with a pulse oximeter and body temperature was measured using a rectal probe.

Images from seven scans taken after contrast injection were prepared in Medx (www.medx-inc.com) to produce a high quality image for identifying the region of interest. Visualized anatomical landmarks were compared to the Mouse Brain Library (www.mbl.org) atlas of horizontal sections from an adult C57Bl male mouse. An outline of the hippocampal formation containing dentate gyrus, CA3, CA1, and subiculum was drawn on the image in which the hippocampal formation was best visualized, corresponding to approximately Bregma -5.64 mm, as well as two sections dorsally and two sections ventrally, spanning from approximately Bregma -3.24 to -6.84. Contours were drawn by an investigator who was trained on a standard set of images and who was blind to treatment.
The area of these contours was calculated in Medx. Left and right contour area values were averaged and treatment groups were compared using a mixed model ANOVA.

**Dendritic morphology**

Male rats from dams injected with 22-25 mg/kg MAM or the equivalent volume of normal saline were used in this experiment. All animals used in this experiment were 45 or 90 days old at the time of sacrifice. To stimulate and promote the growth and maintenance of dendritic spines during cortical development, age-matched rats were placed in an enriched environment containing PVC tubing segments and rodent wheels for two hours per day, five days per week from P21 to sacrifice. Rats were deeply anesthetized with a lethal dose of sodium pentobarbital (80 mg/kg) and decapitated.

Brains were dissected from the skull, and the dura and pia were removed. The brains were wrapped in clean gauze and immersed in 100 ml of NeoGolgi solution in an opaque container. Twenty-four hours later, both the NeoGolgi solution and gauze were changed and incubated for twelve weeks. The tissue samples were then dehydrated using the following protocol: 50% alcohol for 1 hour, 70% alcohol for 1 hour, 95% alcohol twice for 12 hours, and 99% alcohol twice for 12 hours. The tissue samples were then placed in 1:1 99% alcohol and ether for 4 hours, 2% celloidin for 3 days, 4% celloidin for 3 days, 8% celloidin for 3 days, and 12% celloidin for 3 days. To harden the tissue, the brains were immersed in chloroform. The brains were then mounted on embedding blocks and placed in 70% alcohol until they were ready for sectioning. The brains coronally sectioned using a vibratome at 200 μm. All sections were collected in 70% ethanol and rinsed twice in distilled water for 5 minutes. The sections were then immersed in a closed vessel of 19% NH₄OH for 30 minutes.
and rinsed in distilled water twice for 5 minutes. All sections were then placed in Kodak Rapid Fix (diluted 1:7) for 10 minutes, rinsed twice in distilled water for 5 minutes, mounted onto slides and coverslipped.

**Analysis of subicular apical dendrites using single neuron reconstructions.** All analyses were done using a combination of Neurolucida, StereoInvestigator, and Neuroexplorer software (MBF Bioscience). From each subject, two sections containing the ventral hippocampus were randomly selected. Contours of the ventral subicular complex, (containing the subiculum, the presubiculum, and the parasubiculum) were drawn on both the left and right side of these sections. Neurons in these contours were examined for completeness of Golgi impregnation and clarity and completeness of the dendritic tree. Periodic neurons with pyramidal neuron morphology, dendrites denude of spines, and a greater density of periodic axonal swellings (varicosities) were observed. Based on their distinct appearance, these neurons were avoided in our analysis. Neurons chosen for further analysis were traced in their entirety using a 40x objective, and dendritic spines were marked using a 60x objective. Based on the coronal sectioning scheme, it was not possible to obtain the entire subicular apical dendrite, and in particular the distal tuft.

The reconstructed neuron was analyzed by Sholl analysis using 10 micron concentric rings to quantify branching. One to four neurons were analyzed per animal, and values were pooled to obtain one value per animal. Spine counts were obtained in 10 micron bins from the soma. The shortest pooled value of dendritic branching or spine counts extended to 280 microns, thus the analyses were performed on bins up to and including this value. A repeated measures ANOVA was used to analyze both spine densities and dendritic branching. Neuron location within the subicular complex (subiculum, presubiculum,
parasubiculum) was tested as a covariate in the model in the P45 animals, and it was found to have no effect the model of either spine densities or dendritic branching. Adding neuronal location within the pyramidal cell layer on the deep to superficial axis as a covariate in the model also had no effect on either outcome measure in the P45 animals. The resulting model tested for treatment as a significant between subjects factor without any covariates. Values from P45 and P90 animals were pooled and age was added to the model as a between subjects factor.

**Analysis of subicular dendrites using population methods.** Apical and basilar dendrites were analyzed by population in the ventral subiculum. Three to five serial sections per brain were identified with an intact subiculum. Care was taken not to include the most caudal subiculum sections where the laminar structure disappears. Contours were drawn around the subiculum following anatomical landmarks depicted in a standard stereotaxic atlas (Paxinos & Watson 1998). Close attention was paid to the border between CA1 and subiculum, with the subiculum identified by the expansion of the pyramidal cell layer. The subiculum border with the presubiculum was defined by shorter apical dendrites, more variability in apical dendrite orientation, and less laminar organization. As with the neuron reconstruction study, brains from animals P45 and P90 were pooled.

To distinguish proximal from distal apical dendrites, the contours of the inner and outer molecular layers of the subiculum were defined as follows. The height of the subiculum excluding the pyramidal cell layer and a 60 micron buffer was determined. The outer molecular layer was considered to be the half of the remaining height closest to the hippocampal fissure. The inner molecular layer was considered to be the half of the height near the pyramidal cell layer. A 15-micron buffer between the inner and outer layers was
allowed. The outlines were drawn using a 10x objective. Basilar dendrites quantification was done in contours that included the pyramidal cell layer.

Spine density was calculated in the inner and outer molecular layers. A systematic sampling method, using principles of the optical fractionator and L-Cycloids approaches, was used for quantification of spine density in this region. A 120 micron x 120 micron grid was superimposed on the contour, and the 20 micron x 20 micron region in the lower right corner of each grid box was visualized with a 60x objective. After focusing 40 um from the slide surface, the first dendrite that intersected either the right or lateral grid line, or both, within 20 um of the intersection was chosen for spine quantification. Spines along a 10-15 micron length of the dendrite starting from its intersection with the box were marked. Spine density was calculated by dividing the total number of spines counted by the total length of dendrite segments examined for each animal. Dendritic branch density was investigated in the inner molecular layer using the Space Balls probe. Intersections of dendrites into the probe were assessed using a 60x objective. Grid size was 90 by 90 microns, and sphere radius was 20 microns.

**In vitro electrophysiology**

Male rats from dams injected with saline vehicle or 25mg/kg MAM were used in these experiments. All averages are reported as the mean followed by the standard error of the mean. The average age of the subjects was 78 ± 2.2 days for control animals and 79 ± 1.7 days for MAM-E17 animals. The average weights were 421 ± 12.0 g and 443 ± 12.7 g for control and MAM animals, respectively. Neither age nor weight was different between
treatment groups in independent samples t-test (p > 0.5 in both cases). Rats were taken from eight control litters and six MAM litters. No more than four slices were used from one animal in the same recording location. Not all recording locations were tested in all animals.

Slice preparation. Animals were anesthetized with isoflurane and decapitated using a guillotine. The brain was rapidly removed, hemisected, and immersed in ice cold cutting solution for 1 minute. Cutting solution was composed of the following (in mM): 1.25 Na$_2$HPO$_4$, 26.0 NaHCO$_3$, 2.5 KCl, 10.0 NaCl, 200 sucrose, 10 d-glucose, 2.0 MgCl$_2$, and 1.0 CaCl$_2$ and bubbled continuously with 95% O$_2$ and 5% CO$_2$. After chilling, the front half of the brain was cut off and a section of the dorsal surface was removed to provide a flat surface. The brain was then glued to the vibratome specimen disc using cyanoacrylate glue. The specimen disc was placed in ice cold bubbled cutting solution for slicing. Horizontal slices 400 μm thick were cut from the ventral hippocampus (approximately 5.8 to 6.8 mm ventral from Bregma) using a Leica VT1000s vibratome. Slices were immediately trimmed rostral to the hippocampal formation and transferred to the recovery vessel containing room temperature, bubbled artificial cerebral spinal fluid (ACSF). ACSF was composed of the following (in mM): 1.25 Na$_2$HPO$_4$, 26.0 NaHCO$_3$, 2.5 KCl, 126 NaCl, 10 d-glucose, 1.0 MgCl$_2$, and 2.0 CaCl$_2$. After a 1.5 hour recovery period, slices were transferred to an interface recording chamber held at 29 degrees C with ACSF perfused at a rate of 1-2 mL/min.

Recording and stimulation. Field extracellular recordings were performed using a vertically pulled (ESF Patch Pipette Puller PA-10, Warner Instruments, www.warneronline.com) glass micropipette (World Precision Instruments) with a 1.5/1.12 mm outer/inner diameter and filled with ACSF (resistance 5 MΩ). A concentric bipolar
platinum/iridium electrode (FHC, Inc., http://www.fh-co.com) was used for stimulating afferents. Stimulating pulses (100 μsec duration) were generated by a square pulse stimulator (S88, Grass Technologies, www.grasstechnologies.com) and isolated from grand with a battery-powered stimulus isolation unit (SIU5, Grass Technologies). Data were amplified (Intracellular Electrometer IE-210, Warner Instruments) and lowpass filtered at 5,000 Hz and digitally recorded (Digidata 1322A, Molecular Devices, www.moleculardevices.com) with a sampling frequency of 10,000 Hz. Data were saved for offline analysis in pClamp (Molecular Devices).

Stimulus and recording locations are show in Figure 2-2.

![Figure 2-2](image)

**Figure 2-2.**
*Stimulation and recording locations used in *in vitro* extracellular electrophysiology experiments in ventral hippocampal slices.* a) Stimulate at CA1 stratum oriens-alveus border and record in subiculum inner molecular layer. b) Stimulate perforant path and record in subiculum outer molecular layer. c) Stimulate CA3 Schaffer collaterals and record in CA1 stratum radiatum. d) Stimulate perforant path and record in CA1 stratum lacunosum-moleculare.

**Inputs to subiculum:** CA1 afferents to subiculum were stimulated on the border between stratum oriens and the alveus. Location of the subiculum was identified visually by the widening of the pyramidal cell layer. Recordings were performed in the area of the subiculum proximal to CA1. The tip of the recording electrode was located in the proximal apical dendritic zone where the response was maximal to test pulses. The perforant path was stimulated by placing the stimulating electrode in the visible white matter on the border.
between the subiculum and dentate gyrus. Responses to perforant path stimulation were recorded in the OML of the subiculum, containing the most distal apical dendrites of the pyramidal cells.

**Inputs to CA1:** The site for stimulation of the Schaffer collaterals was located at the visible insertion of the fiber bundle into CA1. Responses to Schaffer collateral stimulation were recorded in CA1, midway between CA2 and subiculum in the stratum radiatum. This location was empirically optimized for the presence of a FV followed by the negative-going EPSP which occasionally had a population spike superimposed on it. The perforant path input to CA1 was stimulated in the bundle locally in CA1 and responses were recorded in stratum lacunosum-moleculare. The order of stimulation manipulations was varied; there was no observed effect of stimulation order between or among regions.

**Drugs.** All drugs for these experiments were purchased from Tocris Bioscience (www.tocris.com). CNQX was aliquoted in dimethyl sulfoxide (DMSO) before use, all other drugs were aliquoted in normal saline. Drugs were bath-administered at final concentrations as follows: 10 μM CNQX, 10 μM Gabazine (hydrobromide), 50 μM D-AP5, and 1 μM TTX.

**Data analysis.** Data was analyzed in Clampfit Version 9 (Molecular Devices). Data was filtered with a Gaussian filter with a lowpass cutoff of 5 kHz. Traces from the same recording at the same stimulus intensities were averaged (typically three responses per stimulus intensity). Prestimulus baseline was calculated as the average voltage of the trace during a 150 μsec interval approximately 1 msec before stimulus onset. The maximal fiber volley (FV) response was defined as the local minimum between two points containing the FV. This value was subtracted from the prestimulus baseline to obtain the FV amplitude. The slope of the field excitatory postsynaptic potential (fEPSP) was calculated as the
maximum slope between 10-90% of the fEPSP. See Figure 2-3 for example trace. Both FV amplitude and fEPSP slope values were multiplied by -1 to obtain positive values for statistical and graphing purposes. Input-output curves were obtained from the responses to increasing stimulus intensity (5, 10, 15, and 25 V) at a stimulation frequency of 0.05 Hz. Initial experiments used a greater number of stimulus intensities (up to 18) from 1 to 75 V, but no significant responses were observed at > 5 V, and no increase in response was observed at 75 V. Thus a coarser input-output curved was adopted for later experiments. The extra stimulus intensities were dropped from the analysis of the early experiments. Input-output ratios were calculated by dividing EPSP slope by FV amplitude.

![Graph](image)

**Figure 2-3.** Example trace. Fiber volley amplitude was calculated from the minimum value obtained between the black stippled lines (a) subtracted from the baseline obtained approximately 1 msec before stimulus artifact (not shown). fEPSP initial slope was the maximal slope between 10 and 90% of the downward slope, as shown in (b).

**Statistical analysis.** Statistical analyses were conducted in Microsoft Excel 2007 (Microsoft Corporation, http://office.microsoft.com) or SPSS (IBM Corporation, http://www.spss.com). Responses to stimulation levels of 10, 15, and 25V are shown. Intensities of 5 V were also tested, but the 95% confidence intervals for absolute values of the FV amplitudes and fEPSP slopes did not exceed 0 for these stimuli, indicating that the synaptic responses at the 5V level did not reliably exceed noise. Thus these data were
Values from 10, 15, and 25 V stimulus intensities were dropped from subsequent analyses. Outliers were removed if the value was more than two standard deviations from the group mean calculated without the value in question. Data were inspected to ensure no apparent difference in batch distribution between the treatment groups, and batch was used as a covariate in all analyses. In the final data set, files were weighted to equalize the contribution of multiple slices per animal when one brain region was tested in more than one slice from the same subject. The sum of the weights for each brain region per equaled one.

Because of the non-linearity of the stimulus-response function and non-normal distribution of the data at each stimulus intensity, the data were log-transformed. The log-transformed FV and EPSP data were analyzed using a mixed ANOVA for each region. The data are shown as a function of stimulus intensity in Figures 2.4 and 2.5. The transformed data were distributed normally and the stimulus intensity functions were linear. There were no significant interactions between stimulus intensity and treatment group except in one region (IML) where the stimulus intensity function continued to fail normality tests. Given this anomaly and the overall lack of interaction between stimulus intensity and treatment group, the repeated measure of stimulus intensity was eliminated from the final analyses and averages of the natural log-transformed FV and EPSP measurements (across stimulus intensities 10-25 V) were used as the dependent measures.
Figure 2-4
Fiber volley amplitude. Log-transformed fiber volley amplitude as a function of stimulus intensity from the four tested brain regions. a) Inner molecular layer of subiculum following CA1 stimulation b) outer molecular layer of subiculum following perforant path stimulation c) stratum lucidum of CA1 following Schaffer collateral stimulation d) stratum lacunosum-moleculare of CA1 following perforant path stimulation.
In vivo electrophysiology

Male and female offspring from rat dams administered saline or MAM (22-25 mg/kg) at E17 were used for this experiment. The average age of the subjects was 110 ± 6.7 days for control animals and 114 ± 4.2 days for MAM-E17 animals.

**Surgical procedures:** Animals were anesthetized with isoflurane mixed with oxygen or anesthetized with chloral hydrate (400 mg/kg i.p. induction divided into two injections and supplemented approximately every hour as necessary). Depth of anesthesia was monitored by breathing rate and the presence of whisking. Animals were placed in a stereotaxic apparatus (David Kopf Instruments, www.kopfinstruments.com) and the skull was leveled. Body temperature was monitored with a rectal probe (BAT-10, Physitemp Instruments, www.physitemp.com) and maintained at 37 degrees Celsius with a
thermostatically controlled heating pad. Breathing rate and blood oxygen saturation were monitored using a pulse oximeter (Surgivet model V3402, www.surgivet.com). Stereotaxic coordinates were taken from the atlas and modified empirically to target the ventral subiculum and the entorhinal cortex. The scalp was incised and a burr hole was drilled in the skull overlaying the target region(s). Recordings were targeted to the subiculum between 6.0 and 6.7 mm posterior from Bregma. Entorhinal cortex stimulation (0.2 msec pulse width, 0.4 Hz) was targeted to pyramidal cell layers II and III at 7.8 mm posterior from Bregma.

**Extracellular recordings:** Tungsten electrodes (0.005 inch diameter, 5 M Ohms resistance, A-M Systems, www.a-msystems.com) were used for recordings in offspring of dams injected with 22 mg/kg MAM. A pulled glass micropipette (World Precision Instruments, 1.5/1.12 outer/inner diameter thin-walled glass capillary) was used for experiments on offspring of dams injected with 25 mg/kg MAM. The micropipettes were filled with 2 M sodium chloride containing 2% Pontamine sky blue dye. Silver wire was inserted for signal acquisition. Recordings were made against a reference electrode placed between the skin and the skull. The signal was amplified, bandpass filtered between 0.1 and 10,000 or 20,000 Hz and acquired at 10,000 Hz. The data were digitized and stored using custom-designed computer software (Neuroscope) running on an Intel-based personal computer with a data acquisition board interface (Microstar Laboratories, www.mstarlabs.com). Experiments including stimulation of the entorhinal cortex utilized a bipolar stimulating electrode (NE-100X, Rhodes Medical Instruments, Inc., Summerland, Cal.) sunk into the entorhinal cortex at a 6.5 degree angle lateral (as measured by the stereotaxic apparatus) to the midline. This angle prevented the electrode holders from touching.
The recording electrode was advanced within the ventral subiculum until a single unit could be clearly discriminated on the basis of a large-amplitude, highly consistent waveform. This unit was defined as the “Main unit” (see Data Analysis). In recordings made with Tungsten electrodes, other units, in addition to the Main unit, with smaller-amplitude waveforms could also be discriminated and were defined as “Background units.” The combined spikes of the Main unit and the Background unit were dubbed “Population units” (see Data Analysis, below). Infrequent test stimulation pulses (separated by at least 30 seconds) in the entorhinal cortex were delivered once the recording electrode was in the subiculum. The stimulating electrode was advanced to its final location based on observed unit responses in the subiculum.

**Histology:** At the end of each experiment, the animal was deeply anesthetized and decapitated. The brain was removed and fixed in formalin for at least 24 hours. Brains were immersed in a solution of 30% sucrose in phosphate buffer until saturated. Frozen brains were sectioned coronally in 45 micron sections using a cryostat. Sections were mounted on a gelatin-coated slide, stained with cresyl violet/neutral red, and examined under a microscope to determine the recording and stimulation sites (Figures 2-4 and 2-5).
**Data analysis**

Data was analyzed offline using a combination of Neuroscope, Matlab (The Mathworks, Inc) and Prism 5 (GraphPad Software, Inc.). Data files were one to three minutes in length. All data are displayed as the mean ± standard error of the mean.

**Spontaneous spiking analyses:** Data was highpass filtered at 1000 Hertz. Spikes were identified by setting a user-defined threshold (threshold values did not vary between treatment groups). Spikes were sorted using Chronux ((Mitra & Bokil 2008) and http://chronux.org) and custom Matlab scripts. The spike cluster identified in Chronux with
the greatest amplitude showing consistent spike activity and stable waveform characteristics across the entire trace was designated as the “Main unit”. Other clusters were combined and designated “Background units”. All units together are designated “Population units”. For these classes of units, the following outcome measures were defined:

1. Average amplitude of the Main unit was calculated as the local minima of the spikes.

2. Instantaneous firing rate was calculated as the inverse of the average time in seconds between spikes.

3. Phasic burst-like activity was detected in the Main unit by empirically setting the threshold for burst-like activity at 40 msec interspike interval (ISI) and examining the characteristics of two or more consecutive spikes exhibiting more or more consecutive 40 msec ISIs.

4. Population phasic activity was operationally defined using the same criteria as phasic burst-like activity but applied to the Population spikes.

5. Contemporaneous activity between the Main and Background units was explored as additional proxy of phasic population activity. Main-Background contemporaneous activity was determined by expressing the instantaneous firing rate (calculated in 5-msec bins) of the Background units compared to the average instantaneous firing rate of the Background Units of the entire file in a 40 msec window centered on the firing of a Main unit to. Increases in Background unit activity before or after the Main unit firing suggest the degree to which one particular unit (Main) is tuned to the activity of the surrounding units (Background).

Spontaneous field potential analyses: Data was lowpass filtered at 300 Hertz and downsampled to 2500 Hz. Files were analyzed using custom Matlab scripts. Power spectra
were calculated using the Welch method with the following parameters: 2 second moving window, window overlap of 40%, and 5000 nFFTs. From these periodograms we calculated overall power (area under the curve), peak power, and the frequency of peak power. Autocorrelations were created for a subset of files, but not examined further as no differences were observed between treatments. Spectrograms were constructed to allow for visual exploration of the variations in power across time. Periodic power increases were identified by filtering the data from 1 to 5 Hz, identifying the mean power at one second intervals and plot the means across time. Mean power that was at least one standard deviation above the grand mean of the file was considered the onset of a periodic power event. Consecutive means one standard deviation or more above the grand mean were considered part of the same event. The frequency of these events was calculated as number of events in the file divided by the file length in seconds, or events per second.

Evoked spiking analysis: A Tungsten electrode was used to record evoked activity in the ventral subiculum after entorhinal cortex stimulation. An average of 31 ± 1.5 stimulations was performed for each tested stimulus intensity. Only files with evidence of monosynaptic responses were analyzed. Evidence of monosynaptic responses was both spike latency less than 9 msec after stimulus and increasing spike probability with increasing stimulus intensity. Traces were analyzed in Neuroscope. Waveforms of spikes were not large or detailed enough to be reliably identified as belonging to a single unit due to the low impedances of the electrodes. Therefore, the monosynaptic excitatory response in a given location was operationally determined as follows: At the maximal stimulus intensity, the modal spike probability across nine 1.0-msec bins following the stimulus artifact was required to exceed 0.5. An additional 1 msec was then added to the modal latency, creating a
2-msec latency window in which the probability of an evoked response (one or more spikes) was determined across a range of stimulation intensities (0.1 to 1.0 mA). Each trace was fitted to a sinusoidal curve following the Boltzmann equation using Prism 5. The estimated maximum value and estimated half-maximal value were calculated for each trace.

**Locomotion in response to ketamine in the MAM E17 mouse**

Locomotion to a novel open field was assessed in Plexiglas activity chambers (model ENV-520, Med Associates, Inc., www.med-associates.com). Chambers were 17x17 inch square enclosures equipped with infrared detectors to track horizontal and vertical animal movement. Measure of total horizontal distance covered during locomotion was used as the index of activity. After two bouts of handling in the days leading up to the experiment, individual mice from MAM E17 or control treatment groups (n = 12) were placed in the center of the chamber and allowed to explore freely for 30 minutes. Mice were then injected i.p. with 2.5 mg/kg ketamine dissolved in isotonic saline (injected at 10 mg/kg) and returned to the chamber. Post-drug activity was recorded for 60 minutes. On a subsequent day, a subset of the animals (n = 8) was retested for response to vehicle injection. Data were analyzed using a mixed ANOVA with drug and treatment as the between subjects factors.

**Cerebral blood volume measurements**

Magnetic resonance imaging on two sets of mice was performed as detailed in the “anatomy” section. The parameters used on the two sets were slightly different. The second set is described above. The first set utilized previously a published protocol (Moreno et al.
2007) that differed from the second in the following ways: Axial T2-weighted images were acquired with repetition time of 2000 msec and time to echo 80 msec. Eight slices were acquired with a acquisition matrix of 256 x 256; field of view 20 mm; slice thickness, 0.6 mm; slice gap, 0.1 mm). Voxel size was 0.078 mm x 0.078 mm x 0.6 mm (0.0037 mm$^3$) with an in-plane resolution of 0.0061 mm$^2$. One pre-contrast image was collected and six post-contrast images were collected.

Relative cerebral blood volume (CBV) was mapped as the average change in the region of interest of the transverse relaxation rate induced by the contrast agent. To control for differences in levels of contrast administration, cardiac output, and global blood flow, the derived raw CBV values were normalized to the maximum 4-pixel signal value of the posterior cerebral vein. Regions of interest were dentate gyrus, CA3, CA1, subiculum, and entorhinal cortex. All regions were examined for basal metabolic rate. The second set of animals was assayed for both baseline and ketamine-evoked CBV. In this set, ketamine (30 mg/kg diluted in normal saline) was injected via the catheter after three post-contrast images were acquired and four more image sets were acquired.

A mixed analysis of variance was done using regions of interest as the within subjects factor and treatment (MAM E17 or control), set number (1 or 2), and sex as between subjects factors. The only between subject factor of interest was treatment. Only the second set of animals received ketamine injections. For the effect of ketamine on CBV data, a repeated measures analysis of variance was done for each subregion with treatment as the between groups factor.
Chapter 3
Anatomical changes of the hippocampal formation in the MAM E17 rat model

Introduction

Reduced hippocampal volume is a highly replicated finding in schizophrenia (Shenton et al. 2001; Shenton et al. 2001; Wright et al. 2000). These volume changes are observed in pools of subjects at ultra-high risk for developing psychosis and may predict its eventual onset (Lawrie et al. 1999; Schobel et al. 2009a; Pantelis et al. 2003).

Previous studies of the MAM E17 model have reported reduced hippocampal size, as measured in vivo, in freshly dissected tissue, and in reduced thickness or area measurements postmortem (Moore et al. 2006; Featherstone et al. 2006; Matricon et al. 2010; Flagstad et al. 2004; Sanderson et al. n.d.).

We performed estimates of the right hippocampal volume in MAM E17 rats from our laboratory conditions and then went on to focus the investigation to the ventral hippocampal region since our neurophysiological and functional studies (see Chapters 5-7) targeted the ventral hippocampal formation. We compared area measurements that included the ventral hippocampus in both Nissl- and Golgi- stained tissue, collected in both the horizontal and coronal planes (see Figure 3-1c for schematic). We complemented our hippocampal size studies in rat by inducing the MAM E17 model in mouse and examined the effect of prenatal MAM treatment on the size of the hippocampal formation in MAM
E17 mice. We examined horizontal sections of mouse brain obtained *in vivo* using magnetic resonance imaging.

**Results**

*Postmortem measurement of hippocampal size in the MAM E17 rat*. In MAM E17 rats, a reduction of whole hippocampal area from the right hippocampus was evident in horizontal sections (paired T-test, \( p < 0.05 \), \( n = 4 \) rats per group) (Figure 3-1a). This finding was replicated in a set of rats with sections in the horizontal plane taken only from ventral hippocampus. The area measurements from these sections spanned approximately 2.5 mm of the ventral hippocampus, and they were significantly reduced between treatments (repeated measures ANOVA, \( F(1,6) = 16.32 \), \( p = 0.007 \), \( n = 3 \)) (Figure 3-1b). A decrease in area when investigated in the coronal plane, however, was not evident (\( F(1,8) = 0.001 \), \( p = 0.98 \), \( n = 5 \)) (Figure 3-1c), despite these sections partially overlapping the region of interest contained in the horizontal sections (as shown in Figure 3-1d). Since we were particularly interested in the ventral subiculum, we investigated the laminar structure of this area more closely.
Figure 3-1.

**Volume and area of hippocampal formation.** a) Whole hippocampal volume of the right hippocampus was significantly decreased in MAM E17 animals ($p < 0.05$, $N = 4$ animals/group). b) Hippocampal area from horizontal sections showed a significant difference in area between groups ($p < 0.01$). c) Hippocampal area from coronal sections were unchanged between groups. d) Schematic of the volume of tissue contained in the horizontal slices (green band, representing Figure 3-1b) and the coronal slices (blue band, representing Figure 3-1c).

In a separate set of brains processed with the Golgi process instead of perfused with paraformaldehyde and Nissl-stained, we calculated the thickness of the molecular layer of the ventral subiculum, contained between the pyramidal cell layer and the pial surface adjacent to the dentate gyrus. We hypothesized that this method would capture changes in area/volume driven by the size of dendritic arbors of pyramidal neurons and/or changes in lamination of the subiculum, but not the length of the hippocampus. Moreover decreases in cortical (interlaminar) thickness in paralimbic cortical regions have been reported previously (Moore et al. 2006; Gourevitch et al. 2004). In this sample of the ventral subiculum, however, no difference in the thickness of the molecular layer was evident (control animals...
In vivo measurements of hippocampal size in the MAM E17 mouse. In MAM E17 mice, six horizontal sections containing the hippocampus were examined in the same area as examined in rats: dentate gyrus, CA3, CA1, and subiculum (Figure 3-2). The slices spanned the majority of the hippocampal formation, from approximately Bregma -3.24 to -6.84. While a mixed ANOVA did not show a significant overall effect of treatment (p = 0.203, n = 5), there was a nearly significant decrease in MAM E17 mice in the dorsal-most section (p = 0.052).

Discussion

We found in the MAM E17 rat that the volume of the hippocampus was significantly reduced. This finding was in line with the MAM E17 literature, specifically decreased weight of the post mortem hippocampal formation (Flagstad et al. 2004; Featherstone et al. 2006) and decreased in vivo size (Chin et al. 2010) in MAM E17 animals.
When the cross-sectional area of the ventral hippocampal formation was specifically measured, area in the horizontal plane was significantly reduced compared to controls. Surprisingly, two measurements taken in the coronal plane did not reveal differences between groups: hippocampal formation cross-sectional area in the coronal plane of a mid-to-rostral sample of the region did not uncover group differences and measurements of the height of the molecular layer showed no reductions in the MAM E17 rats. In the MAM E17 mice imaged in vivo, we sampled horizontal area of nearly the entire hippocampal formation and found evidence for decreased hippocampal size only in the dorsal-most slice.

There are a few caveats to consider in interpreting the reduced hippocampal area and volume in the MAM E17 animals. In the whole hippocampal volume study, the N is low, only 4 animals per group and each group was comprised of only one litter. Thus litter effects cannot be ruled out. However, the direction of the effect was the same as was seen in the area measurements of the ventral hippocampal formation. In that study, at least three litters were represented per treatment. In both cases, the observations of significant treatment effects in small samples suggest that the overall treatment effect should hold when the cell size is increased.

Taken as a set, the studies in rat were performed either in Nissl body-stained sections or Golgi-impregnated sections from either the horizontal (Nissl) or coronal (Golgi) plane. The positive results in the Nissl/horizontal experiments contrast with the negative results in the Golgi/coronal experiments. This discrepancy between findings and stain could be due to differences in tissue processing or the plane or section, or an interaction between the two. For example, the observed results could be explained if Golgi processing induced more shrinkage in control tissue than MAM E17 tissue, or if Nissl processing disproportionately
caused shrinkage in MAM E17-exposed brains. A differential effect of Golgi on the population of neurons that were impregnated is possible (see below), but its impact on dorsoventral height of the molecular layer is unlikely since relatively few filled neurons are necessary to determine the borders of the region of interest. Only if Golgi incompletely filled the distal dendrites in a systematically biased way based on treatment would the heights of the region be affected.

Since the discrepancy between findings and stain is not very likely, the different groups between the two sets of experiments could have been due to the plane of sectioning. Furthermore, the results could be interpreted to shed light on the nature of the size reduction. The overall size of the hippocampal formation was observed to be decreased in MAM E17 rats, and a thorough sampling of the tissue should reveal results no matter the slice orientation. The dissociation between results in the two planes suggests that the volume change does not occur equally in all dimensions. Specifically, the transverse axis of the ventral hippocampal formation is fully captured in the horizontal but not coronal sections. The plane both methods had in common was the mediolateral plane. Changes occurring in the mediolateral extent of the hippocampal slices, if present, must have been small enough that they did not impact coronal area measurements. The plane captured uniquely, though incompletely, by the set coronal sections was the dorsoventral plane, which contains much of the longitudinal axis of the hippocampus. From these data we can conclude that there were minimal changes in the dorsoventral extent of the caudal hippocampal formation. These data do not inform whether there is an overall shortening of the longitudinal axis occurred. Specifically, either the septal pole or temporal pole, or both, may not extend as far rostrally in MAM E17 animals as in controls. If this were the case, it would contribute to the
finding of decreased overall hippocampal volume. It is also possible, given these results in rat, that there is a preferential effect of volume loss in the ventral but not dorsal hippocampal formation. This would help explain why coronal sections containing both parts of the hippocampal formation did not show changes while horizontal sections containing only the ventral hippocampal formation did show a change.

In the MAM E17 mouse study of horizontal sections of the hippocampal formation, technical limitations arising from the image acquisition protocol prevented a finer interslice resolution. This method represents a more complete reconstruction compared to the rat sampling areas, but the inter-slice resolution of 0.7 mm is not as good. At this interslice resolution, the dorsal and ventral extremes of the hippocampus were often missing in the slices. The study was further limited by small dorsoventral differences between subjects depending on how the images were taken. Thus one subject’s sampling could start more dorsal than another’s, and this would impact the measurement of the dorsal hippocampal formation in particular because of the rapid area change in this region. Finally, while the in-plane resolution of less than 100 square microns was more than sufficient to see changes in our region of interest which was on the order of hundreds of square millimeters, the accuracy of the contours was still limited by the level of contrast on the images that distinguish regions of high blood volume from low. The above issues can be partially addressed by increasing the N (i.e. the cell size) of the sample. This project is ongoing and will utilize the animals from the original imaging protocol. A thorough histological examination of the tissue will be performed postmortem on the animals scanned in the second set. Despite these limitations, this study holds value as the first examination, to our
knowledge, of the effects of E17 MAM treatment in mice. Thus this initial evidence provides a rationale for continuing to investigate the model in this species.

Further studies might specifically investigate the thickness of the ventral subicular molecular layer in the horizontal plane, corresponding to the orientation of the majority of the pyramidal cells. These experiments would allow for determination of which axis or axes of the hippocampus were impacted by MAM E17 treatment and which were not. Further examination of the set of sections used for whole hippocampal volume studies could reveal the presence of a foreshortened septal or temporal pole. It is quite possible, given the above results, that an overall but non-uniform shrinkage of the MAM E17 rat brain will be observed. This finding would be consistent with some studies of schizophrenia showing a more marked volume loss in the anterior than posterior pole (Narr et al. 2004; Szeszko et al. 2003) and of showing shape changes along the longitudinal axis (Csernansky et al. 1998).

Conclusions

Reduced area in the horizontal plane across much of the ventral extent of the hippocampal formation in MAM E17 rats and in the dorsal extreme of the MAM E17 mouse animals is evidence that the model was reliably induced after in utero exposure to 25 mg/kg MAM. The identification of hippocampal size decreases in the horizontal plane is consistent with volume changes observed in the whole structure in other studies but is the first study to use slicing at this orientation. The possible dissociation of findings between the horizontal and coronal planes suggests that hippocampal volumetric changes may be directional. Quantification of volume and inter-laminar thickness of the entire MAM E17 hippocampus in both rat and mouse is necessary to fully understand which dimensions of the hippocampus
are most impacted and if these changes are consistent with three dimensional changes in the hippocampus in schizophrenia patients.
Chapter 4
Changes in dendrite morphology in the ventral subiculum of the MAM E17 rat model

Introduction

A number of studies have examined neuronal morphology within the hippocampal formation in schizophrenia. In schizophrenia patients, a prominent reduction in dendritic spine density has been demonstrated in the apical dendrites of the subiculum (Rosoklija et al. 2000), and there is evidence of decreased somal size in the subiculum (Arnold et al. 1995). Furthermore, protein and gene expression studies point to changes that could relate to structural remodeling in the subiculum (Eastwood et al. 1995; Law et al. 2004; Arnold et al. 1991).

A study in the MAM E17 model investigating cellular organization of the dorsal and ventral hippocampal formation found localized areas of aberrant pyramidal cell organization and orientation (Gourevitch et al. 2004) in CA1/subiculum. In the ventral but not dorsal subiculum, moreover, there is reduced parvalbumin expression in MAM E17 animals (Lodge et al. 2009). Together, these data point to cellular-level alterations in the ventral hippocampal formation of the MAM E17 model.

To assess whether the MAM E17 model exhibited cell morphological changes relevant to schizophrenia, we investigated the dendritic spine density and dendritic branching in the ventral subiculum in MAM E17 rats.
Results

We investigated both spine density and dendritic branching using two different methods. The first method utilized reconstructions of the apical and basilar dendrites on subicular pyramidal cells (Figure 4-1). In the second method, dendritic spine density was assessed at the population level. Focusing first on the apical dendrites in the single neuron reconstructions, we examined spine counts in 10 micron bins from the half-maximal point of spine counts to 280 microns from the soma. MAM E17 animals show significantly decreased spine counts in this span (repeated measures ANOVA on bins from 70 to 280 microns, between groups factor $F = 10.74$, $p = 0.007$, $n = 8$ animals per group) (Figure 4-2a). In the same reconstructed neurons, dendritic branching was measured using a Sholl analysis comprising 10 micron concentric rings. MAM E17 treated animals exhibited significantly reduced dendritic branching from the half-maximal (50 micron) to 280 micron span (repeated measures ANOVA on bins from 50 to 280, between groups factor $p = 0.024$, $n = 8$ animals per group) (Figure 4-2b).

Next we examined basilar dendrites for treatment differences. There was no significant effect of treatment, though there direction of the effect was consistent with the findings in the apical dendrites (repeated measures ANOVA, between groups factor $F = 3.06$, $p = 0.108$, $n = 6$ or more animals per group).
Figure 4-1
Ventral subicular neurons filled with Golgi. Scale bar is 100 microns in a; 10 microns in b.

Figure 4-2
Spine counts and intersection counts in ventral subiculum in single neuron reconstructions (A-C) and spine population measurements (D). a) Spine counts on reconstructed apical dendrites from 10 to 280 microns. The half-maximal value was achieved at 70 microns from the soma. There was a significant effect of treatment. b) Dendritic intersections on reconstructed apical dendrites from 10 to 280 microns from the soma. The half-maximal value was achieved 50 microns from the soma. There was a significant treatment effect. c) No differences between treatment groups in the spine number on reconstructed basilar dendrites. d) When spine density was measured in different layers of the subiculum, no treatment differences were observed.

The coronal slice orientation used in the neuronal reconstructions impacted how much of the dendritic tree was preserved for each neuron. Since the orientation of the dendritic tree of ventral subicular cells is more in the rostrocaudal plane than the
dorsoventral plane, many neurons were truncated. To circumvent this artifact of tissue preparation, as well as to minimize any selection bias in the process of reconstructing neurons, we devised a method to sample subicular spine density based on population measurements. In this second method of dendritic spines investigation, we utilized pseudorandom sampling of dendrite segments in three different layers of the subiculum: the pyramidal cell layer, the inner molecular layer, and the outer molecular layer. Dendrite segments (10-15 microns) were identified in each layer and the spines marked. No differences between treatment groups were identified using this method (Figure 4-2d). In the pyramidal cell layer, containing the pyramidal cells and their basilar dendrites, the independent samples t-test revealed a p value of 0.165 (n = 11 animals per group); in the inner molecular layer, containing the proximal apical dendrites, the p value was 0.279 (n = 8 or more animals per group); and in the outer molecular layer, containing the distal apical dendrites, the p value was 0.887 (n = 10 or more animals per group).

**Discussion**

In single neuron reconstructions, there was a significant deficit in apical dendritic spine counts of ventral subicular cells in the MAM E17 animal accompanied by a significant decrease in dendritic branching. There was no evidence of a treatment effect in the basilar dendrite spine counts, though the graph in Figure 4-2c suggests that, if anything, the MAM E17 basilar dendrites had fewer spines. Despite these results, an unbiased population sampling of spines did not show evidence of a treatment difference in any layer.
These studies were performed on Golgi-impregnated tissue in which, as noted above, no gross morphological differences in the hippocampus have yet been observed. Since Golgi method are poorly understood (Rosoklija et al. 2003), it is possible that the uptake staining of MAM E17 animals was biased in some way compared to controls. For example, staining could preferentially occur in a subpopulation of MAM E17 neurons that is distinguished by a factor that both increases the probability of being stained and that displays morphological changes that are not representative of the neuronal population as a whole. This cannot be ruled out. Additionally, Golgi could impregnate MAM E17 neurons incompletely compared to control neurons, and this could result in fewer spines being visible. Our selection criteria, however, specifically avoided neurons that appeared to be incompletely impregnated with the Golgi method or that were substantially aspiny, a population of cells that a rater blind to treatment group found was more numerous in the MAM E17 group. By avoiding these cells, we biased ourselves away from under-counting spines in MAM E17 animals.

One limitation of these experiments could arise from including animals at two different ages, P45 and P90. However, where we observed effects of prenatal MAM, they did not interact with age. Studies investigating developmental models of schizophrenia other than the MAM E17 model have found changes in CA1 decreases in spine density (Zehr et al. 2008) or dendritic branch (Baharnoori et al. 2009) over an age span that included P45. Both studies focused on the dorsal hippocampus, and neither study investigated the subiculum. Thus it is unclear if and to what extent age-related post-adolescent spine changes might occur in the ventral subiculum.

The single neuron reconstruction studies and population spine quantification employed different methods, each with their strengths and weaknesses. Single neuron
reconstructions allowed for more precise characterization of spines and dendrites but included relatively few neurons. Furthermore, the use of coronal slicing in the experiment could have impacted the selection of neurons to only those whose dendrites projected out of the horizontal plane of the hippocampal formation. In contrast, the population sampling methods employed here does not rely on intact dendritic trees, however it may be not be sensitive enough to changes that occur in only a subpopulation of neurons.

There are at least two explanations to reconcile the finding of decreased spine density in MAM E17 animals found in the single neuron reconstruction studies with the null finding in the population studies. The first is that one or the other method is not sound, and it resulted in over- or under-counting of spines in one treatment group. We believe the regions sampled for the population studies were equivalent between groups because there was no treatment difference in the size of the contour or in the number of dendritic segments analyzed. Moreover, our neuron selection criteria for single neuron reconstructions, which excluded cells with aspiny or ‘sickly’ dendrites, were biased away from under-counting spines and dendrites in the MAM E17 animals. Furthermore, an analysis of placement of the reconstructed cells on the deep-to-superficial axis of the cell layer as well as on the medial-lateral axis spanning the subiculum through the pre-subiculum revealed no differences between the groups. Thus, we believe that the results from both methods reflect true spine counts in the ventral subiculum. The second and more plausible explanation for the discrepancy between spine densities using the two methods arises from the coronal sectioning scheme. The choice of neurons for reconstruction was limited to neurons whose dendritic tree extended a considerable distance into the molecular layer. Since most neurons’ dendritic trees were cut during tissue preparation, only the subset of neurons with cell bodies
in the pyramidal layer was available for possible reconstruction. The available neurons represent a population whose dendritic trees extend out of the rostrocaudal plane that most dendritic trees occupy. Thus the neurons chosen represent a non-uniform sample of all subicular neurons. Among this specific subset of neurons, there is evidence for a deficit of spines in MAM E17 animals. The contribution these neurons make to the overall regional counts of spines, though, appears to be too small to affect the population measure of spine density. For that reason, there is not an overall spine deficit in MAM E17 neurons when investigated at a population level. Increasing the number of brains cut coronally that are available for single neuron reconstruction would strengthen the conclusion of spine density changes on a subset of neurons. Preparing brains cut on the horizontal plane would be the preferred way to investigate single neuron reconstructions, however, to determine the extent of the neuronal population affected by spine deficits.

The observed spine deficit in MAM E17 animals could be mediated through effects on specific genes related to spinogenesis during development and/or plasticity. In the first case, the deficit would be established early in life and observed as a constant deficit throughout the animal’s life. In the second case, the spine deficit could emerge at a later age with dysregulation of mechanisms of plasticity would become apparent with maturation of the circuit, perhaps before puberty. The two scenarios could also interact by, for example, a small deficit of early postnatal spinogenesis growing more apparent with the emergence of later plasticity-driven spine alterations. These hypotheses on the timing of the emergence of the deficit could be tested by examining differences in spine density at earlier postnatal time points.
Regardless of the developmental trajectory of the spine loss, the functional impact would likely be decreased glutamatergic input on a subset of neurons in the subiculum, particularly on the proximal apical dendrites. This region receives CA1 input, as do the basilar dendrites. The evidence presented here is not conclusive about changes in the outer molecular layer since single neuron reconstructions did not extend to the distal apical dendrites. The population study, however, did not find evidence for treatment differences in this region.

The spine loss in the MAM E17 treated animals is in line with evidence of spine loss in the subiculum of schizophrenia patients (Rosoklija et al. 2000). The sectioning schema for human study was coronal, the orientation in the human in which the dendritic tree of subicular pyramidal cells is preserved. In a subsequent study by this group in which spines were quantified as a function of distance from the soma, the deficit seemed to be more pronounced in the region of the apical dendritic arbor residing in the inner molecular layer (about 100-200 microns from the soma) (A. Dwork and G. Rosoklija, personal communication). These results are consistent with our findings in the MAM E17 animal, with the caveat that the distal dendritic tree of the subiculum remains underexplored.

**Conclusions**

Spine deficits observed in the MAM E17 model in a subpopulation of ventral subiculum occur with changes in dendritic branching. These morphological changes are likely restricted to a subpopulation of the neurons, and the subpopulation may be small enough that the changes are not detected in studies of the entire population. The MAM E17 spine loss on
pyramidal cell proximal apical dendrites in subiculum suggests that these neurons will have a loss of functional input from CA1. The findings reported here support the utility of the MAM E17 model for studying hippocampal anatomical changes relevant to schizophrenia. Moreover, the findings provide a basis for targeting potential functional alterations in the model.
Chapter 5
Evoked activity in ventral subiculae neurons in the MAM E17 rat model

Introduction

Several experimental approaches have been used to provide evidence of deficits in glutamatergic transmission in schizophrenia, including altered glutamate receptor subunit composition and expression in the hippocampus as well as altered behavioral effects of NMDA-antagonists (see Chapter 1: General Introduction). Decreased expression of parvalbumin-positive interneurons in the medial temporal lobe is associated with schizophrenia (Zhang & Reynolds 2002); these interneurons potently modulate glutamatergic signaling (Freund & Buzsaki 1996). In addition, functional magnetic resonance imaging studies point to hypoactivation in tasks that recruit the hippocampus (Liddle et al. 1992), and the degree of hippocampal activation has been correlated with hallucinations (Silbersweig et al. 1995).

The MAM E17 model exhibits changes that may be indicative of alterations in glutamatergic neurotransmission. Administration of the non-competitive NMDA antagonist MK-801 leads to hyperlocomotion and neurochemical alterations compared to control animals (Le Pen et al. 2006; Lena et al. 2007). Anatomical evidence as detailed in the previous chapter points to subtle changes in spine density in the ventral subiculum of the MAM E17 model which would lead to decreased glutamatergic input on these neurons. Decreased parvalbumin immunoactivity in the MAM E17 ventral hippocampus (Penschuck
et al. 2006; Lodge et al. 2009) could further dysregulate glutamatergic transmission. Thus a direct assessment of glutamatergic transmission in the hippocampus was needed. We examined synaptically evoked excitatory responses in the ventral subiculum and ventral CA1 in a slice preparation, characterizing an index of signal propagation (fiber volleys, FV) and a measure of the population excitatory postsynaptic response to afferent stimulation.

Specifically, we performed extracellular recordings in vitro in slices of tissue containing the ventral hippocampal formation of MAM E17 or control animals to examine the relationship between evoked inputs (FV) and the subsequent population responses (field excitatory postsynaptic potentials, fEPSP) in ventral subiculum and ventral CA1. Inputs preferentially targeting the either the proximal or distal apical dendrites were examined; the evoked responses in these targeted areas were examined separately. Specifically, recording positions in CA1 were in stratum lucidum (SLu) to measure the response to Schaffer collateral stimulation and in stratum lacunosum-molecular (SLM) to measure the response to perforant path stimulation. In a similar pattern, recordings in subiculum were performed in the inner portion of the molecular layer (IML) to measure the response to CA1 stimulation and in the outer portion of the molecular layer (OML) to measure the response to perforant path stimulation. In a separate set of experiments, we examined evoked responses in vivo to better understand the behavior of the MAM E17 intact circuit.

We hypothesized that MAM E17 ventral hippocampal formation, and specifically the subiculum, would exhibit changes in signal propagation (as seen by alterations of the FV), the excitatory response (as seen by changes in the fEPSP), or both, in response to afferent stimulation.
Results

**Determination of FV and fEPSP.** Most of experiments described in this section were performed in artificial cerebrospinal fluid (ACSF) without any drugs added to the bath. To understand the components that the evoked responses contained, however, we performed a subset of experiments in ACSF with various pharmacological manipulations. Without any drugs added to the ACSF, the responses were similar to the averaged trace shown in Figure 5-1a recorded from subiculum IML after CA1 alveus stimulation. We then added CNQX, AP5 and Gabazine to block AMPA, NMDA, and GABA_A receptors, respectively, to block post-synaptic responses dependent on these receptors. This manipulation resulted in a trace similar to the trace shown in Figure 5-1b: the slower component of the response, occurring approximately 5 msec after stimulus onset, was abolished. When we then added TTX to block voltage-gated sodium channels which propagate the action potential volley along the afferent fibers, the remaining fast-onset component, the presumed FV, disappeared. The remaining signal, shown in Figure 5-1c contained only the stimulus artifact, denoted by the arrow in Figure 5-1a, which was reversed by altering the polarity of the current. The component that met standard criteria for an EPSP (e.g. polarity, latency) and was abolished by CNQX, AP5, and Gabazine and thus dependent on post-synaptic responses was determined to be the fEPSP. The component that was insensitive to CNQX, AP5, and Gabazine but sensitive to TTX, thus dependent on sodium channels, was determined to be the FV.
Pharmacology. a) Average of 5 responses to stimulation performed in ACSF. Arrow points to stimulus artifact. b) Average of 5 responses to stimulation performed when AMPA, GABA\_A, and GABA\_B receptors were blocked. The gray trace is a reproduction of the trace in a. The missing component of the black trace, occurring about 5 msec after stimulus onset, is considered the fEPSP. c) Average of 5 responses to stimulation when sodium channels as well as AMPA, GABA\_A, and GABA\_B receptors were blocked. The missing component in c compared to b, occurring about 3 msec after stimulus onset, is considered the fiber volley.

Perforant path inputs to the subiculum and CA1. Because the perforant path that crosses the distal dendritic fields of subiculum and CA1 is composed of fibers arising from layer III entorhinal cortex, we combined FV measurements taken from both regions. Using the average of natural log transformed FV amplitude (see Materials and Methods), we found that MAM-treated animals displayed reduced FV amplitude compared to controls (F(1,1,49) = 6.02, p < 0.05) (Figure 5-2a). When the regions were analyzed separately, the effect was in the same direction but was significant only in stratum lacunosum-moleculare of CA1 (F(1,1,21) = 4.7, p < 0.05).

Evoked synaptic responses to perforant path stimulation. Since we saw a decrease in FV amplitude, a measure of input strength, in MAM-treated animals compared to controls, we wanted to know if the net synaptic response, as measured in the field EPSP (fEPSP), was altered. We investigated the slope of the evoked field EPSP in the OML of subiculum and the stratum lacunosum-moleculare of CA1. The data showed region-specific deficits in MAM-treated animals. Namely, the response recorded in subiculum to perforant
path input was significantly decreased in MAM E17 animals while no differences were observed between groups in CA1 stratum lacunosum-moleculare (OML: F(1,1,1,23) = 4.3, p < 0.05, SLM: F(1,1,1,20) = 0.58, p > 0.4). The data are graphed in Figure 5-2b and c.

![Graph](image)

**Figure 5-2.**
**Pre- and post-synaptic responses in distal dendrites of subiculum and CA1.** a) Fiber volley amplitude of fibers originating from CA1 and recorded in subiculum outer molecular layer of subiculum or stratum lacunosum-moleculare of CA1 were significantly decreased in MAM E17 animals (p < 0.05). b) Evoked fEPSP slope in subicular distal dendrites was significantly decreased in MAM E17 animals (p < 0.05) but not in CA1 distal dendrites, as shown in c.

**Afferents to the proximal apical dendritic regions in subiculum and CA1.**

Having observed both pre- and post-synaptic deficits in MAM-treated animals in the distal dendrites of subiculum, we wondered if similar deficits would be found in the proximal dendrites of subiculum or CA1. The main innervation of proximal subicular dendrites is the CA1 axons whereas the main innervation of proximal CA1 dendrites in layer stratum lucidum is the Schaffer collaterals. Thus to investigate subicular inputs, we stimulated the CA1 alveus and examined the FV amplitude in the IML of subiculum. To investigate CA1 inputs, we stimulated Schaffer collaterals and examined the FV amplitude in stratum lucidum. Neither the subiculum nor CA1 recordings revealed differences between the
groups (subiculum: F(1,1,29) = 1.16, p > 0.29; CA1: F(1,1,18) = 1.3, p > 0.2). See Figure 5-3a and b.

Figure 5-3
Pre- and post-synaptic responses in proximal dendrites of subiculum and CA1. a,b) There were no differences between groups in the average fiber volley amplitude recorded in the inner molecular layer of subiculum (a) or the stratum lucidum of CA1 (b). c) The average evoked fEPSP slope recorded in the subiculum inner molecular layer was significantly decreased in the MAM-treated group (p < 0.05). d) The average evoked fEPSP slope recorded in CA1 stratum lucidum was not different between treatment groups.

Evoked synaptic responses in the proximal dendritic regions of subiculum and CA1. Despite no group differences in the apparent strength of inputs to the proximal dendrites of subiculum pyramidal cells, MAM E17 offspring showed a significant decrease in the slope of the evoked fEPSP (F(1,1,1,28) = 7.1, p < 0.05). No group differences were observed in CA1 proximal dendrites (F(1,1,1,17) = 1.6, p > 0.2). See Figure 5-3c and d for graphs.

Figure 5-4 summarizes the significant effects of prenatal MAM treatment by recording measurement and region. The FV in MAM-treated animals from entorhinal cortical fibers was weaker, and, in the subiculum, the fEPSP evoked by stimulation of those
fibers was also blunted. The response of subicular neurons to CA1 input was also affected. Specifically, while the strength (as measured by the FV) of the input from CA1 was not different, the evoked synaptic response at the proximal dendrites of subicular neurons was significantly smaller in amplitude. The synaptic responses of ventral CA1 did not appear to be different in MAM E17 and control rats.

![Diagram of synaptic connections]

**Figure 5-4**
Summary of findings of recordings in subiculum and CA1. Arrows represent significant decreases of MAM E17 measurements compared to control.

**Evoked responses in vivo.** Given the evidence of deficits in the perforant path, we decided to use *in vivo* recordings to investigate the intact entorhinal projections to the subiculum. In anesthetized animals, we stimulated the entorhinal cortex and recorded unit responses in the pyramidal layer of ventral subiculum. This experiment was designed to test the generation of a subiculum “output signal”, the action potential, in response to activation of its entorhinal inputs. After fitting the data points to a sigmoidal curve, we calculated the estimated half-maximum and maximum spike probability for each experiment (Figure 5-5). Despite this circuit showing deficits in the *in vitro* experiments, no differences between groups in the sensitivity or maximal response to stimulation of entorhinal input were observed *in vivo*. The average curves for each group are shown in Figure 5-6.
Evoked in vivo experiments. The entorhinal cortex was stimulated and recordings were taken in the ventral subiculum. a) Estimated half-maximum monosynaptic spike probability after entorhinal cortex stimulation. b) Estimated half-maximum spike probability.

Average fitted stimulus-response curves. Curves and the data points that they fit are plotted.

Discussion

The results obtained in this set of field potential recordings in the ventral subiculum and ventral CA1 show that E17 MAM treatment led to deficits in both FVs and fEPSPs. There were two main findings: a FV deficit and fEPSP deficits, both in MAM treated
animals. First, the FV amplitude in the outer molecular layer, thought to measure the depolarization of perforant path fibers originating from the entorhinal cortex, was decreased in MAM-treated animals. This deficit was observed when FV data from OMLs of the subiculum and CA1 were averaged. Since both the subiculum OML and the CA1 stratum lacunosum-molecular receive input from the same fibers, this analysis likely reflects the most valid and reliable result. By contrast, the FV amplitudes in terminal regions in subiculum and CA1 of axons originating, respectively, from CA1 (the alveus) or CA3 (Schaffer collaterals) were not affected. This suggests that MAM treatment does not cause universal changes in white matter track density or that it does not cause wide-spread deficits in the ability of these fibers to depolarize. Since tests of the Schaffer collateral inputs were less powered than the other inputs, more experiments would be necessary to strongly conclude that there were no deficits in this bundle in MAM-treated animals.

The second main finding was that evoked fEPSPs were altered selectively in the subiculum of MAM E17 rats. Subicular fEPSPs were significantly decreased in both the IML and OML and in the IML were independent of afferent strength changes. Thus, these changes may indicate an abnormality in subicular neurons that affects excitatory synaptic responses throughout the dendritic tree. Although the CA1 experiments may have been statistically under-powered, we note that synaptic responses in the CA1 did not differ between MAM E17 and control groups. This suggests regional heterogeneity and a preferential effect on subicular processing in MAM treated animals.

We presented two outcomes measures for each in vitro recording location. The FV measurement reflects the strength of the input available to the target structure while the fEPSP reflects the net effect of the release of glutamate and the ability of the post-synaptic
dendrite to respond. Taken together, the data suggest an abnormality in the density or excitability of axons derived from the entorhinal cortex and a subiculum-specific deficit in synaptic transmission that may be primarily mediated post-synaptically.

In contrast to the in vitro experiments, recordings performed in the intact preparation did not exhibit group differences. The in vivo electrode placements were qualitatively equivalent to those used in vitro: stimulating in the entorhinal cortex (directly in the pyramidal layer in vivo and via the output fibers of the perforant path in vitro) and recording in the ventral subiculum (the pyramidal cell layer in vivo and the dendritic layers in vitro).

While the methods employed here are advantageous for observing the behavior of the circuit under evoked conditions, there are a few drawbacks. In the case of both slice and intact preparation experiments, only summed population-level activity can be observed. The stimuli engage a relatively large bundle of axonal fibers or population of cells to propagate an electrical impulse that then excites a large number of dendrites arising from a population of cell bodies. How these cells as a population or individually would respond to a stimulus in the physiological range is uncertain. It is possible for a population-level investigation to not be sensitive to changes at the level of individual neurons, as was seen in the spine study presented in a previous chapter. This could be due in part to the specific subpopulation of neurons being investigated. The subpopulation studied here were those neurons lying on the same plane as the slicing was performed. We believe that the angle was such that a majority of connections were preserved (Witter & Amaral 2004) but without labeling and staining cells there remains uncertainty. As a further caveat, the cells targeted by the stimulus could be excitatory or inhibitory. The proportion of inhibitory connections is likely small, however. Reconstructions of entorhinal efferents on subicular neurons revealed the majority
of synapses were asymmetric and a minority (approximately 6%) was symmetric (Baks-te Bulte et al. 2005). Furthermore, a small but reliable GABA$_A$-mediated current was observed intracellularly in subicular cells in response to entorhinal cortical stimulation. Thus, both anatomical and physiological evidence point to a small inhibitory component of the projection from entorhinal cortex. In the present study, the inhibitory and excitatory components remain undistinguished.

Some cautions must be taken in interpreting the in vivo responses specifically. One limitation is the low number of animals per group. This low number arose partly from the desire to keep stimulation and recording sites within a restricted area and well-matched between groups due to the dorsoventral and mediolateral specificity in connectivity between the entorhinal cortex and subiculum (Andersen et al. 1971). Thus the results of a number of successful recording sessions could not be analyzed due to the lack of histological confirmation of the stimulus and recording locations. Other caveats arise from limitations of the recording method used. The choice of a Tungsten electrode allowed us to analyze fEPSP responses (data not shown) but not to confidently identify a unit response based on waveform. For that reason the analysis relied on latency of the responses, a method which has the potential to both over count the responses (in the case of two spikes occurring within the same time bin) and to under count the responses (in the case of a small jitter of a unitary response causing it to be binned in the non-counted bin). Furthermore, not all stimulation intensities were tested at every recording session. Consequently the data were analyzed by comparing equal spike probabilities to the stimulus intensity required, instead of the more traditional way of comparing the stimulus intensities to see the resultant spike probabilities.
Despite the regional similarities in stimulating and recording sites in the two preparations, the differences in the targeted neuron compartment could have contributed to the findings of group differences in vitro alongside no group differences in vitro. Specifically, in vitro stimulation targeted a bundle of axons while stimulation in vivo was targeted to the pyramidal layer. This resulted in a greater dependence in vivo on the precise location within both the entorhinal cortex and the subiculum because not all areas of the entorhinal cortex are equally connected to all areas of the subiculum, as detailed in the introduction. While some spread of stimulating current is assumed, the spread may not have been widespread enough to equalize small positional differences. Thus subtle differences in anatomical location could have a large effect on stimulation efficacy. The group sizes were likely not large enough to overcome the resultant statistical noise due to placement differences.

The findings of decreased perforant path input to subiculum and CA1 in MAM E17 animals can only obliquely be related to human findings. Diffusion tensor imaging can be applied in vivo to measure white matter integrity. Variations of the technique with the sub-millimeter resolution necessary to measure the perforant path were recently applied to schizophrenia patients and matched controls (Yassa et al. 2010). The study showed significantly decreased anisotropy but not volume measurements in the entorhinal cortex of patients, suggesting white matter but not gray matter changes in the disease. Postmortem evidence points to subtle and not always consistent cell morphological changes in the entorhinal cortex (Arnold et al. 1991; Arnold et al. 1997), which could contribute to the white matter deficits found by Yassa and colleagues.
The idea that the MAM E17 subiculum is disconnected from its afferents fits well with the current conceptions of the model and its relevance to schizophrenia (Grace 2000; Lodge & Grace 2011). It could be argued that, at least on a functional level, the potential attenuation of entorhinal cortical input observed in the slice preparation matters less than abnormalities in how the subicular cells translate the input into spike firing, since it is the spike firing that drives the downstream targets of the subiculum. In light of that, the trend for increased responsivity of the subiculum to entorhinal cortex could have a large effect on downstream targets. Ventral hippocampal activity is known to have a role in promoting dopamine spike firing (Floresco et al. 2001) and increasing dopamine content in the ventral striatum (Legault et al. 2000). Given how these results fit within a framework of functional implications on dopamine circuits modulated in schizophrenia (Laruelle et al. 2003), further experiments to investigate these conclusions are merited. Future studies will increase the number of animals and utilize glass electrodes to better distinguish unit firing in vivo.

Conclusions

Overall, the MAM E17 model in vitro shows an attenuation of synaptically evoked responses in the ventral subiculum. Perforant path input was decreased to subiculum and CA1. While these results were not replicated in vivo, further studies are needed to fully understand the behavior of the circuit in the intact preparation. These circuit-level changes expand our knowledge of the MAM E17 model and are relevant to theories of schizophrenia neuropathology related to intrinsic hippocampal activity and hippocampal regulation of other circuits implicated in the disease.
Chapter 6
Spontaneous activity in the ventral subiculum of the MAM E17 rat model

Introduction

Since schizophrenia is a chronic disease, it is reasonable to propose the presence of changes in basal brain functioning of the brain accompany the disease. The presence of resting state activity in various regions of the brain has been shown to be reliably correlated or anticorrelated with other regions in healthy individuals (Fox et al. 2005; Gusnard & Raichle 2001). This resting state network shows evidence of widespread dysconnectivity in schizophrenia (Liang et al. 2006). While studies investigating these regional changes based on magnetic resonance imaging are likely investigating a variable that relates to neural activity (Logothetis 2003), obtaining evidence of alterations on the level of the single neuron is not feasible. Understanding basal neuronal activity in regions of known pathology, though, may be important for understanding disease mechanisms.

The MAM E17 model exhibits a number of pathologies that are relevant to schizophrenia and point to deficits in the ventral hippocampal formation. Reduced spine counts in the ventral subiculum and the putative loss of glutamatergic input on these neurons could affect spontaneous spike firing, and alterations in evoked activity in the ventral subiculum could be indicative of changes of intrinsic properties of the cells that could impact spontaneous activity. While the slice preparation described in the previous chapter allows for fine control of recording and stimulating locations, the preparation does not inform on spontaneous activity generated by the intact circuit. This activity is the total of all
inputs to the subiculum, not just those inputs preserved in the slice. Furthermore, only population activity can be inferred in the slice preparation when the recordings are done extracellularly.

To better understand spontaneous spike firing patterns, we performed *in vivo* extracellular recordings and collected both unit (spike firing) and population (field potential) spontaneous activity.

**Results**

**Population unit activity.** Recordings were made in the ventral subiculum in the intact animal under isoflurane anesthesia. Care was taken to avoid recordings discovered post-mortem to be located in the presubiculum or CA1. Among these recordings, there was no difference in the instantaneous firing rate of populations of units in MAM E17 or control rats (48.7 ± 6.05 and 49.1 ± 7.74 Hertz, respectively; n = 21 files from 11 animals) (Figure 6-1a). To investigate the possibility that the waveforms were different between groups or that recordings in one group were performed at a different distance from the population activity, the average amplitude of the units was measured. No differences were revealed (0.044 ± 0.0014 and 0.043 ± 0.0018 mV for MAM E17 and control rats, respectively) (Figure 6-1d).
Figure 6-1

**Instantaneous firing rate of spontaneously firing cells in ventral subiculum.** All bar graphs display mean and standard error of the mean. Blue bars denote control animals, red bars denote MAM E17 animals. There were no significant differences between groups. a) Instantaneous firing rate (iFR) of population activity. b) iFR of Main unit. c) iFR of Background units. d) Amplitude of spikes from all units. e) Amplitude of spikes from the Main unit only. f) iFR of Main unit in MAM E17 animals injected with 25 mg/kg MAM and recorded with a glass electrode.

**Main and background activity.** It is possible that the instantaneous firing rate calculated based on all activity in a recording area could mask differences of the firing rate of individual units. Since electrode position for each recording was optimized to include what was judged a single unit, we were able to discriminate a unitary waveform using the spike sorting algorithm of Chronux. Other waveforms were discriminable but could not be clustered reliably. Clustering was done by a single investigator blind to treatment condition, and the resultant spikes are referred to as “Main unit” activity. Units that were discriminated but not clustered are referred to as “Background” units. Main and background unit instantaneous firing rate was compared between groups. Main units showed no evidence for differing firing rates between groups (Figure 6-1b): MAM E17 units fired at 2.4 ± 0.37 Hz and units in the control group fired at 2.9 ± 0.34 Hz (two-tailed t-test, unequal variances assumed, p = 0.321). The Background unit instantaneous firing rate was similarly unchanged.
between groups (46.4 ± 6.0 and 46.0 ± 7.7 Hz for MAM E17 and control, respectively) (Figure 6-1c). To confirm that Main unit waveforms did not differ between treatments, the amplitude of these waveforms were compared between groups. There was no difference between groups for the average amplitude of the Main units (0.13 ± 0.010 and 0.12 ± .009 mV for MAM and control, respectively) (Figure 6-1e).

**Phasic activity.** Despite not seeing differences between groups in the overall firing rates, we wondered if the treatments affected the patterning of this spike activity. We analyzed sets of spikes with interspike intervals of 40 msec or less between spikes. We asked if a greater proportion of spikes were contained in these phasic increases of activity and, furthermore, if the firing rate within these phasic increases was different between the groups. Examining the Main unit alone as well as all units combined from each file, we found that on average there was no treatment difference in the percent of total spikes of either category falling within the phasic activity increases. For Main units, the percent of spikes occurring within 40 msec of each other was 27 ± 4.4% for MAM and 31.7 ± 5.7% for control (n = 6 animals and 9 files). When all units were combined (Main and Background), the percent of spikes occurring within 40 msec of each other was 73 ± 5.0% for MAM and 75 ± 5.2% for control (n = 6 animals and 10 files) (Figure 6-2a). There were no differences between treatments in the rate of spiking within these increases (Main unit average instantaneous firing rate, MAM and control: 68 ± 5.5 and 72 ± 12.0 Hz; all units, MAM and control: 98 ± 9.8 and 90 ± 8.8 Hz) (Figure 6-2b).
Main and background spike patterning. Changes in how the Main and Background units fire together could happen independently from changes in the firing rates of the Main and Background units alone, and this change would not be captured by the phasic activity measurements described above. We analyzed the recordings to examine where the Background spikes occurred in relation to the Main spikes. By calculating how numerous the background spikes would be in 5 msec bins if the spikes were distributed evenly in the file, we compared the expected value to the actual number of spikes in a 40 msec window centered on each Main spike (Figure 6-2c). Repeated measures ANOVA revealed that treatment had no significant effect on the fold change of background spikes from the expected value ($p = 0.361$, $n = 9$ animals, 21 files).

Chloral hydrate experiments. Since all of these recordings were performed under isoflurane anesthesia, it was possible that a differential effect of the anesthesia between groups could have masked differences between groups in firing rate. Since a previous study utilized chloral hydrate anesthesia for a similar investigation of MAM E17 rats and reported an increase in spontaneous firing rates in the MAM E17 animals in a broader but
overlapping region of interest, we investigated whether our choice of isoflurane anesthesia was masking differences between groups. Using the same methods as were used in the experiments performed under isoflurane anesthesia, we recorded extracellularly in the ventral subiculum of MAM E17 and control rats under chloral hydrate anesthesia. We examined the instantaneous firing rate of the Main unit and of the combined Main and Background unit activity, and we found no differences between treatment groups (Main units, MAM and control: 14.2 ± 2.65 and 21.6 ± 2.43 Hz, n = 3, p = .148; all units, MAM E17 and control: 63.4 ± 14.43 and 72.8 ± 21.78 Hz, n = 3, p = .740) (Figure 6-3a and b). We alternated the chloral hydrate experiments with isoflurane experiments and compared the firing rates of these interleaved experiments. We found that the rate of all spikes was not different between the two anesthetics for either treatment (p = .687 and .950 for MAM and control, respectively) (Figure 6-3a). Despite the population activity being the same rate between the anesthetics, the rate of the Main unit firing was significantly lower under isoflurane anesthesia compared to chloral hydrate for both treatment groups (p = .009 and .007 for MAM and control, respectively) (Figure 6-3b).

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**Figure 6-3**

*Instantaneous firing rate (iFR) of spontaneously firing cells in ventral subiculum in animals anesthetized with chloral hydrate.* No significant differences were observed between groups. a) iFR of Main units. b) iFR of all units.
Glass electrode recordings. Since the results obtained in the set of animals dedicated to in vivo recordings were from model animals generated with 22 mg/kg MAM dosing and the results from the in vitro recordings were from 25 mg/kg dosing, we wanted to confirm that the small increase in dosage wasn’t the reason for seeing differences in one type of electrophysiological recording and not the other. We also wanted to investigate spontaneous firing with a glass electrode instead of Tungsten in order to directly compare these results to other experiments. Thus in a small set of animals we set out to understand if our recording electrode and model induction were affecting the spontaneous firing rate. Since only minimal background activity was recorded from the glass electrodes, we did not use the clustering algorithm in Chronux but instead identified the comparable Main unit activity using thresholds of spike amplitude and width using Neuroscope. We found no difference in instantaneous firing rate between MAM and control animals in these experiments (MAM and control: 3.7 ± 1.01 and 3.9 ± 1.12 Hz, n = 3 animals and 7 recordings, p = 0.91) (Figure 6-1f). Indeed, the unit activity recorded with the glass electrodes was not significantly different than that recorded with the Tungsten electrodes (compare Figure 6-1f to Figure 6-1b).

Local field potentials. Since changes in MRI signal in schizophrenia could relate to local field potential changes (Logothetis 2003), we were interested in whether the MAM E17 model showed changes in LFPs. In the same recordings from which we extracted spiking data, we also extracted LFP data. We analyzed the LFPs for the power of frequencies in the files and for occurrences of phasic increases in power.

Welch periodograms were performed on all files to examine the distribution of power across the frequency spectrum. No differences were found in the maximum power
achieved or the total power of the signal from 1 to 100 Hz were observed (Figure 6-4a). We examined spectrograms to look for changes in the signal across time, and regular periods of increased power across all frequency bands from 1 to 200 Hz were observed. Since the greatest power in the files was observed in the lowest frequency bands (Bédard et al. 2006), we filtered the signal from 1 to 4 Hz to examine the frequency of these transient increases in power (Figure 6-4b). We found no differences between treatment groups in the frequency of the transient power increases (Figure 6-4c), nor a difference in the cutoff frequency used to find the power increases (Figure 6-4d).

**Discussion**

These results examining the spontaneous firing rates of putative single units as well as population activity strongly point to the conclusion that the MAM E17 model rats have no differences in their ventral subiculum spontaneous firing rate under anesthesia. This finding
was observed with two different types of anesthesia, two different types of recording electrodes, two different doses of MAM induction, and multiple types of spike analyses. A shortcoming of extracellular recordings investigating spontaneous activity is that cells must be active in order to be analyzed. This leaves open the possibility that the kind of cells spontaneously active in one group of animals is not the same as the kind of cell spontaneously active in the other group. Though we never noted a difference in difficulty in finding active cells, we can’t discount the possibility that we were sampling from different populations.

All of these experiments were carried out in anesthetized animals, thus confounds of anesthesia cannot be ruled out. Since more than one anesthesia was used, though, it is unlikely that our choice of anesthesia had an effect on the experiments. Rather, the use of an anesthetized preparation instead of an awake preparation could mask differences in the neurophysiology of MAM E17 animals compared to controls.

The results reported here are in contrast to the only other investigation of unit activity in the hippocampus of the MAM E17 model. In a 2007 report by Lodge and Grace (Lodge & Grace 2007) an examination of ventral hippocampal extracellular activity identified significantly increased spontaneous single unit firing in the anesthetized MAM E17 animal compared to control. The discrepant results between these studies could be due to the more specific recording target in the present experiments (ventral subiculum only, while in Lodge and Grace 2007 the ventral subiculum as well as the ventral CA1 were included). Furthermore, their experiments utilized a very small set of animals (n of 4 animals). Thus compared to those findings, the experiments presented here represented a more focal recording area and a larger group of animals.
This lack of difference in spontaneous spike activity occurs despite small changes in dendritic morphology and alterations in excitatory synaptic transmission in major glutamatergic pathways to the subiculum in the MAM E17 rat. The changes in dendritic spine density and synaptic responses were consistent with a possible decrease in ‘activation threshold’ for subicular neurons, but a decreased capacity to follow moderate-strong input from CA1 or entorhinal cortex. A lack of difference in spontaneous spike activity under anesthesia may also be consistent with this picture. Under these conditions, we would expect afferent activity to be low.

Future experiments could investigate the intracellular properties of ventral subicular cells. Examining the cells’ responses to excitatory and inhibitory current injection would generate hypotheses about how the individual cells respond within the network. Experiments performed intracellularly in the prefrontal cortex of MAM E17 animals using sharp electrodes revealed a more depolarized resting membrane potential in these cells (Moore et al. 2006). Similar changes in MAM E17 ventral hippocampal cells could affect the ability of the subiculum to propagate its inputs. Intracellular recordings would have the benefit of being unmistakably from a single cell, in contrast to the extracellular results reported here. Furthermore they would have less potential sampling bias as extracellular spontaneous activity as cells don’t have to be spontaneously active in order to be recorded. Filling of the cell with biocytin after the recording would allow for later morphological examination of branching and spine patterns. This could be useful data in light of the anatomical findings reported above. Filling the cell would also inform on its location within the pyramidal layer, both on the deep-to-superficial axis and on the proximodistal axis, as both directions have been associated with the presence of bursting or regular-spiking induced firing patterns.
(Staff et al. 2000; Jarsky et al. 2008; Greene & Totterdell 1997; Kim & Spruston 2011b). The role these kinds of cells play in the subiculum network properties remains poorly understood (Menendez de la Prida 2006).

Direct comparisons of these results with the schizophrenia literature are difficult. MRI experiments putatively examine neural activity, but the activity recorded in the BOLD signal and other related signals such as CBV is probably not directly related to synaptic activity but likely local field potentials (LFPs), at least in evoked MRI activity (Logothetis 2003; Viswanathan & Freeman 2007). Thus whether spontaneous spiking activity or spontaneous LFPs are affected in schizophrenia is unknown. The findings in the MAM E17 model, however, suggest that the existing phenotype in the model do not support changes in spontaneous subicular neuronal activity in schizophrenia.

Conclusions

The results presented here suggest that differences in spontaneous spiking and field potential activity do not exist in the ventral subiculum of MAM E17 animals recorded under anesthesia. The finding of normal spontaneous neural activity in ventral subiculum of the MAM E17 model helps to isolate the finding of alterations in evoked neuronal activity in the absence of resting activity as presented in the previous chapter. Whether changes in spontaneous single unit activity exist in schizophrenia is unknown. The lack of results in the present experiments does not rule out more global changes in resting activity or alterations in connectivity between regions in the MAM E17 model. Changes such as these can be
tested experimentally using *in vivo* magnetic resonance imaging, as presented in the following chapter.
Chapter 7
Behavior and brain metabolic function of the MAM E17 mouse model

Introduction

Evidence of increased hippocampal metabolic activity at baseline in patients with schizophrenia has been found in both cerebral blood flow studies (Friston et al. 1992; Liddle et al. 1992) and positron emission tomography studies (Medoff et al. 2001). A recent investigation into the cerebral blood volume (CBV) showed reliable increases in baseline hippocampal values, especially in the CA1 subregion (Schobel et al. 2009a). Increases in CA1 CBV, furthermore, correlated with a measure of positive symptom severity, suggesting that brain metabolic function is relevant to disease outcomes.

Another hallmark of schizophrenia besides increased metabolic activity at rest may be hyperresponsivity to NMDA-receptor antagonists such as ketamine (Lahti 2001; Malhotra et al. 1997). This finding has provided new avenues for therapeutic targets (reviewed in (Coyle 2006).

Experiments in the MAM E17 model have shown that this model may be relevant to both the behavioral as well as neurochemical effects of NMDA antagonists. Namely, MAM E17 rats have been shown to hyperlocomote following administration of MK-801, another non-competitive NMDA antagonist (Lena et al. 2007). This effect emerges post-pubertally (Le Pen et al. 2006), in support of the neurodevelopmental relevance of the model. MK-801 administration leads to increased extracellular glutamate in the prefrontal cortex in the MAM E17 model after (Lena et al. 2007). These pieces of evidence suggest that the MAM
E17 model could give insight to the effect of ketamine on the hippocampal formation in schizophrenia.

We set out to test the effects of ketamine administration on brain metabolic activity in the hippocampal formation of the MAM E17 model, using the same CBV technique used in human patients (Schobel et al. 2009a). Due to size limitations of the magnet used for rodent imaging, we induced the MAM E17 model in mice instead of rats. After confirming in vivo a slight deficit in hippocampal volume in the model (see previous chapter), we investigated the behavioral and neurometabolic effects of acute ketamine administration compared to controls.

**Results**

**Behavioral data.** Locomotion in response to ketamine injection was tested in MAM E17 mice in an open field chamber. Baseline activity was calculated as the average of the three bins preceding injection. In the first analysis, a repeated measures ANOVA, the pre-ketamine baseline and the bin with the maximal response to ketamine (contained within the first three bins after drug injection) utilized both treatment and injection type (ketamine or vehicle) as the between subjects factors. The analysis showed a main effect of MAM treatment ($p = 0.028$, $n = 16$) and of injection ($p < 0.001$) (Figure 7-1).
MAM E17 mice display hyperlocomotion in response to ketamine (2.5 mg/kg). A mixed ANOVA comparing the baseline locomotion to the peak locomotion following drug injection revealed a significant effect of treatment, p = 0.017. Mice injected with vehicle are also shown.

**Figure 7-1**

MAM E17 mice display hyperlocomotion in response to ketamine (2.5 mg/kg). A mixed ANOVA comparing the baseline locomotion to the peak locomotion following drug injection revealed a significant effect of treatment, p = 0.017. Mice injected with vehicle are also shown.

Magnetic resonance imaging. We examined basal CBV values in hippocampal subregions as well as these regions’ responses to ketamine. Contours in a single section containing the mid-hippocampal formation were made around the dentate gyrus, CA3, CA1, subiculum, and entorhinal cortex. First, volumes of the hippocampus (subregions summed) were calculated and no group differences were noted (data not shown). The CBV values of the hippocampal complex and the individual subregions were then analyzed (Figure 7-2). Data from two datasets of mixed males and females were pooled, and the subsequent ANOVAs were controlled for scan batch and sex. The only effect of interest was MAM E17 treatment. The univariate ANOVA revealed a weak trend for a significant treatment difference (F = 2.33, p = 0.142, n = 12). When the subregions were analyzed separately in a mixed ANOVA, no region showed a significant between-subject effect.
CBV images, subregion contours, and baseline CBV values. a) Horizontal mouse brain slice (Bregma -5.64) stained with cresyl violet. b) Representative image used to draw contours for CBV calculations. c) Magnification of white box from (b) in which subregional contours are overlayed. d) Average CBV values of all subregions (dentate gyrus (DG), CA3, CA1, subiculum (SUB), entorhinal cortex (EC)) The effect of treatment was not significant (p = 0.142, n = 12). e) CBV values of each subregion.

The second set of animals scanned received acute ketamine injection and the change in hippocampal CBV values was recorded. A repeated measures ANOVA of the pooled response of the subregions (Figure 7-3a) showed a significant increase following ketamine administration (F = 5.219, p = 0.043, n = 5) but no effect of treatment. Thus subregions were not analyzed individually; however, the subregion data are shown in Figure 7-3b through f.
Effect of ketamine administration on regional CBV values in subregions of the hippocampal formation. Shown are the baseline is the values of the average value in the hippocampus (a) or in the subregions individually (b-f) before drug injection (baseline) and the values of the three subsequent 16-minute scans following injection.

**Discussion**

The experiments in this chapter were aimed at providing an initial characterization and validation of the MAM E17 model in the mouse. This would allow genetic manipulations, most commonly applied to the mouse, do be used to understand the genetic mechanisms underlying the susceptibility or resistance to the effects of MAM on the developing brain. However, although we found some behavioral abnormalities consistent with the rat model, overall we found the C57B6 mouse is much more resistant to the effects of prenatal MAM exposure than the rat. We did find increased locomotion in the MAM E17 mouse model in response to systemic ketamine, which is in line with previous accounts of hyperlocomotion in the rat model in response to MK-801, another non-competitive NMDA-receptor antagonist. The gross morphology of the brain and the volume of the hippocampus did not appear to be affected in the MAM E17 mice. Moreover, the basal metabolic activity
of the hippocampal formation exclusive of the entorhinal cortex showed a weak trend for overall increases, but this effect was not significant at any one subregion of the region. Following ketamine administration, changes in metabolic activity were significant overall but non-specific to the MAM E17 mice, despite the treatment-specific behavioral effects of the drug.

The finding of ketamine-induced hyperlocomotion in MAM E17 mice is in line with previous reports of MK-801-induced hyperlocomotion in MAM E17 rats (Le Pen et al. 2006; Le Pen et al. 2010; Lena et al. 2007). The dose chosen for the study (2.5 mg/kg) had been previously optimized in our laboratory to reliably induce locomotion without causing stereotypies. The effect was clear but short-lived. The drug-induced hyperlocomotion in rats generally lasted longer, on the order of 30 minutes or more, perhaps relating in part to the subcutaneous instead of intraperitoneal injection site.

The lack of treatment group differences in the CBV response to ketamine is not consistent with the altered behavioral response to this drug observed in the MAM E17 group. Obvious differences between the two studies were the state of the animal (awake versus anesthetized) and the higher dose of ketamine used in the imaging study. Although the dose used in the imaging study has been shown to increase in CBV in limbic and paralimbic cortex and detect differences in mouse models (Duncan et al. 1999; Gaisler-Salomon et al. 2009), it may not be relevant to the behaviors induced in awake animals at the lower dose. We also note that we did not include an acute saline treatment group, so the change in CBV after ketamine may be confounded by changes in contrast signal stability over time. However, a previous study investigating the effect of contrast showed that the overall level did not decrease by a significant amount over the time course we investigated.
(Moreno et al. 2006). Thus it is unlikely that subtracting the value of the signal over time would result in the emergence of a treatment difference. Since the behavior was recorded with a temporal resolution of 5 minutes and the CBV data was collected with a temporal resolution of 16 minutes, it is possible that treatment differences in response to ketamine were too fast to be captured by the scans. Lastly, it is also possible that the behavioral differences are mediated by activity in regions other than the hippocampus. Since previous microdialysis experiments in the MAM E17 rat showed changes in extracellular glutamate in the frontal cortex, future analyses of the post-ketamine CBV data will investigate metabolic responses in this region. A dissociation of response is possible between the hippocampal formation and frontal cortex, it was seen previously in a mouse model of altered glutamate transmission with relevance to schizophrenia (Gaisler-Salomon et al. 2009). Frontal cortical changes in metabolic activity could be anticipated in the MAM E17 mouse model because increased extracellular glutamate was observed in the MAM E17 rat model following MK-801 administration (Lena et al. 2007).

Conclusions

These studies are the first to our knowledge to explore schizophrenia-relevant phenotypes in the MAM E17 model induced in mice. A MAM E17 mouse would theoretically be useful for future genetic and imaging studies in which methodological concerns limit the choice of species. It is possible that this mouse model has similar relevance to schizophrenia as the rat model given the treatment-specific response to NMDA receptor antagonist and potential hippocampal area loss. Overall, however, the effects we
observed were very small relatively to the effect of this manipulation in the rat. Future studies of disease-relevant phenotypes will be necessary to assess the usefulness of this mouse model.
Chapter 8
General discussion and conclusions

Summary of results

The experiments presented here were undertaken to determine the contribution of the ventral subiculum in the schizophrenia-relevant phenotypes of the MAM E17 model. This goal required the characterization of the structure and function of the subiculum in this model. A broader goal was to further assess the validity of the MAM E17 model and, if validated, use the model to think about regulation of the hippocampus and its downstream targets in schizophrenia. Thus, with the MAM E17 model I attempted to address: 1) if the developmental origins of the model lead to reductions in hippocampal size and morphological abnormalities as seen in the hippocampus and subiculum in schizophrenia; 2) if the MAM E17 rodent model shows alterations in glutamatergic synaptic transmission in the ventral CA1 and subiculum; 3) whether changes in synaptically evoked activity are reflected in spontaneous single unit and population firing characteristics in the intact MAM E17 brain; and 4) if there are changes in the resting cerebral blood volume in the MAM E17 hippocampus that are consistent with changes observed in schizophrenia using similar methods.

I found reductions in the whole volume of the hippocampal formation complemented by area reductions in horizontal sections of the MAM E17 rats. In MAM E17 mice, the observed areal deficit assessed in the same plane in vivo was observed exclusively in the dorsal hippocampal. Both mouse and rat studies were underpowered, but taken together they suggest a full quantification of hippocampal volume would reveal a reliable reduction,
consistent with volume/area loss in the hippocampal formation seen in previous studies (Moore et al. 2006; Featherstone et al. 2006; Matricon et al. 2010; Flagstad et al. 2004). Regionally selective changes in the hippocampal shape and/or size are consistently observed in vivo in schizophrenia (Wang et al. 2001; Narr et al. 2004; Csernansky et al. 1998). Thus, the changes reported in the current set of experiments are in line with the previous findings in the MAM E17 model and reflect the heterogeneous nature of volume changes in the hippocampal formation in schizophrenia.

I observed selective spine loss in the ventral subiculum of MAM E17 rats accompanied by dendritic branching decreases using single-neuron reconstructions but no loss using population-based principles of stereology. The evidence suggests a spine loss only on a subset of neurons that may consequently suffer a loss of glutamatergic input from CA1 projections. The spine loss in the ventral subiculum is in line with observations in schizophrenia (Rosoklija et al. 2000).

I found and characterized alterations in glutamatergic transmission in the MAM E17 model. Attenuated evoked activity in the ventral subiculum was observed in the model in the slice preparation. These findings were not accompanied by changes in in vivo evoked activity, spontaneous field activity, or unit activity in the ventral subiculum in the anesthetized preparation. Taken together, the apparent neurophysiological abnormalities in the ventral subiculum suggest a mechanism by which ventral subicular output not regulated normally by its primary inputs, possibly resulting in ventral subicular output to downstream targets that is ‘dysconnected’ from the hippocampal trisynaptic circuit and other regulators of the subiculum. This is consistent with evidence for excessive drive of the dopaminergic
system by ventral CA1/subiculum in this model (Lodge & Grace 2007) and with theoretical conceptualizations of the disease process (Lodge & Grace 2011).

Finally, I obtained a global measure of brain metabolic processes using cerebral blood volume mapping. While the results were not significant, all subregions showed that MAM E17 metabolic activity increased over controls. Findings in schizophrenia using similar methods show significantly elevated resting metabolic activity in hippocampal subregions (Schobel et al. 2009b), and a number of studies show resting hyperperfusion of the hippocampal region specifically and the temporal lobe more generally (Liddle et al. 1992; Friston et al. 1992; Molina et al. 2003). The human and animal modeling data pose the question of what mechanisms underlie this increase in resting metabolic activity and how it may interact with the symptomatology of the disease. Since the human studies are only correlative, and since we have an incomplete idea of the causal factors of increased metabolic activity, future investigations will need to probe the directionality of the correlation and mechanisms that give rise to it.

These experiments contribute to the profile of schizophrenia-relevant hippocampal deficits observed the MAM E17 model. This investigation of the model lays the groundwork for the use of this neurodevelopmental model for revealing possible mechanisms linking the structural and histochemical changes to the functional abnormalities of the hippocampal formation seen in schizophrenia. Many of findings can be used to inform on some of the current discrepancies and mechanistic questions in the schizophrenia literature and generate new hypotheses about the disease.

These findings must be interpreted within the limitations of the MAM E17 model, which are considered at the end of this section.
Gross anatomical changes in the model and their relevance to schizophrenia.

The anatomical abnormalities of the MAM E17 model presented here have similarities to those seen in the disease, and further investigations may help shed light on some of the inconsistencies found in the schizophrenia literature. In the MAM E17 model, we observed evidence for spatially restricted hippocampal volume loss in the MAM E17 mouse model *in vivo* and evidence for overall hippocampal volume loss in the MAM E17 rat model postmortem. The present results suggest that the volume loss observed in the model may not be detectable in all axes in all regions, despite it being present in the hippocampus as a whole (Flagstad et al. 2004) and in the dorsal area specifically (Moore et al. 2006) and others).

The ability to investigate hippocampal volume *in vivo* and postmortem in the mouse and to investigate postmortem the same MAM E17 rat brain sliced at different orientations (for example, one hemisphere cut horizontally and one coronally) are two examples of studies that would be difficult to accomplish in schizophrenia patients. In the case of the mouse model, the benefit is obtaining pre- and postmortem volume estimates within the same subject, which would be a long and expensive longitudinal study in humans. In the case of the rat model, the matched set of a hippocampal formation cut at different angles will allow an almost three dimension reconstruction of the brain at a spatial resolution not possible using imaging techniques. Since human postmortem tissue is sliced at standard angles and researchers don’t always have access to the entirety of the structure, the rat model can be used to examine how slice orientation can affect measurements. These experiments have the potential to inform on two outstanding issues of the human hippocampal volume data: first, why are volume reductions consistently seen *in vivo* but inconsistently observed
postmortem and secondly, does the angle of slicing and the potential of incomplete regional representation affect the outcome. Because our postmortem data will represent the entirety of the structure, we can investigate if only examining part of the structure would give a false impression of the whole. Because our mouse data will encompass pre- and postmortem data, we can examine if changes seen premortem are preserved postmortem or if the process of brain preservation nullifies differences between treatment groups.

**Dendritic morphological changes in the subiculum and their relevance to schizophrenia.** Reduced spine counts on subicular pyramidal cells have been seen in schizophrenia (Rosoklija et al. 2000) and now in the MAM E17 model. The spine loss in the MAM E17 model seems to occur only in a subpopulation of pyramidal cells in ventral subiculum and could have important functional consequences. The subpopulation of pyramidal neurons implicated in the results reported here are those pyramidal cells whose dendrites project dorsally out of the proximodistal or transverse plane and were thus preserved in coronal slices. Intriguingly, the physiological connections between CA1 and entorhinal cortex and back to CA1 were shown in one study to be organized in the dorsal to ventral direction (Vorobyov & Brown 2008). Specifically, antidromic activation of CA1 in response to entorhinal cortex stimulation was found in relatively dorsal or septal CA1 locations while orthodromic activation was observed in fields ventral to the first set. This was interpreted as the transmission of information flow between the hippocampus and entorhinal cortex and back again to be in the dorsal to ventral direction. Whether the same physiological organization holds true in the subiculum to entorhinal cortex connections is unknown. The existence of this dorsal to ventral pattern is supported anatomically as projections of CA1 cells innervate subicular areas that are generally at the same dorsoventral
location or more ventral locations (Amaral et al. 1991). This directionality may extend to some subicular cells whose dendritic trees could extend dorsally or ventrally relative to the cell body. Dendritic trees that extended dorsally would have a greater chance of receiving afferents from CA1 cells located dorsally. Since ventral subicular cells have differential targets than dorsal cells, this arrangement could expose cells projecting to paralimbic and hypothalamic regions to more dorsally processed CA1 information. These cells could “fast-forward” the flow of information to the ventral subiculum. The extent to which this is an organizing principle of the hippocampal formation is unknown. It could be investigated electrophysiologically using an intact preparation as used by Vorobyov and Brown applied to CA1 and subiculum. Anatomical investigations using an anterograde tracer could reveal the degree to which CA1 projections target more ventral areas of subiculum.

**Cerebral blood volume measurements and their relevance to schizophrenia.** The CBV imaging methods we used on MAM E17 mice are similar to those used in human studies (Schobel et al. 2009b) in which increased CBV was also predictive of later onset of psychosis. Despite the lack of statistical significance, the direction of the findings suggest that the MAM E17 mouse can be used, potentially with a greater number of animals per group to bring out significance, as a model to investigate a putative biomarker of the disease. Longitudinal studies of the MAM E17 mouse would reveal if the increased CBV is a developmental process or if it exists prepubertally. Initial data collected to answer this question (not shown) suggest that the MAM E17 mouse shows elevated hippocampal CBV even prepubertally. The MAM E17 model could thus be applied to investigation developmental interventions that could impact CBV and suggest novel mechanisms that contribute to the elevation in the disease state.
Neurophysiological findings and their relevance to schizophrenia. The neurophysiology data suggest weaker signaling from entorhinal cortex to both subiculum and entorhinal cortex. The weaker signaling could be a deficit in fiber number or integrity. Decreased number of fibers could occur if prenatal MAM treatment impacts the developing entorhinal cortical cell in such a way to affect cell number or affect the ability of these cells to project to their appropriate target. Indeed, one group observed cellular disarray in the entorhinal cortex of MAM E17 rats (Matricon et al. 2010). The integrity of these projections could also be impacted if, for example, the fibers had decrease myelination, an effect observed in the corpus callosum and cingulum of MAM E17 animals (Chin et al. 2010).

Luxol staining to test the density of myelinated fibers near the pial surface of the MAM E17 CA1 and subiculum can be used to investigate myelination of the perforant path. Regardless of the amount of signal arriving at the postsynaptic cell, both the in vivo and in vitro evidence suggests that the MAM E17 subiculum could exhibit a lower threshold of response to entorhinal cortex input. It is unclear whether this same alteration occurs in the case of CA1 input in the MAM E17 rats.

When the spontaneous firing rate of the subicular cells are observed in the intact circuit there is no change in either the rate or the patterning of neuronal output. This finding could suggest compensatory mechanisms in response to the loss of major inputs like entorhinal cortex. Two potential contributing effects to maintain normal spike output could be increased input from other subicular afferents such as the amygdala and/or alterations in intrinsic membrane properties such as a depolarized resting membrane as seen in the MAM E17 prefrontal cortex (Moore et al. 2006; Lavin et al. 2005). The first possibility could be tested by chemically ablating the basolateral amygdala and recording in the ventral
subiculum. If increased input from the amygdala is driving subicular cells in the MAM E17 animal, then the chemical ablation should have a greater effect on spontaneous firing of subicular neurons in MAM E17 animals compared to controls. The second hypothesis of altered intrinsic membrane properties could be tested by intracellular recordings in the ventral subiculum.

These electrophysiological results do not have a simple corollary in the disease state due to ethical and technical considerations in human studies. However, the value of disease modeling using models such as the MAM E17 rat or mouse is the ability to test hypotheses that can link data acquired through different techniques. To do that, a conceptual framework of the model is necessary.

**A conceptual model of MAM E17 hippocampal abnormalities**

The data presented here and elsewhere allows for the construction of a model that displays a loss of input to the subiculum and CA1 from entorhinal cortex with a potentially lower threshold of evoked firing in the subiculum to this input. These changes in evoked activity were observed in the absence of changes in spontaneous activity of subicular cells. These physiological changes are coupled with the anatomical finding of decreased dendritic spines on a subpopulation of subicular cells. Taken together, the evidence points to subicular neurons that are disconnected from their major inputs yet maintain their spontaneous activity. They could do this because of extra input from other areas such as the amygdala or by altered intrinsic cell properties, or both. They could also be reflecting the physiological consequences of decreased parvalbumin expression which could disinhibit the pyramidal neurons. Another mechanism might be altered response to dopamine modulation as was
reported in medial prefrontal neurons. Since the subiculum but not CA1 displays dopaminergic fibers (Amaral & Witter 1995), this alteration would leave the subiculum especially vulnerable to the increased population dopamine cell firing observed in MAM E17 animals (Lodge & Grace 2007). These findings and hypotheses place the MAM E17 model in a strong position to test hypotheses about hippocampal, glutamatergic, and dopaminergic alterations in schizophrenia.

A previous study in the MAM E17 model showed that chemical ablation of the ventral CA1/subiculum normalized aberrant dopaminergic activity (Lodge & Grace 2007). This study raises the possibility that more targeted and subtle manipulations could attenuate increased dopamine cell activity. For example, if the MAM E17 ventral subiculum is deficient in GABAergic tone because of a deficit of parvalbumin interneurons, then local infusion of a GABA receptor agonist may help normalize activity in the region. Another question that remains unexplored in the model is the effect of increased dopamine cell population activity has on the hippocampus. Since the subiculum is likely more heavily influenced by dopamine than other parts of the hippocampal formation, then the process of a positive feedback loop is possible: aberrant subicular activity drives aberrant dopamine release which in turn feeds back to the subiculum. Local infusion of dopamine receptor agonists and antagonists into the ventral subiculum of the MAM E17 animals while recording in that area and in the ventral tegmental area would inform on this hypothesis.

Another projection target of the ventral subiculum that has been implicated in schizophrenia is the prefrontal cortex (Moghaddam 2002). Aberrant drive from the ventral subiculum could contribute to pathologies observed in this target. Thus future
electrophysiology studies could uncover the extent to which subicular activity impacts the prefrontal cortex.

Despite the number of neuropathological elements of schizophrenia modeled by the MAM E17 model, the extent to which the method of inducing the model has relevant to schizophrenia remains unexplored. The model arises from chemical exposure timed for a critical time window of temporal cortical development. The resultant brain changes seem to include changes that are established early in development (spine density changes in MAM E17 animals are seen at postnatal day 25, data not shown) and changes that emerge later in life, as is the case for behavioral and neurochemical sensitivity to amphetamine (Moore et al. 2006). Thus the chemical exposure must alter genes and gene pathways that determine the development quite distal to the time of the exposure. This relationship between proximal and distal developmental changes is highly relevant to schizophrenia, a disease in which anatomical changes and perhaps brain metabolic changes are seen before first onset of the disease while psychosis emerges after puberty. The MAM E17 model may thus be useful to focus neurodevelopmental theories of schizophrenia by suggesting gene candidates and brain regions that increase the susceptibility of the disease.

**Application of the model to test hypotheses of the pathophysiology of schizophrenia**

The results presented here have the ability to shape the current understandings of disease process in schizophrenia. These results emphasize the ventral subiculum as a nexus between hippocampal circuits and downstream circuits involving the limbic system, dopamine regulation, and stress response. As such, pathologies of the ventral subiculum could have an outsized influence on these schizophrenia-relevant circuits. Discrete,
reversible circuit manipulations of the ventral subiculum and its upstream and downstream partners can reveal how converging information in the ventral hippocampus affects the subiculum’s output. Probing glutamate function as well as dopamine-glutamate interactions is a promising use of the model as alterations of both systems with relevance to schizophrenia have been observed. The model can be applied to increasing our understanding of resting metabolic rate in the hippocampus and how it is modulated by treatment strategies. As new and untested drug therapies move through the pipeline, their efficacy in blunting psychosis-related neurophysiology and behavioral phenotypes in the MAM E17 model could be an important screening tool.

Collectively, the findings reported here and much of the published data on the MAM E17 model must be interpreted within the limitations of the model. A major limitation is the unknown mechanism of model induction. While the timing of the induction has relevance to the timing of prenatal insults that increase the risk for schizophrenia, the mechanism by which MAM administration interacts with neurodevelopmental processes leading to long term alterations in limbic and dopamine systems is unknown. Ongoing analyses of gene chip expression data are investigating which genes and gene families are significantly altered in the prefrontal cortex and hippocampus of MAM E17 animals at different developmental time points. These studies have the potential to generate candidate genes or gene pathways that lead to the effects observed in the model. Without a thorough understanding of these mechanisms, interpretations of the model are strongest 1) when there is evidence of similarities between the changes seen in the model and those seen in the disease and 2) when the model links biological with behavioral or neurochemical phenotypes in order to guide hypothesis generation in the disease.
Future directions

The consistent inconsistencies of the schizophrenia literature underscore the idea that there is not a single “schizophrenia” but rather a constellation of symptoms that show heterogeneity between people and even within affected individuals across their lifetime. Keeping that in mind, the task of modeling the disease is daunting, if not outright impossible. However, the usefulness of a model lies in its reduction of heterogeneity to predictable signatures: what commonalities of the disease underlie the most fundamental pathologies? These are the changes that should be modeled. When the changes that the animal model exhibits span anatomical, neurophysiological, and neurochemical outcomes, this model can be applied to questions that touch all of these domains in the disease. Indeed, it is only by understanding all of these alterations together in the disease can researchers and clinicians make rational decisions about treatments, both established and speculative. The changes observed in the MAM E17 model span these domains and make it a strong choice for continued research into the pathophysiology of schizophrenia and for the rational development of new treatments for the disease. The ultimate goal of a disease model is obsolescence: that new intervention and treatment strategies for the disease are so effective that further development is unnecessary. While the schizophrenia modeling field is far from that point, the MAM E17 model can be justifiably applied to the goal.
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