The intrinsic and synaptic properties of inverted pyramidal cells within the neocortex

Robert Michael Steger
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The Intrinsic and Synaptic Properties of Inverted Pyramidal Cells within the Neocortex

by

Robert Steger

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Abstract
THE INTRINSIC AND SYNAPTIC PROPERTIES OF INVERTED PYRAMIDAL CELLS WITHIN THE NEOCORTEX

by
Robert Steger

Adviser: Professor Joshua Brumberg

Within the nervous system, the cortex is the area of the brain where higher order sensory, motor and cognitive processing occurs. The cortex contains a diverse array of cell types which form complicated and intricate circuits which gives rise to higher order sensory, motor and cognitive functions. The majority of neurons found in the cortex are pyramidal cells. While pyramidal cells differ based on soma size, dendrite span and cortical position, almost all share a noticeable defining characteristic: their apical dendrite extends toward the pial surface. However, there also exists a class of pyramidal cell where the apical dendrite extends in the opposite direction, toward the cortical white matter; these pyramidal cells appear to be upside down, or inverted.

Utilizing physiological and histological techniques, inverted pyramidal cells (IPC) within neocortex layer VI of the somatosensory cortex were examined and compared to the more common upright pyramidal cells (UPC). This research produced a number of key findings: 1) the intrinsic physiology of IPCs differs from UPCs on a number of measures including input resistance, and action potential threshold and half-width; 2) IPCs, beyond the orientation of the apical dendrite, are morphologically dissimilar as compared to UPCs and 3) Stimulation of the underlying cortical white matter revealed IPCs are either integrated into different cortical circuits or process inputs differently. The main conclusions emphasize a need for further examination
and classification of cortical neuronal cell types. These data are relevant to models of information processing through micro- and larger neocortical circuits and indicate that different cell types found within similar lamina can have different functional properties.

**Key Words:** Inverted, Pyramidal Cell, Neocortex Layer VI
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Chapter One: Literature and Background Review

1. General Introduction

All complex life initially stems from a single cell. From a single fertilized ovum begins a rapid and immense differentiation and diversification of cell tissue. The brain itself emerges into three main anatomical structures: hindbrain, midbrain and forebrain. The evolutionarily most recent area of the brain is the neocortex, which arises from the forebrain. Along with these developmental changes in anatomy, the nervous system also produces billions of cells including neurons and glia (Azevedo et al., 2009; Williams and Herrup, 1988). Over the course of development, these basic cell types exhibit an immense amount of diversity and specificity. Neurons differ based on their physiology, neurotransmitters, receptors, synaptic connections and morphology. While the prototypical neuron contains a soma, axon and dendrites, individual neurons vary in the shape and size of these components. The varying and intricate dendritic morphologies give neurons their own distinct appearance. However, dendritic morphology has a more functional purpose, to receive and integrate synaptic inputs. Integration patterns are heavily influenced by the span of their dendritic trees (Johnston and Narayan, 2008). The most common type of neuronal cell is the pyramidal cell, in which the dendritic trees can be differentiated based on morphology and spatial location: basal, oblique, apical and tuft, which receive different synaptic inputs (reviewed by Palmer, 2014). Even within neurons with similar morphological features, there exists immense diversity. The pyramidal cell, for instance, can vary in cell body size, dendrite length, dendrite geometry and/or dendrite thickness. These variations are often associated with different physiological properties (Schubert et al., 2006). Given the immense diversity even within neurons of the same morphological type, it is therefore important to better investigate the intrinsic properties and differences of these individual neurons.
A small percentage of cells within the pyramidal cell subtype exhibit a simple but dramatic morphological change; an inversion in the orientation of their primary dendrite. Pyramidal cells are typically characterized as having a single primary (apical) dendrite which ascends toward the pia mater (brain surface), there are also inverted pyramidal cells in which the primary dendrite points toward the cortical white matter. This report seeks to review how complex neocortical circuits arise from individual neurons. Neuronal phenotypes will be examined in relation to the laminar organization of the cortex. Phenotypic arrangement will be further refined to identify morphological correlates of electrophysiological responses. This will then lead to the description of different circuits based on laminar position, morphology and physiology. More specifically, I will examine the morphological, physiological and synaptic differences that exist within these two classes of pyramidal cells, upright and inverted. I will also examine these differences across rodent species, mouse and rat, to determine the degree of neuronal similarity across taxa. The understanding of these specific circuits is important in establishing how the cortex carries out its functioning.

1.1: Laminar Organization of the Neocortex

The mammalian neocortex is the outermost layer of the cerebral hemispheres and is organized into five distinct lobes (frontal, temporal, parietal, occipital and insular) which perform different specialized functions. For example, the occipital lobe contains the primary visual cortex and the temporal lobe houses the primary auditory cortex. While overall lamination can vary, the neocortex can often be organized into six layers, which can further be organized into sublayers depending on cortical area and function. Separated into gray matter (cell bodies and dendrites) and white matter (myelinated axons), the human neocortex contains billions of neurons which form complex connections, which also number into the thousands of billions.
Most sensory experience is processed through the subcortical brain area known as the thalamus before reaching its cortical target. The connections between the cortex and thalamus are often reciprocal allowing the thalamus to refine and filter information being passed through to the cortex (Robertson, 1997; McFarland and Haber, 2002). In this respect, higher order sensory processing does not only involve cortico-cortico connectivity, but cortico-subcortical as well including thalamo-cortico, cortical-striatal, and cortical-spinal circuits. In order to further understand the overall functioning and processing of the brain, it is important to comprehend how morphologically and physiologically distinct cells form circuits with each other. Within the cortex, there is a diverse array of neuronal cell types. These cortical neurons feature a great number of morphological phenotypes including pyramidal, stellate and basket cells. Differences in morphology often correlate with differences in physiology and synaptic connectivity (Grudt and Perl, 2002; Kim and Connors, 1993) in which in turn impact their function. These morphologically distinct cell types vary depending on the area of the brain and are found in different densities throughout the cortex, and integrate into distinct circuits (see figure 1).
Figure 1: Schematic representation of laminar organization within the cortex. Each cell represents the predominant cell type found within each layer and their outputs. PC = pyramidal cell. BC = basket cell. SC = stellate cell. STPC = slender tufted pyramidal cell. TTPC = thick tufted pyramidal cell. IPC = inverted pyramidal cell.

Layer I of the cortex, also known as the molecular layer or acellular layer, consists mostly of myelinated axons as well as the distal ends of the apical dendrites of underlying pyramidal neurons. This layer contains sparse populations of cells including Cajal-Retzius cells. Cajal-Retzius cells arise from the preplate, which exists prior to the establishment of the cortical plate (Hestrin and Armstrong, 1996). These cells may assist with the organization of the other cortical layers as they synthesize the glycoprotein reelin, which when absent disrupts cortical migration and causes cells to become backed up at the cortical plate (Frotscher, 1998; Ogawa et
al., 1995). It is known that neuronal phenotypes are not randomly distributed throughout the cortex, but rather are found differentially in certain layers. Spiny stellate cells, for example, are found almost exclusively in sensory areas in layer IV whereas large pyramidal cells, known as Betz cells, are only found in layer V of the primary motor cortex. Likewise, the dendrites and axons of these cells are not randomly distributed and show biases toward particular layers. While this appears to place constraints on cortical connectivity, it may serve to optimize neuronal circuits. Theoretical models have shown that the evolution of cortical modules serves to maximize synaptic connectivity while minimizing conduction delays between connecting neurons (Chklovski et al., 2002). It has also been theorized that if the cortex did not have a laminar organization, then the volume of the cortex would have to be much larger to retain the same level of connectivity (Mitchison, 1992).

While layer I contains a very sparse population of cells, the subsequent layers have large amounts of gray matter with differences in neuronal populations. Layers II and III form the supragranular layer and contain mostly small pyramidal cells which are excitatory, as well as GABAergic cells including chandelier, bipolar and double bouquet cells (Van der Linden and Lopes Da Silva, 1998; Werner, 1986; Peters and Kara 1985). Layer II/III also specifically express the transcription factors Cux1, Cux2 and Lhx2 (reviewed by Molyneaux et al., 2007). Pyramidal cells in this layer express Rgs8, a regulator for G-protein signaling. The Rgs gene family is known for its involvement in synaptic plasticity (Lein et al., 2007). It also appears that inhibitory interneurons are found in a greater concentration and diversity in these layers than in the other layers. Many of these GABAergic cells have horizontally oriented axon arbors forming local connections with neighboring neurons (Kawaguchi, 1995). In contrast, excitatory cells with vertically oriented axons are capable of forming interlaminar connections as well as forming
circuits to numerous subcortical regions including the tectum, pons and cerebellum as well as from neurons projecting from the corpus callosum (Molyneaux et al., 2007; Ramos et al., 2008).

Layer IV, the granular layer, has the highest density of cells compared to the other layers in the cortex. Excitatory neurons are a defining characteristic of neurons in the sensory cortices of this layer. This layer specifically expresses the gene KCNIP2 (Molyneaux et al., 2007), which codes for the rapidly inactivating A-type potassium channel regulating the excitability of this layer (An et al., 2000). Layer IV receives inputs from the thalamus, most prominently within the sensory modalities (Shatz et al., 1977; Jensen and Killackey, 1987; Jones 1998). For example, in the rodent whisker somatosensory system, known as the barrel cortex, the majority of input into layer IV originates from the ventral posterior medial nucleus of the thalamus (VPM; Lu and Lin, 1993). There is also evidence that axons from the posterior medial area of the thalamus (POm) are also present early in development (Kichula and Huntley, 2008). The composition of layer IV of barrel cortex is largely (~ 80%) made up of spiny stellate cells and small pyramidal cells (Simons and Woolsey, 1984). As the majority of thalamic afferents first synapse in layer IV of the cortex, layer IV traditionally represents the initial stage of intracortical processing.

Layers V and VI, collectively known as the infragranular layers, represent the major source of cortical output. Layer V contains a heterogeneous population of neurons that project to many different cortical and subcortical areas. Within the barrel cortex, layer V can be divided into two sublayers, Va and Vb, which differ in their synaptic connectivity. PlxnD1, which aids in neuronal guidance, is expressed in layer Va, but not in layer Vb (Wakatabe et al., 2006). Layer Va receives input from the posterior medial nucleus of the thalamus (POm) and projects callosally to the contralateral hemisphere as well as projecting intracortically to the ipsilateral striatum. Layer Vb expresses genes found in subcerebral neurons including Er81, Nfh and Lmo4
Layer Vb projects largely to subcortical regions including the POm, trigeminal nuclei, pons and spinal cord (Mercier et al., 1990; Hoffer et al., 2005; Bureau et al., 2006). These two sublayers also differ morphologically and physiologically. Pyramidal cells in layer Va have comparatively small cell bodies with thin tufted dendrites whereas pyramidal cells in layer Vb have larger cell bodies and thick tufted dendrites. Callosal neurons also have shorter apical dendrites and fewer bifurcations than the other projection neurons (Hattox and Nelson, 2007; Ramos et al., 2008; Oberlaender et al., 2011). Finally, layer VI, the multiform layer, is the most heterogeneous layer of the cortex containing various neuronal phenotypes (Chen et al., 2009; Kumar and Ohana, 2008). This diversity in cell types appears to be reflected in its diversity in connectivity. Within the cortex, Layer VI exclusively expresses the growth factor CTGF (Heuer et al., 2003; Rossini et al, 2011; Watakabe et al., 2006), as well as the transcription factor FOXP2 (reviewed by Molyneaux et al., 2007; Ferland et al., 2003). Like layer IV, layer VI receives thalamic input. However, layer VI contains reciprocal connections to the thalamus providing cortical feedback. While layer V also contains cortico-thalamic connections, it does not target sensory specific areas, but rather the interlaminar and association nuclei (reviewed by Deschênes et al., 2003). Layer VI also receives cortico-cortico inputs and is involved in contralateral signaling. Morphologically, layer VI contains various types of pyramidal cells that differ in cell body size and dendritic architecture (Chen et al., 2009). Once again, different types of pyramidal cells appear to be segregated into sublayers based not only on morphological characteristics but also in terms of their afferent and efferent connections. In this regard, laminar organization of different morphological and physiological neuronal subtypes gives rise to specific cortical circuits. As inverted cells are found mostly in the infragranular layers and thus may form circuits specific to their cortical location.
1.2: Properties of Cortical Neurons

1.2.1: Phenotypic Diversity of Cortical Neurons: Morphology

As previously discussed, the cortex contains a large variety of cell types that can be characterized by their morphology. Cells can be characterized by size of their cell bodies, the length of their axon as well as their dendritic structure. More recently, the presence and density of dendritic spines have also been emphasized in classifying neurons. Morphological classification often falls into two broad categories: pyramidal and nonpyramidal. Nonpyramidal cells can be further divided based on dendritic branching including bipolar, multipolar and bitufted (Kriegstein and Dichter, 1983). Neurons can also be subdivided based on spine density as well as axonal projection pattern and post-synaptic target(s). Dendritic spines are small protrusions found extending from the surface of dendrites (Miller and Peters, 1981). Spines often receive input from neighboring axons and aid in neuronal signaling (Alvarez and Sabatini, 2007). The presence and concentration of spines vary according to neuronal cell type, in which pyramidal cells and layer IV cells have a high spine density while GABAergic cells are described as being medium spiny or aspinous (McCormick et al., 1985; Tarczy-Hornoch et al., 1998). Finally, neurons can be classified by their connectivity; if they target a particular cortical layer, subcortical region or cell type.

The predominant cell in the neocortex is the pyramidal cell which accounts for approximately 65-70 % of the total neuronal population. These cells are characterized by their triangular, or pyramid shaped, cell body. Pyramidal cells also contain a primary apical dendrite which originates from the apex of the soma and projects toward the pial surface. They also contain numerous basal dendrites which branch as the distance away from the soma increases.
Pyramidal cells have high densities of spines along their dendrites as well as having greater dendritic length as compared to nonpyramidal cells (Kriegstein and Dicther, 1983). While the majority of pyramidal cells have their primary dendrite oriented toward the pia, a small percentage possess an atypical orientation in which the primary dendrite does not extend towards the pia, but rather are completely inverted, extending toward the cerebral white matter. Apical dendrite guidance appears to be regulated largely by the neurotrophic factor, semaphorin-3A. Grown in culture, cortical pyramidal cells lacking semaphorin 3A are more likely to have an inverted morphology (Polleux et al., 2000). Semaphorin 3A has also been shown to affect axonal guidance (Shwarting et al., 2004; Shelly et al., 2011). However, The axons of inverted cells, like their upright counterparts, project toward the cortical white matter (Mendizabal-Zubiaga et al., 2007).

Nonpyramidal multipolar cells such as spiny stellate cells are also found in high densities in layer IV of the primary sensory areas of the cortex. These cells are classified by the shape of their dendrites as well as the presence of dendritic spines. In contrast to pyramidal cells, the dendrites surrounding the cell body do not show a specific orientation. Rather, the dendrites radiate around the cell body giving them their star shape. Spiny multipolar cells also show a greater degree of dendritic branching than aspiny multipolar cells and are most similar to small pyramidal cells physiologically (McCormick et al., 1985; Elston et al., 1997).

While all cells can be defined by their dendritic morphology, there also exists a great diversity in axonal patterns. Spiny stellate cells, for example, vary in axonal arborization depending on species as well as location within the cortex. For example, in the mouse barrel cortex, numerous axon collaterals within a barrel ascend to layer I whereas the axons of spiny stellate cells in cat visual cortex descend toward the cortical white matter and form numerous
horizontal collaterals which can reach one mm in length (Valverde, 1986) as well as have axon collaterals extending into layers II/III (Gilbert and Wiesel, 1983).

In addition to the excitatory pyramidal cells and spiny stellate cells, the neocortex also contains numerous sparsely spiny and aspinous interneurons which are predominantly inhibitory cells. Once again, these cells can be characterized by their dendritic morphology. Martinotti cells, for example, have a bitufted dendritic morphology with extensive branching and contain a sparse density of spines (Wang et al., 2004). However, their unique anatomical feature appears to be in their axonal arborization. Axons from these cells generally emerge from the first or second dendritic branch and rise toward the pia where they cluster in layer I and send collaterals to neighboring columns (Markram et al., 2004).

Other major classes of interneurons found in the cortex are cortical basket cells and chandelier cells. These cells are aspinous and have either a multipolar or bitufted morphology, respectively. However, these cells are best characterized by their axons. Basket cells, for example, have a wide horizontal spread of their axons which contact the soma of pyramidal cells. By contrast, the axons of chandelier cells form vertically oriented arrays of terminals, known as cartridges (Lewis and Lund, 1990), which make numerous axoaxonic connections with pyramidal cells (Somogyi, 1977, see Table 1 for an overview of several known neuron classes).
Aside from morphological features, neurons can also be described by their physiological properties. A functional property of all neurons is the excitable membrane which, with sufficient depolarization, can initiate an action potential. Typically, a neuron is able to affect the activity of neighboring neurons through the release of neurotransmitters. In most cases, glutamate serves as an excitatory neurotransmitter generating a depolarizing potential on its post-synaptic neuron known as an excitatory post-synaptic potential (EPSP) (Meldrum, 2000). This increase in the post-synaptic neuron’s excitability brings its voltage closer to threshold and thus increases its probability of firing an action potential. Conversely, GABA-ergic cells are the most common type of inhibitory neuron (Roberts, 1980). These cells cause a depression in a post-synaptic cell’s excitability by hyperpolarizing the cell with an inhibitory post-synaptic potential (IPSP). See Table 1 for an overview of neuron types.

**Table 1: Cortical Neuron Classes**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Discharge Pattern</th>
<th>Primary Neurotransmitter</th>
<th>Co-Peptide</th>
<th>Location of Cell Body</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyramidal</td>
<td>RS, IB</td>
<td>Glutamate</td>
<td>SST, CCK</td>
<td>Layers II-VI</td>
</tr>
<tr>
<td>Spiny Stellate</td>
<td>RS</td>
<td>Glutamate</td>
<td></td>
<td>Layer IV</td>
</tr>
<tr>
<td>Basket</td>
<td>FS</td>
<td>GABA</td>
<td>PV</td>
<td>Layer II - V</td>
</tr>
<tr>
<td>Chandelier</td>
<td>FS</td>
<td>GABA</td>
<td>PV</td>
<td>Layer III, V</td>
</tr>
<tr>
<td>Cajal-Retzius</td>
<td>FS(?), FS</td>
<td>GABA</td>
<td></td>
<td>Layer I</td>
</tr>
<tr>
<td>Double Bouquet</td>
<td>FS, CB</td>
<td>GABA</td>
<td>VIP</td>
<td>Layer II/III</td>
</tr>
<tr>
<td>Martinotti</td>
<td>FS, CB, IS</td>
<td>GABA</td>
<td>NPY, CCK, SST</td>
<td>Layers II-VI</td>
</tr>
</tbody>
</table>

**1.2.2: Phenotypic Diversity of Cortical Neurons: Physiology**

Aside from morphological features, neurons can also be described by their physiological properties. A functional property of all neurons is the excitable membrane which, with sufficient depolarization, can initiate an action potential. Typically, a neuron is able to affect the activity of neighboring neurons through the release of neurotransmitters. In most cases, glutamate serves as an excitatory neurotransmitter generating a depolarizing potential on its post-synaptic neuron known as an excitatory post-synaptic potential (EPSP) (Meldrum, 2000). This increase in the post-synaptic neuron’s excitability brings its voltage closer to threshold and thus increases its probability of firing an action potential. Conversely, GABA-ergic cells are the most common type of inhibitory neuron (Roberts, 1980). These cells cause a depression in a post-synaptic cell’s excitability by hyperpolarizing the cell with an inhibitory post-synaptic potential (IPSP). See Table 1 for an overview of neuron types.

**Regular Spiking**
In addition, neurons can be further classified by their action potential firing properties including regular spiking, intrinsic bursting, or fast spiking. Spike discharge pattern corresponds with the type of neuron: whether it is excitatory or inhibitory. Regular spiking (RS) cells are found throughout the cortex, from layers II to VI, and are most often classified as excitatory neurons. These RS cells exhibit an adapting train of action potentials in response to a constant current injection. Following a train of spikes, these cells feature a prolonged afterhyperpolarization in which the membrane potential falls below the original resting potential. These cells also adapt, displaying a decrease in discharge rate following periods of sustained depolarizing current injection (McCormick et al., 1985). Rates of adaptation may differ among RS cells. The majority of RS cells are slow adapting (~80%) in which these cells fire consistently throughout the presentation of a stimulus and when depolarized to just above threshold will only fire a single action potential (Nunez et al., 1993). But some RS cells have fast adapting properties which results in their only firing at the onset of a depolarizing pulse. The majority of slow adapting cells feature a fast afterhypolarization following each spike. Fast adapting RS cells show a steady train of spiking activity at the onset of current pulse injection, but their membrane voltage ultimately reaches a depolarizing plateau in response to prolonged stimulation and they do not show spontaneous firing. While adaptation patterns may differ in RS cells, there appears to be no clear laminar distribution corresponding to type(s) of RS cells (Nunez et al., 1993; Dégenétaiis et al., 2002).

**Intrinsic Bursting**

While some nonpyramidal cells can be classified as regular spiking, the majority of RS cells are pyramidal. There are two broad classes of pyramidal neurons: Regular spiking and Intrinsic bursting. Intrinsic bursting (IB) neurons are another group of physiologically distinct
cells that can be characterized by their discharge pattern. Like RS calls, IB cells are also excitatory neurons. Whereas RS cells only produce a signal spike when depolarized to just above threshold, IB cells fire bursts of at least three action potentials in response to a “just threshold” depolarizing stimulus (McCormick et al., 1985; Nunez et al., 1993; reviewed by Contreras 2004). There are, however, conditions in which IB cells will not burst. For instance, applying a stimulus when the membrane is held at a depolarized level causes an IB cell to fire a train of single action potentials. (McCormick et al., 1985). RS cells, too, can alter their firing pattern and have been shown to fire in doublets or triplets if the applied depolarizing current is strong (Kayano and Kang, 1994).

While the majority of RS cells are pyramidal neurons, there are also nonpyramidal cells with regular spiking properties. These nonpyramidal RS cells are mostly spiny stellate cells or sparsely spiny multipolar or bipolar cells which are excitatory. However, the vast majority of interneurons are inhibitory and have different spike discharge patterns.

**Fast Spiking**

While there are many inhibitory cell types in the cortex, they can all be divided into one of three groups: cells expressing either parvalbumin (PV), somatostatin (SST) or those containing the serotonin receptor 5HT3a (Rudy et al., 2011). Inhibitory PV+ interneurons display fast spiking (FS) properties. These cells do not show spontaneous activity at rest and when active display trains of action potentials in quick succession with a fast repolarization phase that is not evident in RS cells (McCormick et al., 1985; Kawaguchi, 1995). For the most part, the PV+ interneuron group displays fast spiking properties and includes basket cells and chandelier cells. Basket cells represent the dominant form of inhibitory interneurons in the cortex whereas SST
and 5HT3a positive cells have much more diverse firing patterns including burst spiking and late spiking (reviewed by Rudy et al. 2010).

The different classes of interneurons arise from different areas of the developing cortex. However, these three groups are broadly defined and form numerous subgroups based on co-expression patterns. Early in development, cortical interneurons arise primarily from transitory central telencephalic structures including: the medial ganglionic eminence (MGE), and the caudal ganglionic eminence (CGE). Interneurons arising from these structures differ in expression patterns as well as firing properties. Cells emerging from the MGE express the LIM-homebox gene \textit{Lhx6}. LIM-homeobox gene expression is important during early periods of development and is highly conserved across species (reviewed by Hobert and Westphal, 2000). Cells migrating from the MGE express parvalbumin or somatostatin, but not calretinin (CR) (Lavdas et al., 1999). In contrast, CR positive interneurons arise from the CGE (Xu et al., 2004) and have different firing properties from cells originating from the MGE. CR containing cells do not display fast spiking properties and are of a more heterogeneous population unlike PV positive neurons which are all fast spiking (Zaitsev et al., 2005; Cauli et al., 1997).

These expression patterns are associated with differences in ion channel properties. For instance, there is a large diversity of potassium (K\textsuperscript{+}) channels and their expression patterns are correlated with the family of interneuron. CR interneurons are found to coexpress the calcium binding K\textsuperscript{+} channel SK2 and the voltage gated K\textsuperscript{+} channel Kv3.4. In contrast, PV neurons and SST neurons have high expression of Kv3.1 and Kv3.2 channels, respectively (Chow et al., 1999; Toledo-Rodriguez et al., 2004). These channels are associated with different firing properties in which SK2 channels are commonly expressed in RS firing cells and Kv 3.1 and Kv3.2 neurons display FS properties.
The physiological properties of these different cell types are related to their expression patterns. Expression patterns of different ion channels are also correlated to morphological properties as well as their laminar location. These factors taken together may give rise to the development of specific cortical circuits. Currently, there is little research regarding the specific physiological properties of inverted pyramidal cells which is an important factor when considering a possible functional role for this phenotype within the cortical circuit.

1.2.3: Associations between Morphology and Electrophysiology

The classification of a neuron’s morphological and electrophysiological properties overlaps such that morphology can often predict physiology and vice versa. For instance, pyramidal cells display regular spiking or bursting properties whereas many inhibitory interneurons are fast spiking. A neuron’s morphology is often able to predict its physiological role in the circuit.

Indeed, response properties in the visual and somatosensory sensory areas are correlated with cell morphology and laminar position. In the visual cortex, cells in a specific layer have certain receptive field properties. In general, the receptive field properties in the visual cortex can be divided into simple cells, which have distinct ON/OFF regions, and complex cells which do not have segregated ON and OFF zones (Hubel and Weisel, 1962). Cells in layer IV and the upper layer of layer VI receive direct thalamic input and have simple receptive fields which are orientation specific whereas layers further removed from thalamic input have complex receptive fields which lack specific excitatory and inhibitory zones. In general, layer IV spiny stellate cells are almost all simple cells while layers II/III, V and VI consist mostly of complex cells and are mostly populated by pyramidal cells. Moreover, these cells do not only differ in regard to laminar position, but also in morphology as well as their projections (Gilbert and Wiesel, 1979).
Simple cells receive input primarily, or exclusively, from the thalamus, whereas complex cells receive input from the thalamus as well as from intrinsic and corticofugal afferents, which mediate cortical output. Complex cells also form subcortical connections more often than simple cells (Singer et al., 1975). More specifically, layer IV simple cells in the visual cortex are reflective of the overlapping response properties of their thalamic inputs. The convergence of these afferents onto simple cells appears to give simple cells their orientation specific receptive field properties. Moreover, morphological differences may also account for differences in receptive field size. The afferents to the superficial sublayers of layer IV(a and b) have wider terminal arborizations, and thus larger receptive field sizes, than cells in the inferior sublayer of layer IV(c). The receptive fields found in pyramidal cells in layer VI can be simple or complex which may be related to the dendritic structure of those cells (Gilbert and Wiesel, 1979; Martinez et al., 2005).

Similar results have been observed in the barrel cortex of the rodent in which two classes of pyramidal cells in layer V give different responses to whisker stimulation, either regular spiking action potentials or short latency bursts (de Kock et al., 2007). These two classes of layer V pyramidal cells have also been observed in the visual cortex (Mason and Larkman, 1990). These two classes of pyramidal cells can be described in terms of their dendritic morphology. Cells in one class have thick apical dendrites which extend into layer I and have large cell bodies whereas cells in the other class have slender dendrites which can extend to layers II/III and have comparatively smaller somata. These cells will be referred to as thick tufted and slender tufted dendrites, respectively. These differences in morphology also reflect differing firing patterns and functional connectivity (Schubert et al., 2006). The slender tufted cells in layer Va make mostly intracortical connections and display regular spiking properties whereas the thick tufted cells in
layer Vb make numerous subcortical connections, including with the superior colliculus, pons and POM (Hoffer et al., 2005; Bureau et al., 2006), and have intrinsic bursting properties. In hippocampal pyramidal cells, bursting appears to be mediated by calcium currents (Wong and Prince, 1978; Johnston et al., 1980) and so a similar mechanism may be responsible for burst firing in cortical pyramidal cells (Kim and Connors, 1993, Robinson et al., 1993). However, it has been suggested that sodium conductance is responsible for some bursting behavior in some neocortical cells (Brumberg et al., 2000). It is also possible to initiate bursting behavior in nonbursting cells by blocking potassium currents, indicating both inward and outward currents are involved in bursting behavior (Mason and Larkman, 1990).

Receptive field properties appear to be related to a neuron’s projection target. Corticotectal neurons in layer V of the visual system have broad apical dendrites (Koester and O’Leary 1992) which contributes to their large receptive fields (Lemmon and Pearlman, 1981; Palmer and Rosenquist 1974) and are considered complex with binocular receptive fields (Harvey, 1980). In contrast, corticogeniculate cells found in layer VI are largely simple cells (Swadlow and Weyand, 1987) and have relatively narrow dendritic fields (Brumberg et al., 2003). The relationship between receptive field properties, cortical target, and discharge pattern, displays a high degree of specificity. For instance, X and Y geniculate cells (also known as magnocellular and parvocellular cells in the primate) have very different receptive field responses. While both classes of cells have a center-surround organization; X cells have smaller receptive field size and display a sustained response to constant stimulation, Y cells have a larger receptive field and display a more transient response (Van Hooser et al., 2003). Both classes of cells also form monosynaptic connections to simple cells in layer IV of the striate cortex, albeit to different sublaminae where X cells largely target IVa and Y cells mainly target layer IVb.
However, the ability to connect to a simple cell is not only dependent on receptive field properties, but also on the cell’s response pattern (Alonso et al., 2001, Alonso and Swadlow, 2005). Indeed, the axonal conduction velocity of projection neurons are related to their receptive field properties in which simple cells have typically slowly conducting axons as compared to complex cells (Swadlow and Weyland, 1987).

Similar results have been observed in the rodent barrel cortex. Neurons within this region receive thalamic input either from the ventral posterior medial nucleus of the thalamus (VPM) or the posteromedial nucleus of the thalamus (POm). Cortico-thalamic cells projecting to the VPM nucleus have rapid conduction velocities whereas POm projecting neurons have much slower axonal velocities (Kelly et al., 2001) which is presumably related to the morphology of their axons.

Receptive field properties are also determined by intracortical connectivity. Specifically within the barrel cortex, a neuron’s receptive field size is shaped by inhibitory connections. The administration of bicuculline methiodide, a GABA antagonist, causes an increase in receptive field size (Hicks and Dykes, 1983; Kyriazi et al., 1996). Receptive field size is also determined by the axonal projections they receive. In layer II/III of the barrel cortex, some neurons only respond to a single, principle whisker. These neurons receive column restricted layer IV inputs, which in turn restricts layer II/III receptive field size. However, layer II/III cells that respond to multiple whiskers receive cross columnar axonal projections from neighboring cells and axonal segments frequently overlap (Brecht et al., 2003).

The underlying nature of these anatomical pathways of morphologically distinct cells may be revealed in a neuron’s biochemistry. Indeed, two genotypically defined populations of layer V pyramidal cells in the barrel cortex and visual cortex have very similar properties. In
layer V, a subset of slender tufted and thick tufted cells express a promoter for the transcription factor \textit{ETV-1} (etv), and a promoter for a glycotransferase \textit{Glt25d2} (glt), respectively, in which etv neurons represent corticostriatal cells and glt neurons project to the thalamus and other subcortical areas. Once again, it was found that cells with specific projections also have distinct morphological and physiological properties (Groh et al., 2010; Doyle et al., 2008).

Furthermore, synaptic connections are not random; axons arborizing in a particular cortical layer preferentially connect to certain cell types within that layer. Numerous anatomical studies have revealed that sensory input from the thalamus to layer IV strongly connect to FS cells within that layer (Staiger et al., 1996; Cruikshank et al., 2010). It has also been revealed that FS cells within this layer are also likely to be interconnected (Gibson et al., 1999). While IB cells within layer V receive excitatory input from all layers in a cortical column, RS cells are more interconnected via intracolumnar connections (Schubert et al., 2001).

Cortico-thalamic cells within the sensory cortices (visual, auditory and somatosensory) of the adult rat express \textit{(GAP)-43} almost exclusively within layers V and VI (Feig, 2004). GAP-43 is initially expressed in the growth cone of developing neurons and aids in neurite outgrowth (Benowitz and Routtenberg, 1997; Aarts et al., 1998) and is associated with axonal regeneration and synaptic plasticity (Benowitz and Routtenberg, 1987). Knockout mice lacking GAP-43 die within a few days following birth. While it was found that GAP-43 was not necessary for the formation and outgrowth of the growth cone, knockouts had failed to create appropriate neuronal connections (Strittmatter et al., 1995). Notably, mice lacking in GAP-43 failed to develop barrels and had abnormal thalamocortical projections (Maier et al., 1999).

Cortico-thalamic cells within the top of layer VI of the somatosensory cortex target the VPM nucleus whereas neurons within the bottom of layer VI and within layer Vb target the POm.
(Killackey and Sherman., 2003; Bourassa et al., 1995). This pattern is similar to the organization of the visual cortex in which the lateral geniculate nucleus receives information from layer VI and the pulvinar receives input from layer VI and Vb (Bourassa and Deschenes, 1995).

Cortico-thalamic cells are glutamatergic and therefore can excite relay thalamic neurons. As stated previously, the receptive fields of simple cells found within the visual cortex are driven by the convergence of their thalamic afferents. However, in computational models, 200 thalamic cells must be activated synchronously in order to drive a single spiny stellate cell. But this can only occur if the thalamic cells have already been excited by cortical activity (da Costa and Martin, 2009). Within the barrel cortex, cortiothalamic cells originating from layer V have larger axons, with larger terminals, as compared to layer VI cortico-thalamic cells (Landisman and Connors, 2007). The larger the axonal terminal, the more neurotransmitter can be released and the greater the efficacy of that synapse (Pierce and Lewin, 1994). In this regard, layer V cortico-thalamic cells drive thalamic activity (Theyel et al., 2010). In this view, morphological features are correlated with projection targets which in turn influence the neuron’s physiology and synaptic partners. As inverted pyramidal cells have a very distinct morphological feature compared to the more common upright pyramidal cell, they may display physiological differences as well.

2. Cortical Circuitry

2.1: Formation of cortical circuits

Complex cortical processing relies on the ability of individual cells to form precise synapses which form initially during development. This process involves a combination of genetic factors, molecular cues, electrical activity and sensory experience.
The dendrites of a neuron are the principle means by which a neuron receives incoming information from nearby neurons. Furthermore, the integration of electrical activity by the dendrites can lead to the initiation of action potentials. Therefore, in order to understand the creation of cortical circuits, we too must understand the mechanisms which affect dendrite structure. During the course of development, dendritic arborization increases and branching becomes more complex. As dendrites grow out from the soma, they form numerous synapses with neighboring neurons. Indeed, it appears that increased neural activity leads to increased complexity in dendritic arborization (McAllister et al. 1996).

These changes appear to be mediated by the level of excitation the dendrite receives. In several studies, the presence of a glutamate antagonist leads to decreased arborization. This process appears to be mediated through the NMDA receptor (NMDAR) activation as blocking NMDAR’s alters dendritic arborization whereas blocking AMPA receptors does not reduce dendrite growth rates (reviewed by Cline 2001). Similarly, NMDARs are also involved in dendritic spine plasticity. During development, and throughout adulthood, many sensory areas go through periods of spine production and elimination. However, blocking NMDARs with the antagonist MK801 reduces the rate of spine elimination within the barrel cortex (Zuo et al., 2005).

Abnormal development of dendritic spines is a characteristic of some developmental disorders such as fragile X syndrome (Rudelli et al., 1985; Irwin et al., 2000). Fragile X syndrome is an inherited single gene disorder which affects the *Fragile X mental retardation 1* (*FMR1*) gene found on the X chromosome. This disorder is marked by severe deficits in cognitive, social and sensorimotor development. The *FMR1* gene is involved in the regulation of
protein translation which includes dendritic spine development and is characterized by excessive amounts of immature spines, indicating deficits in pruning as well as maturation.

This increase in immature spines appears to be at least partially regulated by glutamergic signaling as blocking metabotropic glutamate receptors (mGluRs) can lead to increases in spine immaturity (Cruz-Martin et al., 2010). Numerous studies have revealed that mGluRs directly activate and enhance NMDA responses (Awad et al., 2000; Pisani et al, 2001) suggesting a further role for NMDA in spine plasticity (Chen et al., 2012). Indeed, this role of NMDA can be further observed in the developing barrel cortex. Located in layer IV of the somatosensory cortex, the barrel cortex is a well defined model of cortical circuitry as each barrel represents a single whisker on the rodent’s mystacial pad. However mice which do not express the NR1 gene, which is essential for NMDARs, fail to develop barrels completely (Iwasato et al., 2000). Furthermore, studies within the frog visual system have observed that NMDA is involved in dendritic tree branching (Rajan and Cline 1998). This lends strong support for the role of glutamate in the process of guiding dendritic growth and thus the formation of cortical circuits.

However, dendritic growth is only the first phase in circuit development. Stabilization of dendritic arbors as well as synaptic strength must also occur for circuits to develop normally. The slowing of dendritic arborization in developing neurons appears to be mediated by Calcium/Calmodulin-dependent Protein Kinase II (CamKII) (Zou and Cline 1999). The regulation of dendritic growth due to CamKII signaling has been found across species: the tectum of the frog (Wu and Cline 1998), the barrel cortex of the rodent (Wilbrecht et al., 2010) and the visual cortex of the rodent (Mower et al. 2011).
The shape of the dendritic arborizations may in turn affect the neurons’ physiological properties. A computational model revealed that bursting activity increased as dendritic trees became larger whereas smaller dendritic trees produced fast spiking activity (Eyal et al., 2014; Mainen and Sejnowski, 1996). Thus the span of a dendritic tree, and its distribution of ion channels, may represent the differences in a neuron’s ability to integrate incoming activity.

There are numerous sources of activity which drive neuronal development and assist in establishing cortical microcircuits. Neurons appear to start the early circuit formation process while still in the subplate. The subplate is a transient layer that lies between the cortical white matter and layer VI of the cortex. Neurons in this layer are the first physiologically mature cells to enter the cortex. These subplate neurons are of a heterogeneous populations having numerous morphological subtypes including pyramidal, fusiform and inverted pyramidal. These cells differ in not only morphology, but in molecular expression as well which consist of several subclasses of growth, transcription and guidance factors (reviewed by Kanold and Luhmann, 2010). These cells have complex axonal projections which span the cortex and extend towards the thalamus. Subplate neurons may represent a transient relay between the thalamus and cortex and thus leads to the development of thalamocortical circuits. Removal of the subplate disrupts proper thalamocortical responses within the somatosensory cortex (S1) of the mouse as well as prevents the development of the barrels (Tolner et al., 2012). Similar effects can also be observed in the visual cortex of the cat (Kanold et al., 2003).

2.2: Connectivity

2.2.1: Cortical targets

Pyramidal cells can be classified into two major classes based on their targets: cortico-cortico projection neurons and subcortical projection neurons. The determinants of where a
neuron will project are based on morphological and physiological properties as well as laminar position. Cells in layers II/III, for example, make largely cortico-cortico connections whereas neurons in layers V and VI make subcortical connections. Recently, several genetic factors have been implicated in whether a neuron will project subcortically or callosally. Neurons in layer V which express \textit{Fezf2} and \textit{Ctip2} project to subcortical areas. \textit{Fezf2} also appears to be expressed only in pyramidal neurons and not in GABA-ergic neurons (Chen et al., 2008). In knockout mice studies, neurons which do not express these genes fail to make the appropriate connections. Interestingly, the physiological properties underlying these neurons were also affected. Indeed, knockout \textit{Fezf2} mice display firing patterns similar to callosal projecting neurons. Furthermore, knockout mice also had an abnormal dendritic morphology (Inoue et al., 2004). This lends strong support that neuronal physiology is related to its projection target.

\textbf{2.2.2: Cortical circuitry}

Cortical functions emerge from the diversity of cortical neurons and the dynamic and plastic properties of their synaptic connections (Griffen and Maffei, 2014; Espinosa and Stryker, 2012). These complex functions arise through interconnected circuits between cortical and subcortical areas, including the basal ganglia, hippocampus and thalamus. Cortical activity is facilitated by concurrent responses of excitation and inhibition (Wilent and Contreras, 2004; Tan et al., 2004; Atallah and Scanziani, 2009). For instance, a deflection of a rat’s whisker leads to a consistent response within the barrel cortex which consists of an initial EPSP followed by an IPSP (Wilent and Contrares, 2004).

The cortical column is a fundamental unit of cortical anatomy, in which all cells within a column have the same receptive fields indicating they receive similar inputs running vertically through the cortical layers (Mountcastle et al., 1957). However, neurons are also group
horizontally through intracortical connections (Lorente de No, 1949). Pyramidal neurons form selective intracortical synaptic partners based on their axonal targets. For instance, it was found that a cortico-tectal neuron is more likely to have feed forward connection to a neighboring cortico-tectal neuron versus a neighboring cortico-cortico neuron (Brown and Hestrin, 2009).

Not surprisingly, deficits in cortical function due to developmental disruption, injury, or genetic mutation underlie many neurological disorders such as epilepsy and cognitive impairment. An examination in a mouse model for Alzheimer’s revealed disorganized cortico-cortico circuits whereas fibers originating from the thalamus did not show as severe a degradation (Delatour et al., 2004). Greater knowledge of cortical neurons and their connections is therefore critical toward the understanding of the mechanisms of cortical function in the normal and diseased brain.

3. Inverted Pyramidal Cells

Recent years have seen an explosion of anatomical and physiological studies detailing the diversity of cortical cell-types including GABAergic interneurons (Ascoli et al., 2008; Ma et al., 2006; Xu et al., 2006) and pyramidal cells (Brumberg et al., 2003; Hattox and Nelson, 2007; Ramos et al., 2008; Staiger et al., 2004). Gene and protein expression studies have also revealed previously unknown cell-types (Hevner et al., 2003; Hevner, 2007; Nelson et al., 2006; Watakabe et al., 2007; Yamamori and Rockland, 2006). Continued discovery of novel cortical cell populations and subpopulations emphasizes the need for further quantitative studies examining individual cortical cells and their interconnected neuronal circuits.

Radially-oriented apical dendrites pointing toward the pial surface are a characteristic feature of nearly all pyramidal neurons. However, cortical pyramidal neurons with atypically-oriented apical dendrites pointing toward the cortical white matter have been recognized since
the time of Cajal and in every mammalian species examined (Ramon y Cajal, 1911).

Nevertheless the physiology and anatomy of these “inverted” pyramidal cells (IPCs) remains poorly understood (reviewed in Mendizabal-Zubiaga et al., 2007). Found almost exclusively in the infragranular layers (V and VI) of the cortex, IPCs are known to form intracortical and callosal projections but lack the cortico-fugalfugal projections to subcortical targets such as those made by other infragranular cells (Bueno-López et al., 1999; Reblet et al., 1992; reviewed in Mendizabal-Zubiaga et al., 2007). Thus, despite only representing a small percentage of cells in the cortex (depending on species and area examined: 1-8.5%; Globus and Scheibel, 1967; Parnavelas et al., 1977; Bueno-Lopez et al., 1991, Qi et al., 1999), IPCs are capable of participating in important cortical functions via local as well as interhemispheric projections.

In order to assess the physiological properties of IPCs, we recorded from layer VI IPCs and UPCs in the mouse and rat somatosensory system using the whole cell patch clamp method. The somatosensory cortex of the rodent was chosen as it contains the barrel cortex. The barrel cortex which receives input from the rodent’s whiskers and represents their primary source of sensory processing. The barrel cortex is also a model system of neuronal circuitry as it has a 1:1 correspondence between the cortex and the mystical pad (Woolsey and Van der Loos, 1970; Feldman and Brecht, 2005; Wu et al., 2011). This topography allows us to see clear patterns of cortical development as well as the circuitry underlying sensory processing. We investigated intrinsic properties by directly injecting current of varying intensity (0-300 pA) and examining the cells response. We further assessed synaptic properties by once again recording from layer VI cells, but stimulating the underlying cortical white matter and measured the cells response. Finally, we also examined specific morphological differences that exist between IPCs and UPCs beyond the direction of the apical dendrite.
Chapter Two: Aims

Aim 1-A: To identify the intrinsic physiological properties of inverted pyramidal cells in the mouse and rat compared to upright pyramidal cells

Coronal slices of primary somatosensory cortex (300 μm thick) were prepared from P14-21, CD1 mice or Sprague-Dawley rats of either sex on a vibratome. Slices were placed in a chamber perfused with oxygenated artificial cerebral spinal fluid. Neurons were visualized with infra-red differential interference contrast microscopy. Whole-cell current clamp recordings were conducted. Injection of depolarizing and hyperpolarizing current steps of increasing amplitudes will be used to measure intrinsic membrane properties.

Aim 1-B: Three-dimensional morphological reconstructions will be done using a microscope equipped with the digital reconstruction software, Neurolucida (MBF Biosciences Inc.). Morphological measurements of neuronal dendrites and somata were made using the associated NeuroExplorer software package (MBF Biosciences Inc.).

Aim 2: To assess and compare the interhemispheric and thalamocortical properties of inverted and upright pyramidal cell circuits in the mouse and rat. If they have similar physiological properties, this suggests that IPCs form distinct circuits which remain consistent across species.

Coronal sections were prepared as described above. We characterized synaptic properties by electrically stimulating the cortical white and gray matter and recording the excitatory responses from layer VI IPCs. The characteristics of the evoked Excitatory Post Synaptic Potentials (EPSPs) onto IPCs were analyzed in response to the different stimulation locations. Measures to be analyzed include: threshold for activation, latency, magnitude, duration, rise and fall times. We
statistically compared the responses between stimulation locations, and the results were also
compared to a set of upright pyramidal cells recorded from in the identical manner. To look at
synaptic dynamics, in addition to single pulses, trains of eight pulses were applied at varying
frequencies (1-20Hz) to see if summation onto IPCs is any different than onto upright pyramidal
cells or if it varies as a function of stimulation location. If IPCs do form functionally different
circuits than upright cells, then we expect there to be differences in the synaptic responses
between the two cell types due to their integration into distinct cortical circuits.
Chapter Three: Introduction:

Intrinsic Physiology and Morphology of Layer VI Inverted

Pyramidal Cells in the Somatosensory Cortex

The predominant neuronal cell in the cortex, and the major source for cortical output, is the pyramidal cell. As first observed by Ramon y Cajal, pyramidal cells feature a primary apical dendrite projecting towards the pial surface with many basal dendrites surrounding the soma. The apical dendrite is thicker and longer than the basal dendrites. However, a certain percentage of pyramidal cells feature atypically oriented primary dendrites. In the rat visual cortex, for instance, completely inverted pyramidal cells (IPCs) represent 1% of the neurons within the region (Parnavelas, et al. 1977). These inverted cells have their primary dendrite going towards the white matter, rather than the pial surface. These atypical pyramidal cells are present across sensory systems and species. Inverted pyramidal cells have been found in the cat visual cortex (Matsubara et al 1996), the anteater (Sherwood et al. 2009) and the chimpanzee sensorimotor system (Qi et al. 1999). In the case of the chimpanzee sensorimotor cortex, less than 1% of pyramidal cells were classified as inverted, but up to 8% had an atypical organization. The majority of the inverted pyramidal cells were found in layer VI of the chimpanzee cortex. Similarly, inverted pyramidal cells were also found mostly in layer V and VI in the cat auditory cortex (Prieto and Winer 1999) as well as in the rat somatosensory cortex (Valverde 1989). However, the potential function of these neurons remains largely unknown (reviewed in Medizabel-Zubiaga et al. 2007).

Inverted pyramidal neurons (IPCs) have also been found in abundance in several neurological disorders. Mutant reeler mice, for instance, are characterized by having abnormal
cortical development wherein neurons do not migrate to their proper laminar locations. As opposed to wild-type mice, reeler mice have significantly more IPCs. These IPCs are also found not only in greater number, but spread throughout all layers of the cortex (Landrieu and Goffinet 1981). IPCs have also been found in persons with agyria, a chromosomal disorder which is characterized by lissencephaly. In this case, IPCs were found in abundance in the superficial layers of the cortex (Bordarier et al. 1986). Once again, however, what role IPCs play in these disorders is not yet established.

An electrophysiological study examining sideways oriented pyramidal cells found that these atypical cells behave similarly to pyramidal cells within layer II/III of the juvenile rat (Brederode et al. 2000). However, the synaptic connectivity of these cells may be vastly different than pyramidal cells. IPCs for instance only appear to make cortico-cortical and cortico-claustrum connections (as reviewed by Mendizabal-Zubiaga 2007) while upright pyramidal neurons also project to non-telencephalic structures (Hallman et al. 1988, Kasper et al. 1994). IPCs are therefore capable of participating in important cortical functions via local as well as interhemispheric projections.

In this section, we quantitatively examined for the first time the intrinsic electrophysiological properties of IPCs and radially-oriented pyramidal neurons found in infragranular layers of the mouse and rat cortex. It is of interest to examine both species in order to better determine the function of these cells. That is, if they have similar physiological properties, it could demonstrate that IPCs form distinct circuits which remain consistent across species. Additionally, we utilized biocytin reconstructions in order to quantitatively analyze and compare the dendritic morphology of IPCs. Finally, we used perfusion of artificial cerebral spinal fluid lacking extracellular magnesium in order to test the role of inverted neurons during
periods of increased spontaneous activity. We observed both morphological and intrinsic physiological differences in IPCs between species as well as differences indicating that IPCs are integrated in distinct synaptic networks in rat versus mouse. Our results provide important data on the intrinsic properties of IPCs, reveal novel species differences in IPCs previously assumed to be homogeneous, and are relevant to models of information processing through micro- and larger neocortical circuits.
Chapter Four: Materials and Methods:

Intrinsic Physiology and Morphology of Layer VI Inverted
Pyramidal Cells in the Somatosensory Cortex

4.1. Preparation of slices

Coronal slices of primary somatosensory cortex (300 μm thick) were prepared from P14-21, CD1 mice or Sprague-Dawley rats (Charles River Laboratories) of either sex on a vibratome (VT1000S, Leica) in accordance with Queens College of the City University of New York IACUC and the National Institutes of Health guidelines for the use of laboratory animals and as described previously (Brumberg et al., 2003, Ramos et al., 2008). While there is evidence that hypopigmented mammals have abnormal visual pathways (Guillery et al., 1973; Guillery et al., 1989), the somatosensory cortex remains largely unaffected (Ramos et al., 2008). Animals were anesthetized with an intraperitoneal injection of Euthasol (Virbac AH Inc) until unresponsive to noxious stimulation (toe-pin). Following decapitation, the brain was quickly removed, blocked, and placed into ice-cold (4°C) oxygenated artificial cerebral spinal fluid (ACSF). ACSF contains (in mM) 125 NaCl, 2.5 KCl, 1 MgCl₂, 1.25 NaH₂PO₄, 2 CaCl₂, 25 NaHCO₃, and 25 d-glucose and was aerated with 95% O₂-5%CO₂ to a final pH of 7.4. Where indicated, slices were perfused with modified ASCF containing zero extracellular Mg²⁺ in order to elicit spontaneous bursting according to the protocol of Flint and colleagues (Flint and Connors, 1996; Flint et al., 1997).

4.2. Electrophysiological recordings

Neurons were visualized with infra-red differential interference contrast (IR-DIC) microscopy (Olympus BX51WI). Patch pipettes (4–7 MΩ tip resistance) were pulled on a Flaming/Brown microelectrode puller (P-97, Sutter Instruments). Pipettes were filled with (in
mM) 120 KGlUt, 10 NaCl, 20 KCl, 10 HEPES, 2 Mg-ATP, 0.3 Na-GTP, 0.5 EGTA, and 0.3–1%
iocytin (wt/vol) for subsequent visualization of the neurons (see following text). Once a stable
recording was obtained (resting $V_m$ of $<-55$ mV, overshooting action potentials, ability to
generate repetitive action potentials to a depolarizing current pulse), neurons were classified
according to discharge pattern in response to a constant depolarizing current pulse (1000 ms) as
intrinsically bursting, regular spiking, etc. (McCormick et al., 1985; Brumberg et al., 2000,
Ramos et al., 2008). Injection of depolarizing and hyperpolarizing current steps of increasing
amplitudes (10pA increments) were used to measure intrinsic membrane properties. Off-line
analysis of action potential and passive membrane properties was performed using Clampfit9
(Molecular Devices).

4.3. Histology and Neuronal Reconstruction

Following recordings slices were placed in cold fixative (4% paraformaldehyde in 0.1 M
phosphate buffer) and kept at 4°C for no more than 2 weeks. Biotin-avidin-HRP histochemistry
was preformed as described previously (Ramos et al., 2008). Slices were not re-sectioned. For
three-dimensional morphological reconstructions, the Neurolucida system (MBF Biosciences Inc)
was used in conjunction with an Olympus BX51 microscope using 4× (0.1 numerical aperture
(NA)), 10× (0.4 NA) and 60× (1.4 NA, oil) objectives. Digital images were taken using an
Optronics Microfire camera attached to a dedicated PC. Morphological measurements of
neuronal dendrites and somata were made using the associated NeuroExplorer software package
(MBF Biosciences Inc). Cells were classified as inverted if its principle dendrite was descending
towards the cortical white matter. The principle dendrite was determined for both upright and
inverted cells by examining dendrite diameter. The thickest dendrite to emerge from the soma
was considered to be the principle, or apical, dendrite. These measurements were made by using
the “quick measure line” tool within the Neurolucida program and placing a line across the
dendrite as it emerged from the soma, values for the four cell types were averaged for subsequent
analyses (see Table 2).

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<th>Soma</th>
<th>Basilar Dendrites</th>
<th>Apical Dendrites</th>
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<tr>
<td>Mouse IPC</td>
<td>44.7±1.27</td>
<td>1.53±0.05</td>
<td>4.38±0.46</td>
</tr>
<tr>
<td>Mouse UPC</td>
<td>41.86±3.59 *</td>
<td>1.33±0.06</td>
<td>3.7±0.57</td>
</tr>
<tr>
<td>Rat IPC</td>
<td>49.11±1.58 ††</td>
<td>1.39±0.07</td>
<td>7.39±1.02</td>
</tr>
<tr>
<td>Rat UPC</td>
<td>43.02±0.59 †</td>
<td>1.40±0.05</td>
<td>5.38±0.91</td>
</tr>
</tbody>
</table>

Table 2. Summary of somatic and dendritic characteristics of mouse and rat inverted and upright pyramidal cells. All data are reported as means ± standard error of the mean (SEM). Statistical significance (p<0.05) is reported with the symbols * † †.

4.4. Gogli staining and quantification of dendritic spines

Animals (CD1 mice, N = 13) of either sex at p80-90 were randomly selected. The brains
were immediately removed and rinsed in 0.1 M phosphate buffer (pH 7.13) for 3 minutes. After
the PB rinse, retrieved brains were immersed in a Golgi-Cox solution (FD Rapid Golgi Stain Kit,
FD Neurotechnologies Inc.) comprising potassium dichromate, mercuric chloride, and potassium
chromate. This mixture of solutions was replaced once after 12 hours of initial immersion, with
storage at room temperature in darkness for 2–3 weeks. After the immersion period in the Golgi-
Cox solution, the embedded brains were transferred to a cryoprotectant solution (FD Rapid Golgi
Stain Kit) and stored at 4°C for at least 1 week in the dark before cutting. The brain slices were
sectioned in the coronal plane at approximately 200–250 μm thickness on a freezing cryostat
(approximately −25°C). To prevent ice crystal damage, tissues were rapidly frozen with dry ice and quickly embedded in optimal cutting temperature (OCT) medium. Sliced tissues were transferred onto triple-dipped gelatin slides and were coated with additional cryoprotectant solution. Cut sections were air dried at room temperature in the dark for at least 2–3 weeks before further processing. After drying, sections were rinsed with distilled water and were subsequently stained in a developing solution (FD Rapid Golgi Stain Kit) and dehydrated with 50%, 75%, 95%, and 100% ethanol. Finally, the sections were defatted in xylene substitute and coverslipped with either Permount (Fisher Scientific, Fair Lawn, NJ) or SHUR/Mount (Triangle Biomedical Sciences, Inc.). Neurons were reconstructed using the Neurolucida software (see above) and only the cells that exhibited complete Golgi impregnation with a limited amount of staining artifacts were traced. The number of spines per dendrite were quantified using a 100x oil immersion lens (NA=1.40) and the length of the apical and basilar dendrites were measured. Spine density was determined by dividing spine number by dendritic length in microns and multiplying by 100 to present the data as spines/100 µm.

4.5. Sholl Analysis

A Sholl analysis was performed in order to determine if there were differences in apical and basal dendritic branching patterns between inverted and upright mouse and rat pyramidal cells. Neuroexplorer (MBF Biosciences Inc.) was utilized to conduct the analysis. Dendrites were analyzed in increasing distances (at 10µm intervals) from the soma using concentric rings centered on the soma. Quantitative measurements were taken including dendritic branch length, branching points (nodes), intersections and endings.

4.6. Quantitative Comparisons
Statistics were computed using the Statistica software package (StatSoft) for within-group and between-group analyses. One-way and repeated measures ANOVAs were conducted and Tukey's HSD post hoc test were used to determine the source of the variance, if any. Specific comparisons were made between different cell types found in the same species (i.e. rat IPCs vs. rat UPC) as well as similar cell types found between the two species (i.e. rat IPCs vs. mouse IPCs). Statistical significance was set at \( p < 0.05 \). All data are reported as means ± standard error of the mean (SEM) unless otherwise noted.
Chapter Five: Results:

Intrinsic Physiology and Morphology of Layer VI Inverted Pyramidal Cells in the Somatosensory Cortex

5.1. Intrinsic properties of IPCs in infragranular layers of rat and mouse cortex

IPCs in both rat and mouse cortical slices were readily identifiable using IR-DIC (Figure 2A) and could be unequivocally confirmed following 3D morphological reconstruction of biocytin-filled neurons (see below). A representative photomicrograph of an IPC in a mouse cortical slice is shown in Figure 2A. We recorded from a total of 40 and 24 IPCs in the mouse and rat neocortex (respectively) and for comparison 38 and 46 upright pyramidal cells (UPCs) in the mouse and rat neocortex (respectively).

A number of intrinsic membrane properties of these cells were examined such as the resting membrane potential, which was determined soon after whole-cell configuration was achieved. Resting membrane potential data for all cell types are shown in Figure 2B. Mouse IPCs exhibited an average resting membrane potential of $-69.68\pm0.89$ mV, which was similar to that observed in rat IPCs ($-67.48\pm1.04$ mV) and mouse UPCs ($-67.94\pm0.67$ mV). A one-way ANOVA revealed no significant difference between the resting membrane potential of mouse and rat IPCs nor were there differences between mouse IPCs and UPCs, or between mouse and rat UPCs ($-69.99\pm0.83$ mV). Significant differences in resting membrane potential were found between IPCs and UPCs in rats ($p < 0.04$) as well as between mouse and rat UPCs ($p < 0.02$) suggestive of differences in conductances that typically regulate resting membrane potential such as those mediated by $K^+$ ions (reviewed in Lesage, 2003).
The input resistance of all recorded neurons was calculated by the slope of a line fitted to the current vs. voltage relationship for small amplitude hyperpolarizing currents steps (25pA increments). Calculations were derived from peak voltage responses. As shown in Figure 2C, the average input resistance calculated for mouse IPCs was 454.95±35.98 MΩ, whereas the average input resistance for rat IPCs was 609.85±57.15 MΩ. A one-way ANOVA revealed no significant difference between the input resistance of mouse and rat IPCs nor where there differences between mouse IPCs and UPCs (388.98.83±35.73), or differences between mouse and rat UPCs (289.73±26.64). Significant differences in input resistance were found between rat IPCs and UPCs (p < 0.001) and between mouse and rat IPCs (p < 0.04). Thus, both input resistance and resting membrane potential are different between rat IPCs and UPCs.
Figure 2. Visualization and recording of inverted pyramidal neurons. (A) IR-DIC photomicrograph of a mouse slice maintained in vitro where the somata (asterisks) and apical dendrites of numerous UPCs (up arrows) can be seen as well as an IPC (down arrow). Measurements of the resting membrane potential (B) and input resistance (C) of the recorded neurons grouped by species and cell-type. Asterisks denote significant differences between groups (p<0.05). The boundary of the box closest to zero indicates the 25th percentile while the boundary of the box farthest from zero indicates the 75th percentile. Solid lines within the boxes mark the median while dashed lines mark the population mean. Error bars above and below the box indicate the 90th and 10th percentiles. Scale bar in A: 30μm.
A number of suprathreshold response properties were investigated in IPCs in both rat and mouse cortex in response to depolarizing current steps including action potential threshold, half-width, and amplitude. As shown in Figure 3A, IPCs in mouse displayed an average action potential threshold of $-38.60\pm1.41\text{mV}$ while IPCs in rat displayed a similar average threshold of $-38.51\pm0.84\text{mV}$. These data were not significantly different nor were comparisons made between rat UPCs ($-42.69\pm0.85\text{mV}$) and mouse UPCs ($-42.64\pm1.13\text{mV}$). In contrast, significant differences were only observed when comparisons were made between rat IPCs and UPCs ($p < 0.001$). The finding that rat IPCs displayed more depolarized action potential thresholds are indicative of differences in cellular excitability (see below).

The average action potential width at half-amplitude was computed for all cell types (Figure 3B). Action potential widths of mouse IPCs (recordings done at $\sim22^\circ\text{C}$) was $2.36\pm0.09\text{ ms}$ compared to $2.25\pm0.09\text{ ms}$ which was observed for rat IPCs. Significant differences were observed between mouse UPCs ($1.56\pm0.06\text{ ms}$) and IPCs ($p < 0.001$) as well as comparisons between rat UPCs ($1.79\pm0.06\text{ ms}$) and IPCs ($p < 0.001$). We did not find differences between rat and mouse IPCs or between rat and mouse UPCs. Thus, both action potential threshold and width were found to be different in both species based on cell-type.

As shown in Figure 3C, action potential amplitude was also measured in mouse ($82.57\pm1.44 \text{ mV}$) and rat ($77.13\pm1.92 \text{ mV}$) IPCs as well as in mouse ($82.40\pm0.90 \text{ mV}$) and rat ($80.16\pm1.04 \text{ mV}$) UPCs. Comparisons between all groups revealed no significant differences.

Action potential rise times were calculated by subtracting the time for the action potential to reach peak voltage from the time just before threshold. Rise times were only calculated for the first action potential generated in response to a depolarizing current pulse that just exceeded threshold in mouse ($0.95\pm0.02\text{ ms}$) and rat ($0.94\pm0.01\text{ ms}$) IPCs as well as in mouse ($0.97\pm0.02$
ms) and rat (0.93±0.08 ms) UPCs. Comparisons between all groups revealed no significant
differences. In contrast, significant differences were observed in comparisons of action potential
fall times. Fall times were calculated by measuring the time between peak voltage of the action
potential and its return to baseline. In particular, the fall times of rat IPC (0.5±0.04 ms) were
faster and differed significantly (p<0.01) from mouse IPCs (0.75±0.04 ms). In addition, the fall
times of rat UPCs (0.52±0.06 ms) were faster and differed significantly (p<0.02) from mouse
UPCs (0.81±0.09 ms). All other comparisons were not significantly different. Similar to the
findings from our analysis of resting membrane potential, we observed differences among the
cell-types for conductances that are typically carried by the potassium ion.
Figure 3. Measures of active membrane physiology to electrical stimulation. Comparisons of action potential threshold (A), half-width (B), and amplitude (C) reveal differences among the groups. Asterisks denote significant differences between groups (p<0.05). The boundary of the box closest to zero indicates the 25th percentile while the boundary of the box farthest from zero indicates the 75th percentile. Solid lines within the boxes mark the median while dashed lines mark the population mean. Error bars above and below the box indicate the 90th and 10th percentiles.
To assess potential differences in excitability, the number of action potentials elicited by suprathreshold current steps was measured in all cell types. Examples of repetitive spiking in response to increasing stimulation in a mouse and rat IPC is shown in Figure 4A. All cell-types displayed a regular spiking phenotype in both species. As shown in Figure 4B, all cell types exhibited increases in the number of spikes elicited by increasing current injection. Repeated measures ANOVA indicated that all cell-types displayed significant increases in action potential number with increasing current steps (all comparisons: p < 0.001) and a significant interaction was observed in current injection amplitude vs. cell-type (p < 0.001). Interestingly, as shown in Figure 4B, rat IPCs displayed asymptotic levels of firing to current steps >100pA while all other groups continued to display increases in action potential number up to ~200pA. Mouse IPCs discharged more action potentials than rat IPCs and a repeated-measures ANOVA revealed a significant difference in the number of spikes elicited to increasing current steps between rat and mouse IPCs (F(31,33)=3.60, p <0.001) as well as a significant interaction between current injection amplitude vs. cell-type (p < 0.001). Similar significant differences were found between rat UPCs and rat IPCs ((F(31,38)=2.68, p < 0.01), with UPCs discharging more action potentials than IPCs. Significant differences were also observed between rat UPCs and mouse UPCs (F(31,65)=4.28,p<.0001). In contrast, no differences were observed between mouse IPCs and UPCs. Moreover, rat IPCs emitted the fewest numbers of action potentials even in response to strong stimulation intensities.
Figure 4. Response properties of neurons to increasing electrical stimulation. (A) Representative example of repetitive firing to increasing 1 sec depolarizing current steps (left-right: +80, +110, +150 in pA) in a mouse and rat IPC (baseline membrane voltage = -70mV and -72mV for mouse and rat, respectively). (B) Measures of increasing action potential discharge and maximal firing frequency (C) of recorded neurons, means, and one standard error of the mean are shown. Calibration in A: 200ms, 40mV.
The maximum firing frequency (Hz) recorded in all cells was compared in response to increasing current steps. For each cell, the maximum firing frequency was always observed at the beginning of each response (i.e. the first inter-spike interval that was recorded). As shown in Figure 4C, increases in the maximum firing rate were exhibited by all cells in response to increasing current injection. A repeated measure ANOVA performed on these data indicated significant increases in firing with increasing current intensity for all groups (p < 0.001) as well as a difference between cell types (p < 0.01). Moreover, a significant interaction was observed between current injection amplitude vs. cell-type (p < 0.001). We examined these differences further and performed analyses comparing specific pairs of cell types. These analyses revealed significantly greater frequency firing displayed by mouse IPCs compared to UPCs (F(29.49)=7.7, p<0.001). Similar differences were observed between mouse IPCs vs. rat IPCs (F(29,34)=1.910, p<0.001). Significant differences were also observed between the maximum firing frequency between rat IPCs and UPCs (F(29,63)=3.465, p<0.001). No significant differences were found in comparisons of mouse UPCs with rat UPCs (F(29,78)=1.022, p=0.45). Overall, mouse IPCs had the highest maximum firing frequency and thus were capable of firing faster frequency action potentials compared to all other cell-types.

5.2. Synaptic properties of IPCs in 0[Mg$^{2+}$] ACSF studies

We used 0mM Mg$^{2+}$ in the extracellular ACSF in order to induce spontaneous activity in cortical slices as was first described by Connors and colleagues (Flint and Connors 1996; Silva et al., 1991) and has been shown to increase glutamatergic transmission via NMDA receptors that would be otherwise blocked by Mg$^{2+}$ ions. While often used as an in vitro model of epilepsy, we used 0mM Mg$^{2+}$ as a tool to assess cortical activity in all cell types under periods of increased synaptic activity. Under control conditions, all recorded neurons from both species did not
exhibit spontaneous action potential discharge. Furthermore, spontaneous subthreshold postsynaptic potentials were not of sufficient frequency for quantitative analysis. However, following the perfusion of slices with 0mM Mg\(^{2+}\) ACSF, we observed action potential bursting in a subset of neurons from both cell types and both species despite a lack of change in the resting membrane potential indicative of a synaptic mechanism underlying bursting (Flint and Connors, 1996; Flint et al., 1997). Figure 5 contains a representative example of bursting in a mouse IPC with varied numbers of action potentials during 0Mg\(^{2+}\) ACSF bath perfusion. We observed bursting in 8 of 23 (34.78%) mouse IPCs, 7 of 21 (33.33%) rat IPCs, 4 of 16 (25%) mouse UPCs, and 8 of 30 (26.67%) rat UPCs. Chi-square analyses of the number of cells that showed bursting in each group revealed no significant differences.

Figure 5. Burst firing in response to removal Mg\(^{2+}\) from the ACSF. (A) Representative example of burst firing in a mouse IPC following perfusion of 0mM Mg\(^{2+}\) ACSF (Baseline membrane voltage = -71mV). (B-C) High magnification of two segments shown in A, which reveal different numbers of action potentials present during burst events. Calibration: A = 10secs, 50mV; B = 500ms, 50mV.
The frequency between bursts containing action potentials was calculated and is shown in Figure 6A. Although all groups revealed average inter-burst intervals (IBI) below 1Hz, significant differences in average IBI were observed between mouse IPCs and UPCs (p<0.05; one-way ANOVA) with mouse IPCs (0.38±0.04 Hz) bursting less frequently than mouse UPCs (0.22±0.04). We also found significant differences between mouse IPCs and rat IPCs (p<0.001; one-way ANOVA) with rat IPCs (0.18±0.02 Hz) bursting more frequently than mouse IPCs. Thus, under conditions of increased synaptic activity, action potential bursting varied according to cell-type and species which may indicate differences in the network configurations in which these respective cells are embedded.

Figure 6. Properties of burst firing during 0mM Mg\(^{2+}\) ACSF experiments. Average time between bursts (A), average number of action potential during bursts (B), average firing (C), and maximum firing (D) frequency during bursts. Asterisks denote significant differences between groups (p<0.05). Plots represent population means and error bars indicate standard error of the mean.
The number of action potentials observed during bursting also varied widely between the different cell types. A representative example of a mouse IPC that exhibited from two up to nine action potentials per burst is shown in Figure 5. The average number of action potentials observed during bursting for all cell types is shown in Figure 6B. Mouse UPCs displayed an average of slightly more than 4 action potentials per burst (4.35±1.71) compared to all other cell types which displayed less than 4 action potentials per burst (mouse IPCs=3.47±0.37; rat IPCs 3.38±0.55; rat UPCs=3.72±0.56). However, comparisons between groups did not reveal any significant differences in the number of action potentials per burst. We also examined the average and maximum frequency of action potential discharge during bursts (F(3,22)=0.29, p=0.83). These data are shown in Figure 6C and 6D, respectively. Both types of cells in the rat displayed greater average and maximum action potential discharge during bursts compared to both mouse cell types. However significant differences were found for comparisons of average (F(3,22)=13.28, p<0.0001) and maximum frequency(F(3,22)=11.46, p<0.0001) discharge bursts. Tukey’s HSD was used to evaluate post hoc differences. Rat and mouse IPCs revealed significant differences for both average (p<0.001) and maximum (p<0.01) frequency discharge during bursts. Similar differences were found for comparisons between rat and mouse UPCs for measures of average (p<0.01; One-way ANOVA) and maximum (p<0.01; One-way ANOVA) frequency discharge during bursts. No differences were observed for comparisons between rat UPCs and rat IPCs or for comparisons between mouse IPCs and mouse UPCs. These data indicate that regardless of cell-type, rat neurons emit faster frequency action potentials during bursting compared to mouse cells even though all cell-types in both species have similar numbers of action potentials per burst.

5.3. Morphological properties of biocytin-reconstructed IPCs in rat and mouse cortex
Three-dimensional reconstruction of biocytin-filled neurons was used in order to determine more detailed morphological characteristics of physiologically-identified IPCs in rat (n = 17) and mouse (n = 21) as well as upright pyramidal cells in rat (n=16) and mouse (n=10). Representative examples of biocytin-filled and reconstructed mouse and rat cells are shown in Figure 7 and 8, respectively. As multiple cells were recorded from a single slice prior to fixing the tissue, our biocytin-filled cells were such that they did not allow us to quantify dendritic spines, but previous work (Chen et al. 2009) has shown that these cell types do possess dendritic spines and are assumed to be excitatory. Other anatomical studies have also confirmed the presence of spines on IPCs in rat (Parvenalas et al., 1997), rabbit (Mendizabel-Zubiaga et al., 2007) and chimpanzee (Qi et al., 1999). Perhaps due to not immediately fixing slices following recordings, several cells may have incomplete dendritic trees. However, all cells were recorded similarly and processed in an identical fashion and still morphological differences were observed despite this limitation. We examined a number of morphological metrics related to somatic and dendritic compartments including both apical and basilar dendrites and those features found to be significantly different are shown in Table 2. In order to determine whether a pyramidal cell was upright or inverted, we examined the diameter of the dendrite emerging from the apical shaft. Apical dendrites are thicker than basal dendrites and their thickness can vary from a diameter of 1 µm (Larkamn and Mason 1990; Lederberger and Larkum, 2010) to greater than 10 µm (White and Hersch 1982). IPCs had an average diameter of 2.33±0.19 µm and 2.54±0.14 µm for the mouse and rat, respectively. Similar results were found for mouse UPCs (2.67±0.1 µm) and rat UPCs (2.47±0.11). Statistical analysis showed no significant differences between groups (One-way ANOVA; F(3,39)=0.620, p=0.6).
Figure 7. Representative photomicrographs of biocytin-filled IPCs and UPCs. Filled neurons following physiological experiments in the mouse (IPC: A-B; UPC: C-D) and rat (IPC: E-F; UPC: G-H) cortex. Black line above neurons indicated layer V/VI border. Micrographs are taken at single focal plane. Scale bars in A, C, E, G = 250; B, F = 60; D, H = 30 (all in μm).
Figure 8. Morphological reconstructions of inverted and upright pyramidal neurons following biocytin histochemistry. Representative reconstructions of mouse (A) and rat (C) IPCs. Representative reconstructions of mouse (B) and rat (D) UPCs. Black line below cells indicates layer VI-white matter border. All scalebars = 100µm.

We observed significant differences in the somatic perimeter measurements between rat and mouse cells (One-way ANOVA; F(3,63)=4.469, p<0.01). Reconstructed rat IPCs had larger somatic perimeters (49.11±1.58 µm) compared to that seen in mouse (44.70±1.27 µm) IPCs. However, post hoc analysis (Tukey’s HSD) revealed this was not a significant difference. There were also no significant differences between the mouse (41.86±1.14 µm) and rat (43.02±1.49 µm) UPCs or between mouse IPCs and UPCs. However, rat IPCs had larger soma perimeters as compared to rat UPCs (p<0.03) as well as compared to mouse UPCs (p<0.05). Interestingly, comparing somatic area of rat (152.61±9.21) and mouse (138.44±8.46) IPCs and UPCs yielded no significant differences (F(3,65)=1.95, p=0.13).
Qualitatively, rat IPCs and UPCs appeared to have more numerous and elaborate dendritic processes in both apical and basilar dendrites which was confirmed by quantitative analyses. Specifically, we observed a greater number of dendritic nodes (branches) on apical dendrites (6.17±1.15) as well as basilar dendrites (7.39±1.02) in rat IPCs and UPCs (apical: 4.88±0.56; basilar: 5.38±0.91) than in mouse IPCs (apical: 3.86±0.55; basilar: 4.36±0.46) and UPCs (apical: 3.9±0.92; basilar: 3.7±0.67). Statistical comparisons (one-way ANOVA) of these metrics between rat and mouse revealed no significant differences for number of nodes in basilar dendrites (F(3,70)=2.35, p=0.08) or in apical dendrites (F(3,62)=1.66, p=0.18). We also observed greater total length of apical (840.07±149.81 µm) and basilar dendrites (1037.97±117.28 µm) in rat IPCs than in mouse (apical: 400.14±61.03 µm; basilar: 496.04±54.95 µm) as well as in rat UPCs (apical: 420.35±54.62; basilar: 521.41±59.29) and mouse IPCs (apical: 325.30±41.07; basilar: 425.19±59.45). Statistical comparisons (One-way ANOVA) of these metrics between rat and mouse cells revealed significant differences for total length of apical (F(3,62)=5.40, p<0.01) and basilar dendrites (F(3,70)=6.42, p<0.001). Post hoc analysis (Tukey’s HSD) revealed both greater apical and basilar dendrite length in rat IPCs as compared to all other groups. Similar significant differences were found for comparisons of mean length of basilar dendrites (F(3,70)=7.39, p<0.01) which were longer in rat (249.72±28.83) than mouse (134.18±14.95) IPCs as well as compared to rat (137.09±19.66) and mouse (97.63±9.94) UPCs. In contrast no significant differences were found between mouse and rat UPCs nor between the IPCs and UPCs of the mouse. Finally, total dendritic surface area was also significantly different between groups (F(3,70)=9.45, p<0.0001). Post hoc comparisons (Tukey’s HSD) revealed rat IPC dendrites (3513.20±379.88) was significantly greater than that in mouse IPCs (1684.45±153.17; p<0.05) and rat (950.94±88.70; p<0.001) and mouse (838.70±126.14; p<0.001) UPCs. Once again, no
significant differences were found between rat and mouse UPCs. Thus, as was observed in analyses physiological properties, rat and mouse IPCs display differences in morphological parameters. Overall, rat cells appeared to have larger somata as well as longer and more branched dendrites.

5.4. Sholl analysis

Sholl analysis was utilized to determine the complexity of the reconstructed dendrites. The number of intersections and dendritic length in 10 μm radii away from the soma were calculated as described in Materials and Methods. Figure 9 illustrates the differences in dendritic length between the mouse and rat as a function of distance from the soma. We compared the results of the Sholl analysis on 22 rat IPCs and 26 mouse UPCs cells using a one way repeated measures analysis of variance (ANOVA) in order to evaluate dendritic complexity. Comparisons were also conducted on 16 rat UPCs and 10 mouse UPCs. We found that there were a significant difference between rat and mice cells in terms of the number of intersections for their apical dendrites (F(90,114.61)=1.55, p< 0.05) as well as basilar dendrites (F(87,126.55)=1.58 p<0.01). Tukey's HSD revealed that both apical and basilar dendrites from mouse IPCs differed significantly from mouse UPCs (apical: p<0.01; basilar: p<0.01). Similarly the apical and basilar dendrites of rat IPCs and rat UPCs were significantly different (apical: p<0.001; basilar: p<0.001). However, mouse and rat IPCs did not differ from each other significantly (apical: p=0.25; basilar: p=0.9) nor did mouse and rat UPCs (apical: p=0.98; basilar p=0.12). However, both IPCs and UPCs had similar branching patterns with apical and basilar dendrites showing no significant differences (apical: F(57,140.96)=0.91, p=.65), p=0.58; basilar: F(54,158.74)=0.94., p=0.60). There were also no significant differences in the number of dendritic endings, as function of distance from the soma, of either the apical (F(93,111.64)=0.88, p= 0.73) or basilar dendrites
(F(84,129.52)=1.03, p=0.43). However, while total dendritic length was also not significantly different for the apical dendrites for comparisons involving UPCs and IPCs (F(102,102.71)=0.1.33, p=0.08), significance was found examining the total length of basilar dendrites (F(105,252.45)=2.24, p<0.001). Mouse IPCs did not differ from rat IPCs (p=0.98), but mouse UPCs were significantly different from rat UPCs (p<0.05). Mouse IPCs also differed from mouse UPCs (p<0.001) and rat IPCs differed significantly from rat UPCs (p<.001). The lack of differences in dendrite morphology may be considered unsurprising as these are reflected in UPCs. While the rat brain is bigger than the mouse, dendritic morphology may be conserved across species (Routh et. al. 2009).

**Figure 9**

*Figure 9. Sholl analysis.* Graph representing average dendritic length of the apical and basilar dendrites of mouse and rat UPCs (A and C) and IPCs (B and D) as a function of distance from the soma (in μm). Error bars represent standard deviation.
5.5. Comparison of spine density between Golgi labeled IPCs and UPCs in the mouse

Many studies have found evidence for dendritic spines on both IPCs and UPCs across species including rat, rabbit and chimpanzee (Parvenalas et al., 1997; Mendizabel-Zubiaga et al., 2007; Qi et al., 1999). Using our Golgi impregnated tissue, we found that mouse IPCs have dendritic spines as well (figure 10A). Here, we have now compared spine density of IPCs to that of UPCs in the mouse (figure 10B). To best quantify the number of spines, 5 IPCs and 8 UPCs were reconstructed from the somatosensory cortex of Golgi stained tissue. An independent measures t-test was used to evaluate any differences between the two cell types. The spine density of basilar dendrites of mouse UPCs were on average 8.64±3.09 spines per 100 µm of dendritic length whereas the density of basilar dendrites on IPCs was 12.34±2.60 spines per 100 µm. However, this difference was not statistically significant (p = 0.42). Similar results were found for the apical dendrites with UPCs having on average 14.26±4.20 spines per 100 µm and IPCs having an average of 13.33±3.29 spines per 100 µm. Once again, there were no statistical differences between the two cell types (p = 0.88). Finally, the mean total spine density (apical+basilar spines) for UPCs was 11.15±3.68 spines per 100 µm and 11.64±6.90 spines per 100 µm for IPCs. Once again, these differences were not statistically significant (p = 0.77). This further confirms that mouse IPCs have spines as previously seen in the rat and other species (Parvenalas et al., 1997; Mendizabel-Zubiaga et al., 2007; Qi et al., 1999). Furthermore, it was found that spine density is also similar between IPCs and UPCs (figure 9C). It was also found that neither apical nor basilar dendritic length of UPCs (883.35±212.95) in our Golgi sample was significantly different from that of IPCs (825.92±157.44; p=0.85) which is similar to our findings from our biocytin filled neurons (see above).
Figure 10. Gogi labeled images of UPCs and IPCs. Representative dendrites and spines of labeled UPCs at low magnification (A; scalebar = 50 μm) and high magnification (insert scalebar = 25 μm) and IPCs at low magnification (B; scalebar = 25 μm) and high magnification (insert scalebar = 25 μm). The graph (C) illustrates the spine density of mouse UPCs and IPCs in the apical and basilar dendrites as well as the density for apical+basilar (total) dendrites. WM = cortical white matter.
Chapter Six: Discussion:
Intrinsic Physiology and Morphology of Layer VI Inverted
Pyramidal Cells in the Somatosensory Cortex

6.1. Intrinsic properties of IPCs

In this part of the dissertation, we describe the physiology and morphology of pyramidal neurons in infragranular layers with apical dendrites pointing toward the white-matter (IPC) versus those pointing toward the pia (UPC) in both rat and mouse neocortex. Numerous differences were observed between IPCs and UPCs within species for sub- and suprathreshold measures. For example, resting membrane potential and input resistance were different between IPCs and UPCs in the rat, suggesting that potential differences in the ion channels regulating this intrinsic membrane physiology exists such as so-called “leak” K⁺ channels (reviewed in Lesage, 2003). In addition, action potential thresholds were higher in IPCs compared to UPCs in both rats and mice. These results suggest possible differences in the activation voltages (Colbert and Pan, 2002) and/or spatial configuration (Grubb and Burrone, 2010) of ion channels which contribute to action potential initiation such as voltage-gated Na⁺ channels. Consistent with potential differences in ion channel expression such as K⁺ channels among the different species, both rat cell-types exhibited faster action potential fall times compared to both mouse cell-types. There were also differences found between cell-types found in the same species.

Measures of repetitive firing revealed differences between cell-types found in the same species, as well as species differences for similar cell-types. For example, among all cell-types examined, rat IPCs generated the fewest action potentials to levels of current injection that strongly excited both mouse cell-types as well as rat UPCs. In contrast, we observed that mouse
IPC cells were capable of greater maximum firing frequencies compared to rat IPCs but were similar to mouse and rat UPCs. These data suggest possible limitations in the integrative properties of rat IPCs to encode high frequency and/or high intensity stimuli (Brumberg, 2002) compared to mouse IPCs. These data are relevant given the fact that IPCs in both species are found in both sensory and motor cortices and likely participate in sensory and motor functions via connection within and between hemispheres.

6.2. Bursting properties of IPCs

ACSF with 0 mM Mg\(^{2+}\) was used as a tool to assess synaptic activity in all cell types under periods of increased activity and as an indirect measure of the cortical circuits in which these cell-types are embedded. We found that a subset of neurons of both cell-types and in both species displayed rhythmic bursts of action potentials. Although all cell-types discharged similar numbers of action potentials per burst, we found differences in the time between bursts and in the frequency of action potential discharge during bursts among different cell-types. These data suggest that the different cell-types are part of distinct intracortical synaptic networks that are differentially activated by perfusion with 0 mM Mg\(^{2+}\). Further studies will be necessary to reveal whether IPC and UPC networks vary within infragranular layers as well as across neocortical lamina. Given that IPCs make interhemispheric projections (reviewed in Mendizabal-Zubiaga et al., 2007), these data are also relevant toward greater understanding of information processing via callosal connections.

6.3. Morphological differences of IPCs and UPCs

We examined the morphology of IPCs in both rat and mouse following biocytin reconstruction which confirmed that we indeed recorded from IPCs. While several filled cells may have incomplete dendritic branching, all cells were recorded similarly and several
differences were found between cell-type as well as species. These analyses revealed that rat IPCs are larger in only some dendritic and somatic measures. However, the differences in these morphological features are not to scale with differences in cortical thickness between rats and mice, which is ~2:1. Larger dendritic architecture and more complex branching likely relate to the number and spatial extent of synaptic inputs that each cell type receives. Therefore, one interpretation of our morphological analyses is that IPCs from the different species have different complement of synaptic afferents, a finding that is supported by the differences we observed in spontaneous bursting recordings where we observed more frequent bursting and faster firing within bursts in rat IPCs compared to mouse IPCs. Furthermore, the smaller size of the mouse IPCs may allow for faster membrane discharge accounting for the increased firing frequency exhibited by these cells. It has been noted that mice whisk at higher frequencies than rats (Mitchenson et al., 2011; Jin et al., 2004) and as their function relates to sensory and motor processing, rat and mouse IPCs may have different neocortical processing domains within and between sensorimotor circuits.

Despite decades of intense investigation, the diversity of cortical neurons continues to be revealed and has been recently aided by novel molecular, genetic, and physiological methods. Comparison of pyramidal cell-types within and across cortical lamina has revealed numerous similarities and important differences between physiological and/or anatomical measures. For example, layer V neurons in the somatosensory cortex that project to subcortical targets (spinal cord, brainstem, tectum), have thick apical dendrites with large dendritic tufts that reach the pial surface and display burst-type electrophysiological properties (Hattox and Nelson, 2007; Kasper et al., 1994; Rumberger et al., 1998). In contrast, layer V neurons that lack subcortical projections have thin apical dendrites with small-medium dendritic tufts and display a regular-
spiking phenotype (Kasper et al., 1994; Rumberger et al., 1998). Thus, neurons within the same lamina can have different morphologies, afferent projection targets, and physiological properties. Conversely, neurons found in different lamina that share similar afferent projection targets can display similar physiology and morphology. Such is the case with callosal projection neurons found in supragranular (II-III) vs. callosal neurons in infragranular (V-VI) layers of the somatosensory cortex (Ramos et al., 2008). Results from the present study add to our understanding of cortical neurons and suggest greater diversity among neurons in infragranular layers in both the rat and mouse (Chen et al., 2009).

It is important to note that our intrinsic results showed different results than our bursting results, where rat cells displayed a higher frequency of action potentials than mouse cells when bursting. Each pyramidal cell receives thousands of synaptic inputs which affect their integrative properties (DeFelipe and Fariñas, 1992). However, network properties often reflect the neuron’s intrinsic properties (Agmon and Connors, 1992; Hu, 1995). Our passive results showed differences in resting membrane potential and input resistance between rat cells and mouse cells which may reflect differences in integrative properties and ion channel distributions. It is therefore not completely surprising that these two species also differ in their bursting dynamics.

Only recently have studies specifically sought to compare similar cell types in both rats and mice. Of particular relevance to our present findings was a study comparing the physiology and morphology of pyramidal neurons in the CA1 region of the hippocampus (Routh et al., 2009). In this study, which compared rats to two different strains of mice, surprisingly few morphological and electrophysiological differences were observed between species. Similar to our results, total dendritic surface area was found to differ between rats and mice (larger in rats) as well as action potential threshold (more hyperpolarized in rats). Unlike our findings, action
potential amplitude was found to differ between species and total dendritic length was not different between species (Routh et al., 2009). Taken together, these data emphasize the need for additional studies that compare important cell-types found in both rats and mice and highlight the care that should be used when extrapolating results from one species to the other.
Chapter Seven: Introduction:

Synaptic Properties of Layer VI Inverted Pyramidal Cells

in the Somatosensory Cortex

Pyramidal neurons throughout the cortex make precise intracortical and subcortical connections allowing the cortex to carry out its complex functions. While pyramidal cells have similar intrinsic characteristics (apical dendrite, excitatory, regular spiking or bursting), they are not of a homogeneous population, but can be grouped based on morphological, physiological, molecular and functional properties. For instance, pyramidal neurons that express Sox5 target subcortical regions including the tectum, pons and thalamus whereas Satb2 is expressed in callosal projecting neurons (Fishell and Hamashima, 2008; Leone et al., 2008).

More specifically, the connection specificity of different neuronal types is often related to firing pattern. Columnar organization generally leads to segregated channels of information. For example, layer II/III cells preferentially connect with layer V cells which share similar firing patterns (Otsuka and Kawaguchi 1997). Within the visual system, layer IV spiny neurons synapse onto layer II/III neurons, where cells which respond to the same orientation stimuli are more likely to be connected (Ko et al., 2011). Similar results have also been seen in the barrel cortex (Peterson and Sakmann, 2000). However, intracortical connectivity also reflects this type of separation. The long range targets of cortical pyramidal cells reflect the responses of both their presynaptic origins and their postsynaptic connections (Brown and Hestrin, 2009; Otsuka and Kawaguchi, 2011).

It has also been shown that the intrinsic physiology of neurons correlates to their synaptic responses (Agmon and Connors, 1992; Zhu and Connors, 1999). For instance, whisker
stimulation often leads to a single action potential in RS cells, but can trigger a burst of 2-3 action potentials in FS cells (Zhu and Connors, 1999). Similarly, examining thalamocortical inputs into the mouse barrel cortex also revealed an association with intrinsic physiology and synaptic response. Most notable is that RS and FS cells have relative short response latencies and are considered monosynaptic. IB cells, however, had greater response latencies and their synaptic responses themselves contained the presence of inhibitory post synaptic currents (IPSC) indicating their lack of monosynaptic connections (Agmon and Connors, 1992). As discussed in the previous section (see section 6.3), inverted pyramidal cells have distinct morphological and intrinsic physiological properties separate from the more common pyramidal cell. In this section, we focus on the synaptic inputs received by IPCs and UPCs in order to determine if they have different synaptic inputs.
Chapter Eight: Methods:

Synaptic Properties of Layer VI Inverted Pyramidal Cells
in the Somatosensory Cortex

8.1. Preparation of slices

Coronal brain slices were prepared from CD-1 mice of either sex at postnatal day (p) 11-21 as previously described in Chapter 4, Section 1. Rats?

8.2. Electrophysiological recordings

Layer VI IPCs and UPCs were visualized and targeted for electrophysiological recordings in mice (IPCs n = 15, UPCs n = 17) and in rats (IPCs n = 9, UPCs n = 11). Patch clamp recordings were done as described previously in Chapter 4, Section 2.

To assess synaptic properties, a concentric electrode (~1 MΩ, Fredrick Hare Inc.) was connected to an isolated pulse stimulator (AM Systems). Digital outputs were used to trigger the stimulator through Clampex version 10.3 (Molecular Devices). The stimulating electrode was placed in the cortical white matter directly below layer VI while simultaneously recording form layer VI cells. The stimulating electrode was placed specifically toward the bottom of the white matter as that has been shown to yield the most consistent response in cortical cells (Woodward et al, 1990). See figure 11A for a schematic representation of electrode placement. Single pulses (250 µs in duration) were used to evoke postsynaptic potentials (PSPs) in neurons in layer VI of the somatosensory cortex (figure 11B). In order to standardize stimulation between neurons, stimulation intensity was gradually increased until obvious PSPs (~1mV) were consistently evoked 50% of the time and then the stimulus intensity was increased to 1.2x that value (+200 µA to + 1 mA). A one-way ANOVA revealed no differences in stimulation
intensity between groups: mouse UPCs (518 ± 150 μA, mouse IPCs (449 ± 201 μA), rat UPCs (575 ± 68 μA) and rat IPCs (430 ± 89 μA; figure 12A). The resulting stimulation intensity was used to elicit EPSPs over a wide range of stimulation frequencies (1-20 Hz) in order to assess both short term (paired pulses) and longer term (trains of 8 pulses) synaptic dynamics. Signals were acquired with a Multiclamp 700B (Molecular Devices) amplifier and collected and analyzed with Clampfit version 10.3 (Molecular Devices). All data were digitized at 10 KHz and filtered at 1 KHz.

**Figure 11.** White matter stimulation. Schematic representation of stimulation protocol. The stimulator is depicted as entering the cortical white matter while the recording electrode lies above it in cortical layer VI (A). Graph (B) of single pulse minimal stimulation showing both PSP failures and successes and an average of n=10 successful stimulations (C). Scale bars = 2 mV and 100 ms.
8.3. Voltage Clamp recordings

In order to assess any possible sources of disynaptic inhibition, mouse UPCs (n=4) and IPCs (n=4) were recorded in voltage clamp configuration and their membrane voltages were held at varying holding potentials (-80 mV, -70 mV, -60 mV, and -50 mV) while the underlying cortical white matter received single pulse stimulation as previously described.

8.4. Histology

Following recordings, slices were placed in cold fixative (4% paraformaldehyde in 0.1 M phosphate buffer) and kept at 4°C for no more than 2 weeks. Biotin-avidin-HRP histochemistry was performed as described previously in section 4.3 to allow for confirmation of IPC versus UPC morphologies.

8.5. Quantitative Comparisons

Stimulus evoked PSP latencies, duration and amplitudes were analyzed from the average of 20 stimuli utilizing single pulse stimulation at an interstimulus interval of 2 seconds. Representations of the 20 stimuli can be seen in figure 11B and the average of those traces in figure 11C. Latency was operationally defined as the time between stimulus offset and PSP onset. The duration (broadness) of the PSP was measured as the half width at half maximum height (in ms) and PSP amplitude was measured at the peak of the resultant PSP relative to the pre-stimulus membrane voltage (in mV). Rates of rise and fall of the PSPs were measured by calculating the difference in time (in ms), from onset (rise times) or to offset (fall times), divided by the change in membrane potential (in mV) measured at the peak amplitude relative to the baseline membrane potential. Synaptic facilitation/depression was assessed by comparing the amplitude of the initial PSP to the last PSP initiated by stimulation for both paired pulse and train stimulation paradigms. In order to assess excitatory post synaptic currents (EPSC’s) recordings
were done in voltage clamp configuration. The evoked EPSC magnitude was measured as the change in current (in pA) from baseline current to the peak of the resultant evoked response. Current versus voltage relationships were also computed by plotting the maximal amplitude evoked by white matter stimulation at the different holding potentials (-80 mV, -70 mV, -60 mV and -50 mV). Statistics were computed using the Sigmaplot software package version 10 (Systat) for within-group and between-group analyses. One-way and repeated measures ANOVAs were conducted and Tukey's HSD post hoc test were used to determine the source of the variance, if any. Specific comparisons were made between different cell types found in the same species (i.e., rat IPCs vs. rat UPC) as well as similar cell types found between the two species (i.e., rat IPCs vs. mouse IPCs). Statistical significance was set at $p < 0.05$. All data are reported as means ± one standard error of the mean (SEM) unless otherwise noted.
Chapter Nine: Results:

Synaptic Properties of Layer VI Inverted Pyramidal Cells
in the Somatosensory Cortex

9.1 Single Pulse

To study synaptic inputs onto layer VI pyramidal cells, a concentric bipolar electrode was placed in the underlying cortical white matter. Stimulation was increased until a consistent EPSP could be evoked (see section 8.2). Several fundamental aspects of the resultant PSP were measured including response latency, rise and fall times and amplitude. The latencies for the mouse cells were 4.30 ± 1.17 ms (UPC) and 4.19 ± 0.86 ms (IPC). Rat cells had shorter latencies, but not significantly so. Rat UPCs had a latency of 3.71 ± 1.42 ms and IPCs had a latency of 3.27 ± 0.85 ms (Figure 12B). Overall, there were no significant differences in response latency between cell types or between species (p’s>0.05).

PSP half width was also measured by calculating the duration of the PSP at half height. Mouse UPCs had the broadest EPSPs (103.58 ± 16.35 ms) compared to mouse IPCs (57.78 ± 8.83 ms); p < 0.05. There were no differences between rat IPCs (66.70 ± 7.53 ms) and rat UPCs (67.25 ± 5.16 ms), p > 0.05 (Figure 12C).

PSP amplitude was also measured. Mouse UPCs had the largest amplitude (3.24 ± 0.48 mV) followed by mouse IPCs (1.36 ± 0.315 mV). Rat IPCs had similar amplitudes as UPCs; 0.85 ± 0.13 mV and 0.86 ± 0.19 mV, respectively. Mouse UPCs had larger amplitudes compared to Mouse IPCs and Rat UPCs (p < 0.05; Figure 12D). There were no other significant differences between groups. See table 3 for a comparison between groups. As a whole, mouse UPCs had the largest PSP amplitudes as well as possessing the broadest PSPs while also having the slowest
rise time compared to mouse IPCs. In general, rat UPCs did not differ from rat IPCs in respect to amplitude, latency, half width or rise and fall times.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>n</th>
<th>Latency (ms)</th>
<th>Rise time (ms/mV)</th>
<th>Fall time (ms/mV)</th>
<th>Half-width (ms)</th>
<th>Amplitude (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse IPC</td>
<td>15</td>
<td>4.50 ± 1.17</td>
<td>0.034 ± 0.087</td>
<td>-0.013 ± 0.003</td>
<td>57.78 ± 8.83</td>
<td>1.36 ± 0.32</td>
</tr>
<tr>
<td>Mouse UPC</td>
<td>17</td>
<td>4.19 ± 0.86</td>
<td><strong>0.074 ± 0.013</strong></td>
<td>-0.015 ± 0.003</td>
<td>103.58 ± 16.35</td>
<td><strong>3.24 ± 0.48</strong></td>
</tr>
<tr>
<td>Rat IPC</td>
<td>9</td>
<td>3.27 ± 0.85</td>
<td>0.034 ± 0.010</td>
<td>-0.0092 ± 0.002</td>
<td>66.70 ± 7.53</td>
<td>0.85 ± 0.13</td>
</tr>
<tr>
<td>Rat UPC</td>
<td>11</td>
<td>3.71 ± 1.42</td>
<td>0.027 ± 0.006</td>
<td>-0.0048 ± 0.0004</td>
<td>67.25 ± 5.16</td>
<td>0.86 ± 0.19</td>
</tr>
</tbody>
</table>

Table 3. Summary of EPSP properties of mouse and rat inverted and upright pyramidal cells. All data are reported as mean ± standard error of the mean (SEM). The symbol (*) indicates statistical significance (p < 0.05) between IPCs and UPCs, and the symbol (†) indicates between species differences for the same phenotype of cell.

Figure 12. PSP properties following single pulse stimulation. Comparisons of pulse intensity (A), PSP latency (B), PSP half-width (C) and PSP amplitude (D) reveal differences among the
groups. Asterisks denote significant differences between groups (p<0.05). The boundary of the box closest to zero indicates the 25th percentile while the boundary of the box farthest from zero indicates the 75th percentile. Solid lines within the boxes mark the median value. Error bars above and below the box indicate the 90th and 10th percentiles.

In order to investigate the kinetics of the evoked PSPs, rise and fall rates were calculated for IPCs and UPCs. The rate of rise for the stimulus evoked PSPs were 0.074 ± 0.0125 ms/mV (mouse UPCs), 0.034 ± 0.087 ms/mV (mouse IPCs), 0.027 ± .006 ms/mV (rat UPCs) and 0.034 ± 0.011 ms/mV (rat IPCs). PSP fall times were -0.015 ± 0.003 ms/mV (mouse UPCs), -0.013 ± 0.003 ms/mV (mouse IPCs), -0.009 ± 0.002 ms/mV (rat UPCs) and -0.005 ± 0.001 ms/mV (rat IPCs). While there were no significant differences in the rate of fall (ANOVAs, p’s > 0.05), there were significant differences in rise times among the neuronal populations (p < 0.05). Post-hoc tests revealed that mouse UPCs had slower rise times than both mouse IPCs and rat UPCs. There were no significant differences between rat IPCs and UPCs (Figure 13A). The ratio of rate of fall over rate of rise was also calculated and yielded significant results (p < 0.05). Post-hoc tests showed significant differences between mouse UPCs (-0.18 ± 0.10) and mouse IPCs (-0.41 ± 0.17) and rat UPCs (-0.38 ± 0.14). There were also significant differences between mouse IPCs and rat IPCs (-0.18 ± 0.08) as well as between rat IPCs and rat UPCs (p<0.05; figure 13B). These results suggest that there are differences in the nature of the synaptic inputs onto IPCs and UPCs in response to white matter stimulation.
Figure 13. **PSP Kinetics.** Comparisons of PSP rate of rise (A), and PSP ratio of fall rate:rise rate (B) reveal differences among the groups. Asterisks denote significant differences between groups (p<0.05). The boundary of the box closest to zero indicates the 25th percentile while the boundary of the box farthest from zero indicates the 75th percentile. Solid lines within the boxes mark the median value. Error bars above and below the box indicate the 90th and 10th percentiles.

9.2 **Voltage Clamp**

Given that the cells tended to rest close to the Cl⁻ reversal potential, we wanted to assess if disynaptic inhibition via activation of GABA_A currents was influencing our PSP measurement. In order to assess any possible inhibitory inputs affecting PSP size, we measured the change in stimulus evoked currents, using voltage clamp, in response to single pulse white matter stimulation at varying holding potentials (-80 mV, -70 mV, -60 mV, and -50 mV) for both mouse UPCs and IPCs (figure 14A-B). No significant inhibitory postsynaptic currents (IPSCs) were observed at any voltage levels for either IPCs or UPCs, which suggests a lack of strong disynaptic inhibition following white matter stimulation. This is consistent with earlier results studying inputs onto identified layer VI corticothalamic neurons (Yang et al. 2014). The observed EPSCs did show a trend towards decreasing in magnitude at more depolarized holding
voltages, but these differences were not significant for either IPCs or UPCs (ANOVA’s, p > 0.05; figure 14 C-D). However, there was a large difference in EPSC size between UPCs and IPCs (figure 14 E). Independent t-tests revealed significantly higher EPSC magnitude in UPCs at holding voltages of -80 mV (UPC: -31.7 ± 8.4 pA, IPC: -5.0 ± 0.9; p < 0.05), -70 mV (UPC: -40.6 ± 8.0 pA, IPC: -3.9 ± 0.9 pA; p < 0.05), and -60 mV (UPC: -27.6 ± 4.5 pA, IPC: -4.4 ± 0.5 pA; p < 0.05). While EPSCs remained larger for UPCs during the -50 mV holding condition, it was not significantly different from IPCs (UPC: -17.3 ± 7.9 pA, IPC: -2.6 ± 0.1 pA; p > 0.05). These results are consistent with our single pulse data from our current clamp recordings where mouse UPCs had significantly higher PSP amplitudes compared to IPCs.

Figure 14. **EPSC response properties.** Graphs represent the average current evoked in response to white matter stimulation at different holding voltage for mouse IPCs (A) and UPCs
(B). Representative EPSCs at each of the holding potentials for mouse IPCs (C) and UPCs (D). Panel E is an overlay of a UPC and IPC in response to white matter stimulation at a holding potential of -80 mV. Green = -80 mV; Red = -70 mV; Blue = -60 mV and purple = -50 mV. Error bars represent one SEM. Scale bars = 5 pA and 20 ms (C) and 20 pA and 20 ms (D).

9.3 Paired pulse

Following the single pulse experiments, cells received paired pulse stimulation, using the same stimulus intensity as for the single stimulation studies. The pairs of pulses were delivered over a wide range of frequencies (1 Hz, 2 Hz, 4 Hz, 10 Hz and 20 Hz). Facilitation or depression was assessed by calculating the paired pulse ratio of the amplitude of the second PSP divided by the amplitude of the initial PSP. A synapse was defined as depressing if the ratio was less than 1.0 and facilitating, if the ratio was greater than 1.0, if the ratio was equal to 1.0 then it was assumed that the synaptic strength was unchanged. Mouse UPCs displayed synaptic depression in response to increasing frequencies. In contrast, mouse IPCs displayed facilitation at lower frequencies, but depressed at 20 Hz. Rat UPCs showed initial depression, but displayed synaptic facilitation starting at 4 Hz. Rat IPCs, however showed depression throughout stimulation (Figure 15 D-E).
Figure 15. **Representative PSPs during paired pulse stimulation** for both mouse and rat at 4 Hz (A), 10 Hz (B) and 20 Hz (C). The ratio of PSP height from the last pulse divided by the first pulse can be seen (D) for IPCs and (E) for UPCs. Solid black lines represent UPCs and colored lines represent IPCs. Scale bars = 2 mV and 200 ms. Error bars represent one SEM.
We examined the ratio of depression/facilitation across cell types and species and those found to be significantly different are shown in Table 4. There were no significant differences between groups at 1 Hz (mouse UPC: 0.73 ± 0.14; mouse IPC: 0.68 ± 0.15; rat UPC: 0.75 ± 0.06; rat IPC: 0.87 ± 0.11) or at 2 Hz (mouse UPC: 0.79 ± 0.13; mouse IPC: 0.85 ± 0.12; rat UPC: 0.91 ± 0.05; rat IPC: 0.80 ± 0.14). However there were significant differences at 4 Hz (p<0.05) where mouse UPCs (0.79 ± 0.14) were significantly more depressed than mouse IPCs (1.24 ± 0.16) and rat UPCs (1.06 ± 0.08). Similarly, rat IPCs (0.72 ± 0.12) were significantly more depressed compared to rat UPCs and mouse IPCs (Figure 15 A).

<table>
<thead>
<tr>
<th></th>
<th>1 Hz</th>
<th>2 Hz</th>
<th>4 Hz</th>
<th>10 Hz</th>
<th>20 Hz</th>
<th>1 Hz</th>
<th>2 Hz</th>
<th>4 Hz</th>
<th>10 Hz</th>
<th>20 Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse IPC</td>
<td>0.68 ± 0.15</td>
<td>0.85 ± 0.12</td>
<td>1.24 ± 0.16</td>
<td>1.17 ± 0.10</td>
<td>0.74 ± 0.06</td>
<td>0.66 ± 0.03</td>
<td>0.66 ± 0.12</td>
<td>0.37 ± 0.06</td>
<td>0.49 ± 0.16</td>
<td>0.28 ± 0.11</td>
</tr>
<tr>
<td>Mouse UPC</td>
<td>0.73 ± 0.14</td>
<td>0.79 ± 0.13</td>
<td>0.79 ± 0.14**</td>
<td>0.72 ± 0.11**</td>
<td>0.42 ± 0.05**</td>
<td>0.65 ± 0.08</td>
<td>0.79 ± 0.08</td>
<td>0.30 ± 0.08</td>
<td>0.33 ± 0.04</td>
<td>0.26 ± 0.01</td>
</tr>
<tr>
<td>Rat IPC</td>
<td>0.87 ± 0.11</td>
<td>0.80 ± 0.14</td>
<td>0.72 ± 0.12**</td>
<td>0.73 ± 0.16**</td>
<td>0.85 ± 0.21</td>
<td>0.86 ± 0.09</td>
<td>0.76 ± 0.11</td>
<td>0.78 ± 0.08**</td>
<td>0.59 ± 0.07</td>
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<tr>
<td>Rat UPC</td>
<td>0.75 ± 0.06</td>
<td>0.91 ± 0.05</td>
<td>1.06 ± 0.08</td>
<td>1.12 ± 0.16</td>
<td>1.22 ± 0.19</td>
<td>0.77 ± 0.13</td>
<td>0.85 ± 0.07</td>
<td>0.71 ± 0.13</td>
<td>0.83 ± 0.19</td>
<td>0.42 ± 0.22</td>
</tr>
</tbody>
</table>

Table 4. Summary depicting ratio of final EPSP amplitude over initial EPSP amplitude in paired pulse and train conditions across stimulation frequencies. All data are reported as means ± standard error of the mean (SEM). The symbol (*) indicates statistical significance (p < 0.05) between IPCs and UPCs and the symbol (**) indicates between species differences for the same phenotype of cell.

Similar results were found at 10 Hz where mouse IPCs and rat UPCs showed significant (p < 0.05) facilitation (1.17 ± 0.10, 1.12 ± 0.16, respectively) compared to mouse pyramidal and rat IPCs (0.72 ± 0.11, 0.73 ± 0.16, respectively) which demonstrated synaptic depression (Figure 15 B).

Finally, at 20 Hz stimulation, rat UPCs once again showed synaptic facilitation (1.22 ± 0.19) whereas all other groups had differing levels of depression (mouse UPCs: 0.42 ± 0.05,
mouse IPCs: 0.76, rat IPCs: 0.85 ± 0.21; figure 15 C). Mouse UPCs were significantly more depressed than mouse IPCs (p < 0.01) and rat UPCs (p < 0.01). However, no other groups differed significantly from each other. Overall, mouse UPCs showed the greatest amount of paired pulse depression whereas rat UPCs showed modest facilitation as function of increasing frequency stimulation. These data further suggest differences in the nature of the synaptic inputs or integration of these different cell types.

9.4 Train Stimulation

Following paired pulse stimulation, IPCs and UPCs of both species received train stimulation of 8 pulses at the same amplitude as the paired pulse condition of increasing stimulus frequencies (1 Hz, 2 Hz, 4 Hz, 10 Hz, and 20 Hz). Once again, a ratio of PSP amplitude was calculated by dividing the amplitude of last (eighth) elicited PSP divided by the amplitude of the initial PSP. Whereas there were differences in facilitation/depression during paired pulse stimulation, all cell types demonstrated depression (figure 16 D-E).

As with the paired pulse results, there were no significant differences between groups at 1 Hz (mouse UPCs: 0.65 ± 0.08; mouse IPCs: 0.66 ± 0.03; rat UPCs: 0.77 ± 0.13; rat IPCs: 0.86 ± 0.09) or at 2 Hz (mouse UPCs: 0.66 ± 0.12; mouse IPCs: 0.70 ± 0.09; rat UPCs: 0.85 ± 0.07; rat IPCs: 0.76 ± 0.11).

However, at 4 Hz rat IPCs (0.78 ± 0.08) were significantly less depressed than mouse IPCs (0.37 ± 0.06; p < 0.05). There were no significant differences in rat UPCs (0.71 ± 0.13) or mouse UPCs (0.50 ± 0.08, figure 16 A).

Finally, at 10 Hz and 20 Hz (figure 16 B-C), there were no significant differences between groups: mouse IPCs: 10 Hz: 0.49 ± 0.16; 20 Hz: 0.28 ± 0.11, mouse UPCs: 10 Hz: 0.33
± 0.04; 20 Hz: 0.26 ± 0.01, rat IPCs: 10 Hz: 0.59 ± 0.07; 20 Hz: 0.50 ± 0.10, rat UPCs: 10 Hz: 0.83 ± 0.19, 20 Hz: 0.42 ± 0.22). See table 4 for comparisons across all cell types.

While there were not many differences in response to train stimulation, rat cells were typically less depressed than mouse cells. Over all, these data indicate IPCs and UPCs process inputs differently or are otherwise involved in different circuits. Additionally, we have also found species differences in synaptic processing within the same neuronal phenotype.
Figure 16

A

4Hz

Mouse
-68 mV

Rat
-70 mV

B

10Hz

Mouse
-68 mV

Rat
-70 mV

C

20Hz

Mouse
-68 mV

Rat
-70 mV

D

E
Figure 16. Representative PSPs during train stimulation for both mouse and rat at 4 Hz (A), 10 Hz (B) and 20 Hz (C). The ratio of PSP height from the eight pulse divided by the first pulse can be seen (D) for IPCs and (E) for UPCs. Solid black lines represent UPCs and colored lines represent IPCs. Scale bars = 2 mV and 1000 ms (A); 2 mV and 200 ms (B); 5 mV and 100 ms (C –upper) and 1 mV and 100 ms (C-lower). Error bars represent one SEM.
10.1 Synaptic Properties of Inverted Pyramidal Cells

Numerous differences were found when examining the stimulus evoked synaptic inputs onto IPCs and UPCs. Specifically, PSP rise times, PSP half-widths and PSP amplitudes were different in the mouse IPCs versus UPCs suggesting that there are differences in how IPCs integrate synaptic inputs compared to UPCs. There were also differences between IPCs and UPCs in encoding different frequencies of synaptic inputs. It was observed that IPCs in the mouse demonstrated more synaptic depression compared to UPCs as a function of stimulus frequency in the paired pulse conditions. However, there were few differences between cell types or species in response to train stimulation where increased depression was observed as a function of stimulus frequency. Overall, mouse UPCs demonstrated the largest amplitude and the longest duration EPSPs along with the greatest paired pulse depression. Rat cells however, had smaller EPSP amplitudes which remained fairly consistent during repeated paired stimulation. Taken together, these data suggest differences in how IPCs and UPCs integrate synaptic inputs and IPCs may play a unique role in sensory processing. It is possible that these cell types receive the same inputs, but integrate them differently. For instance, thalamo-cortical inputs onto RS cells evoke larger PSPs that show greater depression than those same inputs onto FS cells (Agmon and Connors 1992; Shiff and Reyes 2011).
10.2 Single pulse

Using minimal stimulation methods in the cortical white matter, we were able to elicit consistent EPSPs in layer VI IPCs and UPCs in the mouse and rat. While there were no differences in latency to EPSP onset, mouse UPCs were found to have the slowest rise times. However, mouse UPCs also had the greatest amplitude EPSPs. This is in contrast to rat neurons which uniformly had relatively small EPSP amplitudes. These differences may be due to the integrative properties of these specific cell types, and whether inputs to the cell occurred more distally or proximally to the soma. In fact, AMPA receptor responses are most efficient at synapses on proximal dendrites and NMDA mediated responses are larger when the synapses are on more distal dendrites (Lajeunesse et al., 2013). As discussed in an earlier section, 5.3, there are differences in the dendritic geometry between IPCs and UPCs as well as between mice and rats where rat IPCs had the greatest dendritic elaboration and branching. These differences in arborization may also explain how these cell types integrate incoming signals. For example, layer V pyramidal cells display either regular spiking or intrinsic bursting firing properties which are associated with the shape of their dendrites, either slender tufted or thick tufted, respectively (Mason and Larkman, 1990; de Kock et al., 2007). As IPCs have greater dendritic elaboration, they may receive more distal inputs. Distal inputs are often attenuated and considered modulatory (Stuart et al., 1997; Hausser et al., 2000; Spruston, 2008).

10.3 Voltage clamp

In order to assess possible sources of inhibitory input, mouse IPCs and UPCs were held at different voltages ranging from -80 mV to -50 mV while receiving stimulation. We found that EPSC size overall was reduced at more depolarized holding potentials, towards the reversal potential for sodium. This is in agreement with previous research that shows sodium as the main
charge carrier of the cell (Hodgkin and Huxley, 1952; Stuart and Sakmann, 1995). We also observed little evidence of IPSCs at any holding potential for either IPCs or UPCs suggesting minimal disynaptic inhibition on these cells. However, we did find that EPSC size was significantly higher for UPCs compared to IPCs.

**10.4 Paired pulse and train stimulation.**

Whereas synaptic depression as a function of stimulus frequency was similar in both mouse and rat cell types, there were significant differences in the degree of depression or facilitation as a function of frequency. Mouse IPCs showed the strongest synaptic depression as a function of increasing stimulus frequency followed by mouse UPCs. Conversely, Rat UPCs demonstrated a small degree of facilitation as stimulus frequency increased which suggests that IPCs process inputs differently or are otherwise involved in different circuits than UPCs.

As with the previous section (section 5), cells of the same type differed between species. This once again suggests that mice and rats differ from each other physiologically. For instance, GABA_A receptors have different subunit variants across species (Sinkkoken et al., 2000). Furthermore recent fMRI research has shown differences in connectivity between mouse and rat where mice may have less interhemispheric connectivity than rats (Jonckers et al., 2011). Additionally, cell responses in regard to whisker deflection vary between mice and rats. Mouse cells appear to be more sensitive to amplitude changes, and less sensitive to changes in deflection velocity compared to rats (Kwegyir-Afful et al., 2008).

While antidromic activation cannot be ruled out entirely, it is relatively rare, ~3% of layer VI neurons have been shown to display antidromic activity following white matter stimulation, and the majority of those antidromic cells were shown to be cortico-thalamic neurons (Brumberg et al., 2003; Rose and Metherate, 2001). Overall, these data demonstrate the increased need to
better examine the numerous diversity of cells within the cortex to better ascertain their possible role in cortical processing. Furthermore, the differences between species once again reveal that two closely related species can still have large differences in how their cells integrate neural information. These results build on our previously shown intrinsic physiological and morphological differences (section 6.3) and support our view that the inverted pyramidal cell should be considered a separate class of pyramidal cell.
Chapter Eleven: General Discussion and Concluding Remarks

11.1 Limitations of Study

The present work investigated the inverted pyramidal cell and its morphological, physiological and synaptic properties and how they differ from the more common upright pyramidal cell. The purpose of this research is to better understand the myriad of cell types that have yet to be properly characterized and implicated into the functional circuitry that determines cognitive, sensory and motor abilities.

There are some limitations within this report that should be addressed, however. Our data comes from young animals (p11-21). While we assume the majority of cortical synapses has formed by this age (Bender et al., 2003; Inan and Crair 2007), we do not know whether these differences reflect the adult brain. Furthermore, we are treating IPCs as a homogeneous group. Just as UPCs can vary into numerous subgroups, it is unknown if IPCs do as well. IPCs appear to have similar dendritic variance as UPCs (section 5.3; figure 9) along with previous evidence (Chen, 2009; Mendizabal-Zubiaga 2007) suggest that IPCs are not a wholly homogenous group. As UPCs can be defined by their long range projection target, IPCs also vary in their projections including callosal, striatal and claustral. Further morphological analysis including specific differences in apical dendrite length and branching pattern would provide more insight into this issue. Our morphological data only focused on dendrite properties. However, the axon of IPCs may take on different shapes. In reeler mice, IPCs can be divided by their axonal patterning where the axon either emerges from their cell body and bends toward the cortical white matter, or the axon emerges from the apical dendrite and heads directly toward the cortical white matter (Landrieu and Goffinet, 1981). It is unknown if these differences exist in the normal mouse and
if there is any functional significance to these differences. However, the goal of this report was to begin to characterize a novel neuronal phenotype. As complex cortical functioning is derived from the function of many different neuronal circuits, and those neuronal circuits are composed of a diverse and diffuse number of individual neurons, it is important to best understand how each specific neuron functions and to determine what role each neuron has in cortical functioning. This report describes for the first time, the physiology of the inverted pyramidal cell. With this knowledge, we may better discern the complex functioning of the neocortex.

11.2 Functional implications of inverted pyramidal cells

While IPCs account for ~1% of pyramidal cells, we should not discount their potential importance. Other neurons that that have similar relative rarity can have a large effect on cortical circuitry. Chandelier cells, for instance, represent a small percentage of interneurons, but can exert a large effect on their synaptic targets (Smoyogi, 1977; Woodruff et al., 2011; Inan et al., 2013). Chandelier cells have also been implicated in a number of neurological disorders including schizophrenia (Lewis et al., 2000) and epilepsy (DeFelipe, 1999). Similarly, IPCs are in greatest quantities in reeler mice (Landrieu and Goffinet 1981) and persons with agyria (Bordarier et al. 1986) as well as in the sensory cortices of normal brains. However, the functional significance is not well understood. While reeler mice demonstrate abnormal motor and sensory integration, they still form a functional somatotopic arrangement similar to wild-type mice (Wagener et al., 2010, Guy et al., 2014). Further studies have also shown minimal differences between receptive field properties in reeler mice (Drager, 1981; Lemmon and Pearlam, 1981). Thus, it is unknown if IPCs actively contribute to the abnormal behavior of reeler mice. We contend that IPCs are their own functional subclass of cell and have functional significance. As mouse IPCs and UPCs showed greater synaptic differences from rat IPCs and
UPCs, further research should concentrate more on IPC circuitry within the mouse brain. The mouse may also be a better animal model as there is more potential for knockout and other genetic studies within the mouse. For instance reeler mice have a large percentage of IPCs, and may further elucidate the function of IPCs.

As IPCs have similar long range targets as UPCs, IPCs may represent another source of information processing. Recent research suggests that dendrites contribute to sensory processing even if that information is not passed to the soma (Branco and Hausser, 2010). Within the visual system, dendrites appear to aid in orientation selectivity (Smith et al., 2013; Jelinek and Elston 2001). As IPCs have greater dendrite branching than UPCs, IPCs may be able to further enhance or differentiate afferent inputs.

11.3 Conclusion

Investigating the properties of specific cell types found in the cortex enables us to gain greater understanding into how the brain organizes processes and transmits sensory, motor and cognitive information. Each neuron represents a fundamental unit in information processing. In order to understand the complexity involved in the development and function of cortical circuits, it is important to understand how each neuron within that circuit functions, and how each neuron may function in relation to other neighboring and connected neurons. Investigating the myriad of neuronal subtypes that exist within the cortex can lead to better models of circuits and to improved knowledge of how the vast and complex stream of incoming information from our environment is processed and interpreted.
References


