GABAergic Neurotransmission in Prefrontal Cortex
Cover illustration: Morphological and functional properties of synapses. Top left, electron micrographs showing the ultrastructure of one GABAergic (GABA) and several glutamatergic (GLU) synapses. A glutamatergic and a GABAergic synapse converge on one dendrite. Top right, electrophysiological recordings of excitatory postsynaptic currents from pyramidal and fast spiking neurons, extract from chapter 3, Figure 2; Bottom left, Biocytin reconstructed neurons, extract from chapter 3, Figure 1; Bottom right, electron micrographs showing a parvalbumin (PV) labeled dendrite receiving both a glutamatergic synapse (GLU) and a GABAergic parvalbumin (GABA/PV) synapse. Additionally, the GABA/PV bouton synapses on another postsynaptic structure.
GABAergic Neurotransmission in Prefrontal Cortex

door

Diana Codruta Rotaru
geboren te Turnu Severin, Roemenië
promotor: prof.dr. H.D. Mansvelder
# Table of Content

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 1</td>
<td>General Introduction</td>
<td>7</td>
</tr>
<tr>
<td>Chapter 2</td>
<td>Mediodorsal thalamic afferents to layer III of the rat prefrontal cortex: synaptic relationships to subclasses of interneurons</td>
<td>35</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>Glutamate receptor subtypes mediating synaptic activation of prefrontal cortex neurons: relevance for schizophrenia</td>
<td>61</td>
</tr>
<tr>
<td>Chapter 4</td>
<td>GABA transporter GAT1 prevents spillover at proximal and distal GABA synapses onto primate prefrontal cortex neurons</td>
<td>89</td>
</tr>
<tr>
<td>Chapter 5</td>
<td>Functional properties of inhibitory synaptic inputs onto interneurons in the monkey dorsolateral prefrontal cortex</td>
<td>117</td>
</tr>
<tr>
<td>Chapter 6</td>
<td>General Discussion</td>
<td>133</td>
</tr>
<tr>
<td>References</td>
<td></td>
<td>147</td>
</tr>
<tr>
<td>Summary</td>
<td></td>
<td>177</td>
</tr>
<tr>
<td>Nederlandse Samenvatting</td>
<td></td>
<td>181</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td></td>
<td>185</td>
</tr>
<tr>
<td>List of publications</td>
<td></td>
<td>187</td>
</tr>
</tbody>
</table>
THE ROLE OF THE PREFRONTAL CORTEX IN COGNITIVE BEHAVIOR

Cognition has been the subject of study in many disciplines including psychology, neuroscience, philosophy, linguistics, and computer science and it involves mental process such as decision making, problem solving, executive control of action, language understanding or speech generation (Eckardt, 2003). In neuroscience, the study of cognition is linked to one of the most interesting questions, which is how coordinated and purposeful behavior arises from the activity of different brain regions which in turn is depended upon the firing pattern of billions of neurons wired as complex neuronal networks.

The prefrontal cortex (PFC) is one of the brain areas that has been under intense scientific investigation specifically because evidence suggests that dysfunction of this region and its associated circuitry underlies many of the cognitive disturbances associated with major neuropsychiatric disorders such as attention-deficit/hyperactivity disorder (ADHD), autism or schizophrenia (Duncan, 2001; Uhlhaas et al., 2009). Thus intense research in humans, primates and rodents has been addressing specific questions about the role of the PFC in cognition. Multiple functional neuroimaging studies combined with lesion studies of different areas of the PFC linked this brain region with many cognitive processes such as working memory, attention, planning and decision-making (Miller, 2000; Hashimoto and Sakai, 2002; Jones, 2002; Dalley et al., 2004; Vertes, 2006; Euston et al., 2007; Badre and D'Esposito, 2009; Fuster, 2009).

Currently several hypotheses propose mechanisms by which PFC might be involved in cognition (Miller and Cohen, 2001). It is thought that detailed information of the external world is being provided by the cortical sensory - motor systems. Cognitive control implies the coordination of incoming sensory and motor information with representations of internal goals and rules to facilitate a context-appropriate behavioral response that can serve adaptive functions (Miller, 2000; Miller and Cohen, 2001; O'Reilly and Frank, 2006). The PFC, which is extensively interconnected with cortical and subcortical regions (Fig. 1), is thought to be crucial for cognitive function by exerting top-down control of the flow of neural activity between brain regions (Miller and Cohen, 2001).
Figure 1. The PFC connections with other brain regions. The role of PFC in the flow of information (left) and schematic diagram of the extrinsic and intrinsic connections of the prefrontal cortex. The partial convergence of inputs from many brain systems (black lines) and internal connections (red lines) of the prefrontal cortex may allow it to play a central role in the synthesis of diverse information needed for complex behavior. Most connections are reciprocal except the ones indicated by arrows (right). Adapted from (Miller and Cohen, 2001)

To understand how PFC is exercising its top-down control over other brains regions it is important to understand the extrinsic as well as the intrinsic synaptic connectivity of this brain area. It is known that superficial layers, including layers 2-3 of the PFC, receive cortical and subcortical inputs (Krettek and Price, 1977; Swanson, 1981; Giguere and Goldman-Rakic, 1988; Jay and Witter, 1991; Cenquizca and Swanson, 2007; Hoover and Vertes, 2007) which are integrated by the local microcircuit of these layers. Next, the output of superficial layers is fed to nearby PFC areas (Kisvarday et al., 1986; Lewis and Lund, 1990) and to other association areas of the cortex (Fig. 2) (Selemon and Goldman-Rakic, 1988; Barbas and Pandya, 1989; Pandya and Yeterian, 1990) but also to the deeper layers of PFC which in turn control the activity of subcortical centers with important roles in behavior (Levitt et al., 1993; Kritzer and Goldman-Rakic, 1995; Pucak et al., 1996).

Input from PFC to other cortical and subcortical areas involved in sensory representation affects the activity state of these regions, having a strong impact on the way sensory information from periphery is processed (Miller and Cohen, 2001). Therefore, the PFC could play important roles in “choosing” which inputs are processed and transmitted further.

Given that, the middle layers of the PFC are the main recipient of inputs from several brain regions, it could be suggested that these layers represent the place of complex computations with importance in cognitive function. Evidence favoring the idea that middle layers of PFC are involved in cognitive behavior come from studies of schizophrenia. For example, it is well established that one of the core features of schizophrenia is cognitive deficit (Censits et al., 1997; Mohamed et al., 1999; Heaton et al., 2001; Gold, 2004). Additionally, post-
mortem brain studies of schizophrenic patients showed strong cellular and molecular impairments specifically in middle layers of the PFC as compared with deeper ones (Lewis, 2010; Hoftman and Lewis, 2011). Thus an association exists between specific molecular and cellular changes in the middle layers of the PFC and cognitive dysfunction. Understanding the neuronal microcircuit of the PFC middle layers is an important step towards understanding the role of this brain region in cognitive control.

**Figure 2. Schematic diagram of layer 3 output circuitry prefrontal cortex.** After integrating incoming inputs from subcortical regions and other associational cortices, the layer 3 pyramidal neurons (P) gives rise to local axon collaterals that arborize in close proximity to the soma of these cells, intrinsic collaterals that spread horizontally and arborize in distant regions, and associational projections to other cortical regions either within the same or within the contralateral hemisphere. Adapted from (Hoftman and Lewis, 2011)

The activity pattern of neuronal networks in any brain regions, including the PFC, strongly depends on the functional and morphological connectivity between different neuronal subtypes. Two fundamentally different classes of neurons are found in the cerebral cortex, pyramidal cells and interneurons (Fig. 3A), that use glutamate and γ-aminobutyric acid (GABA) respectively as their main neurotransmitters. Pyramidal neurons are the most abundant and mediate the majority of the communication between brain areas, but their activity is controlled by GABAergic interneurons (Fig. 3B).
PYRAMIDAL CELLS AND INTERNEURONS IN CORTICAL NETWORKS

Morphological studies revealed two important differences between pyramidal cells and interneurons. Firstly, GABAergic interneurons represent only 20–25% of cortical neuronal population and secondly, their variability especially at a morphological level is far greater than that of the pyramidal cells (Fig. 4). The morphological diversity of interneurons led to the hypotheses that in the brain there are different classes of interneurons and each class has a specific role in cortical networks (Freund and Buzsaki, 1996; Somogyi and Klausberger, 2005; Soltesz, 2006). Thus an important question is how the large morphological variability of interneurons relates to their function in network activity.
The study of interneurons was initiated by Ramon y Cajal, whose insights into connectivity, in the late 19th century led him to the exploration of different cell types in the brain. Using Golgi impregnation, a method that stained only 1% of the cells in cortical tissue, Cajal revealed the dendritic and axonal processes of single neurons, and described the specific morphological features of pyramidal cells and interneurons. He showed that pyramidal neurons possessed elaborate dendritic arbors (Fig. 4) and asked, in his Histology of the Nervous System, “Why do dendritic trees even exist?”, concluding that, “Dendrites exist solely to allow the cell to receive, and then transmit to the axon, the greatest variety of information from the most diverse sources” (Ramon y Cajal, 1911). In 1899, he presented the cortical interneurons as “the short axon cells,” because their axons projected locally (however, if stretched out these axons are anything but short), thus dragging the attention to the axonal morphology of these cells.

Later, it was proven that functionally, in the adult cortex pyramidal cells are excitatory while GABA-containing (GABAergic) interneurons are typically inhibitory (Krešimir, 2010). Synapse-mediated activity via excitatory (glutamatergic) and inhibitory (GABAergic) receptors leads to patterns of electrical activity in cortical networks that ultimately underlie important behavioral states. Understanding how neurons transform incoming synaptic inputs into output signals depends at least partially, on the study of the functional and morphological synaptic interconnectivity between excitatory and inhibitory neurons.

The pyramidal cells also called principal cells are cell types involved in the flow of information between different cortical layers and regions via their projecting axons that transmit the local computations to other partners in the network. The last two decades of research added significant new knowledge to
the way pyramidal cells codes information. New hypotheses suggest that dendrites of pyramidal cells are able to coordinate a variety of electrical, biochemical and cellular processes, fundamental to neuron physiology and circuit function (Stuart et al., 2000).

Figure 5. Compartmentalization of pyramidal cells (A) Synaptic relationships between different interneuron subtypes with pyramidal cell compartments showing that the axon (light gray) of a chandelier interneuron contacts the axon initial segment of the pyramidal cell, while a large basket cell contacts the soma of pyramidal cells and a double bouquet cell synapses on the dendrites of pyramidal cells. From (DeFelipe and Farinas, 1992a). (B) Computational models of hierarchical parallel processing in dendritic trees: (Ba) the ‘point neuron’ where all inputs are collected integrated at the soma, with an output generated when somatic voltage crosses threshold, (Bb) the two-layer network where synaptic inputs are integrated locally in dendritic subunits, each of which computes the sum of its local inputs and applies a local threshold nonlinearity (e.g. a dendritic spike). The results of multiple dendritic subunits are then summed at the soma, where the final decision to generate output is taken. (Bc) Dendritic release of neurotransmitters and neuromodulators allows local integration in dendritic subunits to also be expressed as local output. Furthermore, there is bidirectional communication between individual subunits and between subunits and the soma. Figure modified from (Branco and Häusser, 2010) (C) Diagrams showing different morphological types of interneurons, which were identified by cluster analyses of the subcellular distribution of axonal projections onto somata and dendrites of postsynaptic pyramidal neurons. Chandelier cells (ChC), small basket cells (SBC), large basket cells (LBC), neurogliaform cells (NGFC), double bouquet cells (DBC) and Martinotti cells (MC) (Ascoli, 2008)

The initial hypotheses proposed by Cajal, that dendrites of pyramidal cells work as input receivers collecting information from different sources and then deliver this information to the soma via electrical signaling has been
confirmed. But recently, a new hypothesis proposes that dendrites are not just simple conductors of information (Spruston, 2008), but via specific properties such as dendritic morphology, voltage gated channels, or synaptic receptors they can affect local signal integration and crucially determine how electrical signals propagate to the soma. Moreover, local dendritic integration might be directly converted into a spatially confined output signal, like dendritic spikes (Sjöström et al., 2008; Spruston et al., 2008). Additionally, the local outputs could bypass the soma and axon (Branco and Häusser, 2010), because dendritic branches can release either classical neurotransmitters such as glutamate and GABA (Ludwig and Pittman, 2003; Margrie and Urban, 2008), as well as neuromodulators like endocannabinoids and BDNF (Regehr et al., 2009). Such dendritic processes seem to be highly compartmentalized at the level of individual branches, and this led to the hypothesis that different types of information can be processed at specific locations within a single dendrite (Branco and Häusser, 2010). Thus, pyramidal cells consist of several compartments made over its soma and the sum of the single dendritic branches. Each compartment represents a major signaling unit—one that integrates electrical and chemical inputs, and can send independent output signals to a variety of targets (Branco and Häusser, 2010) (Fig. 5A, B).

Interestingly, different subtypes of interneurons provide inputs to specific pyramidal cell compartments. Thus some interneurons synapse preferentially on the somatic compartment whereas other ones at different sites along the dendritic tree suggesting that interneurons act as functional parts of the signaling unit (Fig. 5C). It has been proposed that the morphological variability of interneurons is related to the fact that they serve different compartments of the pyramidal cells to achieve their specific function (Soltesz, 2006).

**GENERAL FUNCTIONS OF GABAERGIC TRANSMISSION IN NETWORK DYNAMICS WITH ROLE IN BEHAVIOR**

The functional importance of interneurons in behavioral states is supported by evidence showing that disruption of the excitatory–inhibitory balance maintained by the interplay of pyramidal cells and interneurons (Fig. 6) is linked to the etiology of several neuropsychiatric disorders, ranging from epilepsy (DeFelipe, 1999; Gleeson and Walsh, 2000) to schizophrenia, autism, and bipolar disorder, anxiety and alcoholism (Akbarian et al., 1995; Knable, 1999; Lewis and Levitt, 2002; Powell et al., 2003; Baraban and Tallent, 2004; Lewis et al., 2005). Conversely, genes associated with such disorders have been shown to influence the development of cortical interneurons (Erbel-Sieler et al., 2004; Flames et al., 2007; Fazzari et al., 2010; Wen et al., 2010).
Figure 6. Changes in the balance of excitation to inhibition. Pyramidal neurons (blue) make excitatory outputs (triangles) onto other pyramidal neurons, and also onto inhibitory interneurons (red). These inhibitory neurons in turn feed inhibition (red circles) back onto the pyramidal neurons. In cortical cultures, raising activity for two days produces a coordinated set of changes in synaptic strength that result in reduced feedback excitation and increased feedback inhibition onto pyramidal neurons (lower left). Conversely, blocking activity for two days increases the gain of excitatory feedback and decreases inhibitory feedback. Similar changes in the cortical excitation–inhibition balance are induced by sensory deprivation. (from (Turrigiano and Nelson, 2004)

GABAergic activity is important from early on in development and it has been shown that in immature cortex it regulates developmental processes, such as induction of critical periods in synaptic plasticity (Hensch, 2005). After the cortical maturation, GABAergic interneurons play essential roles in modulating cortical output and plasticity (Whittington and Traub, 2003). In different brain regions GABA neurons regulate the activity of efferent glutamatergic pyramidal neurons (White, 1989; Markram et al., 2004) and one functional consequence of this is the establishment of neural synchrony within cortical networks (Neltner et al., 2000). Synchronous activity in turn was shown to be evoked by direct sensory stimulation (Pinto et al., 2000; Kohn and Smith, 2005; Bruno and Sakmann, 2006) but also by several cognitive processes such as attention (Gruber et al., 1999; Steinmetz, 2000; Bartos et al., 2007) or short term memory tasks (Tallon-Baudry et al., 1997; Tallon-Baudry and Bertrand, 1999). Moreover blockade of cortical GABAergic receptors impairs working memory in monkeys (Sawaguchi et al., 1989) and attention set-shifting in rats (Enomoto et al., 2010). Additionally, it is known that in schizophrenia there is a reduction in the evoked gamma oscillations during cognitive tasks (Cho et al., 2005; Gonzalez-Burgos et al., 2008a) and it has been proposed that this is due...
to a deficit in the GABAergic transmission (Gonzalez-Burgos et al., 2010). Therefore due to its role in plasticity and neuronal oscillations, the GABAergic system has great impact on several cognitive states.

Malfunction at specific levels in the cortical circuits in particular, deficits of GABAergic activation or dysfunction of their inputs onto postsynaptic targets can lead to an imbalance of excitation to inhibition that may underlie the specific cognitive deficits described in these disorders. Thus the study of the synaptic mechanisms implicated in cortical network dynamics serves a double purpose: on one hand, it investigates the understanding of their role in normal behavioral states and on the other hand, the potential development of more efficacious treatments for different psychiatric disorders. The first part of this thesis including chapters 2 and 3 deals with synaptic mechanisms responsible for recruiting inhibitory cells into network activity and the experiments were performed in rodents. The second part of the thesis, including chapters 4 and 5, is addressing questions about the postsynaptic targets of inhibitory cells and the experiments were performed in primate frontal cortex.

SUBTYPES OF GABAERGIC CELLS – FAST SPIKING AND NON FAST SPIKING INTERNEURONS

As previously mentioned, a rich diversity of GABAergic interneurons provides inhibition at different compartments of the pyramidal cells thus temporally-regulating the activity of these cells. A relevant question is how each different subtype of interneuron acts to achieve its functions. Classifying interneurons is an important step in understanding their specific functions, but this apparently easy task has become a well-known problem in the GABAergic field due to debates regarding the important features to be used as classifying criteria (Ascoli, 2008).

Combining the morphological Golgi analysis initiated by Cajal and continued by Lorente de Nó and others, (Szentagothai, 1978; Fairen et al., 1984; Lund et al., 1988a), with new methods ranging from intracellular horseradish peroxidase stainings (Douglas and Martin, 1998; Somogyi et al., 1998) to electrophysiological recordings (Lacaille et al., 1989; Gupta et al., 2000) (Maccaferri and Lacaille, 2003) and finally, molecular and genetic screenings (Kawaguchi and Kubota, 1993; Kawaguchi, 1993b; Cauli, 1997; Markram et al., 2004), researchers provided several classification schemes (Fig. 7 and 8) and insights into GABAergic function.
The remarkable variation in their molecular, anatomical, and physiological features renders a functional classification extremely difficult and
although several classification schemes have been proposed, none of them is satisfactory due to the lack of clear correlation between the intrinsic electrophysiology, synaptic responses and their molecular markers and morphology (Ascoli, 2008) One reductionist way of viewing interneurons is to consider separately their morphological, molecular or physiological aspects. The studies presented in this thesis made use of molecular, physiological or morphological classification (Fig. 9), depending on the specific question addressed in each of the chapters.

Good insights into the functional roles of different types of interneurons have been gained by patch-clamp recordings from GABAergic cells. Electrophysiological experiments testing the membrane properties of different cells showed that one specific subgroup of interneurons called the Fast Spiking (FS) cells can be reliably differentiated from the large group of interneurons. The FS subgroup has homogenous membrane properties and electric criteria are sufficient to differentiate them. By comparison with the FS cells the rest of the interneurons are called Non Fast Spiking (NFS). The large variability of electrophysiological properties observed in the NFS group is underlined by multiple subtypes interneurons comprised under this label. Patch clamp experiments followed by morphological reconstructions and stainings for several molecular markers helped define several important criteria that are summarized below in Figure 9.
on the response of the neurons’ membrane potential to direct injection of current. FS neurons form a relatively homogeneous group of interneurons that contain the calcium-binding protein parvalbumin (PV), contain no neuropeptides, and make synapses onto the perisomatic compartment of the postsynaptic pyramidal cell. FS/PV basket cells synapse onto the proximal dendrites and soma of pyramidal cells and also onto other GABA neurons. FS/PV chandelier cells synapse onto the axon initial segment of pyramidal cells but not onto other GABA neurons. The non-FS cell cluster is largely heterogeneous. An important group of non-FS cells are the Martinotti neurons, which in most cases contain the calcium-binding protein calbindin (CB) and the neuropeptides somatostatin (SST) and neuropeptide Y (NPY). Martinotti cells make synapses onto the distal dendrites of pyramidal cells. The subgroup of non-FS cells that contain the calcium-binding protein calretinin (CR) also express vasoactive intestinal peptide (VIP) and make contacts onto other GABA neurons much more frequently than onto pyramidal cells and thus are called interneuron selective. The non-FS basket cells containing cholecystokinin (CCK) do not contain PV, CB, or CR and make synapses onto the perisomatic compartment of postsynaptic pyramidal cells. From (Gonzalez-Burgos and Lewis, 2008)

THE FAST SPIKING INTERNEURONS

FS interneurons are easily identifiable by patch-clamp recordings because their firing pattern shows high frequency rates with no adaptation during a spike train (Fig. 9) and membrane properties including fast action potentials and fast membrane time constant with a large afterhyperpolarization (AHP). The easily distinguishable firing pattern facilitated the study of FS cells relative to the rest of interneurons which cannot be readily separated from pyramidal cells, for example (Zaitsev et al., 2009). Therefore extensive functional characterization of this cell type was performed in comparison with the rest of the GABAergic cells. Molecular staining following patch clamp experiments proved that these cells express primarily the calcium binding protein, Parvalbumin (PV) (Freund, 2003). The characteristic fast kinetics of the FS PV expressing interneurons are attributed to a large degree to the expression of voltage-gated potassium channels (namely Kv3.1), and sodium channels with rapid activation kinetics, (Rudy and McBain, 2001; Jonas et al., 2004). Biocytin stainings and electron microscopic assessments of the patched cells showed that FS PV cells comprise two clearly distinguishable morphological subtypes, the basket and the chandelier cells, both of which establish their innervations in the vicinity of the somatic compartment of the target pyramidal neurons.

The basket cells mediate perisomatic inhibition in all areas of the cerebral cortex (Ascoli, 2008). The axons of several basket cells converge onto single pyramidal cells and the resulting arrays of boutons gives the appearance of a basket (Halasy et al., 1996; Somogyi et al., 1998; Klausberger et al., 2005). The Chandelier cells synapse on the axon initial segments of pyramidal cells, and for this reason they have also been called axo-axonic cells (Somogyi, 1977). In spite of the similarities in firing pattern and parvalbumin content, chandelier cells are clearly separated from conventional basket cells. In this thesis, I use either the FS or PV term to refer to both morphological subtypes.
Because their innervations either on the soma or the axon initial segment is in close proximity of the action potential initiation site (the axon initial segment), the FS PV interneurons provide strong inhibitory control over the postsynaptic cell firing and are the main class of cells to provide feedforward inhibition and hence precise control of neuronal output (Pouille and Scanziani, 2001). Moreover, PV basket cells contact a vast number of postsynaptic cells being thus able to synchronize the firing of large groups of neurons (Cobb et al., 1995; Pouille and Scanziani, 2001).

Due to their role in controlling the pyramidal cell activity it is important to understand how these cells become active in different brain areas, by describing in detail the sources and the properties of the synaptic inputs received by them. It is also important to understand the mechanism involved in mediating GABAergic inhibition from interneurons onto their postsynaptic target. The four chapters of the thesis address these questions for the FS PV interneurons in the layer 3 of the PFC.

**THE NON FAST SPIKING INTERNEURONS**

The Non–FS (NFS) cell class comprises a multitude of different GABAergic cells. To discriminate between them a combination of several features need to be considered. Their electrical parameters do not always yield separate clusters of cells and a high level of heterogeneity is found at functional and morphological levels of the NFS cells. Studies reveal several labels for their firing patterns such as low threshold spiking, late spiking, irregular spiking or adapting spiking. They display brief single spikes, but in contrast to pyramidal and FS cells, they have slow membrane time constants and their synaptic outputs and inputs are kinetically slower as well (Jonas et al., 2004). Molecular markers combined with morphological features describe Cholecystokinin-containing basket cells, Somatostatin-containing Martinotti cells, Calbindin-containing Neurogliaform cells and Vasointestinal peptide-containing multipolar cells (Porter, 1998; Ferezou, 2002; Markram et al., 2004). Here, we did not study each of these GABAergic cell types separately, but we classified them as a large group of NFS cells (Fig. 9).

It has been suggested that 92% of the GABAergic synapses contacting CA1 pyramidal cells innervate their dendrites and not the soma or the axon initial segment (Megias et al., 2001). This raises important questions about the role of these cells for the types of computational process that occur at different dendritic sites on pyramidal cells (Stuart et al., 2000; Chen and Johnston, 2006; Harvey and Svoboda, 2007; Spruston, 2008). Most of NFS cells innervate pyramidal cells at different dendritic sites away from the somatic compartment or proximal dendrite. For example the Martinotti cells (Martinotti, 1889) have a large axonal arborisation that ends in the upper layers of the cortex suggesting that this cell type innervates mostly the distal dendritic arbor of the pyramidal cells. Another example is the neurogliaform that has a dense axonal arbor around its own soma suggesting it innervates nearby dendrites. As a result of
their axonal distribution pattern which seems to avoid the somatic compartment of pyramidal cells the NFS interneurons appear to be in close proximity with the excitatory inputs onto pyramidal cell, which contact the pyramidal cells via dendritic spine. Moreover, the NFS cells have been shown to provide modulatory control over postsynaptic EPSP integration and plasticity of the glutamatergic synapses.

One important finding is that often, the functional dichotomy of the FS vs. NFS is associated with different connectivity and expression patterns for receptors, transmitters, and modulators (Freund, 2003). Thus, one approach towards understanding their specific roles is to view them in relation with the specific role of different pyramidal cell compartments. An average pyramidal cell receives about 30,000 synaptic inputs and has an elaborated dendritic tree to provide support for their integration. The cell body integrates inputs from the dendrites and receives only GABAergic synapses, the majority of which belong to the FS PV basket. Similarly the axon-initial segment, where action potentials are generated, receives exclusively GABAergic inputs from FS PV chandelier cells. Thus, these two cell types can dictate whether processed information from the entire dendritic tree of the pyramidal cells can be transmitted to other neurons or not. In comparison, the rest of the interneurons, the large NFS group, synapse in close contact to excitatory inputs arriving at different levels on the dendritic tree collaterals Thus they can dictate the fate of plastic changes at these synaptic sites and also block integration of inputs from specific brain regions

GLUTAMATERGIC INPUT ONTO INTERNEURONS – SYNAPTIC MECHANISMS RESPONSIBLE FOR RECRUITMENT OF INHIBITION

To become active and thus able to contribute to network activity, interneurons need to reach firing threshold. One activation modality is via neuromodulators which were shown to be able to depolarize and thus induce spiking activity in GABAergic cells (Parra et al., 1998). Another modality is via glutamatergic inputs that can trigger GABAergic transmission at specific time windows. Importantly, the precise and selective recruitment of GABA-mediated synaptic inhibition leads to precise control of pyramidal cell firing, ultimately resulting in rhythmic cortical activity at different oscillatory frequencies (Buzsaki, 2004a; Buzsaki and Draguhn, 2004b; Buzsaki et al., 2004c; Fries, 2009). Among the synchronized rhythms in the brain, gamma-band (30-80 Hz) oscillations are present in behavioral states ranging from simple sensory stimulation (Gray et al., 1989b) to attention selection (Fries et al., 2001b; Bichot et al., 2005; Womelsdorf et al., 2006) and working memory maintenance. Moreover, in psychiatric disorders like schizophrenia, which is characterized by an important cognitive dysfunction, the deficits in cognitive control exhibited by schizophrenic subjects are correlated with deficits in gamma oscillation production (Cho et al., 2006; Lesh et al., 2011). Understanding the sources of
glutamatergic inputs and the synaptic properties of these inputs is of great importance in further understanding how interneurons become active and contribute to network functions such as synchronization in gamma band oscillations.

**SOURCES OF SYNAPTIC INPUTS ONTO INTERNEURONS**

Excitatory inputs onto interneurons have been previously described (DeFelipe, 1997; Kawaguchi and Kubota, 1997) but it is still unknown what is the exact cellular identity of these sources in each brain area. Within the PFC, synaptic inputs onto interneurons arise from local collaterals of the pyramidal cells or from incoming inputs from specific regions that relay information to the PFC. Strong differences between FS and NFS cells have been described at the level of synaptic input received by them (Fig. 10). For example FS cells are strongly recruited by synapses on their soma (Gulyas et al., 1999) and according to electron microscopic reconstructions, of PV FS cells in hippocampus, they receive three times more asymmetrical, presumed glutamatergic inputs than NFS cells (Gulyas et al., 1999; Mátyás et al., 2004).

![Figure 10. Distribution of excitatory and inhibitory inputs on dendrites of PV-, CB-, and CR-immunoreactive interneurons in CA1 stratum radiatum](image)

Figure 10. Distribution of excitatory and inhibitory inputs on dendrites of PV-, CB-, and CR-immunoreactive interneurons in CA1 stratum radiatum. Note the large difference for the number of excitatory and inhibitory synapses terminating on the three types of dendrites. The surface of the PV-positive dendrite is densely covered by synapses in contrast to the sparse innervation of CB- and CR-positive dendrites. On the other hand, the proportion of inhibitory terminals compared to all synaptic inputs is lowest on the PV and highest on the CB dendrites.(Gulyas et al., 1999)
In the PFC one of the major subcortical inputs arises from the mediodorsal thalamic nucleus. How inputs from this thalamic nucleus recruit inhibitory neurons in PFC constitutes the subject of the second chapter in this thesis.

The thalamus plays a major role in integrating the flow of information to and within the brain because it is richly interconnected with other regions of the brain. Two main classes of thalamic relay nuclei have been described (Fig.
The targets of higher-order relays are other cortical regions, implying that these thalamic nuclei facilitate and modulate signals as they pass from one part of cortex to another. The mediodorsal thalamus is one of the higher order nuclei of the thalamus and the major sources of subcortical input into the PFC. It has been hypothesized that thalamocortical connections are contributing to higher order functions of the cortex given that both PFC and MD receive processed information from the primary cortex. (Guillery and Sherman, 2002). Chapter 2 of this thesis addresses the question of how thalamic inputs synapse onto different types of interneurons in PFC and shows that these inputs have a preference for the PV interneurons but they also synapse onto the rest of the GABAergic cells.

THE ROLE OF THE GLUTAMATERGIC SUBUNITS TO THE FUNCTION OF INTERNEURONS

Not only the sources of glutamatergic inputs are important in determining the activity of interneurons but also the properties of the glutamatergic receptors present at these synapses. The neurotransmitter glutamate acts via several ionotropic receptor subtypes including AMPA, kainate and NMDA receptors which produce significantly different effects when activated at the postsynaptic level (Nakanishi et al., 1998). The peak amplitude of excitatory postsynaptic currents (EPSCs) is several times larger in FS cells compared to NFS cells (Porter et al., 2001; Beierlein et al., 2003) and the short term plasticity of the glutamatergic inputs onto FS shows short term depression and often but not always, short term potentiation in the case of NFS (Gupta et al., 2000; Markram
et al., 2004). This makes recruitment of FS and NFS into feed-forward and feedback circuits, dependent on the firing level of the network. The properties of glutamatergic synapses are important in modulating the EPSP kinetics and thus the synaptic integration of the incoming inputs. Fast vs. slow kinetics are considered important for coincidence detection vs. time integration modes of information processing. In the neocortex, the firing of FS cells precisely follows the presynaptic pyramidal cell activity pattern (Galarreta and Hestrin, 2001a) and this microcircuit is thus able to detect coincident excitatory activity. Because FS PV cells are considered to be responsible for mediating precisely timed signals the composition of synaptic receptors onto these cells is extremely important. Studies of selective knockout of the AMPA receptor subunit, GluA, from PV cells showed a reduction of their phasic excitatory drive (Fuchs et al., 2007) and consequently, PV-positive interneurons spiked less while losing, the temporal precision of these spikes. At the same time the power of gamma oscillations was profoundly decreased. These findings support the hypotheses that recruitment of PV cells via glutamatergic AMPA containing receptors is necessary for gamma oscillations which in turn have important roles in cognition. At the same time, in vivo recordings from PFC using NMDA receptor blockers found an increased activity of the putative pyramidal cells (Homayoun and Moghaddam, 2007) and led to the hypothesis that NMDA receptors onto FS cells mediate disinhibition of the cortical network in PFC. How fast synaptic transmission is controlled and how receptor composition compares with pyramidal cells is addressed in Chapter 3 of the thesis.

POSTSYNAPTIC TARGETS OF GABAERGIC CELLS

The firing activity of GABAergic cells leads to release of GABA and activation of the GABAergic receptors present at postsynaptic targets that include both pyramidal cells and interneurons (Somogyi et al., 1998). GABAergic receptors can be both ionotropic and metabotropic subtypes named GABA_A and GABA_B respectively.

The distribution of GABA_A receptors either at synaptic or extrasynaptic sites can be responsible for two types of effects onto the postsynaptic targets. Firstly the presence of these receptors at synaptic sites can lead to a fast postsynaptic current (IPSC), called phasic GABAergic neurotransmission. The amplitude and kinetics of this current are dependent on the subunits composition and number of GABA_A receptors and on the magnitude and duration of the GABA transient (Mody et al., 1994). Secondly, the activation of the extrasynaptic GABA_A receptors can lead to a continuous and thus tonic form of inhibition (Semyanov et al., 2004), (Fig. 12). The tonic inhibition can be produced by spillover of GABA neurotransmitter into the extracellular space and thus onto extrasynaptic receptors (Wei et al., 2003). The activation of extrasynaptic GABA_A receptors produces a tonic GABA conductance that leads to a shunting effect which alters excitability and the gain of the postsynaptic cell (Semyanov et al., 2004).
Figure 12. The effect of GABA release from GABAergic cells. (a) The release of a single vesicle from a presynaptic terminal activates only those postsynaptic GABA\(_A\) receptors that are clustered in the membrane immediately beneath the release site (yellow). The diffuse blue shading indicates the spread of released GABA. The current record shows an averaged waveform of miniature inhibitory postsynaptic currents (mIPSCs) recorded in the presence of the sodium channel blocker tetrodotoxin. The area beneath the record is shaded to indicate the charge transfer. GAT1 is the GABA transporter that transports GABA back into the cell. (b) Action potential-dependent release of multiple vesicles or evoked release from several terminals promotes GABA 'spillover', and activates both synaptic receptors and perisynaptic or extrasynaptic receptors (blue). The current record shows the larger and much slower averaged waveform of IPSCs evoked by electrical stimulation. The area of the mIPSC is superimposed for comparison. (c) A low concentration of ambient GABA, which persists despite the activity of the neuronal and glial GABA transporters (GAT1 and GAT3), tonically activates high-affinity extrasynaptic receptors. The trace shows the 'noisy' tonic current that results from stochastic opening of these high-affinity GABA\(_A\) receptors, with superimposed phasic currents (in this case, the synaptic events would be arising at sites not depicted in the schematic diagram). A high concentration (10 \(\mu\)M) of the GABA\(_A\) antagonist gabazine (SR-95531) blocks the phasic IPSCs and tonic channel activity, causing a change in the 'holding' current and a reduction in current variance. The shaded area beneath the current record before SR-95531 application represents the charge carried by tonically active GABA\(_A\) receptors. The frequency of spontaneous IPSCs is relatively low and the tonic receptor activity generates a conductance several-fold larger than the averaged conductance that is carried by phasic IPSCs. From (Farrant and Nusser, 2005)
Figure 13. Control of spike timing by shunting and hyperpolarizing inhibition. (a) GABAergic synapse with postsynaptic ionotropic GABA_A receptors permeable to Cl− and HCO3− at synaptic and extrasynaptic locations, and pre- and postsynaptic metabotropic GABA_B receptors, coupled by way of G-proteins to Ca2+ and K+ channels. (b) Spatial distribution of GABAergic synapses. Presynaptic inhibition is mediated by way of GABA_B receptors, and postsynaptic dendritic inhibition is mediated by both GABA_A and GABA_B receptors. By contrast, inhibition at perisomatic sites appears to be mediated exclusively by GABA_A receptors. (c) Kinetics of GABA_A receptor-mediated events. The synaptic conductance change (G_syn) responsible for shunting is short lasting, whereas the time course of the associated membrane potential effect is determined by the membrane time constant of the postsynaptic neuron. The effect on membrane potential can be depolarizing, hyperpolarizing or clamping, depending on the relation between the actual membrane potential (V_m) and the reversal potential of the GABA_A receptor-gated conductance (E_syn). (d) The effect of shunting and hyperpolarisation on spike generation. To emphasize the distinct effects of changes in membrane conductance and membrane potential produced by GABAergic events, this example uses model cells that display intrinsic oscillations driven by a T-type calcium current, which can be observed in several subcortical nuclei. Whereas pure shunting can only veto the generation of an action potential within a brief time window, hyperpolarizing inhibition can also phase reset the oscillation. The interaction between hyperpolarizing inhibition and the T-type calcium current also increases transiently the number of spikes per cycle, which is an example of rebound excitation. (e) Example of rate homogenization and phase synchronization by GABAergic events. The effect of having the reversal potential of the GABA_A receptor-gated conductance within the range of membrane potential fluctuations is explored in a set of three model cells displaying simple Hodgkin–Huxley kinetics, and receiving different levels of tonic excitatory drive. Owing to the clamping effect on membrane potential, following the introduction of rhythmic GABAergic events, both rate and phase of firing is immediately homogenized between the three neurons From (Mann and Paulsen, 2007).

GABA_A receptors mediate their effects by generating an increased permeability to chloride ions. The currents via the ionotropic GABA_A receptors are dependent on the chloride concentration differences between intra- and extracellular compartments which in turn are regulated by different chloride transporters which set the reversal potential of the channels. Thus, depending on
the value of the chloride reversal potential, the GABA postsynaptic current can be either hyperpolarizing (when the chloride reversal potential is below the membrane potential of the postsynaptic cell) or depolarizing (when the chloride reversal potential is above the membrane potential of the postsynaptic cell (Mann and Paulsen, 2007). The postsynaptic events mediated by GABA<sub>A</sub> receptors can have a shunting effect due to an increase of the postsynaptic membrane conductance, and a change in the membrane potential due to the current flowing through this conductance. The pure shunting effect is always inhibitory, as it acts to reduce the membrane depolarizing effect of concurrent excitatory events. This increased conductance during GABA<sub>A</sub> receptor activation limits the depolarization above the reversal potential of chloride and therefore inhibits the membrane potential from reaching action potential threshold. The time course for this form of “shunting” inhibition is dependent on the time course of the GABA<sub>A</sub> conductance change (Staley and Mody, 1992), (Fig. 13).

**GABAergic Inputs onto Pyramidal Cells - Importance of GABA Spill Over on Pyramidal Cells Activity**

GABAergic synapses cover almost the entire surface of pyramidal neurons dendritic arbor, from the tips of their thinnest dendritic shafts to the axon initial segments (Fig. 14). Each type of interneuron innervates its target cell by preferentially distributing multiple synapses with identical dynamics (identical release probabilities and time constants for recovery from synaptic depression and facilitation) (Gupta et al., 2000) onto selected membrane domains, axon initial segments, somata, proximal and distal dendritic shafts and spines, and dendritic tufts (DeFelipe, 1997; Somogyi et al., 1998). The somatic regions are innervated by FS cells that form many putative synapses onto several pyramidal neurons at the same time and tend to have fast kinetics as well as short term depression. The majority of NFS neurons contact the dendrites of pyramidal cells and show physiologically-diverse properties (Gupta et al., 2000). In chapter 4 of the thesis we addressed the specific questions regarding the regulation of differential GABAergic transmission between somatic and dendritic sites.

Most of the experiments in which recordings were made between connected interneuron-pyramidal neuron pairs suggest that GABA synapses mediate their effects predominantly via fast ionotropic GABA<sub>A</sub> receptor which are pentameric ionotropic receptors permeable to chloride ions. Additionally metabotropic GABA<sub>B</sub> transmission was also found at GABAergic postsynaptic sites. Because the GABA<sub>B</sub>-mediated responses were elicited after strong or repetitive or high-frequency stimulation (Benardo, 1994) it was suggested that GABA<sub>B</sub> receptors are located extrasynaptically and are activated by GABA 'spillover' from the synaptic cleft.
Figure 14. Density of inhibitory and excitatory inputs along the dendritic tree. Dendritic structure of a CA1 pyramidal showing the density of asymmetrical (or excitatory) and, symmetrical (or inhibitory) (left and middle numbers, respectively, number/1 μm) and the proportion of symmetrical synapses (right number) are shown (Megias et al., 2001).

Along the dendritic tree, the density of GABA_A receptors is higher closer to somatic regions whereas the GABA_B receptors are expressed mostly at distal pyramidal cell dendrites where they associate with Kir3.2 K⁺ channels (López-Bendito et al., 2002b), (Fig. 15). The FS cells synapsing at perisomatic site (Fig. 15) mediate their inhibition via purely GABA_A receptors (Gonzalez-Burgos et al., 2005a; Freund and Katona, 2007). In contrast, some subtypes of NFS cells such as neurogliaform interneurons, contacting distal dendrites elicit dual IPSPs mediated by both GABA_A and GABA_B receptors (McCormick, 1989; Tamas et al., 2003; Szabadics et al., 2007) (Fig. 15). The dendritic GABA_B-IPSPs powerfully inhibit dendritic Ca²⁺ spikes (Perez-Garci et al., 2007).
2006) and spike backpropagation into dendrites (Leung and Peloquin, 2006) thus contributing to plastic changes in the dendrites. Thus, besides different types of interneurons targeting different dendritic locations, there is a large variability of GABA receptor functions that are separated at different sites along the dendrites of pyramidal cells (Fig. 15).

Figure 15. Schematic of inhibitory inputs along the dendritic tree Distribution of postsynaptic receptors along the dendritic tree with their presynaptic axons belonging to different subtypes of interneurons. (Source, Wikipedia)

Escape of fast neurotransmitters from the synaptic cleft reduces the specificity of synaptic transmission (Bergles et al., 1999), and consequently it can reduce the capacity for information processing of neuronal networks (Barbour, 2001) Synchronous firing of multiple interneurons leads to accumulation of GABA in the extracellular space and thus to unspecific activation of GABA receptors. Thus a cortical state that allows activity at one site to influence activity at surrounding sites reduces synaptic “independence.” At GABAergic synapses, the GABAergic transporter GAT can limit "spillover"
of GABA from the synapse, promoting synapse independence (Isaacson et al., 1993; Rossi and Hamann, 1998; Overstreet and Westbrook, 2003). In chapter 4 we asked if GABA transporters have a differential role in regulating GABA transmission between perisomatic and perydendritic sites and what are subserved by different subtypes of interneurons.

GABAERGIC INPUTS ONTO INHIBITORY NEURONS - IMPORTANCE OF CONTROLLING GABAERGIC OUTPUT

Postsynaptic targets of the inhibitory cells are not only the pyramidal cells but also the interneurons themselves. Studying interneuron to interneuron signaling is not a trivial task because of their scarceness in the cortex. Interneurons are interconnected via gap junctions (Galarreta and Hestrin, 1999; Gibson et al., 1999; Tamas et al., 2000; Galarreta and Hestrin, 2001b) and chemical synapses (Hajos and Mody, 1997; Tamas et al., 2000; Bartos et al., 2001; Szabadics et al., 2001) and also innervate themselves via autapses (Cobb, 1997; Tamas et al., 1997; Bacci et al., 2003a). Paired recordings from different interneurons showed that multiple synapses are established between these cells and that they are highly distributed along the dendrites (Gulyas et al., 1999). FS basket cells have a large numbers of synapses between them (Tamas et al., 1998) and the same holds true for NFS cells (Gibson et al., 1999; Beierlein et al., 2000). This suggests that they form interconnected networks (Fig. 16).

Slice experiments and computational models exploiting this idea conclude that the strength, spatial extent, and anatomical localization of the connections between interneurons may be crucial in modulating complex network oscillations (Beierlein et al., 2000; Tamas et al., 2000; Szabadics et al., 2001), involving the mutually interconnected GABAergic interneurons and glutamatergic pyramidal cells (Fig. 17A). More then their role in mediating oscillations in the cortex it is important that interneurons have mechanism to silence each other when needed. The kinetics of interneuron to interneurons transmission via GABA\textsubscript{A} receptors have been consider of strong importance in their role of mediating oscillatory activity such that slow kinetics impose a lower frequency band of the oscillatory cycle. The kinetics of GABA\textsubscript{A} mediated IPSC depends on the subunit composition of these receptors that are pentameric composed of combinations of multiple subunit subtypes (Vicini, 1999; Cherubini and Conti, 2001; Rudolph et al., 2001), (Fig. 17)
Figure 16. Networks of neocortical interneurons Distinct GABAergic networks in which cells belonging to a particular subtype tend to couple to each other more strongly through electrical and chemical synapses (Beierlein et al., 2000)

Figure 17. The kinetics of inhibitory responses have an important role in oscillatory rhythms (A) Schematic showing inhibitory inputs onto both pyramidal cells and
interneurons. (B) Inputs from interneurons to pyramidal cells synchronize the firing of large groups of pyramidal cells to specific frequency detected by local field potential recordings (C) Firing of interneurons generates IPSC of certain with kinetics. (D) IPSC kinetics determined by GABA<sub>A</sub> receptor composition determines the frequency of the oscillations. Pharmacological interference with GABA<sub>A</sub> receptors has a strong effect on the frequency of oscillations both in slices and in computational models. (Whittington et al., 1995)

Morphological studies (Gulyas et al., 1999) showed that the ration between excitatory and inhibitory inputs is lower in NFS cells compared with FS cells, suggest that the inhibitory drive onto Non FS is stronger then the excitatory one. Perhaps one important function of inhibition onto interneurons is to provide silencing of certain cell types in order to facilitate the flow of information from one site to another, in the brain. In chapter 5 we asked whether the kinetics of GABA responses onto interneurons is cell type specific and whether this specificity is dependent on the subunit composition of GABA<sub>A</sub> receptors.

**AIM OF THE THESIS:**

In this thesis four specific questions have been addressed, in order to better understand the functions of GABAergic interneurons in neuronal circuits of middle layer PFC. We used electron microscopy and electrophysiology approaches to assess the properties of synaptic inputs onto and from GABAergic interneurons. Then we investigated the glutamatergic inputs onto interneurons, in the second and the third chapters and the GABAergic inputs from interneurons in the fourth and the fifth chapters.

1. **How are different PFC inhibitory interneurons recruited in neuronal network activity by thalamic inputs?**

Glutamatergic inputs are the main ways of depolarizing neurons in a temporally controlled manner. The number of excitatory inputs onto interneurons is much lower compared with pyramidal cells and there are also differences in the number of inputs each subtype of interneuron receives. Moreover, different brain regions connect with each other via glutamatergic inputs but it is not yet known how specific these connections are. The mediodorsal thalamus (MD) is the main subcortical structure sending a strong glutamatergic input to the prefrontal cortex. We investigated whether MD afferents to the PFC form selective synaptic connections with different subtypes of local circuit neurons identified by their immunoreactivity for calcium-binding proteins.

2. **What are the respective roles of AMPA and NMDA receptors in activating PFC FS interneurons?**

Glutamatergic synapses are complex structures containing both AMPA and NMDA receptors with different roles in mediating excitatory transmission. It has been hypothesized that the NMDA receptor hypofunction in PFC is
responsible for the cognitive deficits described in Schizophrenia and thus reversely, it is thought that synaptic transmission via NMDA receptors is very important for cognitive function. It is not known though, onto which cells types NMDA has the strongest impact. Given that among interneurons the FS subgroup received the greatest number of excitatory inputs we asked how synaptic NMDA receptors contribute to the activation of FS and pyramidal cells in the PFC network. Answering this question may reveal possible mechanisms linking NMDA receptor hypofunction with gamma oscillation abnormalities in schizophrenia. We use patch clamp recordings from both of these cell types and compared the AMPA and NMDA subunit contribution to their synapses.

3. **How are ambient levels of GABA in the PFC neuronal network regulated?**

Once GABAergic cells become active in a network their intense firing generates release of GABA in the extracellular space that may lead to unspecific activation of GABA receptors in the brain. One of the protective mechanisms against extracellular GABA accumulation is represented by the plasma membrane GABA transporters 1 (GAT1). This transporter is localized in neuronal and glial membranes near synapses and transports GABA back into the neurons or glia. GAT1 shows important changes during adolescence, a period of development which is associated with maturation of the cognitive function. Therefore GAT1 might serve an important role in cognitive development by its way of controlling GABA levels. The functional role of GAT1 has not been investigated in the human or non-human primate. We determined how GAT1-mediated uptake regulates GABA spillover in primate neocortical circuits, by recording from layer 3 pyramidal neurons of monkey DLPFC and testing the effects of the GAT1 blocker (NO711) on proximal (perisomatic) versus distal (dendritic) GABA synapses.

4. **How are PFC interneurons affected by activity of other interneurons?**

Interneurons regulate the pyramidal cell activity but also the activity of other interneuron. Because they have a strong impact on the way pyramidal cells integrate incoming inputs one can ask whether interneuron to interneuron signaling is also important in regulating interneuron activity. Studies that investigated how interneurons regulate their own activity showed a higher connectivity rate between interneurons from one specific subclass then between interneurons from different subclasses. This led to the conclusion that there are separate networks of interneurons in the cortex. Furthermore it was suggested that these separate networks are important in the maintenance of oscillatory rhythms at several requires in the cortex. Additionally it was proposed that the kinetics of GABA (A) mediated inhibition which are dependent on the receptor subunit composition are also responsible in mediating certain oscillatory frequencies. We investigated inhibitory inputs onto physiologically-identified interneurons in primate neocortex to determine if there is a cell type-specific GABA_A mediated transmission.
Chapter 2

Mediodorsal thalamic afferents to layer III of the rat prefrontal cortex: synaptic relationships to subclasses of interneurons.

Published in J Comp Neurol. 2005 Sep 26;490(3):220-38.
Rotaru DC, Barrionuevo G, Sesack SR.
Department of Neuroscience, University of Pittsburgh, Pittsburgh, PA 15260, USA.

The mediodorsal nucleus of the thalamus (MD) represents the main subcortical structure that projects to the prefrontal cortex (PFC) and it regulates key aspects of the cognitive functions of this region. Within the PFC, GABA local circuit neurons shape the activity patterns and hence the “memory fields” of pyramidal cells. Although the connections between the MD and PFC are well established, the ultrastructural relationships between projecting fibers from the MD and different subclasses of GABA cells in the PFC are not known. In order to address this issue in the rat, we examined MD axons labeled by tract-tracing in combination with immunogold-silver to identify different calcium-binding proteins localized within separate populations of interneurons. Electron micrographic examination of PFC sections from these animals revealed that MD terminals made primarily asymmetric synapses onto dendritic spines and less commonly onto dendritic shafts. Most of the dendrites receiving MD synaptic input were immunoreactive for parvalbumin (ParV), whereas MD synapses onto dendrites labeled for calretinin or calbindin were less frequent. We also observed that some MD terminals were themselves immunoreactive for calcium-binding proteins, again more commonly for ParV. These results suggest that the MD exerts a dual influence on PFC pyramidal cells: direct inputs onto spines and an indirect influence mediated via synapses onto each subclass of interneurons. The apparent preferential input to ParV cells endows MD afferents with a strong indirect inhibitory influence on pyramidal neuron activity by virtue of ParV cell synapses onto soma, proximal dendrites, and axon initial segments.
INTRODUCTION

The thalamus constitutes the main gateway through which the cerebral cortex receives information about the external world, and a detailed knowledge of thalamocortical interactions is important for understanding how information is represented and processed in the brain (Castro-Alamancos and Connors, 1997). Three different types of thalamic relay nuclei allow for the segregated transmission to the cortex of distinct types of information: external sensory signals are projected from principal relay nuclei to primary cortical areas (Freund et al., 1985; Bernardo and Woolsey, 1987; Jensen and Killackey, 1987; Chmielowska et al., 1989; Romanski and LeDoux, 1993); “second-order nuclei” connect with association cortices and function during more complex stages of information processing (Bender, 1983; Diamond et al., 1992; Guillery, 1995; Castro-Alamancos and Connors, 1997; Deschenes et al., 1998; Guillery and Sherman, 2002) and midline and intralaminar thalamic nuclei maintain the awake state and levels of arousal and awareness (Moruzzi and Magoun, 1959; Kinomura et al., 1996; Van der Werf et al., 2002).

The mediodorsal thalamus (MD) is an example of a second-order nucleus and has relatively selective projections to the prefrontal cortex (PFC), an association region involved in the cognitive control of behavior (Fuster, 2009). The way in which the reciprocal connections between the PFC and MD (Krettek and Price, 1977; Giguere and Goldman-Rakic, 1988; Groenewegen, 1988; McFarland and Haber, 2002; Lewis et al., 2004) affect cognition is not fully understood, although studies suggest that deficits associated with MD lesions resemble those observed after damage to the PFC (Hunt and Aggleton, 1998a, 1998b). Such impairments include object recognition (Mumby et al., 1993), working memory (Freeman et al., 1996), planning and prospective coding (Daum and Ackermann, 1994), and executive processing (Floresco et al., 1999).

In addition to thalamic inputs, the normal functioning of cortical circuits depends on an inhibitory network created by GABA local circuit neurons (DeFelipe, 1997; Kawaguchi and Kubota, 1997). The main functions of interneurons are to shape the receptive fields of pyramidal cells via lateral inhibition (Sillito, 1975; Eysel, 1992; Kisvarday et al., 1994; Petersen and Sakmann, 2001) and to control the overall level of pyramidal cell excitability by feedforward and feedback inhibition (Cobb et al., 1995; Pouille and Scanziani, 2001). Furthermore, it has been suggested that the synchronous activity associated with different states like sensory perception, arousal, or sleep are achieved and maintained by networks of inhibitory interneurons that are connected with each other via electrical synapses (Steriade, 1997).

Several subtypes of inhibitory interneurons can be defined on the basis of their content of calcium-binding proteins (Kawaguchi and Kubota, 1997). These cells differ in their physiological, morphological, and immunohistochemical characteristics in ways that appear to correlate with
different functions. Interneurons containing parvalbumin (ParV) exhibit “fast spiking” properties and include two morphological subtypes, basket and chandelier cells, which innervate the somata and axon initial segments of pyramidal cells, respectively (Kawaguchi and Kondo, 2002). Other subtypes include calretinin- (CalR) or calbindin- (CalB) containing double bouquet cells (DeFelipe et al., 1990; Miettinen et al., 1992; DeFelipe and Jones, 1992b) and CalB-positive Martinotti cells. These local circuit neurons can be either burstspiking or regular-spiking (Kawaguchi, 1995; Kawaguchi and Kubota, 1997), and their ascending and descending axonal arbors may provide inhibition within cortical columns.

The inhibitory influence of GABA cells on pyramidal neurons is ultimately driven by excitatory inputs to the local circuit neurons (DeFelipe, 1997; Kawaguchi and Kubota, 1997), making it essential to identify their main sources of activation. In primary sensory areas, GABA cells receive direct input from the principal thalamic relay nuclei (Keller and White, 1987) and these inputs include synapses onto the ParV subtype of interneurons (Staiger et al., 1996). In the PFC, the main excitatory inputs that drive local circuit neurons have not been fully defined. In the monkey PFC, the local collaterals of pyramidal cells innervate predominantly ParV and less often CalR interneurons (Melchitzky and Lewis, 2003). Similarly, in the rat PFC pyramidal cell local collaterals synapse onto ParV cells (Sesack et al., 2001).

In an extensive series of electron microscopic studies, (Kuroda et al., 1993; Kuroda et al., 1995a; Kuroda et al., 1995b; Kuroda et al., 1996b; Kuroda et al., 1998a; Kuroda, 1998b) described the main recipient of MD projecting fibers in the PFC as being the spines of pyramidal neurons. More recently, (Kuroda et al., 2004) have shown that the dendrites of GABA local circuit neurons also receive synaptic input from the MD. However, it is still unknown whether the MD innervates all classes of GABA interneurons or synapses selectively onto a specific population.

Therefore, the purpose of the present study was to investigate whether MD afferents to the PFC form selective synaptic connections with different subtypes of local circuit neurons identified by their immunoreactivity for calcium-binding proteins. Given the observations of similar studies in other cortical areas (Staiger et al., 1996; Porter et al., 2001), we hypothesized that the main recipient of MD inputs to local circuit neurons in the PFC would be the ParV cells.

**MATERIALS AND METHODS**

All animal procedures were performed with the approval of the Institutional Animal Care and Use Committee at the University of Pittsburgh and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.
**Tract-tracing procedures**

For this study, 10 male Sprague-Dawley rats (Hilltop Laboratories, Frederick, MD) weighing 300–400 g were used. The animals were kept in colonies on a 12-hour light/dark cycle and had unlimited access to water and chow. The rats were anesthetized with an intramuscular (i.m.) injection of 34 mg/kg ketamine, 1 mg/kg acepromazine, and 7 mg/kg xylazine and placed in a stereotaxic frame. Seven animals received tracer injections: three rats received biotinylated dextran amine (BDA; 10,000 MW, Molecular Probes, Eugene, OR) dissolved as a 10% solution in 0.01 M phosphate-buffered saline (PBS), pH 7.4, whereas four rats received Phaseolus vulgaris leucoagglutinin (PHA-L; Vector Labs, Burlingame, CA) as a 2.5% solution in PBS, pH 8.0. The unilateral injection site coordinates were centered within the medial subdivision of the MD, corresponding to 2.1 and 2.4 mm posterior to bregma, 0.6 mm lateral to the midline sinus, and 5.2 and 5.7 mm, respectively, ventral from the skull surface (Paxinos and Watson, 1986). Injections were made using glass micropipettes with 50 μm tip diameters. The tracer was iontophoretically delivered by using a positive 5 μA current pulsed 7 seconds on/off for 15 minutes. Pipettes were left in place for 10 minutes before removal.

In order to label the thalamocortical projections by anterograde degeneration, three other animals received unilateral electrolytic lesions of the MD using an anodal current of 0.5 mA for 15 seconds passed thorough an Epoxylite-coated stainless steel monopolar electrode exposed for 1.0 mm at the tip. The optimal survival time for anterograde degeneration was determined to be 3 days based on our prior analysis of projections from the paraventricular nucleus of the thalamus (Pinto et al., 2003) and our empirical observations of dense degenerating axons from the MD in the present study at this same time point.

**Perfusion**

After a survival period adapted for each of the three conditions (5 days for BDA, 10 days for PHA-L, and 3 days for electrolytic lesions), the rats were deeply anesthetized with an intraperitoneal (i.p.) injection of 60 mg/kg sodium pentobarbital. The rats were also treated with sodium diethyldithiocarbamate (1 g/kg, Sigma, St. Louis, MO) as a zinc chelator to prevent silver binding to endogenous zinc (Veznedaroglu and Milner, 1992). All rats were then killed by perfusion through the aorta with 10 ml of a heparin-saline solution (1,000 U/ml) followed by 50 ml of 3.75% acrolein in 2% paraformaldehyde and then 250–300 ml of 2% paraformaldehyde in 0.1 M PB, pH 7.4. The brains were extracted and postfixed for 1 hour in 2% paraformaldehyde. Brain blocks containing the PFC or MD were sectioned at 50 μm using a vibratome and collected in 0.1 M PB.
Immunocytochemistry

After vibratome sectioning, brain slices were incubated for 30 minutes in a solution of 1% sodium borohydride in 0.1 M PB followed by rinses in 0.1 M PB and 0.1 M Tris-buffered saline, pH 7.6 (TBS). Sections were then incubated for 30 minutes in blocking solution containing 3% normal goat serum, 1% bovine serum albumin, and either high detergent concentration (0.2% Triton X-100) for light microscopy or low detergent (0.04% Triton) for electron microscopy. Brain sections were then processed differentially depending on the type of anterograde tracing.

PFC sections from rats that received BDA injections or electrolytic lesions of the MD were incubated overnight in polyclonal antibodies raised in rabbit (Swiss Antibodies, Swant, Bellinzona, Switzerland) against different calcium-binding proteins and diluted in blocking solution: anti-ParV (1:1,000), anti-CalR (1:1,000), or anti-CalB (1:2,000). After rinsing, sections containing BDA transport were then incubated for 2 hours in 1:100 avidin-biotin peroxidase complex (ABC). Peroxidase was visualized by incubation in 0.02% 3,3-diaminobenzidine (DAB) as a chromogen, and 0.003% hydrogen peroxide as a cofactor. Sections were then incubated for 30 minutes in a washing buffer containing 0.8% bovine serum albumin and 1% fish gelatin in 0.01 M PBS before being incubated overnight in a 1:50 dilution of 1 nm gold-conjugated goat antirabbit antiserum (Amersham, Arlington Heights, IL) in washing buffer. Sections were then rinsed in washing buffer followed by PBS and then postfixed for 10 minutes in 2% glutaraldehyde, followed again by rinsing in PBS. Sections were then rinsed in 0.2 M citrate buffer and incubated for 3–7 minutes in silver solution (Amersham) to enhance gold particle size.

For the cases that involved electrolytic lesions of the MD, no immunoperoxidase reaction was run and sections were processed only in primary antibodies and immunogold-silver labeling as described above.

For the cases that received injections with PHA-L, we used a different combination of antibodies. In our experience with PHA-L tracing, we have determined that the antibody raised in rabbit provides greater sensitivity than that raised in goat. Hence, this necessitated switching the antibodies against calcium-binding proteins to ones raised in a species other than rabbit. Alternate sections through the PFC were incubated overnight in a combination of rabbit anti-PHA-L (1:1,000, Accurate Chemical, Westbury, NY) and one of three monoclonal antibodies raised in mouse against calcium-binding proteins (1:1,000, Swant). The second day, sections were rinsed in PBS, then incubated for 30 minutes in a 1:400 dilution of biotinylated goat antirabbit IgG (Vector) followed by ABC incubation and DAB reaction. Then the sections were blocked in washing buffer and incubated overnight in a 1:50 dilution of 1 nm gold conjugated goat antimouse antibodies (Amersham). Rinsing, postfixation, and silver enhancement were then conducted as described above.

All the antibodies were tested for specificity and potency in previous studies. These tests involved preadsorption controls, Western blot analyses, or
radioimmunoassay (Celio and Heizmann, 1981; Celio, 1986; Rogers, 1987; Celio et al., 1988; Schwallor et al., 1993; Condé et al., 1994; Zimmermann and Schwallor, 2002). Some of these antibodies (rabbit anti-ParV and rabbit anti-CalR) were also used in our prior published studies (Sesack and Pickel, 1995; Sesack et al., 1998). For the polyclonal antiserum directed against CalB, a 10% crossreaction with CalR in immunoblots has been reported by the manufacturer. However, such crossreaction is not observed with the mouse monoclonal antibody. The implications of this crossreaction for the present results are addressed in the Discussion section.

**Light and electron microscopy**

For light microscopic verification of injection sites and transport, some sections through the MD and PFC were processed solely for ABC reaction (BDA cases) or immunoperoxidase (PHA-L cases). For examination of MD lesion placement, sections through the MD were stained for Nissl substance. All sections for light microscopy were mounted onto glass slides, dehydrated in ethanol and xylene, and coverslipped. Digital light micrographic images were adjusted in Adobe PhotoShop (San Jose, CA) to achieve optimal contrast, illumination, and sharpness.

All sections for electron microscopy were postfixed for 1 hour in 2% osmium tetroxide, dehydrated in increasing concentrations of ethanol and propylene oxide, and incubated overnight in a 1:1 solution of epoxy resin (EMBed-12, Electron Microscopy Sciences, Fort Washington, PA) and propylene oxide. The next day, sections were embedded in straight resin and polymerized at 60°C for at least 18 hours. Ultrathin sections were cut from the infralimbic or prelimbic divisions of the PFC (Krettek and Price, 1977), collected onto copper-mesh grids, counterstained with heavy metals, and examined on a Zeiss 902 or a Phillips Morgagni transmission electron microscope. The PFC was then examined for evidence of MD axon terminals in synaptic relationship to dendrites immunoreactive (-ir) for ParV, CalR, or CalB.

Analog micrographs were printed to match brightness and contrast before being digitized. Digital electron micrographs captured from the Morgagni microscope using an AMT XP-60 camera were adjusted using Adobe PhotoShop to match the contrast and illumination intensity of the analog micrographs.

**Analysis of immunostained tissue**

For each animal, one or two sections through the PFC were analyzed for each of the three calcium-binding proteins. The pattern of axonal labeling for BDA or PHA-L within the PFC guided our selection of regions for electron microscopic analysis. For electrolytic lesions, the regions analyzed were those most expected to contain anterograde degeneration based on the location of the MD lesion site. For each vibratome section the ultrathin sections examined were separated by 10–15 other sections in order to avoid sampling the same axons. We limited our
analysis to the surface of the tissue where it interfaced with the plastic resin in order to assure proper penetration of both immunoperoxidase and immunogold-silver reagents. Within the area of examination, all MD terminals labeled by anterograde tracer or degeneration were counted if they were in the same field (13.8 μm² at 20,000× magnification) with profiles immunoreactive for calcium-binding proteins. This further ensured that all immunoreagents had equivalent access to the region analyzed.

Every incidence of synapses or direct appositions without synaptic specializations between MD terminals and labeled or unlabeled dendritic structures were counted. All the observed synapses between MD terminals and PFC structures were considered to be asymmetric, as thalamocortical axons forming symmetric synapses have never been described (White, 1978; Kuroda et al., 1998a). Nevertheless, in a few cases the postsynaptic densities appeared to be thin, most likely due to the plane of section being near the edge of the synapse. We differentially classified the MD synapses as being onto either spines or dendritic shafts. The latter were further classified as being either labeled or unlabeled for calcium-binding proteins. A dendrite was differentiated from a spine by its varicose aspect, presence of one or more mitochondria, receipt of one or more synaptic inputs, and a larger size compared to the heads of spines (Keller and White, 1986; Gabbott et al., 1986a; Gabbott and Somogyi, 1986c; Chmielowska et al., 1988). Spines were recognized by their small size, spine apparatus, cup shape, and the receipt of asymmetric synapses onto the head. In some cases only the latter two features were present in the analyzed sections. All direct appositions of MD terminals onto dendritic shafts were followed in serial sections where possible to determine whether a synapse was present. Both synapses and appositions are reported in the results to account for the fact that some synapses might be missed due to oblique planes of section or excessive accumulation of peroxidase product. A total of 1,881,097 μm² of tissue was analyzed within the PFC: 673,668 μm² for ParV-ir sections, 525,443 μm² for CalR-ir sections, and 681,986 μm² for CalB-ir sections.

RESULTS

Light microscopy

Tracer injections and electrolytic lesions

Although we used 10 animals for this study, we focused only on those cases that had optimal injection or lesion sites within the medial MD (one case for BDA, three cases for PHA-L, and two cases for anterograde degeneration). Three animals that received tracer injections were not included in our analysis either because the injection site did not include the medial division of the MD (one case with PHA-L) or retrograde transport from the MD was observed (two cases with BDA). One case that received an electrolytic lesion was excluded from the study because the injection site extended beyond the MD.
For the cases that were used in the present analysis, tracer deposits of either BDA (Fig. 1A) or PHA-L were confined to the MD. In three of the four cases the deposit was restricted to the medial segment. The largest injection site extended 2.1–2.9 mm caudal to bregma and the smallest one 2.3–2.5 mm. In the fourth case the tracer filled the rostral MD and included both the medial and lateral divisions. The two electrolytic lesion cases resulted in ablation of the entire MD in one case or ablation of the rostral part of the MD in the other case (Fig. 1B). For tracer cases, anterograde transport was observed within the prelimbic and/or infralimbic divisions of the PFC (Fig. 1C). In accordance with prior tracing studies (Krettek and Price, 1977; Groenewegen, 1988; Kuroda et al., 1993), the densest projection was to Layers I and III (Fig. 1C) with lighter projections also observed in Layer V. For lesion studies, light microscopic analysis of degenerating fibers in the PFC was not performed. However, based on the size of the lesions in the MD, it was presumed that they included the portions projecting to the medial PFC, an assumption confirmed by electron microscopy.

Figure 1. Light micrographic images of coronal sections through the rat MD or PFC. A: An injection of BDA into the dorsomedial MD just lateral to the paraventricular nucleus of the thalamus (PV). Some leakage of tracer is evident in the medial but not the lateral habenula (LHb). B: A large electrolytic lesion of the MD is evident ventral to the LHb and lateral to the PV. The habenula is also damaged in this case. C: The anterograde transport of BDA to the PFC from the injection site shown in A. Labeled axons are visible in all layers but are most dense in Layers I and III. D–F: Immunogold-silver labeling for ParV (D), CalR (E), or CalB (F) within soma (large black arrows) and dendrites (small
arrows) in the rat PFC. In F, the white arrow indicates a probable pyramidal neuron with light immunoreactivity for CalB. 3V, third ventricle; fm, forceps minor. Scale bar = 31.25 μm in D (applies to D–F); 500 μm for B; 250 μm for A,C.

**Distribution of calcium-binding proteins within the medial PFC**

The light microscopic distribution of calcium-binding proteins within the medial PFC of the rat has been previously described in detail (Gabbott et al., 1997) and was not specifically analyzed here. Moreover, the intensity of immunolabeling for these proteins was suboptimal for light microscopy due to the use of low detergent concentrations for electron microscopy and the application of immunogold-silver, which is an order of magnitude less sensitive than immunoperoxidase (Celio, 1990a). Nevertheless, we did detect immunolabeled perikarya and proximal dendrites for each calcium-binding protein, and more distal portions of their dendrites were further distinguished by electron microscopic examination. The morphological features and laminar distribution of these cell populations matched previous descriptions (Fig. 1D–F). Within Layer III, ParV-ir cell bodies and dendrites were more numerous than profiles labeled for CalR or CalB. Soma labeled for CalB included both intensely labeled profiles that appeared to be nonpyramidal and more lightly labeled cell bodies with the appearance of pyramidal neurons (Fig. 1F). These presumed CalB-ir pyramidal cells were concentrated in Layer II but were also represented at a lower density in Layer III.

**Electron Microscopy**

**General description of immunolabeled elements within the medial PFC**

For this study, ultrathin sections through the infralimbic and/or prelimbic cortices were analyzed at the electron microscopic level. The datasets generated by analysis of these two divisions were combined, as no significant differences between them were found. Within each ultrathin section the boundaries of Layer III were recognized by the presence of a large number of tightly packed cell bodies in the deep part of Layer II and an increased density of myelinated axons in the superficial part of Layer V. Layer III itself was characterized by a more homogeneous distribution of cell bodies and neuropil. Our analysis focused on Layer III, although parts of deep Layer II and superficial Layer V were probably also included.

Within Layer III a large number of MD profiles were detected with either of the two tracing methods employed. Some MD axons in lower Layer III and upper Layer V exhibited myelination (not shown). For anterograde transport of BDA or PHA-L, MD axons were recognized by the presence of a flocculent, dark immunoperoxidase reaction product (Fig. 2A,C,D). Neither BDA nor PHA-L was observed within cell bodies or dendrites, suggesting that they did not undergo retrograde transport in the cases analyzed. For the electrolytic lesions, MD axons undergoing anterograde degeneration were recognized by their electron-dense appearance, swollen mitochondria, and disrupted vesicles (Fig. 2B,E). Many degenerating MD terminals and their synaptic targets were surrounded, at least in part, by glial processes (Fig. 2E).
Labeled by either method, MD profiles consistently formed asymmetric synapses (Fig. 2). For simplicity, we will refer to axons labeled by tract-tracing or degeneration that formed synapses within the medial PFC as MD terminals.

*Figure 2. MD axons in the rat PFC.* Electron micrographs of the rat PFC illustrating axon terminals labeled either by immunoperoxidase for BDA or PHA-L anterogradely transported from the MD (MD-t in A,C,D) or by anterograde degeneration following MD lesions (dMD-t in B,E). With either method, MD-ts make primarily asymmetric synapses (large arrows) onto spines (s). Some of the spines postsynaptic to MD-ts exhibit a spine apparatus (sa in A,D). In the case of degenerating MD-ts, some postsynaptic spines are partially enveloped by glial processes (asterisks in E). MD-ts less commonly synapse onto dendritic shafts (d in C), some of which receive additional asymmetric synapses from unlabeled terminals (t). Scale bars = 0.5 μm in C; 0.5 μm in E (applies to A,B,D,E).

The different calcium-binding proteins were recognized by black particles that resulted from silver-enhanced immunogold. This immunolabeling was observed within somata, large and small diameter dendrites, and in axons, although we noted that the density of particles was higher in axon terminals and
somata as compared to dendrites. We detected a difference in the density of immunogold-silver labeling for each of the three calcium-binding proteins, with the highest density found in tissue immunolabeled for CalB and the lowest density observed in tissue immunostained for CalR. Moreover, in the PHA-L cases the density of immunogold-silver labeling for each of the three calcium-binding proteins was lower than in the BDA cases. This most likely reflects a lower sensitivity of the primary antibodies raised in mouse that had to be used in combination with the rabbit primary antibody against PHA-L.

**Synaptic relationships of MD terminals within Layer III of the medial PFC**

The main focus of this study was to assess the synaptic relationships between MD terminals and the three major types of local circuit neurons within Layer III of the medial PFC. GABA interneurons in the PFC are only sparsely spiny (Gabbott et al., 1985, 1986b; Gabbott et al., 1997) and receive most of their synaptic contacts onto their dendritic shafts, whereas pyramidal cells receive synaptic contacts of the asymmetric type predominantly onto dendritic spines (Smiley and Goldman-Rakic, 1993). We therefore focused our analysis on synaptic inputs and synapses onto dendritic shafts versus dendritic spines. To this end, we defined three types of spatial associations between MD axons and dendritic structures that have functional implications for this study (Fig. 3). The most common spatial relationship was the presence of MD terminals synapsing onto dendritic spines within the same field as profiles immunoreactive for calcium-binding proteins (Fig. 3A,B). The second type of spatial arrangement was a nonsynaptic apposition between MD terminals and dendritic shafts (Fig. 3B,C). Such relationships often involved the MD terminal synapsing onto an adjacent dendritic spine (Fig. 3B). Because our analysis focused mainly on synapses, this situation was classified as an axo-spinous synapse without also including it in the “appositions” category. Only MD terminals that were apposed to dendrites without synapsing on any other structure were counted as appositional contacts (Fig. 3C). The third type of spatial arrangement involved an MD terminal making a synapse onto a dendritic shaft (Fig. 3D,E). In some of these cases the MD terminal also formed a synapse onto a dendritic spine in the vicinity (Fig. 3E). However, because we wished to identify as many axo-dendritic synapses as possible and to count each terminal only once, the latter contacts were categorized only as synapses onto dendrites. In a few cases we also detected synaptic contacts of MD terminals onto cell bodies (Fig. 3F). For simplicity, we included these axo-somatic contacts together with axo-dendritic synapses.

A total of 5,737 MD profiles were counted in the medial PFC that included both preterminal axons and synaptic boutons. The MDboutons varied in size from small to medium (0.1–1.5 μm), with the largest varicosities having one to three mitochondria. All MD terminals contained densely packed clear, round vesicles. Of all the MD axons observed, 2,399 (42%) formed synapses onto identifiable structures (Table 1). This observation was based on analysis of single sections or a short series of adjacent sections whenever MD terminals apposed labeled or unlabeled dendritic shafts. The great majority (2,336;
97.5%) of synaptic MD axons formed asymmetric axo-spinous synapses (Fig. 2A,B,D,E). In many cases, one single MD terminal contacted more than one spine (Fig. 2D,E). Only a small number of synapses (n = 60) were found between MD terminals and dendritic shafts (Fig. 2C). This represents 2.5% of all the synapses observed.

Figure 3. Categories of spatial associations formed by MD axons in the rat PFC. For quantitative purposes, MD axons were assigned to one of three different categories defined by their synaptic (syn) or appositional (app) relationships. MD axons synapsing onto spines (s) were counted as axo-spinous contacts when they made no other contacts with adjacent neuronal elements (A) or were also apposed to nearby dendrites (d; B). MD axons were considered to be apposed to dendrites only when they exhibited no synaptic relationship to other neuronal elements (C). MD axons that synapsed onto dendrites were counted only as axo-dendritic contacts (D) even if they also synapsed onto a spine (E). In this way, double counting of axons was avoided. Finally, MD axons synapsing onto perikarya (p) were included together with axo-dendritic contacts (F).

Table 1. Synaptic Relationships between MD Afferents and Somatodendritic Targets Immunoreactive for Calcium-Binding Proteins in the Rat PFC

<table>
<thead>
<tr>
<th>Tissue labeled for</th>
<th>ParV</th>
<th>CalR</th>
<th>CalB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of MD axons and terminals observed</td>
<td>2,027</td>
<td>1,822</td>
<td>1,889</td>
</tr>
<tr>
<td>Number (%) of MD terminals forming synapses</td>
<td>829/2,027 (41%)</td>
<td>764/1,822 (42%)</td>
<td>806/1,889 (43%)</td>
</tr>
<tr>
<td>Number (%) of MD synapses onto spines</td>
<td>809/829 (97.6%)</td>
<td>744/764 (97.4%)</td>
<td>786/806 (97.5%)</td>
</tr>
<tr>
<td>Number (%) of MD synapses onto labeled and unlabeled dendrites or somata</td>
<td>20/829 (2.4%)</td>
<td>20/764 (2.6%)</td>
<td>20/806 (2.5%)</td>
</tr>
<tr>
<td>synapses onto labeled dendrites or somata</td>
<td>15/20 (75%)</td>
<td>6/20 (30%)²</td>
<td>9/20 (45%)³</td>
</tr>
</tbody>
</table>

¹Includes 30 CalB+ spines.
²Includes 2 CalR+ somata.
³Includes 3 CalB+ somata.

**Relationship of MD terminals and ParV-ir structures**

Within the infralimbic and prelimbic cortices of sections stained for ParV, we counted 2,027 MD terminals, of which 829 (41%) formed synapses. Of these synaptic MD terminals, 809 (97.6%; Table 1) synapsed onto unlabeled dendritic spines (Fig. 4A–C). We never detected ParV labeling in spines, although two large structures with spine-like necks were seen to emerge from ParV-ir dendrites. One of these is shown in Figure 5D.
As previously described, we categorized two different types of contacts between MD terminals and ParV-ir dendrites within the PFC. The first type was represented by a dendritic apposition that showed no evidence of synaptic specialization in serial sections. A total of 99 MD terminals apposed to dendritic shafts were counted in this tissue set; 64 (65%) were apposed to ParV-ir dendrites and 35 (35%) were apposed to unlabeled dendrites. In other cases, MD terminals apposed to labeled dendrites synapsed onto one or more spines (Fig. 4A–C) and were counted as axo-spinous synapses in the quantitative analysis (Table 1).

For the second type of contact, 20 MD terminals made axo-dendritic synapses (Figs. 4D, 5). Of these, 15 (75%) were onto ParV-ir structures (Fig. 5), two of which appeared to be spine-like protrusions emerging from ParV-labeled dendrites (Fig. 5D). These structures exhibited extensions of cytoplasm through neck-like attachments, but they contained mitochondria and lacked other classical characteristics of spines, including the spine apparatus. MD inputs had no obvious preference for the caliber of ParV-ir dendrites contacted, which included large, medium, or small caliber shafts. Five MD terminals (25%) made
 synaptic contacts onto unlabeled dendrites in the vicinity of ParV-ir structures (Fig. 4D).

Figure 5. MD axons synapsing onto ParV-ir dendrites. Electron micrographs of the rat PFC showing immunogold-silver labeling for ParV in dendrites (ParV-d) that receive asymmetric synapses (large arrows) from MD axons labeled either by tract-tracing (MD-t) or anterograde degeneration (dMD-t). A: The MD-t is dual-labeled for BDA and ParV. C: The ParV-d receives an additional asymmetric synapse from an unlabeled terminal (t). D: The dMD-t synapses onto a part of the ParV-d that appears spine-like, although its large size and the presence of a mitochondrion (m) are uncharacteristic of pyramidal cell spines. Scale bar = 0.5 μm in D (applies to A–D).

Relationship of MD terminals and CalR-ir structures

In the second set of tissue we analyzed the relationship of MD terminals with CalR-ir dendrites. Within this tissue, 1,822 MD terminals were counted, of which 764 (42%) formed synapses. From this sample, 744 MD terminals (97.4%; Table 1) made axo-spinous synapses (Fig. 6A,B). We also found in this tissue the same pattern of spatial arrangements with dendritic shafts as previously described for ParV-ir. In these sections, 85 MD terminals were apposed to dendrites without forming synapses, and 16 (18%) of these
nonsynaptic appositions included CalR-ir dendrites. Other MD terminals apposed to CalR-ir dendrites still preferred to synapse onto spines in the vicinity (Fig. 6A).

**Figure 6. MD axons in the vicinity of CalR-ir structures.** Electron micrographs of the rat PFC illustrating immunogold-silver labeling for CalR in dendrites (CalR-d), axons (CalR-a), or perikarya (CalR-p) and their relationship to MD terminals labeled by degeneration (dMD-t) or tract-tracing (MD-t). **A, B:** MD-ts make asymmetric synapses (large arrows) onto spines (s). In A, the dMD-t is in close apposition (small arrow) to a CalR-d. In B, the MD-t is dual-labeled by immunoperoxidase for PHA-L and immunogold-silver for CalR. **C:** An MD-t synapses onto an unlabeled dendrite (d) in the same field as a CalR-a. **D:** A dMD-t is closely apposed to a CalR-d, and the point of contact appears to be associated with a postsynaptic thickening on one side (arrow). **E:** A dMD-t makes an asymmetric synapse onto a CalR-p; a small part of the nucleus (n) is
visible, and the dMD-t makes an additional synapse onto a spine. Scale bars = 0.5 μm in C (applies to B,C); 0.5 μm in E (applies to A,D,E).

A similarly low number of axo-dendritic synapses formed by MD terminals were observed in tissue labeled for CalR as for ParV. In this tissue, 20 MD terminals formed synapses onto dendritic shafts or soma, and of these, 14 synapses (70%) contacted unlabeled dendrites in the vicinity of CalR-ir structures (Fig. 6C; Table 1). Four MD terminals synapsed onto CalR-ir dendrites (Fig. 6D; Table 1), and two additional MD terminals made synapses onto CalR-ir perikarya (Fig. 6E).

**Relationship of MD terminals and CalB-ir structures**

The relationship of MD terminals to CalB-ir structures was more complex compared to what was observed in the other tissue sets labeled for ParV or CalR. This is because a population of pyramidal cells within Layers II–III is lightly labeled for CalB in addition to the more heavily labeled CalB-ir interneurons (Gabbott et al., 1997). Within this tissue we counted 1,889 MD terminals, of which 806 (43%) formed synapses and 756 (97.5%) of the terminals making synapses contacted spines (Table 1). Out of this latter number, 30 synapses (4%) were onto spines that were immunoreactive for CalB (Fig. 7A,B); these probably belonged to the dendrites of pyramidal cells. We also observed axon terminals immunoreactive for CalB synapsing onto CalB-ir spines (Fig. 7C). In some cases the immunonegative spines receiving synaptic inputs from MD terminals emerged from CalB-ir dendrites (Fig. 7D). This suggests that the extent of MD synaptic input to the spines of CalB-containing pyramidal cells was probably underestimated.

![Figure 7. MD axons synapsing onto CalB-ir spines.](image)

Electron micrographs of the rat PFC illustrating immunogold-silver labeling for CalB in dendrites (CalB-d), spines (CalB-s), or axon terminals (CalB-t) and their relationship to MD terminals labeled by...
immunoperoxidase (MD-t) or degeneration (dMD-t). A, B: The dMD-ts make asymmetric synapses (large arrows) onto CalB-labeled spines. C: A CalB-s receives an asymmetric synapse from a CalB-t. D: An MD-t makes an asymmetric synapse onto a spine that is unlabeled but arises from a CalB-d. Scale bars = 0.5 μm in C (applies to A–C); 0.5 μm in D.

In this last set of tissue, we found 57 appositions of MD terminals with dendritic shafts, of which 23 (40%) involved appositions with CalB-ir dendrites. As was typical, many other MD terminals made axo-spinous synapses while apposed to a CalB-ir dendrite (Fig. 8A).

**Figure 8. MD axons in the vicinity of CalB-ir structures.** Electron micrographs of the rat PFC illustrating immunogold-silver labeling for CalB in dendrites (CalB-d) or perikarya (CalB-p) in relationship to degenerating (dMD-t) or immunoperoxidase-labeled MD terminals (MD-t). A: A dMD-t is apposed (small arrow) to a CalB-d that receives asymmetric synaptic input (large arrow) from an unlabeled terminal (t). As is typical, the dMD-t synapses onto an adjacent unlabeled spine (s). B: An MD-t that is dual-labeled for BDA and CalB synapses onto an unlabeled dendrite (d) in the vicinity of a CalB-p with a
deeply invaginated nucleus (n). C,D: CalB-ds receive asymmetric synaptic inputs from MD-ts. Scale bars = 0.5 μm in A; 0.5 μm in D (applies to B–D).

Axo-dendritic synapses formed by MD terminals numbered 20 for this dataset. Of these, 11 synapses (55%) were onto unlabeled dendrites (Fig. 8B) and only six synapses were onto CalB-ir dendrites (Fig. 8C,D; Table 1). We also detected three MD terminals synapsing onto CalB-ir perikarya (Fig. 9).

Figure 9. MD axon synapsing onto a CalB-ir perikaryon. Electron micrograph of the rat PFC illustrating immunogold-silver labeling for CalB in a perikaryon (CalB-p) that receives asymmetric synaptic input (large arrow) from a degenerating MD terminal (dMD-t). The insert shows this synapse at higher magnification. The CalB-p contains a large, relatively noninvaginated nucleus (n). Scale bars = 2 μm at high magnification; 0.5 μm in the inset.

Comparison between calcium-binding proteins

The relative extent to which MD terminals were found to synapse onto nonspiny structures immunoreactive for the different calcium-binding proteins was analyzed for statistical significance. The two synapses onto CalR-ir perikarya and three synapses onto CalB-ir soma were included. A 2 × 3 chi-square analysis revealed that the pattern of MD inputs to labeled versus unlabeled somatodendritic structures was significantly different between calcium-binding proteins ($\chi^2 (2, n = 60) = 7.2, P < 0.05$). Post-hoc analyses were then conducted by Fisher's exact test, which is more sensitive than chi-square for 2 × 2 comparisons (Matthews, 1996). In addition, P values for comparisons involving ParV-ir targets were determined using one-tailed tests, because the a priori hypotheses was that MD terminals would synapse more frequently onto this cell class. The difference in frequency of MD terminal synapses onto ParV-ir versus CalR-ir structures was clearly significant ($P = 0.005$), whereas the difference in synaptic frequency onto ParV-ir versus CalB-ir targets just reached significance ($P = 0.05$). There was no significant difference in the extent to which MD axons synapsed onto somatodendritic structures immunoreactive for
CalB versus CalR ($P = 0.5$, two-tailed). Repetition of these statistical tests with the exclusion of the MD synapses onto soma produced higher significance values for the main effect and all post-hoc comparisons, except for the comparison of inputs to CalB-ir versus CalR-ir dendrites, which was still not significant.

**Presence of calcium-binding proteins in MD terminals**

In cases in which we used BDA or PHA-L to label MD terminals within the PFC, we detected dual labeling for immunoperoxidase and immunogold-silver in a relatively small number of cases. The highest proportion was found in the tissue immunolabeled for ParV, in which 3% of MD terminals were dual-labeled for ParV and the anterograde tracer (Fig. 5A). In CalR immunostained tissue, only 1% of the MD terminals were found to be double-labeled (Fig. 6B), while for CalB immunoreacted tissue sections, 2% of the fibers were dual-labeled (Fig. 8B).

**DISCUSSION**

In this study we assessed the synaptic relationships between axons from the MD and GABA interneuron subtypes identified by their immunoreactivity for ParV, CalR, or CalB in Layer III of the rat medial PFC. Previous anatomical and electrophysiological studies have provided evidence for MD afferents making synapses onto GABA interneurons within the PFC (Floresco and Grace, 2003; Kuroda et al., 2004). However, the present study represents the first ultrastructural analysis of MD synaptic connections onto different classes of local inhibitory interneurons within this region. We found an apparent preference of MD terminals for the ParV subclass of interneurons. However, the other two subclasses of cells also received inputs from the MD at a lower frequency. Given that each type of interneuron subserves different functions in information processing, these results suggest an important role of the MD in controlling the types of information that are processed within the PFC via these connections to interneurons. It can be expected that the feedforward inhibition transmitted via MD synapses onto ParV cells will be profound and efficient, because these interneurons themselves have a strong influence on pyramidal neurons via contacts with proximal dendrites, cell bodies, and axon initial segments (Williams et al., 1992; Melchitzky et al., 1999).

**Methodological considerations**

The reliability of the analysis required for this study depends on the inclusion of a substantial portion of the thalamocortical pathway via tract-tracing, the sensitivity and specificity of the immunostaining, and the morphological integrity of the tissue.

Two important advantages of using anterograde tracers is that they preserve the normal morphology of MD axons (Wouterlood and Groenewegen, 1985; Veenman and Reiner, 1996) and allow some degree of presynaptic
double-labeling (Veenman et al., 1992) as an initial assessment of the presence of calcium-binding proteins within MD axons. Although BDA and PHA-L produce relatively equivalent anterograde transport (Wouterlood and Jorritsma-Byham, 1993; Reiner et al., 2000; Novikov, 2001), BDA is more likely to undergo transport in the retrograde direction (Veenman et al., 1992; Reiner et al., 2000), an occurrence that was observed in some animals in our study. These animals were omitted and preference was then given to PHA-L, which never produced retrograde transport from the MD to the PFC.

Compared to tracers, lesion-induced anterograde degeneration provides a more sensitive way of labeling projecting fibers (White, 1978; Pinto et al., 2003). Indeed, we achieved a greater proportion of labeled MD terminals within the PFC by anterograde degeneration, consistent with our prior observations involving projections from the paraventricular thalamus to the PFC (Pinto et al., 2003). For our purposes, the electrolytic lesion technique had the additional advantage over PHA-L of being compatible with the more sensitive rabbit antibodies against calcium-binding proteins. The principal disadvantages of this method are that it generates abnormal morphology of the MD axons, and does not allow presynaptic double-labeling. Therefore, the optimal approach to our study was to combine both tract-tracing and degeneration methods in order to maximize the advantages of each technique.

The second important technical issue was the sensitivity of immunogold-silver staining for the three calcium-binding proteins. In addition to the lower sensitivity associated with the antibodies raised in mouse, the penetration of all antibodies was decreased by the low detergent levels used to maintain morphological integrity. We attempted to minimize this limitation by performing our analysis at the tissue surface in areas of the neuropil that exhibited optimal labeling for both immunoperoxidase and immunogold-silver. Moreover, we found no evidence to indicate that immunolabeling of any one calcium-binding protein was compromised relative to the others. Hence, although the values obtained in this study are likely to be lower than the actual incidence of MD contacts onto different structures in the PFC, the comparison of the relative extent of MD synaptic inputs to different local circuit neurons is still valid.

As described in Materials and Methods, the evidence supporting antibody specificity was previously provided in several studies (Celio and Heizmann, 1981; Celio, 1986; Rogers, 1987; Celio et al., 1988; Schwaller et al., 1993; Condé et al., 1994; Sesack and Pickel, 1995; Sesack et al., 1998; Zimmermann and Schwaller, 2002). However, by immunoblot analysis the polyclonal rabbit anti-CalB antibody was estimated to crossreact by 10% with CalR. It is not known whether a comparable extent of crossreaction occurred in fixed brain tissue. However, in the event that some crossreaction did occur, our findings regarding MD inputs to CalB-labeled structures in tissue using this immunoreagent must be viewed with some caution. Future studies using a different marker for the class of interneurons containing CalB will help to resolve this issue. Nevertheless, the observation of one MD axon synapsing
onto a dendrite with varicose morphology and labeled for CalB by the more specific mouse monoclonal antibody (Celio et al., 1990b) is consistent with some degree of MD innervation to this cell class (Fig. 8B).

**MD inputs to Layer III of the rat PFC**

Our finding that the great majority of inputs (97.5%) from the MD to Layer III of the rat PFC make asymmetric synapses onto dendritic spines provides the first quantitative estimation of the prevalence of axo-spinous synapses in this pathway. The results are consistent with previous studies showing that the main recipients of MD fibers are the dendritic spines of pyramidal cells (Kuroda et al., 1995a; Kuroda et al., 1995b; Kuroda et al., 1996a; Kuroda et al., 1996b). The results also agree with studies reporting principal thalamic synapses onto the dendritic spines of pyramidal cells in other cortical areas (Peters et al., 1976b; Vogt et al., 1981; Hersch and White, 1981b).

The main finding of this study is the observation of MD synapses onto the dendritic shafts of each cell population labeled for calcium-binding proteins. These results agree with the prior reports by Kuroda (Kuroda et al., 1993; Kuroda et al., 2004) of MD inputs to GABA interneurons. Previous studies suggest that local circuit neurons, being primarily aspiny cells, receive the majority of their synaptic inputs onto dendritic shafts (Gabbott and Somogyi, 1986c; Chmielowska et al., 1988; Smiley and Goldman-Rakic, 1993; Sesack and Pickel, 1995). Hence, the presently observed synapses of MD terminals onto labeled and unlabeled dendrites are likely to involve mainly the shafts of interneurons and not pyramidal cells. Like others, we recognized that certain morphological criteria can differentiate between dendrites belonging to pyramidal cells versus interneurons, namely, a spiny aspect being indicative of pyramidal cell dendrites and a varicose morphology, absence of spines, and multiple synaptic inputs indicating inhibitory interneuron dendrites (Smiley and Goldman-Rakic, 1993; Sesack and Pickel, 1995). However, these criteria are not absolute, and it is not always possible to assess each of them (e.g., when dendritic shafts are cut in cross-section). Moreover, it is important to note that Kuroda (Kuroda et al., 1993) observed a few cases of MD terminals synapsing onto the dendritic shafts of pyramidal cells retrogradely labeled from the MD. Hence, it must be acknowledged that some MD axo-dendritic synapses involve inputs to pyramidal cells. This caveat applies in particular to MD axon synapses onto CalB-ir and unlabeled dendrites, as ParV and CalR have not been localized to pyramidal cells in rats.

It should also be acknowledged that some of the dendritic shafts contacted by MD axons in Layer III may have arisen from cells in adjacent layers. Moreover, it is possible that the MD innervation to other layers of the PFC might exhibit target specificity that differs from that observed in Layer III. In this regard, it is important to note that immunostaining for calcium-binding proteins may have been more robust in relatively more proximal dendrites due to limited antibody penetration into the smallest caliber structures, although CalB immunoreactivity did extend into the spines of pyramidal neurons.
Therefore, the data obtained in our study probably underestimates the connections between MD axons and PFC interneurons. Moreover, because ParV somata and their proximal dendrites are better represented within Layer III (Gabbott et al., 1997), we may have underestimated MD axon synapses onto the distalmost dendrites of CalB and CalR cells whose somata lie in adjacent layers. Nevertheless, it is likely that the underestimation of immunoreactivity in distal dendrites affected all classes of interneurons uniformly.

An important functional aspect of our study is the small number of MD inputs onto dendrites versus spines. The fact that the input from MD onto the dendrites of presumed interneurons is sparse may be compensated by other features of these cells. For example, the activation of thalamocortical axons produces synaptic potentials with 3-fold larger amplitude in fast-spiking interneurons as compared to pyramidal cells (Beierlein and Connors, 2002; Beierlein et al., 2003). Inhibitory interneurons respond at lower thresholds and with shorter latencies (Simons and Carvell, 1989; Welker et al., 1993) and are metabolically more active than pyramidal cells (Nie and Wong-Riley, 1995; McCasland and Hibbard, 1997). The fact that some MD axons synapse onto cell bodies may also provide a more potent influence of this input on some classes of local circuit neurons.

MD associations with structures immunoreactive for calcium-binding proteins

ParV-ir structures

The finding that ParV-ir dendrites received the majority of MD synapses onto dendritic shafts in the PFC is in accordance with the results of prior anatomical and electrophysiological studies of principal thalamic projections to other cortical regions (Staiger et al., 1996; Gibson et al., 1999; Porter et al., 2001). To the extent that some axo-dendritic synapses formed by MD axons might have been missed, it is interesting to note that appositions between MD profiles and dendrites were also more frequently observed for ParV than for CalR or CalB dendrites. Our findings also lend support to a recent in vivo electrophysiological study of the PFC in which MD stimulation was hypothesized to activate fast-spiking interneurons (Floresco and Grace, 2003). Interestingly, a number of reports indicate that nontalamic synapses in the PFC also preferentially contact the ParV-ir class of local circuit neurons, including afferents from the hippocampus (Gabbott et al., 2002) and the local collaterals of PFC pyramidal cells (Melchitzky et al., 2001; Sesack et al., 2001; Melchitzky and Lewis, 2003).

The ParV-labeled dendrites that were targeted by the MD also often received multiple synaptic inputs from NMD axons, a morphological feature associated with interneurons (Smiley and Goldman-Rakic, 1993; Sesack and Pickel, 1995). Recent studies of the mouse and monkey somatosensory or motor cortex reported the presence of ParV in some pyramidal cells (Preuss and Kaas, 1996; Jinno and Kosaka, 2004). However, ParV labeling in the rat cortex has
only been described in interneurons (Kawaguchi, 1995; Kawaguchi and Kubota, 1997; Wang et al., 2002). Moreover, in our analysis of Layer III of the rat PFC we never encountered ParV-ir in classical spines. We therefore conclude that the population of ParV-ir dendrites receiving MD inputs belonged exclusively to interneurons. In future studies it will be important to determine whether the MD maintains preferential contacts onto the two major subpopulations of ParV neurons, the chandelier and basket cells (Zhu et al., 2004).

Within the rat medial PFC, it has been estimated that the density of ParV cells is higher than CalR and CalB interneurons both across all layers and within Layer III (Gabbott et al., 1997). Therefore, the apparent preference of MD synapses for ParV local circuit neurons may, in fact, result from a higher representation of ParV cells within the layer analyzed. However, in other brain areas and in other layers within the PFC, excitatory afferents also show a preference for ParV compared with CalB or CalR neurons (Gulyas et al., 1999; Porter et al., 2001; Gabbott et al., 2002).

**CalR-ir structures**

CalR-ir dendrites that received MD inputs represented the smallest proportion of synapses onto immunolabeled structures in the present study. To date, no evidence suggests the presence of CalR within pyramidal cells of the rat cortex. Rather, CalR-ir has been associated with double bouquet cells having both dense local axons contacting mainly other inhibitory cells and a thinner vertical bundle of axons that sometimes spans more than one layer and mainly contacts the spines of pyramidal cells (Kawaguchi and Kubota, 1997; Tamas et al., 1997; Tamas et al., 1998). Hence, we consider it most likely that the small number of MD inputs onto these cells represents synapses onto local circuit neurons.

Previous anatomical studies have shown excitatory inputs onto CalR-ir cells in different regions of the cortex. In the monkey PFC the local collaterals of pyramidal cells synapse occasionally onto CalR-ir dendrites (Melchitzky and Lewis, 2003). In the rat somatosensory cortex, thalamic inputs sometimes synapse onto cells immunoreactive for vasoactive intestinal peptide (Staiger et al., 1996), which is often colocalized with CalR (Kawaguchi and Kubota, 1997). In the rat hippocampus, unidentified excitatory synapses contact CalR-ir dendrites at a lower frequency than inputs to ParV-ir dendrites (Gulyas et al., 1999).

**CalB-ir structures**

The observation that CalB-ir structures received MD inputs in the present study agrees with a previous report of the somatosensory cortex showing that some of the inhibitory interneurons firing action potentials at monosynaptic latencies following thalamocortical stimulation were immunoreactive for CalB (Porter et al., 2001). Therefore, our finding was not totally unexpected, although the same study also suggested that the main type of interneuron showing thalamocortical evoked firing was the ParV class.

Three main points suggest that the CalB-ir dendrites that received MD inputs in our study may have represented more than one class of cells. First,
observation of CalB in a subpopulation of Layers II–III pyramidal cells (Hayes and Lewis, 1992; DeFelipe and Jones, 1992b; Condé et al., 1994; Gabbott et al., 1997) suggests that some of the CalB-ir dendrites that received MD inputs may have belonged to pyramidal cells. Second, CalB and ParV are coexpressed in a subset of neurons included in the fast spiking category (van Brederode et al., 1991; Kubota et al., 1994; del Rio and DeFelipe, 1997). Kubota et al. (Kubota et al., 1994) estimated that such colocalization occurs in about 90% of ParV cells, with 80% showing weak immunoreactivity for CalB and only 10% showing strong labeling. Third, CalB is also reported to be colocalized with CalR in a small number of double bouquet cells (Kubota et al., 1994; del Rio and DeFelipe, 1997). Finally, the potential crossreaction of the polyclonal rabbit anti-CalB antibody discussed above may have caused the erroneous inclusion of some CalR-ir dendrites in the CalB category. Additional studies are needed to examine other markers for the CalB class of local circuit neurons, such as somatostatin or neuropeptide Y (Kubota et al., 1994). Nevertheless, the results obtained in tissue labeled with the specific monoclonal mouse anti-CalB antibody suggest that MD inputs probably do occur onto some CalB local circuit neurons. These cell types are likely to include double bouquet cells forming a possible microcolumnar inhibitory system (DeFelipe et al., 1990; DeFelipe and Jones, 1992b; del Rio and DeFelipe, 1995; DeFelipe, 1997; del Rio and DeFelipe, 1997; Kawaguchi and Kubota, 1997) and Martinotti cells that send their axons to Layer I (Condé et al., 1994; Gabbott et al., 1997; Kawaguchi and Kubota, 1997; Gupta et al., 2000).

**Calcium-binding proteins in MD axons**

In the monkey thalamocortical system, it has been reported that a matrix of CalB-ir neurons extends over wide thalamic areas and provides diffuse inputs to superficial layers of the cortex, whereas a core of ParV-ir neurons restricted only to certain thalamic nuclei projects to middle layers and provides more specific information (Melchitzky et al., 1999; Jones, 2001). The same level of thalamocortical organization and input segregation may vary across species. However, the presence of calcium-binding proteins within some MD terminals in the present study suggests the possibility that a similar type of organization may also be present in rats. The observation that MD axons most commonly expressed ParV is consistent with the scheme proposed by Jones (Jones, 2001) and verifies prior hypotheses that at least some ParV-labeled axons in the PFC derive from the MD (Melchitzky et al., 1999; Lewis et al., 2001). The verification of all three calcium-binding proteins in MD neurons and a more exact quantification of their prevalence will require future studies utilizing more sensitive methods.

**Functional considerations**

The results of our study have important implications for understanding working memory processes. The MD has been shown to contribute to the normal functioning of memory processes, both in rats and monkeys (Freeman et al.,
Mediodorsal thalamic inputs onto interneurons

1996; Hunt and Aggleton, 1998b; Floresco et al., 1999; Gaffan and Parker, 2000; Tanibuchi and Goldman-Rakic, 2003) and MD neurons are reported to fire during working memory tasks (Hunt and Aggleton, 1998b; Tanibuchi and Goldman-Rakic, 2003; Watanabe and Funahashi, 2004). Hence, it is possible that MD inputs onto both pyramidal and nonpyramidal cells in the PFC contribute to specific stages of the working memory mechanism. It could be speculated that activity in Layers V–VI pyramidal neurons recruit MD cells (Kuroda et al., 1993), and that these in turn drive ParV interneurons to feed inhibition forward onto the pyramidal cells that are not relevant for the specific task. Pyramidal cell recovery from the inhibitory state may be accomplished by MD inputs onto CalR cells that are able to inhibit other interneurons. The role of MD inputs onto CalB cells is more difficult to hypothesize, but such connections may help to suppress “irrelevant” information (i.e., nontask-related) arriving at Layer I by hyperpolarizing the distal dendrites of pyramidal cells. Further anatomical and electrophysiological studies are needed to test these various hypotheses.

Our data also have significance for understanding cortical pathology, such as the dysfunction of the PFC in schizophrenia, which might be related to abnormalities in MD afferents. Although the role of the thalamus in schizophrenia has recently been questioned (Cullen et al., 2003; Dorph-Petersen et al., 2004), the fact that several studies report a reduction in the volume and number of cells in the MD in the postmortem schizophrenic brain implicates the MD in at least some aspects of schizophrenia pathology (Pakkenberg, 1990; Popken et al., 2000; Young et al., 2000; Volk and Lewis, 2002). Other studies have reported a selective reduction in the number of ParV-related elements in the PFC of schizophrenic patients (Woo et al., 1997; Lewis, 2000; Lewis et al., 2001; Hashimoto et al., 2004). This decrease in the ParV subclass of local circuit neurons may directly reflect their preferential receipt of MD synapses in the normal brain. Therefore, the direct synaptic connection between MD axons and ParV-ir cells described in this study presents an important link in understanding the functional relationship between different network elements and the pathology of schizophrenia.
Chapter 3

Glutamate receptor subtypes mediating synaptic activation of prefrontal cortex neurons: relevance for schizophrenia.

Published in J Neurosci. 2011 Jan 5;31(1):142-56.
Rotaru DC, Yoshino H, Lewis DA, Ermentrout GB, Gonzalez-Burgos G.
Translational Neuroscience Program, Department of Psychiatry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, USA.

Schizophrenia may involve hypofunction of NMDA receptor (NMDAR)-mediated signaling, and alterations in parvalbumin-positive fast-spiking (FS) GABA neurons that may cause abnormal gamma oscillations. It was recently hypothesized that prefrontal cortex (PFC) FS neuron activity is highly dependent on NMDAR activation and that, consequently, FS neuron dysfunction in schizophrenia is secondary to NMDAR hypofunction. However, NMDARs are abundant in synapses onto PFC pyramidal neurons; thus, a key question is whether FS neuron or pyramidal cell activation is more dependent on NMDARs. We examined the AMPAR and NMDAR contribution to synaptic activation of FS neurons and pyramidal cells in the PFC of adult mice. In FS neurons, EPSCs had fast decay and weak NMDAR contribution, whereas in pyramidal cells, EPSCs were significantly prolonged by NMDAR-mediated currents. Moreover, the AMPAR/NMDAR EPSC ratio was higher in FS cells. NMDAR antagonists decreased EPSPs and EPSP-spike coupling more strongly in pyramidal cells than in FS neurons, showing that FS neuron activation is less NMDAR dependent than pyramidal cell excitation. The precise EPSP-spike coupling produced by fast-decaying EPSCs in FS cells may be important for network mechanisms of gamma oscillations based on feedback inhibition. To test this possibility, we used simulations in a computational network of reciprocally connected FS neurons and pyramidal cells and found that brief AMPAR-mediated FS neuron activation is crucial to synchronize, via feedback inhibition, pyramidal cells in the gamma frequency band. Our results raise interesting questions about the mechanisms that might link NMDAR hypofunction to alterations of FS neurons in schizophrenia.
INTRODUCTION

Hypofunction of NMDA receptor (NMDAR)-mediated signaling has been implicated in the disease process of schizophrenia because NMDAR antagonist administration mimics core symptoms of the illness, including cognitive deficits (Javitt and Zukin, 1991; Javitt, 2009). However, identifying potential sites of NMDAR hypofunction has proved elusive, given the modest and often contradictory changes in NMDARs observed in postmortem studies of schizophrenia (Kristiansen et al., 2006), and the challenges of determining the sites where NMDAR antagonists act to mimic schizophrenia symptoms.

Schizophrenia may involve dysfunction of prefrontal cortex (PFC) inhibitory GABA neurons, including the parvalbumin (PV)-containing cells (Lewis et al., 2005), whose activity is essential to generate gamma oscillations (Cardin et al., 2009; Sohal et al., 2009). PV neuron alterations may underlie the impairment of gamma oscillations and thus cognitive dysfunction in schizophrenia (Cho et al., 2006), since gamma band synchrony is important for cognition (Fries, 2009).

Recent studies proposed that alterations of PV neurons in schizophrenia are secondary to NMDAR hypofunction. For example, NMDAR antagonists decrease mRNA expression for PV and the GABA-synthesis enzyme GAD67 (Cochran, 2003; Behrens et al., 2007), resembling the decrease of PV and GAD67 in schizophrenia (Lewis et al., 2005). Interestingly, systemic administration of NMDAR antagonists increases PFC pyramidal cell firing, apparently by producing disinhibition (Homayoun and Moghaddam, 2007). Moreover, NMDAR antagonists decrease disynaptic inhibition onto hippocampal pyramidal cells (Grunze et al., 1996). Therefore NMDAR antagonists, or NMDAR hypofunction in schizophrenia, could alter PFC circuit function by disinhibiting pyramidal cells via primary effects on PV neurons (Coyle, 2004a; Coyle and Tsai, 2004b; Lewis and Moghaddam). A crucial question is therefore whether normally NMDAR-mediated currents are stronger in PV interneurons or in PFC pyramidal cells.

PV-positive neurons have unique fast-spiking (FS) electrophysiological properties (Kawaguchi and Kubota, 1997; Galarreta and Hestrin, 2002; Pawelzik et al., 2002), which are crucial for their role in gamma oscillations (Bartos et al., 2007; Mann and Paulsen, 2007; Doischer et al., 2008). Furthermore, the properties of glutamate synapses onto FS neurons are also critical, since the network mechanisms of gamma oscillations may involve recurrent excitation–inhibition between pyramidal neurons and FS cells (Hájos and Paulsen, 2009).

FS neurons display a remarkably fast synaptic activation (Hu et al.), which may require short-lasting EPSCs, because long-lasting EPSCs produce spikes during prolonged time windows (Fricker and Miles, 2000; Maccaferri and Dingledine, 2002). Fast synaptic activation may involve weak NMDAR
Glutamate receptors on PFC neurons

contribution, because NMDAR-EPSCs typically are long-lasting (Hestrin et al., 1990; Cull-Candy and Leszkiewicz, 2004). Interestingly, in hippocampus and somatosensory cortex FS neurons actually display short-lasting EPSCs with weak NMDAR contribution (Angulo et al., 1997; Geiger et al., 1997; Lu et al., 2007; Hull et al., 2009).

In PFC circuits, the contribution of synaptic NMDARs to activation of specific populations of neurons is poorly understood. This information is important to determine what cell types could mediate NMDAR hypofunction and the possible mechanisms linking NMDAR hypofunction with gamma oscillation abnormalities in schizophrenia. Here, we examined the NMDAR contribution to synaptic activation of PFC FS neurons and pyramidal cells and the influence of AMPARs and NMDARs in the production of gamma oscillations via feedback inhibition.

MATERIALS AND METHODS

Slices

Experiments were conducted in brain slices prepared from the frontal cortex of 6-week- to 4-month-old C57BL/6 mice of either sex (Charles River), using methods described previously (Rotaru et al., 2007). Animals were treated following procedures in accordance with National Institutes of Health guidelines and approved by the University of Pittsburgh's Institutional Animal Care and Use Committee. For electrophysiological recordings, slices were transferred to a recording chamber and superfused at a flow rate of 2 ml/min with artificial CSF (ACSF) containing the following (in mm): 126 NaCl, 2.5 KCl, 1 MgCl₂, 2 CaCl₂, 25 NaHCO₃, 1.25 Na₂HPO₄, and 10 glucose, pH 7.3–7.4 when gassed with carbogen (95% O₂ and 5% CO₂). Chamber temperature was adjusted to 30–32°C. Unless specifically indicated, the standard ACSF routinely contained 10 μm gabazine, to block GABA_A receptor-mediated synaptic transmission.

Electrophysiology

Patch pipette, tight seal whole-cell recordings were obtained from layer 5 pyramidal cells and interneurons in the infralimbic, prelimbic, or anterior cingulate regions of the mouse medial frontal cortex, here collectively referred to as mouse PFC. Cells were visualized using Olympus or Zeiss microscopes equipped with infrared illumination and differential interference contrast videomicroscopy. Pipettes pulled from borosilicate glass had a resistance of 3–6 MΩ when filled with the following solution (in mm): K-gluconate 120, NaCl 10, HEPES 10, EGTA 0.2, MgATP 4.5, NaGTP 0.3, and Na-phosphocreatine 14 and 0.2–0.5% biocytin, and the pH was adjusted to 7.2–7.4 using KOH. Recordings were obtained using Multiclamp 700 amplifiers (Molecular Devices). Signals were low-pass filtered at 4 kHz and digitized at 10 or 20 kHz using Power 1401 data acquisition interfaces (Cambridge Electronic Design).
Data acquisition and analysis were performed using Signal 4 software (Cambridge Electronic Design).

**Voltage clamp**

The pipette capacitance was compensated and series resistance was continuously monitored but was not compensated. Only recordings with a stable series resistance of <20 MΩ were used for analysis. NMDAR contribution to EPSCs is best estimated at positive membrane potentials, to largely relieve NMDAR channels from Mg2+ block. Good quality voltage-clamp recordings at positive potentials require using Cs+-based pipette solutions to improve voltage-clamp conditions. However, Cs+ blocks K+ channels and severely distorts the cells' firing properties, preventing the identification of FS neurons. Therefore, we measured the NMDAR contribution by recording in nominally Mg2+-free conditions using K+-based pipette solutions, and voltage clamping the cells near their resting membrane potential (see Figs. 2, 3). Slices were incubated before recording for 20 min in Mg2+-free ACSF (MgCl2 omitted), and the same Mg2+-free ACSF was used continuously during recordings.

**Current clamp**

Series resistance and pipette capacitance were monitored and cancelled using bridge and capacitance neutralization. To measure the cells' firing properties, incremental depolarizing current steps (500 ms duration) were injected until producing spikes repetitively. Membrane potential measurements were not corrected for liquid junction potential. In experiments estimating the NMDAR contribution to EPSPs, recordings were done in standard extracellular Mg2+ concentration (1 mm), depolarizing the cells to membrane potential values near threshold by constant current injection via the whole-cell recording pipette.

**Extracellular stimulation**

Synaptic inputs were stimulated using electrodes fabricated with theta-type capillary glass pulled to an open tip diameter of 3–5 μm and filled with oxygenated ACSF. Silver wires inserted into the theta glass were connected to a stimulus isolation unit (World Precision Instruments) commanded by TTL pulses. Stimulation electrodes were typically placed within 100–200 μm of the soma of the recorded neuron. Stimuli of 100 μs duration had current intensity adjusted (10–100 μA) to produce apparently monosynaptic responses with no obvious contamination by polysynaptic events. To produce disynaptic IPSPs in the absence of monosynaptic IPSPs (see Fig. 7), horizontal inputs were stimulated placing the stimulation electrode at >300 μm lateral to the recorded neuron.

**Morphological analysis**

Biocytin-filled neurons were visualized using the Vectastain Elite ABC kit (Vector Laboratories) and their axonal and dendritic trees reconstructed using the Neurolucida Tracing System (Microbrightfield Bioscience) as described previously (Povysheva et al., 2008; Zaitsev et al., 2009). Typically, FS cells belong to one of two different morphological classes: chandelier neurons and the so-called basket cells (Markram et al., 2004). All the biocytin-filled FS
neurons morphologically identified in this study were classified as basket cells (Fig. 1).

**Electrophysiology data analysis**

**Neuron classification**

Pyramidal cells and interneurons were identified visually before recording, and their morphology was confirmed subsequently (Fig. 1). Because the NMDAR contribution to excitatory inputs is strong in interneurons of NFS subtypes (Lu et al., 2007; Wang and Gao, 2009), we only included interneurons unambiguously classified as FS. To distinguish FS cells from NFS interneurons, depolarizing current steps were used to analyze the firing response of each interneuron. Single spike properties were determined on spikes elicited by near threshold current injection. Spike-frequency adaptation was quantified by the ratio between the last and first interspike intervals in spike trains evoked by 500 ms depolarizing steps. Cells were classified as FS if they had the following: (1) narrow spikes (duration at half peak amplitude $\leq 0.6$ ms); (2) large afterhyperpolarizing potentials (amplitude $\geq 15$ mV); and (3) absence of significant spike-frequency adaptation (adaptation ratio $\leq 1.2$). These criteria may exclude some FS neurons, but ensure focusing on FS cells (Fig. 1B). As we (Krimer et al., 2005; Povysheva et al., 2008; Zaitsev et al., 2009) and other laboratories (Kawaguchi and Kubota, 1997; Galarreta and Hestrin, 2002; Pawelzik et al., 2002) demonstrated previously, FS electrical properties are highly correlated with PV expression. To verify that our criteria to classify interneurons as FS and to exclude NFS were appropriate, we measured the parameters described above in recordings from green fluorescent protein-positive neurons in slices from G42 mice (see supplemental Table 1, available at www.jneurosci.org as supplemental material), a mouse strain in which the fluorescent protein is expressed exclusively in PV-positive-FS neurons (Chattopadhyaya, 2004).

**EPSC data analysis**

Spontaneous EPSCs (sEPSCs) recorded at $-70$ mV in nominal Mg2+ free conditions were detected using Mini analysis software (Synaptosoft). For each cell, at least 200 events detected in control conditions or in the presence of AP5 were averaged. The peak amplitude of the average spec was measured relative to the baseline current. The decay kinetics was quantified by fitting a double exponential function and computing a weighted decay time constant ($\tau_w$) as follows:

$$\tau_w = \frac{A_{\text{slow}} \times \tau_{\text{slow}} + A_{\text{fast}} \times \tau_{\text{fast}}}{A_{\text{slow}} \times A_{\text{fast}}}$$

where $A_{\text{slow}}$, $A_{\text{fast}}$, $\tau_{\text{slow}}$, and $\tau_{\text{fast}}$ are the amplitudes and decay time constants of slow and fast IPSC decay components. To estimate the AMPA/NMDA sEPSC charge ratio, first we obtained, by waveform subtraction, the NMDA-mediated sEPSC as follows: NMDA waveform = control waveform $-$ AMPA waveform, where the AMPA waveform was that recorded in the presence of the NMDA antagonist AP5. Charge was estimated by the area under the sEPSC waveform curve.
To analyze EPSCs evoked by extracellular stimulation (eEPSCs), at least 20 responses were averaged from recordings in control conditions or in the presence of the AMPAR antagonist CNQX. Analysis of amplitude, decay kinetics, and charge of the average eEPSC was done as for the sEPSCs. To estimate the AMPA/NMDA eEPSC charge ratio, first the AMPA-mediated eEPSC was obtained by waveform subtraction as follows: AMPA waveform = control waveform − NMDA waveform, where the NMDA waveform was that recorded in the presence of the AMPAR antagonist CNQX.

**EPSP data analysis**

Spontaneous EPSPs (sEPSPs) recorded in control conditions or in the presence of AP5 at two different membrane potentials (100–200 events at approximately −80 mV and at least 70 events near threshold) were detected using Mini analysis software (Synaptosoft). An average sEPSP was obtained for each condition (control and AP5, hyperpolarized and depolarized). To analyze EPSPs evoked by focal extracellular stimulation (eEPSPs), 20 consecutive eEPSPs recorded at each membrane potential were averaged (control and AP5). At depolarized potentials, sEPSP and eEPSP decay were not well fit by exponential functions; thus, the effects of depolarization and NMDAR activation were quantified by measuring changes in the EPSP area, computing a ratio between the EPSP area at depolarized and hyperpolarized potentials (or D/H EPSP area ratio, obtained in control conditions and in the presence of AP5). The D/H ratio compensates for the within-cell variability in the depolarization effects on EPSP area, which, especially in pyramidal cells, vary markedly depending on EPSP size and the subthreshold depolarized potential at which each particular neuron was recorded (Stuart and Sakmann, 1995; Gonzalez-Burgos and Barrionuevo, 2001; Rotaru et al., 2007).

To quantify the magnitude of EPSP summation during stimulus trains, first we obtained an average of all the recorded EPSP trains that remained subthreshold (see Figs. 5, 6). In the average traces, we measured the amplitude of the first (EPSP1) and fifth (EPSP5) EPSPs, relative to the membrane potential measured just prior (10 ms) to EPSP1. Summation was estimated by computing the EPSP5/EPSP1 ratio for each neuron in each condition.

**Spike probability during EPSP–spike coupling**

The probability of eliciting spikes by EPSP trains at depolarized potentials was estimated as the proportion of EPSP trains with spikes (independent of the number of spikes per train) out of the total number of trains recorded. Typically, at least 20 consecutive trains were used to estimate the spike probability in control conditions or in the presence of AP5. Spike probability was quantified similarly when testing the effects of the GABA_A receptor antagonist gabazine (2 μm) or of the use-dependent NMDA channel blocker MK801 (10 μm).

**Chemicals**

Biocytin was obtained from Invitrogen; all other chemicals and reagents were obtained from Sigma Chemical.
Statistical analysis

Results are expressed as mean ± SEM. The statistical analysis was performed using Statistica 6.3 software (Statsoft). The significance of differences between group means was determined using Student's t test, one-way ANOVA, two-way ANOVA, or Mann–Whitney U test, as indicated in each case. Differences between group means were considered significant if $p < 0.05$. The results of statistical comparison of differences between group means are shown in the figure legend of each figure.

Computational network model and simulations

The model consists of a network of 200 excitatory (E) cells and 40 inhibitory (I) spiking neurons. Connections are such that each E cell receives inputs from 10% of the other E cells and 75% of the I cells. I cells receive connections from 75% of the E cells and I cells. Connections are either 0 or 1. Each neuron obeys the following dynamics (Izhikevich et al., 2004):

$$\frac{dV}{dt} = \frac{1}{C} \left( I_{\text{appl}} + g_l \frac{(V - V_l)(V - V_T)}{V_T - V_l} - z(V - V_K) - I_{\text{syn}} + \sigma N \right)$$

$$\frac{dz}{dt} = -az,$$

where $z$ represents adaptation, and $N$ is white noise. Each time $V(t)$ hits $V_{\text{spike}}$, $z$ is incremented by $d$ and $V$ is reset to $V_r$. For E cells, $gl = 0.05$, $Vl = -65$, $VT = -45$, $a = 0.01$, $d = 0.2$, $Vr = -52$. I cells are the same, but $gl = 0.1$ and $d = 0$ (no spike-frequency adaptation), $VK = -75$. $C$ is 1 $\mu F/cm^2$, all conductances are in millisiemens per square centimeter, and voltages are in millivolts. Parameters are chosen so that at rest E cells have a membrane time constant of 20 ms and I cells 10 ms. Bias currents are applied only to the E cells, $I_{\text{appl}} = 3 \mu A/cm^2$, so that the I cells can only fire if E cells fire. A small amount of white noise is added to the E cells.

Synaptic currents have the following form:

$$I_{\text{syn}} = (g_{es} + g_{es_n})(V - V_{ex}) + g_{i}(V - V_{in})$$

where $Vin = -70$ and $Vex = 0$. Every neuron to which a given cell is connected contributes its own synaptic current. The synaptic gating variables satisfy the following equations:
\[
\frac{ds_e}{dt} = -\frac{s_e}{\tau_e}
\]
\[
\frac{ds_n}{dt} = a_n s_e (1 - s_n) - \frac{s_n}{\tau_n}
\]
\[
\frac{ds_i}{dt} = -\frac{s_i}{\tau_i}
\]

Here sn is an NMDA synapse, se is an AMPA synapse, and si is GABA. The model for NMDA does not include any magnesium block, and its form is due to (Wang, 1999). Each time an E cell fires, se is incremented by 1; each time an I cell fires, its corresponding si is incremented by 1. For E→E connections, \(\tau_e = 3\) ms, \(g_{ee} = 0.05\), and \(g_{ne} = 0.005\). For E→I connections, \(g_{ei} = 0.4\), \(g_{ni} = 0.002\), and \(\tau_e = 1\) ms. In both cases, \(a_n = 0.5/\text{ms}\) and \(\tau_n = 80\) ms. For I connections, \(g_{ie} = 0.5\), \(g_{ii} = 0.1\), and \(\tau_i = 2\) ms. All simulations were performed using XPPAUT; the code is available from the authors. Power spectra were taken on the “local field potential,” which is the sum of all the excitatory currents into the excitatory cells. Rasters are actually plots of se the E→E synaptic gating variables. Euler's method was used with a step size of 0.05 ms.

**RESULTS**

**Contribution of NMDAR-mediated currents to EPSCs**

We recorded from pyramidal cells and from FS interneurons in layer 5 of the medial PFC (Fig. 1A). FS interneurons are PV-positive cells with narrow spikes that lack spike-frequency adaptation, whereas NFS interneurons are a largely heterogeneous group of PV-negative cells including multiple subclasses (Ascoli, 2008), most of which have significant spike-frequency adaptation and spikes with longer duration. Because several subclasses of NFS interneurons have strong synaptic NMDAR currents (Lu et al., 2007; Wang and Gao, 2009), unambiguous identification of FS cells is crucial. We separated FS from NFS interneurons based on the combined analysis (see Materials and Methods) of the spike duration at half peak amplitude, the spike-frequency adaptation (measured by the adaptation ratio), and the amplitude of the afterhyperpolarizing potential (AHP) observed following individual spikes (Fig. 1B). To confirm that the criteria were appropriate, we performed recordings from green fluorescent protein (GFP)-positive neurons from G42 mice (see supplemental Table 1, available at www.jneurosci.org as supplemental material), in which GFP is expressed exclusively in PV-positive FS neurons (Chattopadhyaya, 2004). As shown in Figure 1B, many NFS cells have spikes significantly shorter than pyramidal cell spikes, together with obvious spike-frequency adaptation and/or relatively small AHP. Therefore, assessment of only the spike duration is insufficient for proper identification of FS neurons. In this study, we recorded
Glutamate receptors on PFC neurons

from a total of 99 pyramidal neurons, 68 FS cells, and 45 interneurons that were classified as NFS and therefore excluded from analysis of synaptic properties. All the recorded FS neurons for which morphology was identified were basket cells with multipolar aspiny dendrites (Fig. 1C). Examples illustrating the heterogeneous properties of the NFS interneurons are shown in supplemental Figure 1 (available at www.jneurosci.org as supplemental material).

![Figure 1](image)

**Figure 1.** PFC pyramidal cells and FS interneurons were identified based on their firing pattern and morphological properties. A, Top, Firing properties of a typical FS interneuron identified in layer 5. Bottom, Firing properties of a typical layer 5 pyramidal neuron. Some pyramidal cells had weakly bursting properties (not shown). B, Left, Adaptation ratio (last interspike interval/first interspike interval, see Materials and Methods) plotted as a function of spike duration at half peak amplitude for 30 pyramidal cells (triangles), 60 FS neurons (black circles), and 45 NFS cells (gray circles). Right, Plot of the AHP amplitude versus spike duration at half-width. Cells classified as FS (see Materials and Methods) had spike duration \( \leq 0.6 \) ms, AHP amplitude \( \geq 15 \) mV, and adaptation ratio \( \leq 1.2 \). For examples of NFS cell properties, see supplemental Figure 1 (available at www.jneurosci.org as supplemental material). C, Examples of the morphological properties of pyramidal cells (left) and FS neurons (right) filled with biocytin during recording. All the FS cells had properties consistent with the basket cell morphological class (no chandelier neurons were found in this data sample). Axons shown in gray, dendrites in black.

We recorded sEPSCs in Mg2+-free conditions (no Mg2+ added) to minimize NMDAR channel block, and found that the sEPSCs decayed more slowly in pyramidal cells than in FS interneurons (Fig. 2A). Because NMDAR-mediated EPSCs are significantly longer-lasting than AMPAR-mediated EPSCs (Dingledine et al., 1999; Cull-Candy and Leszkiewicz, 2004), we blocked NMDARs to test whether the slower sEPSC decay in pyramidal cells was associated with a stronger NMDAR contribution. Ketamine, a noncompetitive
NMDAR antagonist commonly used to model NMDAR hypofunction, was not used here because ketamine has multiple effects unrelated to its action as NMDAR antagonist. For instance, ketamine inhibits the hyperpolarization-activated channels (Chen et al., 2009), which are abundant in pyramidal cells (Kole et al., 2006) and FS neurons (Aponte et al., 2006) and shape the duration of synaptic responses (Magee, 1998; Rotaru et al., 2007). Ketamine also enhances the excitability of pyramidal cell dendrites (Potez and Larkum, 2008). Application of the competitive NMDAR antagonist AP5, significantly accelerated the sEPSC decay in pyramidal cells but not in FS neurons (Fig. 2B,C). The sEPSC peak amplitude was larger in FS neurons than pyramidal cells (Fig. 2A,B), but was not affected by AP5 in either cell type (sEPSC peak amplitude, pyramidal cells, n = 11, control sEPSC: 30.4 ± 2.1 pA; AP5 sEPSC: 29.9 ± 2.5 pA; FS neurons, n = 10, control sEPSC: 42.0 ± 5 pA; AP5 sEPSC: 36.4 ± 4.7 pA; two-factor ANOVA, pyramidal vs FS: F(1,38) = 5.91, p = 0.019; AP5: F(1,38) = 0.686, p = 0.412; interaction: F(1,38) = 0.463, p = 0.499). Waveform subtraction revealed a weaker NMDAR component in FS cell sEPSCs (Fig. 2B). Consequently, the AMPA/NMDA charge ratio (the ratio between the charge flowing through AMPARs and that flowing through NMDARs) was higher in FS neurons (Fig. 2D).

Our experiments using the competitive NMDAR antagonist AP5 may have underestimated the NMDAR contribution to FS cell sEPSCs if AP5 had weaker effects at FS neuron synapses. For instance, multivesicular glutamate release at FS cell synapses (Watanabe et al., 2005) may produce a high glutamate concentration transient in the synaptic cleft, thus displacing more strongly the competitive antagonist AP5 at FS versus pyramidal cell synapses. To estimate the NMDAR contribution independently of AP5 effects, we used CNQX, a high-affinity AMPAR antagonist whose efficacy is less affected by the cleft glutamate concentration (Biro et al., 2005). Because detection of NMDAR-mediated sEPSCs after CNQX application was unreliable (supplemental Fig. 2, available at www.jneurosci.org as supplemental material), we studied EPSCs evoked with focal extracellular stimulation (eEPSCs) at low intensity, to minimize polysynaptic transmission, which in low Mg2+ is enhanced by NMDAR-mediated excitation (Thomson and West, 1986). AMPAR blockade revealed a significant NMDAR eEPSC in pyramidal neurons, whereas in FS cells the NMDAR eEPSC was smaller (Fig. 3A,B). In either pyramidal cells or FS neurons, the EPSC component remaining after applying CNQX in 0 mm Mg2+, was blocked by application of Mg2+ (1 mm), AP5 (Fig. 3A), or Mg2+ and AP5 together (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Moreover, the eEPSCs decayed more slowly in pyramidal cells than in FS neurons (Fig. 3A,B), and waveform subtraction showed that NMDAR currents significantly prolonged the eEPSC decay in pyramidal cells and not in FS neurons (Fig. 3C). In addition, the AMPA/NMDA charge ratio for eEPSCs was significantly higher in FS cells (Fig. 3D). Therefore, as for sEPSCs, eEPSC properties suggest a weaker synaptic NMDAR contribution in FS cells.
Figure 2. Contribution of AMPARs and NMDARs to sEPSCs in PFC neurons. A, Left, Examples of sEPSCs recorded from a pyramidal cell (gray) or a FS interneuron (black). Note the faster decay time course of individual sEPSCs in the FS neuron. Right, Averages of the sEPSCs recorded from the pyramidal cell and FS neuron are shown superimposed. No Mg\(^{2+}\) was added to the extracellular solution. B, Average sEPSCs recorded from a pyramidal cell (left) and a FS neuron (right) in control conditions (thin black), or after 15 min of d,l-AP5 (100 \(\mu\)m) application (gray). The NMDAR-mediated sEPSC waveform obtained by subtraction is shown as well (thick black). C, Bar graphs summarizing the differences in the decay time constant of the control average sEPSCs (AMPAR- and NMDAR-mediated) and the sEPSCs recorded after AP5 application (AMPAR-mediated). AP5 significantly accelerated the sEPSC decay in pyramidal cells but not in FS neurons. *p < 0.05 (sEPSC decay time, pyramidal cells, n = 11, control: 7.36 ± 0.75 ms, AP5: 4.63 ± 0.22 ms; FS cells, n = 10, sEPSC: 2.04 ± 0.20 ms, AP5: 1.84 ± 0.12 ms; two-factor ANOVA, cell type: \(F_{(1,38)} = 89.9\) p < 0.00001; NMDAR: \(F_{(1,38)} = 11.9\)
p < 0.002; interaction: $F_{(1,38)} = 8.7 p < 0.01$; post hoc Fisher LSD tests control versus AP5: pyramidal cells, p < 0.0001, FS neurons: p = 0.736). D, Bar graph showing the AMPA/NMDA charge ratio, estimated from the sEPSC waveforms for both pyramidal cells and FS neurons. *p < 0.01 (AMPA/NMDA charge ratio, pyramidal cells, n = 11: 2.30 ± 0.48; FS neurons, n = 10: 5.05 ± 0.67; Mann–Whitney U test, U = 2.72, p < 0.005).

Figure 3. Contribution of AMPARs and NMDARs to eEPSCs in PFC neurons. A, Left, Plot of the response amplitude versus stimulus number for eEPSCs recorded from a pyramidal neuron, in control conditions and after application before of the AMPAR antagonist CNQX (20 μm). Note that a significant eEPSC component was observed after CNQX application and was blocked by adding d,l-AP5 (100 μm). No Mg2+ was added to the extracellular solution. The inset shows recordings from the neuron depicted in the plot (calibration bars: 50 pA, 20 ms). Right, From recordings in a different pyramidal cell, shown superimposed are the average eEPSCs recorded in control conditions and after
Glutamate receptors on PFC neurons

CNSX addition (thick and thin black, respectively) and the AMPAR-eEPSC waveform obtained by subtraction (gray). B, Left, In an experiment similar to that shown in A, a very small eEPSC was left in a FS neuron after CNSX application. Right, Average EPSCs and AMPA EPSC obtained by subtraction are shown superimposed for an example FS neuron. C, Bar graph summarizing differences in the decay time constant of the control eEPSCs (AMPAR- and NMDAR-mediated) and the AMPAR-mediated eEPSCs, obtained by waveform subtraction. **p < 0.001 (eEPSC decay time constant, pyramidal cells, n = 10, control eEPSC: 8.86 ± 0.98 ms, AMPA eEPSC: 4.78 ± 0.38 ms; FS cells, n = 13, control eEPSC: 2.85 ± 0.46 ms, AMPA eEPSC: 2.15 ± 0.24 ms; two-factor ANOVA, cell type: F(1,42) = 59.4 p < 0.0001; NMDAR: F(1,42) = 17.5 p < 0.0002; interaction: F(1,42) = 8.4 p < 0.01; post hoc Fisher LSD tests control eEPSC vs AMPA eEPSC: pyramidal cells, p < 0.0001, FS neurons, p = 0.704). D, Bar graph summarizing data of the AMPA/NMDA charge ratio, estimated from the eEPSC waveforms for both pyramidal cells and FS neurons. Relative to the AMPA charge, the NMDA charge was significantly smaller in FS neurons, producing a larger AMPA/NMDA charge ratio. *p < 0.05 (AMPA/NMDA charge ratio, pyramidal cells, n = 23, 0.87 ± 0.28; FS neurons, n = 18, 5.21 ± 1.79; Mann–Whitney U test: Z = 2.022, p < 0.05).

Importantly, our data (Figs. 2, 3) show that the AMPAR-mediated EPSCs have a faster decay in FS neurons than in pyramidal cells (sEPSC decay, FS cells, n = 10, 1.84 ± 0.12 ms; pyramidal cells, n = 11, 4.63 ± 0.22 ms, p < 0.001; eEPSC decay, FS cells, n = 13, 2.15 ± 0.24 ms; pyramidal cells, n = 10, 4.78 ± 0.38 ms; p < 0.001). These results suggest that compared with pyramidal cells, at glutamate synapses onto FS neurons fast signaling is optimized by having weaker NMDAR contribution and faster AMPAR currents. The mechanisms producing faster AMPAR currents in FS cells were not investigated here; however, in hippocampus and somatosensory cortex (Geiger et al., 1997; Angulo et al., 1999; Sambandan et al., 2010) as well as in rat PFC (Wang and Gao, 2009), AMPAR-EPSCs in FS cells seem to be mediated by GluR2 subunit-lacking AMPARs, which have fast deactivation and are Ca2+ permeable.

**Contribution of NMDARs to EPSPs**

The AMPA/NMDA charge ratios estimated for EPSCs showed a significantly weaker NMDAR component in FS cells. However, the weaker NMDAR-mediated currents in FS cells may still be significant for EPSP-mediated FS cell excitation. Thus, we next compared the NMDAR contribution to EPSPs in pyramidal cells and FS neurons. In pyramidal cells, NMDAR-mediated currents prolong EPSPs specifically at depolarized potentials that decrease the voltage-dependent Mg2+ block of the NMDAR channel (Thomson and West, 1986; Forsythe et al., 1988; Jones and Baughman, 1988; Thomson et al., 1988; Thomson, 1997). We therefore studied EPSPs evoked by extracellular stimulation (eEPSPs) in standard external Mg2+ (1 mm) at negative potentials (approximately −80 mV) or during depolarization near spike threshold elicited by current injection into the soma of the recorded neuron.

At potentials near −80 mV, the eEPSPs, which decayed with slower time course in pyramidal cells than FS neurons, were unaltered by AP5 application (Fig. 4A,B). In pyramidal cells, depolarization markedly prolonged
the eEPSPs, which decayed in a highly nonexponential manner (Fig. 4B). In contrast, in depolarized FS neurons, eEPSPs were weakly prolonged and decayed nearly exponentially (Fig. 4B). NMDAR blockade attenuated the enhancement of eEPSPs by depolarization in pyramidal cells (Fig. 4A), whereas in FS neurons eEPSPs evoked at depolarized potentials were not significantly affected by AP5 (Fig. 4A). As the variable and highly nonexponential decay of EPSPs in depolarized pyramidal neurons prevented curve fitting, we estimated the effects of depolarization and AP5 measuring the ratio between EPSP area at depolarized relative to hyperpolarized potentials (EPSP D/H area ratio, see Materials and Methods). In pyramidal cells, depolarization produced an about 3-fold increase in eEPSP area, and this effect was significantly attenuated, down to an about 1.5-fold increase, by AP5 (Fig. 4A). In FS neurons, depolarization produced a weaker, about 2-fold, increase in eEPSP area that was not significantly altered by AP5 (Fig. 4B).

**Figure 4.** NMDAR contribution to EPSPs is significantly stronger in PFC pyramidal cells than in FS neurons. A, Left, eEPSPs in a pyramidal cell at negative potentials (approximately −80 mV) and depolarized near spike threshold, in control conditions and after application of d,l-AP5 (100 μm). Here and in B and C, gray traces show individual eEPSPs and black traces the average of at least 10 eEPSPs. The numbers below the traces indicate the mean membrane potential measured 5 ms before stimulation. Right, The effects of depolarization and AP5 were estimated by computing the ratio between the eEPSP area at depolarized and hyperpolarized potentials (EPSP D/H area ratio). D/H area ratio = 1 means an absence of effect of depolarization (see Materials and Methods). The bar graph summarizes the experiments testing the effects of AP5 on EPSP D/H area ratio in pyramidal cells **p < 0.01 (eEPSP D/H area ratio for pyramidal cells, control: 2.98 ± 0.36, n = 17, AP5: 1.61 ± 0.24, n = 15; Mann–Whitney U test Z = 2.63, p = 0.0085). B, Left, eEPSPs evoked in a FS neuron at potentials near −80 mV and at a depolarized potential near spike threshold. Right, Bar graph summarizing the effects of membrane depolarization and d,l-AP5 (100 μm) application on eEPSPs recorded from FS cells (eEPSP D/H area ratio for FS cells, control: 2.10 ± 0.33, n = 11, AP5: 1.45 ± 0.13, n = 10; Mann–Whitney U test Z = 1.619, p = 0.105). FS interneurons were depolarized to similar subthreshold potentials than pyramidal cells (pyramidal cells, control: −57.82 ± 1.79 mV, AP5: −56.15 ± 1.66 mV; FS neurons, control: −55.19 ± 2.72 mV, AP5: −54.11 ± 4.11 mV; two-factor ANOVA followed by Fisher LSD comparisons, p > 0.25). C, Examples of sEPSPs recorded from a pyramidal cell (left panel) or a FS neuron (right panel) at different membrane potentials (average membrane potential values indicated below the
Gray traces show individual sEPSPs and black traces the average of at least 50 sEPSPs. D, Left, The average sEPSPs shown in C are displayed superimposed. Black traces and gray traces show the averages of sEPSPs recorded at hyperpolarized and depolarized membrane potentials, respectively. Right, Bar graph summarizing the effects of membrane depolarization and d,l-AP5 (100 μm) application on the sEPSP D/H area ratio. **p < 0.005 (sEPSP D/H area ratio for pyramidal cells, n = 8, control: 3.28 ± 0.33, AP5: 1.87 ± 0.22, Mann–Whitney U test, Z = 2.83, p = 0.0045; sEPSP D/H area ratio for FS cells, n = 8, control: 1.25 ± 0.51, AP5: n = 8: 1.30 ± 0.12; Mann–Whitney U test, Z = 0.210, p = 0.833). Pyramidal cells and FS neurons were depolarized to similar subthreshold membrane potentials before and during AP5 application (pyramidal cells, control: −53.5 ± 1.4 mV, AP5: −55.0 ± 1.5 mV, p = 0.655; FS cells, control: −55.9 ± 2.5 mV, AP5: −54.4 ± 3.2 mV, p = 0.641).

AP5 nonsignificantly attenuated (from about 2-fold down to about 1.4-fold) the increase in eEPSP area with depolarization in FS neurons (Fig. 4B). Such nonsignificant decrease in the eEPSP area ratio by AP5 suggests that NMDARs contribute to the change in eEPSP area in FS neurons, but that such NMDAR contribution is significantly weaker than in pyramidal cells, as expected based on the sEPSC and ePSC data (Figs. 2, 3). Importantly, extracellular stimulation may produce polysynaptic network activity, and since polysynaptic EPSPs overlap with the decay phase of monosynaptic EPSPs, they could lead to overestimation of the NMDAR contribution, confounding the measurements of EPSP area during depolarization even if the monosynaptic EPSPs are mostly AMPAR mediated. To confirm that NMDARs differentially contribute to monosynaptic EPSPs in pyramidal neurons versus FS cells, we tested the effects of AP5 on sEPSPs, which are produced in the absence of extracellular stimulation. Similar to the sEPSCs, at a hyperpolarized potential the sEPSPs had significantly larger amplitude in FS neurons than in pyramidal cells (FS sEPSP: 1.22 ± 0.17 mV, n = 8; pyramidal cell sEPSP: 0.61 ± 0.09 mV, n = 8; p < 0.01), suggesting that single-synapse or unitary inputs are stronger in FS cells than in pyramidal neurons (Fig. 4C). Moreover, we found that depolarization increased the sEPSP area by about 3-fold in pyramidal cells and by about 1.25-fold in FS neurons (Fig. 4D), thus having effects that were very similar to those produced on eEPSPs (Fig. 4B). Application of AP5 significantly attenuated (down to an about 1.6-fold increase) the sEPSP area ratio in pyramidal cells (Fig. 4D), whereas in FS neurons the sEPSP area ratio was unaffected by AP5 (Fig. 4D). Together, the eEPSP and sEPSP recordings show that the NMDAR-mediated EPSP component is significantly stronger in pyramidal cells than in FS neurons. The decrease of eEPSP D/H area ratio by AP5 in FS neurons, even if not significant, raises some interesting questions given the absence of AP5 effect on sEPSP D/H area ratio. One possibility is that the nonsignificant decrease in eEPSP D/H area ratio by AP5 in FS neurons reflects blockade of the activation of an extrasynaptic pool of NMDARs that is not activated during sEPSPs, which mostly reflect single-synapse- and single-axon-mediated transmission. Extrasynaptic NMDARs may mediate the effects of glutamate spillover or ambient glutamate, both of which could influence FS neuron activation in specific network activity states, a possibility that remains to be investigated.
Importantly, the effect of depolarization on the eEPSP area was not completely prevented by NMDAR blockade (Fig. 4B), suggesting that together with NMDA-mediated currents, additional mechanisms underlie the increase in EPSP area with depolarization. For example, voltage-dependent conductances shape the EPSP area during depolarization of pyramidal cells and interneurons (Stuart and Sakmann, 1995; Magee, 1998; Fricker and Miles, 2000; Gonzalez-Burgos and Barrionuevo, 2001; Galarreta and Hestrin, 2001a; Rotaru et al., 2007). To test the role of voltage-dependent conductances, we generated EPSP-like voltage transients by injecting EPSC-like current waveforms (Rotaru et al., 2007) at hyperpolarized potentials or while depolarizing the cells near spike threshold with steady current injection (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Depolarization produced an AP5-insensitive increase in the area of the EPSP-like voltage transients, of about 2.2-fold in pyramidal cells and about 1.6-fold in FS neurons (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). These results show that depolarization shapes the EPSP area by the combined effects of NMDAR- and voltage-dependent conductances, as in somatosensory cortex pyramidal cells (Markram et al., 1997).

**Contribution of NMDARs to EPSP summation and EPSP–spike coupling**

The prolongation of EPSPs by NMDAR-mediated currents (Fig. 4) may enhance temporal summation in pyramidal cells. To test the NMDAR contribution to EPSP summation, we used focal stimulation to elicit trains of small EPSPs at hyperpolarized potentials or depolarized potentials near spike threshold. At potentials near rest, EPSPs decayed with slower kinetics in pyramidal cells than in FS neurons [decay time constant, pyramidal cell: 27.6 ± 3.8 ms (n = 7); FS neurons: 12.7 ± 2.4 ms (n = 6), Student's t test, t = 3.158, p < 0.01]. Therefore, whereas in pyramidal cells EPSPs showed substantial summation at 20 Hz, in FS neurons 50 Hz stimulation was necessary for summation of the fast-decaying EPSPs (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). As shown in Figure 5, in pyramidal neurons membrane depolarization significantly enhanced EPSP summation, and this effect was attenuated by AP5 (Fig. 5A). In FS neurons, the increase in summation ratio at depolarized potentials was significantly weaker than in pyramidal cells (p < 0.005) and was AP5 insensitive (Fig. 5B).
Figure 5. The NMDAR contribution to subthreshold EPSP summation is significantly stronger in PFC pyramidal cells than in FS neurons. A, Left, Example of average eEPSPs evoked by repetitive stimulation (5 stimuli, 20 Hz) in a pyramidal cell recorded at approximately −80 mV (black trace) and at a depolarized membrane potential near threshold (gray trace). Right, Bar graph summarizing the differences in eEPSP summation at different membrane potentials in the absence or presence of the NMDAR antagonist d,l-AP5 (100 μm). *p < 0.02 (eEPSP5/eEPSP1 ratio, hyperpolarized: 1.82 ± 0.21, depolarized: 3.41 ± 0.62, n = 14, Z = 2.113 p < 0.05, Mann–Whitney U test; eEPSP5/eEPSP1 ratio hyperpolarized+AP5: 2.03 ± 0.46, depolarized+AP5: 2.65 ± 0.37, n = 12, Z = 1.386 p = 0.165, Mann–Whitney U test). The depolarized membrane potential in control and AP5 conditions did not differ significantly (control: −59.5 ± 2.7 mV; AP5: −57.4 ± 3.0 mV, p = 0.619). B, Left, Example of average eEPSPs evoked by repetitive stimulation of excitatory inputs (5 stimuli, 50 Hz) onto a FS cell recorded at approximately −80 mV (black traces) and at a depolarized membrane potential near threshold (gray traces). Right, Bar graph summarizing the differences in eEPSP summation at different membrane potentials in the absence or presence of the NMDAR antagonist d,l-AP5 (100 μm) (eEPSP5/eEPSP1 ratio, hyperpolarized: 1.33 ± 0.23 n = 11, depolarized: 1.59 ± 0.39 n = 10, Z: 0.281, p = 0.778, Mann–Whitney U test; eEPSP5/eEPSP1 ratio hyperpolarized+AP5: 1.24 ± 0.20 n = 9, depolarized+AP5: 1.46 ± 0.45, n = 7, Z = 0.0532, p = 0.958, Mann–Whitney U test). The depolarized membrane potential in control and AP5 conditions did not differ significantly (control: −55.9 ± 1.9 mV; AP5: −55.0 ± 2.9 mV, p = 0.791).
The NMDAR-mediated boosting of EPSP summation described in Figure 5 may potentiate EPSP–spike coupling more strongly in pyramidal cells. To test this possibility, we estimated the probability of eliciting spikes with trains of small EPSPs paired with postsynaptic depolarization produced by somatic current injection. In pyramidal neurons, spikes were evoked with significant delays and variable timing, following EPSP summation (Fig. 6A). In contrast, in depolarized FS neurons spikes typically were produced with short delays, within a narrow time window near the EPSP peak and independently of summation (Fig. 6B). The latency from the onset of the stimulus trains to the first spike produced was significantly shorter in FS cells (p < 0.00001), although the spike latency was not affected by AP5 in FS (p = 0.822) or pyramidal neurons (p = 0.664) (spike latency FS cells control: 39.9 ± 9.9 ms; FS cells AP5: 32.3 ± 9.5 ms, n = 6; pyramidal cells control: 192.9 ± 20.7 ms; pyramidal cells AP5: 208.3 ± 39.8 ms, n = 6). In addition the spike latency showed larger variability in pyramidal cells (SD of spike latency FS cells control: 24.4 ms, FS cells AP5: 23.4 ms; pyramidal cells control: 50.7 ms, pyramidal cells AP5: 97.7 ms). These data show that FS cells fire much earlier and with less variable timing relative to the onset of stimulation. NMDAR blockade with AP5 significantly decreased the firing probability in pyramidal cells (Fig. 6A), but did not affect the probability of FS cell firing (Fig. 6B). The stronger effect of AP5 on EPSP–spike coupling in pyramidal neurons was not attributable to a more depolarized baseline membrane potential in these cells, given that the mean membrane potential 10 ms before the onset of the EPSP trains did not differ significantly between cell types (pyramidal cells, n = 6, control: −55.8 ± 3.2 mV; AP5: −56.6 ± 2.9 mV; FS neurons, n = 6, control: −55.0 ± 4.1 mV; AP5: −57.0 ± 4.4 mV; two-factor ANOVA, cell type: F(1,20) = 0.002, p = 0.965, NMDAR: F(1,20) = 0.145, p = 0.707). Moreover, we found that the membrane potential value before the onset of the EPSP trains was not correlated with spike probability in neither pyramidal cells (Pearson's r coefficient = 0.436, p = 0.156) or FS neurons (Pearson's r coefficient = 0.0832, p = 0.797), suggesting that the actual depolarization before train onset was not a determinant factor for spike probability.

The NMDAR contribution may increase if spikes are initiated by large EPSPs produced by synchronous activation of multiple presynaptic axons. For instance, compared with depolarization by somatic current injection, large EPSPs may depolarize dendrites more efficiently, causing more significant relief of NMDAR channels from Mg2+ and producing, at least in pyramidal neurons, dendritic NMDA spikes (Schiller et al., 2000; Larkum et al., 2009). We tested whether synaptic activation by large EPSPs revealed a significant contribution of NMDARs in pyramidal cells or FS neurons. We found that NMDAR blockade depressed large EPSP–spike coupling more strongly in pyramidal neurons than in FS cells (supplemental Fig. 5, available at www.jneurosci.org as supplemental material), as observed in previous studies of somatosensory and motor cortex (Ling and Benardo, 1995; Karayannis et al., 2007).
Figure 6. The NMDAR contribution to eEPSP–spike coupling is stronger in pyramidal cells. A, Left, Consecutive traces showing eEPSP trains evoked by 20 Hz stimulation in a pyramidal cell at a depolarized membrane potential near spike threshold in control conditions. Middle, Recordings from the same neuron in the presence of d,l-AP5 (100 μm). Here and in B, subthreshold and suprathreshold eEPSP trains are labeled in gray and black, respectively. The spikes are truncated for easier visualization of the eEPSPs. Right, Bar graph illustrating the significant reduction of spike probability by d,l-AP5 (100 μm) in pyramidal cells. *p < 0.05 (probability of spiking, n = 6, control: 0.67 ± 0.12, AP5: 0.36 ± 0.16, Student’s t test, t = 3.14, p < 0.05). B, Left, Consecutive traces showing eEPSP trains evoked by 50 Hz stimulation in a FS neuron at a depolarized membrane potential near spike threshold in control conditions. Middle, Recordings in the presence of d,l-AP5 (100 μm). The spikes are truncated for easier visualization of the eEPSPs. Right, Bar graph illustrating the absence of significant effect of d,l-AP5 (100 μm) on spike probability in FS neurons (probability of spiking, n = 6, control: 0.60 ± 0.14, AP5: 0.63 ± 0.17, Student’s t test, t = 0.233, p = 0.824).

NMDAR antagonist effects on disynaptic IPSP-mediated inhibition of PFC pyramidal neurons

EPSP–spike coupling in pyramidal cells may be counteracted by disynaptic inhibition evoked by glutamate-mediated excitation of interneurons synapsing onto the recorded cell. To test whether NMDAR-dependent disynaptic IPSPs inhibit EPSP–spike coupling in PFC pyramidal cells, next we evoked EPSPs followed by disynaptic IPSPs that partially truncated the EPSP decay phase (Fig. 7A). That the IPSPs were disynaptic was indicated by their variable trial-to-trial latency, significant failures, and their complete blockade by CNQX (Fig.
During stimulus trains at depolarized potentials, spikes were observed in some trials, whereas in other trials spiking was apparently inhibited by the disynaptic IPSPs (Fig. 7B). CNQX application completely blocked EPSPs and IPSPs (Fig. 7A,B) showing minimal contribution from monosynaptic IPSPs. The \( \text{GABA}_A \) receptor antagonist gabazine increased the spiking probability (Fig. 7C), showing that the disynaptic IPSPs inhibited pyramidal cell firing.

**Figure 7. NMDAR contribution to disynaptic inhibition onto PFC pyramidal cells.**

A. Top, Three traces showing that long-distance lateral stimulation of synaptic inputs produced, in a pyramidal cell, a sequence of an EPSP followed by disynaptic IPSPs (arrows), which strongly truncated the decay phase of the EPSP. Note that the disynaptic IPSPs had variable latency from trial to trial. Middle, Three traces showing that in some trials of the same experiment shown above, input stimulation evoked an EPSP in the absence of disynaptic IPSPs. Bottom, Application of CNQX (20 \( \mu \text{m} \)) abolished all responses (EPSPs and IPSPs) evoked by focal stimulation. B. Repetitive input stimulation in some cases produced pyramidal neuron firing as a consequence of EPSP summation.
Glutamate receptors on PFC neurons (gray). In other trials (black), the occurrence of disynaptic IPSPs (arrows) was sufficient to inhibit spiking. C, Left, Graph showing the number of spikes per EPSP train elicited in a pyramidal neuron plotted versus EPSP train number (trains delivered every 10 s, EPSP train frequency: 20 Hz). Note that application of the GABA<sub>A</sub> receptor antagonist gabazine (2 μm) increased firing. Right, Graph summarizing the effect of gabazine application on the spiking probability. *p < 0.05 (spike probability: control, 0.19 ± 0.05, gabazine, 0.51 ± 0.12, p < 0.05, t = 3.618, n = 5, Student's t test). D, Left, Graph showing the number of spikes per EPSP train plotted versus train number. Note that application of the NMDAR channel blocker MK801 (10 μm) decreased firing. Right, Graph summarizing the effect of MK801 application on the spiking probability. *p < 0.05 (spike probability: control, 0.41 ± 0.12, MK801, 0.28 ± 0.10, p < 0.05, t = 3.048, n = 7, Student's t test).

Disynaptic IPSPs are reduced by NMDAR antagonists in hippocampal pyramidal neurons (Ling and Benardo, 1995; Grunze et al., 1996), but are NMDAR-independent in somatosensory cortex (Ling and Benardo, 1995; Hull et al., 2009). Is NMDAR activation required to produce disynaptic IPSPs that control PFC pyramidal cell firing? If so, then NMDAR antagonists should increase the spike probability by removing or weakening the disynaptic IPSPs. To test this possibility, we applied MK801, a use-dependent blocker of the NMDAR channels (Huettner and Bean, 1988), at a concentration (10 μm) that saturates the NMDAR binding sites in equilibrium conditions. MK801 binding and channel block are dependent on NMDAR activation by glutamate (Huettner and Bean, 1988). Therefore, the effect of MK801 is predicted to be stronger at synapses in which the postsynaptic NMDAR contribution is greater or glutamate is released with a higher probability or reaches higher synaptic cleft concentrations. Figure 7D shows that the main effect of MK801 was to decrease pyramidal cell firing, starting at an early time window following the onset of application, when the MK801 concentration is probably still subsaturating. These data suggest that NMDARs are more critical to recruit pyramidal cells than interneurons producing the disynaptic IPSP-mediated inhibition in PFC slices.

**Fast AMPAR-mediated excitation of FS neurons is important for synchronized oscillations in the gamma frequency band**

FS neuron-mediated inhibition is crucial for the production of synchronized gamma band oscillations (Whittington et al., 2000; Bartos et al., 2007; Mann and Paulsen, 2007; Hájos and Paulsen, 2009). Current models suggest that during gamma oscillations FS neurons are recruited by rhythmic EPSCs synchronized with the network oscillation (Mann et al., 2005b; Oren et al., 2006; Cardin et al., 2009; Sohal et al., 2009). Such rhythmic EPSCs recruit FS cells in most cycles of the gamma oscillation, providing the feedback inhibition that synchronizes the pyramidal cells (Whittington et al., 2000; Mann et al., 2005b; Hájos and Paulsen, 2009). Our experiments show that glutamate synapses onto FS neurons have weaker NMDAR contribution and faster AMPAR currents than those onto pyramidal cells. One possibility is that the fast EPSC kinetics in FS neurons is important for interneuron activation during pyramidal cell-FS neuron feedback loops involved in gamma oscillations. To address this issue, we performed simulations in a model network of pyramidal
and FS cells (Fig. 8A), reciprocally connected via excitatory and inhibitory synapses with properties consistent with those mediated by AMPARs, NMDARs, and GABA_A receptors (Fig. 8B). Pyramidal cells continuously received noisy and heterogeneous excitatory drive, which in the absence of feedback inhibition produced network activity with no significant power in the gamma band (data not shown). No excitatory drive was directly applied onto FS cells, which therefore fired exclusively in response to EPSCs produced by pyramidal cell inputs. In the presence of GABA_A receptor-mediated feedback inhibition from the FS cells, we manipulated the strength of the AMPAR- and NMDAR-mediated conductance at the excitatory connections from pyramidal cells onto FS neurons (gei and gni, respectively), with other network parameters remaining constant. With AMPAR-dominated synapses (gei = 0.4 mS/cm²; gni = 0.002 mS/cm²), network activity was highly synchronized (Fig. 8C), showing a dominant component in the gamma frequency band (Fig. 8E). Increasing the NMDAR contribution (increasing gni from 0.002 to 0.008 mS/cm²) decreased rhythmicity (Fig. 8D), the oscillation power decreasing in an NMDAR-dependent manner (Fig. 8E). Importantly, the simulations were performed in the absence of Mg²⁺ block, since otherwise very large gni values were necessary to produce significant effects (data not shown), possibly because the fast time course of FS neuron EPSCs precludes significant relief from Mg²⁺ block.

How does the increase of NMDAR conductance at pyramidal-to-FS synapses decrease the power of the gamma rhythm? To understand how increasing gni disrupts the gamma oscillation, we examined the inputs onto a typical FS cell during the rhythm. Figure 8, F and G, shows the total current entering the FS cell (black trace), along with the synaptic output of that FS cell (red trace), and the activity of the pyramidal cells (the scaled total AMPA output of the pyramidal cell population, green trace). Note that a smaller NMDAR synaptic current at inputs onto FS neurons (gni = 0.002 mS/cm²) (Fig. 8F) leads to a flat and low amount of input between bouts of firing and that the output of the FS cell is closely time-locked to the peaks in the total input (black peaks), which are, in turn, tightly locked to the excitatory activity (green trace). However, with higher amounts of synaptic NMDA current onto the FS cells (gni = 0.008 mS/cm²) (Fig. 8G), the input currents to the FS cell remain elevated between spikes (note the much higher levels of total input current between peaks) and can result in spikes or bursts of spikes from the FS cell that are not locked to the excitatory activity (Fig. 8G, arrows). This extra firing of the FS cells that is not tightly locked to activity disrupts the rhythm and results in the decrease in power and synchrony. The results from our simulations are consistent with experiments in vitro showing that NMDAR blockade does not affect gamma oscillations that are, however, abolished by AMPAR antagonists (Traub et al., 1996; Buhl et al., 1998; LeBeau et al., 2002; Cunningham, 2006; Roopun et al., 2008). Moreover, in some in vivo and in vitro studies NMDAR blockade increased the gamma oscillation power (Pinault, 2008; Roopun et al., 2008; Hakami et al., 2009; Hong et al., 2009; Pietersen et al., 2009), consistent with the effect of lowering gni in our simulations.
Figure 8. Fast AMPAR-mediated FS neuron activation is crucial for production of gamma oscillations via feedback mechanisms. A, Membrane properties of E cells (which represent pyramidal neurons) and I cells (which represent FS neurons) in the model network. B, Time course of synaptic conductances used to model excitatory and inhibitory synapses between model neurons. Note that the decay of the AMPA conductance is faster at excitatory synapses onto I cells (EI) than at excitatory synapses onto E cells (EE). In addition, the decay time of NMDA conductance is significantly longer than the decay of EI or EE. C, Raster plot showing the spike timing of E cells (left columns) and I cells (right columns) during a network oscillation episode produced in conditions of relatively low NMDAR conductance in the excitatory synapses onto I cells (gni = 0.002 mS/cm², see Materials and Methods). D, A raster plot similar to that shown in C, but obtained during an episode of network activity in conditions of higher NMDAR conductance in excitatory synapses onto I cells (gni = 0.008 mS/cm²). E, Plot of power spectral density (PSD) showing the effects of different strengths of gni on network activity. Note that larger values of gni produced a decrease in oscillation in power and synchrony. F, Plot of the time course of the total current entering a typical I cell (black trace), the synaptic output of that FS cell (red trace), and the activity of the pyramidal cells (the scaled total AMPA output of the pyramidal cell population, green trace). These variables, shown in arbitrary units in the y-axis (negative values for the black trace indicate hyperpolarizing/outward current), were computed for network activity produced with a gni = 0.002 mS/cm². Note the rhythmic input onto the I cell and the regular output from this I cell onto other cells in the network. G, A plot equivalent to that shown in F, for network activity produced with gni = 0.008 mS/cm². Note that in this case, the input currents onto the FS cell remain elevated between spikes (note the much higher levels of
the black curves between peaks) and can result in spikes or bursts of spikes from the FS cell that are not locked to the excitatory activity (arrows).

Finally, we addressed the effect of changing the strength of NMDAR conductance onto FS neurons, in a model network including FS cells and a population of NFS inhibitory neurons that may also be involved in the mechanisms of gamma oscillations. Such NFS cells form a population of interneuron-targeting cells that are PV-negative, have spike-frequency adaptation, and synapse among themselves and onto FS neurons but not onto pyramidal cells (Mann et al., 2005b). During gamma oscillations, such interneuron-targeting cells are highly active and fire in a manner tightly coupled to the gamma oscillation cycle (Mann et al., 2005b), although their role in the gamma oscillation mechanisms is poorly understood. In our model, these cells were called FS cell-targeting inhibitory units (FTUs). We run simulations (supplemental Fig. 6, available at www.jneurosci.org as supplemental material) in a modified model network including FTUs that receive the same type and strength of excitatory inputs from pyramidal cells, and target other FTUs and FS neurons, but not the pyramidal cells. Simulations were done in two different cases, changing the strength of NMDA at inputs onto FSU/FTU cells from a “normal” low value to a high value. The results shown in supplemental Figure 6 (available at www.jneurosci.org as supplemental material) indicate that the presence of the FTUs does not change the effect of increasing the NMDA strength to FS neurons, which is disruptive to the gamma power. We can conclude that adding a NFS population of inhibitory cells has no qualitative effect on the outcome of the modeling regarding the role of NMDA currents.

DISCUSSION

Fast synaptic excitation with weak NMDAR contribution in PFC FS neurons

We compared the mechanisms of synaptic activation of FS neurons and pyramidal cells in mouse PFC, focusing on the relative contribution of NMDARs and AMPARs, as this relation is important to determine the possible cellular substrates and network consequences of NMDAR hypofunction in PFC circuits. We found that FS cells had EPSCs with faster decay due to a weaker contribution of NMDAR currents, which typically produce long-lasting EPSCs. These synaptic properties in PFC FS neurons are consistent with findings of short-lasting EPSCs and weak NMDAR component in FS/PV-positive interneurons from other cortical and subcortical areas (Geiger, 1995; Geiger et al., 1997; Angulo et al., 1999; Goldberg et al., 2003; Nyiri et al., 2003; Lamsa et al., 2007; Lu et al., 2007; Hull et al., 2009; Gittis et al., 2010).

Importantly, the NMDARs present in FS neurons predominantly contain NR2A subunits (Kinney, 2006), which, among NR2 subunits, produce the fastest NMDA EPSC decay (Dingledine et al., 1999; Cull-Candy and Leszkiewicz, 2004). NR2A subunits determine strong Mg2+ block, possibly
Glutamate receptors on PFC neurons

explaining the higher sensitivity to Mg$^{2+}$ of NMDAR-mediated EPSCs in FS neurons (Hull et al., 2009). Furthermore, we found that the decay of AMPAR-mediated EPSCs is faster in FS neurons than in pyramidal cells, a finding possibly attributed to the predominance of GluR2-lacking, rapidly deactivating AMPARs (Geiger et al., 1997; Angulo et al., 1999; Hull et al., 2009; Nissen et al., 2010; Wang and Gao, 2010). Therefore, synaptic properties and specific biophysical features of their dendrites (Hu et al., 2010), contribute to a fast and temporally precise synaptic activation of FS neurons.

One possibility is that the NMDAR currents underlying EPSCs in FS neurons, although small, are nevertheless crucial for EPSP–spike coupling. If so, then a reduction of synaptic NMDAR currents in schizophrenia or by NMDAR antagonists would depress FS cell activity more than pyramidal cell firing. However we found that, in contrast to pyramidal cells, NMDARs have a small impact on EPSP–spike coupling in FS neurons. Moreover, in FS neurons EPSP summation required a shorter time window, was more weakly enhanced by depolarization and was less affected by NMDAR blockade. Therefore, our results suggest that EPSP–spike coupling in FS cells occurs via NMDAR-independent coincident detection, whereas in PFC pyramidal neurons EPSP summation and EPSP–spike coupling were consistent with NMDAR-dependent temporal integration.

Are NMDARs important to recruit FS neuron-mediated inhibition in PFC circuits?

It has been proposed that NMDAR hypofunction produces disinhibition based on the assumption that FS neuron excitation is highly sensitive to NMDAR antagonists (Lisman et al., 2008). However, here we show that FS cell excitation is less sensitive to NMDAR antagonists than is pyramidal cell excitation, because FS cells have EPSCs with a weak NMDAR component, possibly contributing to the rapid coupling of excitation with inhibitory output (Jonas et al., 2004; Hu et al., 2010). In fact, robust long-lasting NMDA EPSCs at pyramidal–pyramidal synapses in PFC (Wang et al., 2008) may be critical for recurrent excitation and working memory (Lisman et al., 1998; Wang, 1999). Therefore, acting locally, NMDAR antagonists appear unlikely to produce FS neuron-mediated disinhibition.

To test whether NMDAR antagonists produce disinhibition in PFC circuits, we performed experiments in conditions in which disynaptic IPSPs inhibited EPSP–spike coupling in pyramidal cells. In these conditions, pyramidal cell firing was enhanced by a GABA receptor antagonist, consistent with disinhibition, but was decreased by NMDAR blockade. Consistent with these findings, disynaptic IPSP recruitment is NMDAR-independent in somatosensory cortex (Ling and Benardo, 1995; Hull et al., 2009), although it is NMDAR-dependent in hippocampal circuits (Ling and Benardo, 1995; Grunze et al., 1996).
Here, and in previous studies (Ling and Benardo, 1995; Grunze et al., 1996), the interneuron subtypes mediating disynaptic inhibition were not identified. Other studies showed that FS neurons produce NMDAR-independent disynaptic IPSPs (Hull et al., 2009; Pouille et al., 2009). NMDAR-dependent disynaptic inhibition may be produced by NFS/PV-negative neurons, which have synapses with strong NMDAR contribution (Lamsa et al., 2007; Lu et al., 2007; Wang and Gao, 2009). NFS neurons, including those in PFC, indeed elicit disynaptic IPSPs, but it has not been determined whether these are NMDAR-dependent (Kapfer et al., 2007; Silberberg and Markram, 2007; Berger et al., 2009).

To compare the effects of fast AMPAR-mediated versus slow NMDAR-mediated excitation of FS neurons on the mechanisms of gamma oscillations, we studied a model network producing gamma rhythms via feedback inhibition. We found that AMPAR-mediated FS neuron excitation was sufficient to support gamma oscillations, consistent with experiments showing that AMPAR blockade and AMPAR deficiency in PV neurons, but not NMDAR blockade, strongly attenuate gamma oscillations (Traub et al., 1996; Buhl et al., 1998; Fisahn et al., 1998; LeBeau et al., 2002; Cunningham, 2006; Fuchs et al., 2007; Roopun et al., 2008). Conversely, decreases in the slow NMDA conductance at pyramidal–FS neuron synapses increased gamma power in the network model. Similarly, NMDAR antagonists enhance gamma power in animal models (Pinault, 2008; Roopun et al., 2008; Hakami et al., 2009; Pietersen et al., 2009) or human subjects (Hong et al., 2009). Our simulations therefore suggest that rapid FS neuron activation (Jonas et al., 2004; Hu et al., 2010) is crucial for the production of gamma oscillations.

Implications for models of schizophrenia pathophysiology

Our findings studying the synaptic activation of PFC FS interneurons raise interesting questions about the mechanisms that might link NMDAR hypofunction to reduction of PV and GAD67 expression. Because lower levels of PV facilitate GABA release (Vreugdenhil et al., 2003), we suggested that decreased PV in schizophrenia is a compensatory response to a GAD67 deficit that reduces GABA synthesis and release (Krimer et al., 2005; Gonzalez-Burgos et al., 2008a). Therefore, PV reduction by NMDAR hypofunction may be secondary to a primary decrease of GAD67 levels. If so, a crucial question is how NMDAR hypofunction leads to reduced GAD67 expression in FS cells. One possibility is that changes in network activity are involved, because activity is the main factor regulating GAD67 expression (Jones, 1990; Akbarian and Huang, 2006). For example, deprivation of afferent activity decreases GAD67 levels and inhibitory synaptic strength (Benson et al., 1994; He et al., 2006; Jiao et al., 2006). Conversely, cortical network hyperactivity increases GAD67 (Liang and Jones, 1997; Esclapez and Houser, 1999). Interestingly, NMDAR blockade depresses the so-called up states in vitro (Tu et al., 2007; Kroener et al., 2009) and in vivo (Steriade et al., 1993; Seamans et al., 2003), thus producing network hypoactivity. Cortical up states recruit pyramidal cells
(Lewis and O'Donnell, 2000; Waters and Helmchen, 2006) and FS interneurons (Puig et al., 2008; Gentet et al., 2010) and involve local recurrent excitation and feedback inhibition (McCormick et al., 2003; Hasenstaub, 2005; Waters and Helmchen, 2006). Therefore, NMDAR antagonists may decrease GAD67 via network hypoactivity produced by up-state depression.

Somewhat contrasting with up-state depression, systemically administered NMDAR antagonists can increase PFC pyramidal cell activity in vivo (Suzuki et al., 2002; Homayoun and Moghaddam, 2007; Kargieman et al., 2007). Increased activity could reflect disinhibition, since NMDAR antagonists depress the in vivo activity of putative interneurons (Homayoun and Moghaddam, 2007), although a substantial fraction (about 33%) of PFC cells depressed by NMDAR antagonists are pyramidal (Kargieman et al., 2007). Our finding that FS cells are less sensitive to NMDAR antagonism than pyramidal neurons suggests that the disinhibition produced by NMDAR antagonists acting locally is mediated by NFS interneurons that have strong NMDAR-mediated EPSCs (Lu et al., 2007; Wang and Gao, 2009).

Alternatively, systemically applied NMDAR antagonists could increase PFC neuron activity indirectly, via simultaneous action in multiple brain regions. For instance, PFC pyramidal cell firing in vivo is increased by intrahippocampal infusion of NMDAR antagonists, but is decreased or is not altered by intra-PFC infusion (Suzuki et al., 2002; Jodo et al., 2005). Moreover, NMDAR antagonists may significantly depress the firing of ventral tegmental area neurons (Tseng et al., 2006), whose projections onto PFC inhibit pyramidal cells (Pirot et al., 1992; Lewis, 2000) and excite FS neurons (Tseng et al., 2006).

In a strain of genetically modified mice generated recently, NMDARs can be deleted selectively in GABA neurons, including PV cells (Belforte et al., 2010). NMDAR deletion in these mice failed to produce significant effects unless the deletion was induced during very early development, in which case adult mice developed schizophrenia-like behavioral alterations (Belforte et al., 2010). Interestingly, excitatory inputs onto immature PV neurons have strong NMDAR currents that progressively weaken with age, becoming small or absent in adult PV neurons (Wang and Gao, 2009). Similarly, in the cortex of adult human subjects, about 70% of the PV-positive neurons have undetectable levels of NMDAR subunit mRNA (Bitanihirwe et al., 2009). Because NMDARs are essential for the maturation of excitatory synapses (Waites et al., 2005), NMDAR subunit deletion early in development may persistently affect behavior into adulthood (Belforte et al., 2010) by disrupting the maturation of FS cell connectivity. However, the NMDAR hypofunction hypotheses of schizophrenia is primarily based on the effects of NMDAR antagonists administered to adult animals or adult human subjects (Javitt and Zukin, 1991). Our results and previous data (Wang and Gao, 2009; Belforte et al., 2010) suggest that NMDAR hypofunction induced in the adult state may affect PFC function by acting at glutamate synapses different from those mediating the activation of FS/PV-positive cells.
Chapter 4

GABA transporter GAT1 prevents spillover at proximal and distal GABA synapses onto primate prefrontal cortex neurons.

Gonzalez-Burgos G, Rotaru DC, Zaitsev AV, Povysheva NV, Lewis DA.
Translational Neuroscience Program, Department of Psychiatry, University of Pittsburgh School of Medicine, W1651 Biomedical Science Tower, 200 Lothrop St., Pittsburgh, PA 15261, USA. gburgos@pitt.edu

The plasma membrane GABA transporter GAT1 is thought to mediate uptake of synaptically released GABA. In the primate dorsolateral prefrontal cortex (DLPFC), GAT1 expression changes significantly during development and in schizophrenia. The consequences of such changes, however, are not well understood because GAT1's role has not been investigated in primate neocortical circuits. We thus studied the effects of the GAT1 blocker 1,2,5,6-tetrahydro-1-[2-[[diphenylmethylene]amino]oxyethyl]-3-pyridinecarboxylic acid hydrochloride (NO711) on GABA transmission onto pyramidal neurons of monkey DLPFC. As in rat cortex, in monkey DLPFC NO711 did not substantially alter miniature GABA transmission, suggesting that GAT1 does not regulate single-synapse transmission. In rat cortical circuits, between-synapse GABA spillover produced by NO711 clearly prolongs the inhibitory postsynaptic currents, but whether NO711 also prolongs the inhibitory postsynaptic potentials (IPSPs) is unclear. Moreover, whether spillover differentially affects perisomatic versus dendritic inputs has not been examined. Here we found that NO711 prolonged the GABA<sub>A</sub> receptor-mediated IPSPs (GABA<sub>A</sub>R-IPSPs) evoked by stimulating perisomatic synapses. Dendritic, but not perisomatic, synapse stimulation often elicited a postsynaptic GABA<sub>B</sub> receptor-mediated IPSP that was enhanced by NO711. Blocking GABA<sub>B</sub> receptors revealed that NO711 prolonged the GABA<sub>A</sub>R-IPSPs evoked by stimulation of dendrite-targeting inputs. We conclude that a major functional role for GAT1 in primate cortical circuits is to prevent the effects of GABA spillover when multiple synapses are simultaneously active. Furthermore, we report that, at least in monkey DLPFC, GAT1 similarly restricts GABA spillover onto perisomatic or dendritic inputs, critically controlling the spatiotemporal specificity of inhibitory inputs onto proximal or distal compartments of the pyramidal cell membrane.
INTRODUCTION

The plasma membrane GABA transporter 1 (GAT1) is abundant in neocortex (Guastella et al., 1990) where it is localized in neuronal and glial membranes near synapses (Minelli et al., 1995; Conti et al., 1998). This location suggests that GAT1 regulates GABA transmission by the uptake of synaptically released GABA (Conti et al., 2004). GAT1 blockade actually prolongs the decay of inhibitory postsynaptic currents (IPSCs) evoked by multiple-synapse stimulation (Overstreet and Westbrook, 2003), suggesting that GAT1 activity shortens the IPSC decay. However, miniature IPSCs (mIPSCs), which reflect single-synapse transmission, remain unchanged after GAT1 blockade (Thompson and Gahwiler, 1992; Isaacson et al., 1993; Overstreet and Westbrook, 2003) and in GAT1 knock-out mice (Jensen et al., 2003; Bragina et al., 2008).

The lack of effect of GAT1 blockade on mIPSCs indicates that GAT1 does not regulate transmission at single synapses and that prolongation by GAT1 block of IPSCs evoked by stimulating multiple synapses may result from between-synapse GABA spillover. Therefore GAT1 may help preserve synapse independence and the spatiotemporal specificity of inhibitory transmission, which is essential to maintain the distinct influence of different interneuron subtypes that provide nearby synaptic inputs onto the same membrane compartment (Klausberger and Somogyi, 2008). For instance, although functionally diverse, parvalbumin- and cholecystokinin-containing basket cells both furnish perisomatic synapses onto pyramidal cells (Freund and Katona, 2007). Similarly, multiple interneuron subtypes target dendrites (Somogyi et al., 1998).

The propensity for GAT1-regulated GABA spillover depends on the density of GABA synapses because GAT1 blockade does not affect IPSCs mediated by multiple synaptic contacts that are distant from each other (Overstreet and Westbrook, 2003). Importantly, the density of GABA synapses is significantly lower in dendrites than in the perisomatic membrane of pyramidal cells (Megias et al., 2001; Papp et al., 2001; Andrasfalvy and Mody, 2006). Therefore studies of synapse density suggest that GABA spillover may be less likely during activation of dendritic- than perisomatic-targeting inputs. Alternatively, GABA spillover may occur between nearby GABA synapses onto different neurons. If so, the probability of spillover may be more dependent on the density of axon terminals and dendrites in the neuropil, which determines the distance between GABA synapses onto different neurons. Thus, a lower GABA synapse density onto dendrites compared with soma of individual cells may be a less important determinant of spillover. Previous studies, however, have not determined whether GAT1 blockade has similar effects at dendritic versus perisomatic synaptic inputs.

It is well established that GABA spillover induced by GAT1 blockade increases the duration of IPSCs evoked by multiple synapse stimulation.
GAT1 prevents spillover at GABAergic synapses

However, whether GAT1 block also prolongs the inhibitory postsynaptic potential (IPSP) duration remains unclear. IPSPs typically outlast the underlying IPSCs because the IPSP decay is shaped by the cell's membrane properties (Koch et al., 1996). Thus IPSC prolongation by GAT1 block may not be sufficient to significantly prolong the IPSPs. An IPSP prolongation by GAT1 block may have important functional consequences because it would involve the effects of both the GABA-activated conductance and the associated change in membrane potential. Although hyperpolarizing IPSPs are inhibitory at peak and during decay, depolarizing IPSPs may inhibit at their peak but excite during decay (Gulledge and Stuart, 2003; Bartos et al., 2007) or may be excitatory throughout their duration (Szabadics et al., 2006). Thus examining whether changes in the efficacy of GAT1-mediated uptake affect IPSP duration is important for understanding GAT1's role in cortical circuit function.

In the dorsolateral prefrontal cortex (DLPFC) of human primates, circuit maturation during adolescence is associated with a significant decrease in GAT1 levels in some GABA terminals (Cruz et al., 2003). Moreover, GAT1 mRNA and protein levels are reduced in the DLPFC of subjects with schizophrenia (Pierri et al., 1999; Volk et al., 2001). However, the consequences of such development- and disease-related changes in GAT1 levels, and thus of GABA uptake efficacy, are not well understood because the functional role of GAT1 has not been investigated in the human or Nhuman primate neocortex. One possibility suggested by anatomical studies is that GABA spillover is less significant in primate neocortex, which, compared with rodent neocortex, has lower density of inhibitory synapses in the neuropil, lower neuronal density and higher density of glial cells (DeFelipe, 2002; Herculano-Houzel et al., 2006; Sherwood et al., 2006; Herculano-Houzel et al., 2007). To determine whether GAT1-mediated uptake regulates GABA spillover in primate neocortical circuits, here we recorded from layer 3 pyramidal neurons of monkey DLPFC to test the effects of the GAT1 blocker 1,2,5,6-tetrahydro-1-[[2-[(diphenylmethylene) amino]oxy] ethyl]-3-pyridinecarboxylic acid hydrochloride (NO711). Specifically, we performed current-clamp recordings to determine whether GAT1 blockade affects IPSPs during miniature transmission or during stimulation of proximal (perisomatic) versus distal (dendritic) GABA synapses.

METHODS

Brain slice preparation

Experiments were performed in tissue obtained from ten female rhesus macaque monkeys (Macaca mulatta) and two male long-tailed macaque monkeys (M. fascicularis) supplied by the University of Pittsburgh Primate Research Center. Housing and experimental procedures were conducted in accordance with U.S. Department of Agriculture and National Institutes of Health guidelines and with approval of the University of Pittsburgh's Institutional Animal Care and Use
Committee. All rhesus animals ≤45 mo of age were bred at this facility. All animals were experimentally naïve at the time of entry into this study.

Brain slices were prepared from five prepubertal rhesus monkeys 15–16 mo of age, four postpubertal rhesus monkeys 42–45 mo of age, one adult rhesus monkey 84 mo old, and two long-tailed monkeys 42–60 mo of age. Tissue blocks containing portions of DLPFC areas 9 and 46 were obtained from one or both hemispheres of each animal. Some of the animals were deeply anesthetized and perfused transcardially with a cold artificial cerebrospinal fluid (ACSF) solution of the following composition (in mM): 210.0 sucrose, 10.0 NaCl, 1.9 KCl, 1.2 Na2HPO4, 33.0 NaHCO3, 6.0 MgCl2, 1.0 CaCl2, 10.0 glucose, and 2.0 kynurenic acid; pH 7.3–7.4 when bubbled with 95% O2-5% CO2, and a DLPFC tissue block was rapidly prepared as previously described (Gonzalez-Burgos et al., 2004). For all other animals, an initial tissue block was removed from one hemisphere using a previously described surgical procedure (Gonzalez-Burgos et al., 2004), and then a second DLPFC tissue block was removed 1–2 wk later, following the transcardial cold ACSF perfusion procedure described in the preceding text. When two tissue blocks were removed per animal in separate surgical procedures, the locations of the blocks were off-set in the rostral-caudal axis, so that nonhomotopic portions of the DLPFC were studied from each hemisphere. Previous studies have shown that the first procedure does not alter the physiological or anatomical properties of the neurons and local circuits present in the tissue obtained in the second hemisphere (Gonzalez-Burgos et al., 2000).

Cortical slices (300–350 μm thick) were cut in the coronal plane using a vibrating microtome (VT1000S, Leica Microsystems, Nussloch, Germany) in ice-cold ACSF. Immediately after cutting, slices were transferred to an incubation chamber maintained at room temperature and filled with a solution containing (in mM) 126.0 NaCl, 2.0 KCl, 1.2 Na2HPO4, 10.0 glucose, 25.0 NaHCO3, 6.0 MgCl2, and 1.0 CaCl2, pH 7.3–7.4 when bubbled with 95% O2-5% CO2.

**Electrophysiological recordings**

For recording, slices were submerged in a chamber superfused at a rate of 2–3 ml/min with a solution containing (in mM) 126.0 NaCl, 2.5 KCl, 1.2 Na2HPO4, 25.0 NaHCO3, 10.0 glucose, 2.0 CaCl2, 1.0 MgCl2, 0.02 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX), d,l-amino-5-phosphonopentanoic acid (AP5) 0.1, bubbled with 95% O2-5% CO2, and maintained at 30–32°C. In some experiments, gabazine or bicuculline methiodide (20 μM) was added to block GABA\_A receptors (GABA\_ARs). Whole cell recordings were obtained from visually identified pyramidal neurons in layer 3 of DLPFC areas 9 and 46 using infrared differential interference contrast video microscoposcopy in Olympus BX51 and BX61 microscopes (Olympus), or Zeiss FS Axioskop microscopes (Zeiss). Recording micropipettes pulled from borosilicate glass had a resistance of 3–5 MΩ when filled with a solution containing (in mM) 120.0 KCl, 10.0 NaCl, 0.2 EGTA, 10.0 HEPES, 4.0 MgATP, 0.3 NaGTP, 14.0 NaPhosphocreatine, and
biocytin 0.5% (pH adjusted to 7.2–7.3). Assuming an intracellular bicarbonate concentration of 15 mM (Farrant et al., 2007), a permeability ratio \( \text{PHCO}_3^-/\text{PCl}^- \) of 0.3 for GABA\(_A\)R channels (Farrant et al., 2007) and using the Goldman-Hodkin-Katz equation, we estimated the reversal potential of the GABA\(_A\)R-IPSP (\( E_{\text{GABA}_A} \)) to be near zero (−0.66 mV). On the other hand, the Nernst potential for K\(^+\) (\( E_{\text{K}^+} \)), which determines the reversal potential for GABA\(_B\)R-activated K\(^+\) currents (Lüscher et al., 1997) was estimated at −102 mV. Recordings were performed using Multiclamp 200A or Multiclamp 200B amplifiers (Axon Instruments, Union City, CA) operating in current-clamp (bridge) mode. Signals were low-pass filtered at 4 kHz, digitized at 10 or 20 kHz, and stored on disk for off-line analysis. Data acquisition was performed using Power 1401 data-acquisition interface boards (Cambridge Electronic Design, Cambridge, UK) and Signal 3 software (Cambridge Electronic Design). Throughout the experiments, the series resistance was monitored, and if it exceeded 30 M\( \Omega \), recordings were excluded from data analysis.

**Recording and analysis of mIPSPs**

mIPSPs were recorded from layer 3 pyramidal neurons in slices obtained from postpubertal animals. To block action potentials thus focusing on IPSPs resulting from spontaneous GABA release at single synapses, the voltage-dependent Na\(^+\) channel blocker tetrodotoxin (1 \( \mu \)M) was added to a bath solution that otherwise had the same composition as that used to record IPSPs. The cells were recorded at −80 mV for 20 min and then NO711 (20 \( \mu \)M) or N,N,6-trimethyl-2-(4-methylphenyl)imidazo[1,2-a]pyridine-3-acetamide (zolpidem, 1 \( \mu \)M) was applied for 15 min. For each cell, the mIPSPs were detected using MiniAnalysis (Synaptosoft, Decatur, GA). At least 300 nonoverlapping events were included to automatically generate an average mIPSP for each cell in control conditions and at the last 5 min of the NO711 or zolpidem application. The amplitude and the decay time constant of an exponential function fit to the 10–90% decay phase were determined for the average mIPSPs obtained for each cell.

**Recording and analysis of IPSPs evoked by focal extracellular stimulation**

Monosynaptic IPSPs were elicited by focal extracellular stimulation applied using theta-glass pipettes (tip diameter: 2–3 \( \mu \)m) filled with freshly oxygenated extracellular solution. Chlorinated silver wires placed inside each compartment of the theta glass were connected to a stimulus isolation unit (Model A350D-A, World Precision Instruments, Sarasota, FL) to apply bipolar stimulation. Stimulation electrodes were placed, to activate proximal inputs, about 50–100 \( \mu \)m lateral to the soma of the recorded neurons or, to activate distal inputs, near the border between layers 1 and 2 (see Fig. 1A). Applying stimuli of 100 \( \mu \)s duration at a baseline frequency of 0.1 Hz, the current intensity (20–100 \( \mu \)A) and electrode position were adjusted to elicit IPSPs of the smallest possible amplitude without failures. Typically, eliciting IPSPs with distal stimulation required higher stimulation currents and produced smaller IPSP amplitudes (see...
results). Distal stimulation produced IPSPs with significantly slower 10–90% rise time (Fig. 1, B and C). In many experiments, IPSPs could be elicited with proximal stimulation, but distal stimulation failed to elicit an IPSP in the same neuron. In some individual experiments, IPSPs elicited by distal stimulation had a fast rise time, similar to that of IPSPs evoked in the same neuron by proximal stimulation. When all experiments that yielded both distal and proximal IPSPs were considered, independent of the individual IPSP rise times, statistical analysis demonstrated that distal stimulation was much more likely to elicit slow rising IPSPs (Fig. 1C). As illustrated in Fig. 1A, the distal dendritic tree of the recorded pyramidal neurons was typically well-preserved, displaying several branches intact in layer 1. We previously reported similar findings when studying dendritic spine density in the layer 1 portion of apical dendrites of recorded layer 3 pyramidal cells from monkey DLPFC (Gonzalez-Burgos et al., 2008b). Studies from others also showed that in slices from monkey DLPFC, distal dendrites are also well preserved for many of the recorded layer 5 pyramidal neurons, which have significantly longer apical dendrites (Chang and Luebke, 2007). Together these morphological findings indicate that a significant fraction of the distal apical dendrites is usually preserved in each neuron, providing a substrate for the activation of distal GABA synapses by distal stimulation. For analysis of the effects of the GAT1 inhibitor NO711, data were included only if both proximal and distal stimulation produced IPSPs in the same neuron and only if the distal IPSPs had a 10–90% rise time of $\geq 4.5$ ms (approximately the mean of proximal IPSP rise time plus 2 SD). Unless specified otherwise, IPSPs were recorded at a somatic membrane potential of $-70$ to $-75$ mV, which was either the cells' resting membrane potential or was adjusted by current injection. IPSPs were recorded for $\geq 10$ min in control conditions, before applying NO711 (20 $\mu$M) for 5 min and followed by $\geq 10$ min of drug washout (which typically produced only a very small reversal of the effect, see Fig. 2F).

To determine the effects of NO711 on IPSPs, we averaged the last 20 consecutive control traces recorded before NO711 application and the last 20 consecutive traces before onset of NO711 washout. The average IPSPs were used to measure the peak amplitude and the decay in control and NO711 conditions. The changes in speed of IPSP decay induced by NO711 were measured fitting a single-exponential decay function. Although the decay kinetics of GABA$_A$R-mediated currents and IPSCs recorded in voltage-clamp mode is typically best fit with double-exponential decay functions, in current-clamp experiments, the IPSP decay is shaped by the cells' membrane time constant. Consequently, we found that in most neurons the IPSP decay was well fit by a single-exponential function and that double-exponential decay functions did not improve the fit. The goal of this study was to determine if GAT1 block-induced spillover increases IPSP duration as opposed to examining if there are changes in complex kinetics of IPSC decay. Therefore the NO711 effects on the decay of single IPSPs were estimated by comparing single-exponential decay time constant. To determine the effects of NO711 during repetitive stimulation,
trains of five stimuli at 20 Hz were applied every 10 s. The time course of the decay of the membrane potential at the end of the IPSP trains could not be well fit by single- or multiple-exponential decay functions. Therefore the effects of NO711 were determined measuring the difference, here named ΔVm, between the membrane potential 10 ms before and 300 ms after the onset of IPSP trains. For IPSPs evoked by perisomatic stimulation in control conditions, ΔVm fluctuated around zero, indicating that the membrane potential after perisomatic IPSP trains typically decayed to pretrain values by 300 ms posttrain onset. When dendritic IPSP trains displayed a posttrain hyperpolarizing potential, it typically peaked later than 300 ms posttrain onset, and therefore ΔVm was measured at the peak negative value posttrain, irrespective of time point. For the hyperpolarizing potential observed following single IPSPs, ΔVm was calculated similarly (peak hyperpolarizing Vm post-IPSP, minus Vm at 10 ms pre-IPSP).

**Figure 1. Stimulation of proximal and distal GABA synaptic inputs onto layer 3 pyramidal neurons.** A: reconstruction of 1 of the cells recorded in this study showing the typical location of the stimulation electrodes. Distal stimulation was applied in near the layers 1/2 border. Proximal stimulation was applied 50–100 μm lateral to the soma of the recorded neuron. The pyramidal cell was reconstructed using Neurolucida (Microbrightfield, Williston VT), after staining to visualize the biocytin label was done as described previously (Gonzalez-Burgos 2008). B: example average sweeps showing the differences in rising phase of IPSPs evoked in the same neuron by proximal vs. distal stimulation. C: summary graphs showing the statistically significant differences between the 10–90% rise time of IPSPs evoked by proximal and distal stimulation (rise time proximal IPSPs, 2.43 ± 0.21 ms, n = 30; distal IPSPs, 7.50 ± 0.87 ms, n = 24; independent samples t-test, t = 6.237, P < 0.00001).
Pharmacological compounds

Fast glutamate transmission was blocked with continuous bath application of 100 μM of d,l-AP5 and 20 μM CNQX, to block, respectively, NMDA and AMPA receptors. To block voltage-dependent sodium channels during mIPSP recordings we used tetrodotoxin (1 μM). To block GABA<sub>A</sub>R-mediated transmission, bicuculline methiodide or gabazine (20 μM) was added to the extracellular solution. To block GAT1-mediated GABA transport, we used NO711. NO711, also named NNC 711, is a partially lipophylic compound but has good solubility in water (up to ≥10 mM). GABA<sub>B</sub> receptors (GABA<sub>B</sub>Rs), were blocked with CGP35348. Zolpidem, was dissolved in dimethyl sulfoxide at 5 mM and then diluted to a final concentration of 1 μM. Dimethyl sulfoxide at its final concentration (0.002% vol/vol) did not produce any effect on the mIPSPs (data not shown). Zolpidem, CGP35348, and NO711 were obtained from Tocris Bioscience (Ellisville, MO). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Statistical data analysis

Data are expressed as means ± SE unless indicated otherwise. The statistical significance of the difference between group means was assessed using independent samples t-test, paired-samples t-test or two-way ANOVA, as indicated in each case. Pearson's χ² test was employed to test differences in the proportion of NO711-sensitive versus NO711-insensitive IPSPs. Differences were considered significant when the P value for the statistical parameters was <0.05.

RESULTS

GAT1 block prolongs the IPSPs elicited by stimulation of perisomatic-targeting inputs

Previous studies showed that when multiple synapses are stimulated, GAT1 blockade produces intersynaptic GABA spillover if the stimulated synapses are sufficiently close (Overstreet and Westbrook, 2003). Because the density of GABA synapses is higher near the soma compared with more distal dendrites of single pyramidal neurons, the effects of GABA spillover may be larger for perisomatic-targeting versus dendrite-targeting inputs. To examine whether GAT1 blockade differentially affects perisomatic versus dendritic IPSPs, monosynaptic IPSPs were evoked by focal extracellular stimulation of proximal or distal inputs (Fig. 1A).

IPSPs evoked by proximal stimulation, or perisomatic IPSPs (psIPSPs), had rise times and decay kinetics very similar to those of unitary IPSPs elicited in monkey DLPFC pyramidal cells by perisomatic synapses from presynaptic fast-spiking basket cells and chandelier neurons (Gonzalez-Burgos et al., 2005a). Whereas in our previous study we used near physiological chloride ion
concentrations in the pipette solution (Gonzalez-Burgos et al., 2005a), here the GABA_A-R-mediated IPSPs (GABA_A-R-IPSPs) were made depolarizing (EGABA_A = 0 mV) to improve the detection of small events. The similarities in GABA_A-R-IPSP kinetics therefore suggest that the depolarizing GABA_A-R-IPSPs evoked in this study, possibly due to their small size, did not cause more or less activation or inactivation of voltage-gated conductances than GABA_A-R-IPSPs recorded in physiological intracellular chloride conditions.

Compared with psIPSPs, IPSPs evoked by stimulation near the distal apical dendrite, or dendritic IPSPs (dIPSPs), had a significantly slower rise time (Fig. 1B). The IPSP rise time is a good indicator of distal versus proximal synapse location because it is determined by the degree of distance-dependent attenuation by filtering of IPSPs during propagation to the soma (Williams and Stuart, 2003; Pouille and Scanziani, 2004). Distal stimulation was much more likely to stimulate distal inputs, as indicated by the highly significant difference between the rise times of distally and proximally evoked IPSPs (Fig. 1C).

We next determined the effect of blocking GAT1-mediated uptake on psIPSPs elicited by proximal stimulation (Fig. 2A) at low frequency (0.1 Hz). We found that GABA transport block with 20 μM NO711 produced a significant prolongation of the psIPSPs (Fig. 2B). The magnitude of IPSP prolongation was determined by fitting an exponential decay function to the psIPSP decay (see methods) and comparing the decay time constant in control versus NO711 conditions. A pair-wise comparison revealed that NO711 increased by 57% the psIPSP decay time constant (Fig. 2C; paired samples t-test: t = 3.44, n = 45, P < 0.001). These results support the idea that when multiple perisomatic synapses are stimulated, GAT1 reduces spillover currents that otherwise significantly prolong the psIPSP duration. In addition to the change in IPSP duration, NO711 produced a small (22%, Fig. 2B) but statistically significant decrease in the psIPSP amplitude (control psIPSP amplitude: 5.01 ± 0.44 mV; NO711 psIPSP amplitude: 3.89 ± 0.43 mV, n = 45; paired samples t-test: t = 3.35, P < 0.002).

In most neurons (33 of 45, 74%), NO711 increased the psIPSP decay time constant (NO711-sensitive psIPSPs). In contrast, in a fraction of cells (12 of 45, 26%), NO711 application failed to increase the psIPSP decay by >5% (NO711-insensitive psIPSPs), as illustrated in Fig. 2C (top). NO711-insensitive psIPSPs may reflect a low probability of spillover due to stimulation of distant synapses (Overstreet and Westbrook, 2003). Alternatively, NO711-sensitive psIPSPs could have been due to stimulation of a much larger number of inputs (Isaacson et al., 1993). However, before NO711 application, the peak amplitudes were small and were not different between NO711-sensitive (4.83 ± 0.49 mV, n = 33) and NO711-insensitive (5.57 ± 0.96 mV, n = 12) psIPSPs (independent samples t-test: t = 0.731, P = 0.468), suggesting that similarly small numbers of inputs were stimulated to elicit NO711-sensitive or -insensitive psIPSPs.
Figure 2. Effects of GAT1 blockade with NO711 on IPSPs evoked by stimulation of perisomatic-targeting inputs (psIPSPs). A: scheme showing the arrangement of the recording and stimulation electrodes. B: example experiment illustrating that NO711 application (20 \(\mu\)M) prolonged the decay and slightly decreased the amplitude of psIPSPs. Top: consecutive traces superimposed. Bottom: averages of traces recorded in control and NO711 conditions. Inset: the average traces normalized to the same peak amplitude and superimposed. C: summary graphs showing the statistically significant effects of NO711 (* = \(P < 0.05\)) on the decay time of psIPSPs. Shown at the time constants of single-exponential decay functions fit to the decay of the psIPSPs recorded from each neuron in control conditions and in the presence of NO711. D: example traces illustrating the effects of NO711 on the time course of psIPSP trains evoked by stimulation with input trains (5 stimuli at 20 Hz). Top: consecutive traces superimposed. Bottom: averages of traces recorded in control and NO711 conditions. E: summary graphs showing the statistically significant effects of NO711 (*, \(P < 0.05\)) on the psIPSP trains, measured as the difference \(\Delta V_m\) between the membrane potential 10 ms before and 300 ms after the onset of the stimulus train (see Fig. 2D). F: example of the time course of NO711 effects on psIPSPs, measured through the \(\Delta V_m\) values.

Further analysis of the psIPSP decay revealed that the decay time constant of psIPSPs recorded before NO711 application was not significantly different between NO711-sensitive psIPSPs (34.0 ± 1.7 ms, \(n = 33\)) and NO711-insensitive psIPSPs (31.2 ± 2.8 ms, \(n = 12\); independent samples t-test: \(t = 0.858, P = 0.395\)). One possibility is that NO711-insensitive psIPSPs reflect cases in which spillover is absent or very small, for instance because the
GAT1 prevents spillover at GABAergic synapses

...stimulated synapses are far apart. If this interpretation is correct, then the similar decay of NO711-sensitive and -insensitive psIPSPs recorded in control conditions suggests that GAT1 activity effectively prevents the effects of GABA spillover on psIPSP decay time.

GAT1-mediated uptake may be especially important during repetitive synaptic activity, because a higher release rate may increase the accumulation of GABA in the extracellular space compartment. We therefore examined the effects of NO711 during repetitive activation of proximal inputs to determine whether GAT1 regulates the time course of psIPSP trains. Every 10 s we applied trains of five stimuli at 20 Hz, a frequency that is within the range of firing rates of task-related activity of interneurons recorded in vivo from the neocortex of monkeys performing behavioral tasks (Constantinidis and Goldman-Rakic, 2002; Wang et al., 2004; Mitchell et al., 2007). NO711 application (20 μM) prolonged the duration of each psIPSP in the train as well as the decay of the membrane potential at the end of the stimulus train (Fig. 2D). Exponential decay functions did not accurately fit the decay of individual psIPSPs or the posttrain potential (not shown). Therefore the effect of NO711 on psIPSP trains was estimated through the difference ΔVm between the pre- and posttrain membrane potential (Fig. 2, D and E). In control conditions, the membrane potential decayed back to its pretrain value by 300 ms posttrain (ΔVm control: 0.034 ± 0.035 mV). In contrast, in the presence of NO711, the neurons’ posttrain membrane potential remained significantly depolarized (ΔVm NO711: 1.130 ± 0.183 mV; paired samples t-test: t = 5.79, n = 42, P < 0.0001). Although the effects of NO711 were typically visible shortly after the onset of bath application, reversal of the effect by washout was very slow (Fig. 2F), possibly due to the partially lipophylic nature of the compound (Borden, 1996).

In many experiments with perisomatic stimulation, the effects of NO711 were tested both on single psIPSPs elicited at low stimulation frequency (0.1 Hz) and on psIPSP trains (20 Hz). This made it possible to compare the effects of NO711 on psIPSP trains, when stimulating inputs that produced NO711-sensitive versus NO711-insensitive single psIPSPs. If NO711-insensitive psIPSPs are due to stimulation of synapses lacking GAT1 transporters or expressing other GABA transporters, such as GAT3 (Keros and Hablitz, 2005), then the psIPSP trains evoked by stimulation of the same inputs must also be NO711-insensitive. In contrast to this prediction, NO711 significantly prolonged the psIPSP trains in experiments in which single psIPSPs were NO711-insensitive (ΔVm control: −0.092 ± 0.100 mV; ΔVm NO711: 0.768 ± 0.434 mV; paired samples t-test: t = 1.833, n = 11, P < 0.05), although less so than in cases with NO711-sensitive single psIPSPs (ΔVm control: 0.045 ± 0.043 mV; ΔVm NO711: 1.365 ± 0.193 mV; paired samples t-test: t = 6.695, n = 29, P < 0.0001). By showing that NO711-insensitive inputs become NO711-sensitive in an activity-dependent manner, these results argue against the possibility that NO711-insensitive psIPSPs result from stimulating synapses lacking GAT1 transporters. These data suggest that in certain conditions the propensity for GABA spillover is very small or absent during
low-frequency stimulation but becomes significant when the same group of synapses is activated repetitively. We found that NO711 produced a small but significant decrease in the cells' membrane time constant which slightly accelerated the mIPSP decay (see Fig. 1, B and C). Thus we cannot exclude the possibility that in some cases the IPSPs appeared to be NO711-insensitive because GAT1 block produced a very small IPSP prolongation that was obscured by the simultaneous decrease in membrane time constant.

Previous studies showed significant developmental changes through adolescence in GAT1 levels at some perisomatic synapses in monkey DLPFC (Erickson and Lewis, 2002; Cruz et al., 2003). Because some of the present experiments were performed in slices from prepubertal monkeys (see methods), we determined whether the effects of GAT1 blockade on the psIPSPs were age-dependent. We found that blocking NO711 prolonged the psIPSPs in both age groups (prepubertal, psIPSP decay control: 34.3 ± 1.9 ms and psIPSP decay NO711: 55.9 ± 6.3 ms, n = 25; postpubertal, psIPSP decay control: 31.9 ± 2.3 ms and psIPSP decay NO711: 47.3 ± 8.9 ms, n = 20). Two-factor ANOVA revealed a significant effect of NO711 [F(1,43) = 14.2, P < 0.0005], no effect of age [F(1,43) = 0.817, P = 0.371], and no significant interaction between age and NO711 effect [F(1,43) = 0.408, P = 0.526]. In addition, we found that NO711 had significant effects on the psIPSP trains in neurons from both pre- and postpubertal animals (prepubertal, ΔVm control: −0.039 ± 0.049 mV and ΔVm NO711: 0.997 ± 0.235 mV, n = 28; postpubertal, ΔVm control: 0.071 ± 0.047 mV and ΔVm NO711: 1.385 ± 0.269 mV, n = 21). As with single psIPSPs, the effect of NO711 application was significant [F(1,43) = 13.6, P < 0.001], and we found no significant effect of age [F(1,43) = 0.0093, P = 0.924] and no significant age × NO711 effect interaction [F(1,43) = 0.0383, P = 0.844]. These data show that the effects on psIPSPs of GAT1-controlled spillover do not change significantly with age.

**GAT1 block does not enhance miniature GABA transmission**

If GAT1-mediated uptake limits the amount of GABA available for synaptic receptor activation, then NO711 application should enhance miniature synaptic events (increase their size and/or duration). However, in rodent hippocampus and neocortex, GAT1 blockade does not affect the amplitude or duration of mIPSCs, suggesting that GAT1-mediated uptake does not affect transmission at single GABA synapses. To determine whether single-synapse GABA transmission is also independent of GAT1 activity in primate neocortical circuits, we studied miniature GABA transmission onto layer 3 pyramidal neurons of monkey DLPFC. mIPSPs were recorded first in control conditions and then in the presence of the GAT1-selective transport inhibitor NO711 (20 μM). This NO711 concentration is saturating for its effects on GAT1 (Borden, 1996) but does not induce the GABA AR desensitization seen with higher (i.e., 100 μM) NO711 concentrations (Overstreet et al., 2000; Overstreet and Westbrook, 2003). The depolarizing mIPSPs recorded at potentials between −75
GAT1 prevents spillover at GABAergic synapses and $-70 \text{ mV}$ using a high-chloride pipette solution ($E_{\text{GABA}_A} = 0 \text{ mV}$, see methods) were completely abolished by GABA$_A$R antagonists (Fig. 3A).

**Figure 3.** Effects on miniature inhibitory postsynaptic potentials (mIPSPs) of applying the GABA transporter 1 (GAT1) blocker 1,2,5,6-tetrahydro-1-[[diphenylmethylene]amino]oxy] ethyl]-3-pyridinecarboxylic acid hydrochloride (NO711) or the benzodiazepine site agonist N,N,N,N,6-trimethyl-2-(4-methylphenyl)imidazo[1,2-a]pyridine-3-acetamide (zolpidem). A: depolarizing mIPSPs recorded from layer 3 pyramidal neurons in control conditions (high chloride pipette solution), were completely abolished by applying the GABA$_A$ receptor (GABA$_A$R) antagonist gabazine (10 $\mu$M). B: examples of average mIPSPs recorded in control conditions and following application of the GAT1 blocker NO711 (20 $\mu$M) show that NO711 did not significantly change the average mIPSP shape. C: summary graphs showing the absence of effects of NO711 on mIPSP amplitude (paired samples $t$-test $t = 0.500, P = 0.627, n = 14$) and a slight but significant acceleration of the mIPSP decay time constant (paired samples $t$-test, $t = 3.046, P = 0.01, n = 14$), which was correlated with a small NO711-induced acceleration of the cells' membrane time constant (see results). D: examples of average mIPSPs recorded in control conditions and following application of the benzodiazepine site agonist zolpidem (1 $\mu$M) show that zolpidem increased the mIPSP amplitude and prolonged mIPSP duration, as illustrated by the normalized traces in the inset. E: summary graphs showing the significant effects of zolpidem on the mIPSP amplitude and duration.
amplitude (paired samples \( t \)-test, \( t = 3.554, P < 0.02, n = 8 \)) and the mIPSP decay time constant (paired samples \( t \)-test, \( t = 2.849, P < 0.05, n = 8 \)).

We determined the effects of GAT1 blockade on miniature transmission by obtaining average mIPSPs for each neuron in control and NO711 conditions (Fig. 3, B and C). A paired-samples \( t \)-test analysis revealed that NO711 application failed to significantly alter the amplitude or to prolong the duration of the average mIPSPs (Fig. 3, B and C). However, NO711 slightly (12%), but significantly accelerated the average mIPSP decay (paired samples \( t \)-test, \( t = 3.046, P = 0.01, n = 14 \)). An acceleration of IPSP decay is contrary to the idea that GAT1 activity shortens the IPSP duration but is consistent with the possibility that NO711 decreases the cells' membrane time constant by enhancing a tonic GABA current after GAT1 block increases ambient GABA levels (Farrant and Nusser, 2005). Indeed in several neurons, NO711 application produced a small depolarizing shift in the membrane potential (data not shown), consistent with the enhancement of a tonic GABA current, which in our experimental conditions should be depolarizing. This NO711-induced depolarization was not further studied and, when present, was compensated by current injection. We found that NO711 produced a small (19%) but significant acceleration of the membrane time constant in the same neurons (membrane time constant control: \( 21.3 \pm 1.2 \) ms, membrane time constant NO711: \( 17.4 \pm 0.9 \) ms, paired samples \( t \)-test, \( t = 3.70, P = 0.003, n = 14 \)). Moreover, the acceleration of mIPSP decay by NO711 was strongly correlated with the acceleration of the membrane time constant in the same neurons (Pearson's correlation coefficient \( r = 0.67001, P = 0.008, n = 14 \)) as expected if the mIPSP decay acceleration by NO711 was due to a decrease in the membrane time constant.

The absence of mIPSP enhancement by NO711 may be explained if the GABA concentration transient in the synaptic cleft saturates the synaptic GABA\(_{A}\)Rs (Edwards, 2007). GAT1 transporters have high affinity for GABA (Borden, 1996) and transport GABA at a slow rate (Mager et al., 1996; Bicho and Grewer, 2005). Therefore if the synaptic cleft GABA concentration transient saturates the GABA\(_{A}\)Rs, it should largely saturate the GABA uptake capacity, making GAT1 block irrelevant. Whether the cleft GABA transient produces GABA\(_{A}\)R saturation appears to be cell type- and synapse-specific (Hajos et al., 2000; Mozrzymas, 2004; Szabadics et al., 2007). Therefore we determined the effects, on mIPSPs, of zolpidem, a compound that like other benzodiazepine site ligands increases the affinity of the GABA\(_{A}\)Rs for GABA (Lavoie and Twyman, 1996; Mozrzymas, 2004) and does not enhance transmission if there is GABA\(_{A}\)R saturation (Perrais and Ropert, 1999; Hajos et al., 2000; Szabadics et al., 2007). As shown in Fig. 3, D and E, in monkey DLPFC layer 3 pyramidal neurons, zolpidem (1 \( \mu \)M) significantly increased both the amplitude and duration of mIPSPs (paired-samples \( t \)-test, mIPSP amplitude: \( t = 3.55, P < 0.01, n = 8 \) and mIPSP decay time constant: \( t = 2.85, P < 0.05, n = 8 \)), a finding consistent with sub-saturating concentrations of synaptic cleft GABA. These results favor the conclusion that during single-
synapse transmission, GAT1-mediated transport does not restrict the amount of neurotransmitter available to activate GABA$_A$Rs even if the cleft GABA transient is sub-saturating.

**Complex regulation by GAT1-mediated uptake of IPSPs elicited by stimulation of dendrite-targeting inputs**

To examine the effects of GAT1-mediated uptake on dendritic IPSPs (dIPSPs), we applied NO711 during activation of dendrite-targeting inputs with focal stimulation of axons near the distal apical dendrite of the layer 3 pyramidal cells (Fig. 4A). Similar to psIPSPs, NO711 application slightly but significantly reduced the dIPSP amplitude (Fig. 4B) by 24.4% (control dIPSP amplitude: 2.17 ± 0.26 mV; NO711 dIPSP amplitude: 1.64 ± 0.25 mV, n = 32; paired samples t-test: t = 2.64, P < 0.02). However, in contrast to psIPSPs, GAT1 blockade did not significantly increase the dIPSP decay time (Fig. 4, B and C), as revealed by analysis of the exponential decay time constant (paired samples t-test: t = 1.12, P = 0.269, n = 32). NO711 application prolonged the dIPSP decay in 15 of 32 experiments but failed to increase the dIPSP decay time constant by >5% in 17 of 32 experiments (Fig. 4C). Interestingly, the proportion of NO711-insensitive IPSPs was significantly larger for dIPSPs than for psIPSPs (dIPSPs: 53%, 17 of 32; psIPSPs: 26%, 12 of 45; Pearson’s $\chi^2$ test, P < 0.02). Although these data favor the conclusion that GABA spillover is less significant for dendritic than perisomatic IPSPs, the results of experiments with repetitive stimulation of distal GABA inputs revealed further complexity, as described in the following text.

Repetitive activity of dendrite-targeting inputs in control conditions induced, in many experiments, a hyperpolarizing potential after the end of the depolarizing dIPSP trains (Fig. 4D). Such hyperpolarization was not observed after the end of psIPSP trains in which the membrane potential decayed back to pretrain values at about 300 ms posttrain onset (Fig. 2, D and E). NO711 application strongly increased the hyperpolarizing potential post dIPSP trains in 14 of 30 experiments, thus shortening the depolarization produced by dIPSP trains (Fig. 4D). However, the effects of NO711 were heterogeneous (Fig. 4E) and, consequently, the overall effect of NO711 on dIPSP trains was not statistically significant ($\Delta V_m$ control: $-0.285 \pm 0.098$ mV; $\Delta V_m$ NO711: 0.318 ± 0.352; paired samples t-test: $t = 1.816$; $P = 0.08$, n = 30), in contrast to the significant prolongation of the posttrain depolarization in psIPSP trains (Fig. 2, D and E). These results suggest that the differences in the effects of GAT1 block on psIPSPs versus dIPSPs may be due, at least in part, to the presence of the hyperpolarizing potential in the latter. Therefore as described next, we characterized the mechanisms underlying the hyperpolarizing potential, to isolate it from the depolarizing GABA$_A$R-IPSPs, and to compare the effects of GAT1 blockade on GABA$_A$R-IPSPs elicited by stimulating dendritic- versus perisomatic-targeting inputs.
Figure 4. Effects of GAT1 blockade with NO711 on IPSPs evoked by stimulation of dendrite-targeting inputs (dIPSPs). A: scheme showing the arrangement of the recording and stimulation electrodes. B: example experiment illustrating that NO711 application (20 μM) did not change the decay but slightly decreased the amplitude of dIPSPs. Top: consecutive traces superimposed. Bottom: averages of traces recorded in control and NO711 conditions. C: summary graphs showing the absence of statistically significant effects of NO711 on the decay time of dIPSPs. Shown at the time constants of single exponential decay functions fit to the decay of the dIPSPs recorded from each neuron in control conditions and in the presence of NO711. D: example traces illustrating the effects of NO711 on the time course of dIPSP trains evoked by stimulation with input trains (5 stimuli at 20 Hz). Top: consecutive traces superimposed. Bottom: averages of traces recorded in control and NO711 conditions. E: summary graphs showing the absence of statistically significant effects of NO711 on the dIPSP trains, measured as the difference $\Delta V_m$ between the membrane potential 10 ms before and at the peak hyperpolarization after the onset of the stimulus train. Note that, typically, the hyperpolarization following dIPSP trains peaked later than 300 ms posttrain onset (see Fig. 4D).

**Stimulation of dendrite-targeting inputs produces a GABA$_B$R-mediated potential that is enhanced by GAT1 block**

Throughout these experiments, the cells' somatic membrane potential was typically maintained at $-70$ to $-75$ mV, a range of values significantly more positive than the estimated K+ reversal potential ($E_{K+} = -100$ mV, see methods). Because GABA$_B$Rs produce hyperpolarizing IPSPs by activation of K+ channels (Lüscher et al., 1997), we hypothesized that a K+ current activated
GAT1 prevents spillover at GABAergic synapses

by postsynaptic GABA_BRs could mediate the hyperpolarizing potential observed during stimulation of dendrite-targeting inputs. This hypotheses was tested first in experiments in which the posttrain hyperpolarizing potential was recorded in control conditions and then the GABA_B antagonist CGP35348 (50 μM) was applied. We found that GABA_B blockade abolished the hyperpolarizing potential (Fig. 5A), as revealed by a paired t-test comparison (control: $-0.42 \pm 0.11$ mV; CGP35348: $0.03 \pm 0.10$ mV; n = 4, t = 2.865, P < 0.05). If a K+ current is involved in generating this potential, then increasing the K+ current driving force by membrane depolarization should enhance the hyperpolarizing potential amplitude. We found that the amplitude of the hyperpolarizing potential postdIPSP trains was significantly increased by membrane potential depolarization (Fig. 5B), consistent with its mediation by a K+ current. In contrast, when dendrite-targeting inputs were stimulated in the presence of CGP35348 (50 μM), no significant posttrain hyperpolarizing potential was observed at hyperpolarized potentials, nor after increasing the K+ current driving force with depolarization (Fig. 5B). The pharmacological and biophysical properties of the hyperpolarizing potential are therefore consistent with the idea that stimulation of dendrite-targeting inputs produced a GABA_A-IPSP and a K+ current- and GABAB-R mediated IPSP (GABAB-R-IPSP).

Although the GABA_B-IPSP was detectable mostly with repetitive stimulation of dendrite-targeting inputs, in some experiments (11 of 30), it was also observed during application of a single stimulus, following the decay of the GABA_A-dIPSP (Fig. 5C1). The presence of a GABA_B-IPSP was not associated with weaker or stronger stimulation of dendrite-targeting inputs because the amplitude of the GABA_A-IPSP did not differ between responses with and without detectable GABA_B-IPSP (GABA_A-IPSP without GABA_B-IPSP: $2.05 \pm 0.33$ mV, n = 19; GABA_A-IPSP with GABA_B-IPSP: $1.99 \pm 0.43$ mV, n = 11; independent samples t-test, t = 0.102, P = 0.919). Consistent with a postsynaptic GABA_B-mediated response, the hyperpolarizing potential elicited by a single stimulus was long-lasting (with duration of about 0.5 and $\leq$1.0 s). The GABA_B-IPSP elicited by a single stimulus was typically smaller than that observed after the end of stimulus trains (Fig. 5C2). Because the GABA_B-IPSP was long-lasting, part of the posttrain hyperpolarization was due to the GABA_B-IPSP elicited by the first stimulus in the train (Fig. 5C2). To separate the contribution, to the posttrain GABA_B-IPSP, of the first stimulus relative to subsequent stimuli in the trains, we subtracted from the dIPSP trains, traces with a single dIPSP recorded from the same neuron. Subtraction analysis showed that about 80% of the posttrain GABA_B-IPSP was elicited by GABA released by stimuli after the first in the train (Fig. 5C2). The GABA_B-IPSP elicited by stimulation of dendrite-targeting inputs was typically increased by NO711 application (Fig. 5C3). These results suggest that activation of postsynaptic GABA_BRs probably was facilitated, or did not depress, by repetitive stimulation of dendrite-targeting inputs and by GAT1 blockade. GABA spillover may affect GABA_A-IPSPs in a substantially different manner than GABA_A-IPSPs because GABA_BRs bind
GABA with much higher affinity than GABA_ARs. Indeed many of the GABA_BRs in dendrites appear to be extrasynaptic (see discussion), suggesting that escape of GABA from the synaptic cleft followed by GAT1-controlled diffusion may represent a significant physiological source of GABA_BR activation (Scanziani, 2000).

Figure 5. Stimulation of dendrite-targeting inputs elicits a GABA_BR-dIPSP. A: example traces showing that the GABA_BR antagonist 3-aminopropyl)(diethoxymethyl)phosphinic acid (CGP35348, 50 μM) markedly reduced the hyperpolarizing potential observed during the decay of dIPSP trains. B: in control conditions, the hyperpolarizing potential amplitude significantly increased (*, P < 0.05) following membrane depolarization (from -79.2 ± 3.3 to -61.7 ± 3.1 mV). In contrast, in the presence of CGP35348, membrane depolarization (from -77.8 ± 2.3 to -65.4 ± 1.5 mV) did not significantly change the posttrain potential. C1: example trace showing that in some experiments, single shock stimulation elicited GABA_BR-dIPSPs. C2: subtraction analysis demonstrated that typically the GABA_BR-dIPSP produced by stimulus trains was larger than that produced by single stimuli. C3: summary graphs showing the peak hyperpolarizing GABA_BR-dIPSP amplitude produced by single stimuli (IPSP1), by trains of 5 stimuli (train) and after subtraction from the trains of the GABA_BR-IPSP produced by a single stimulus (train –IPSP1). Two-way ANOVA indicated that there was a significant effect of stimulus \(F(2,60) = 4.236, P < 0.02\). Bonferroni and Scheffe post hoc tests showed that the GABA_BR-dIPSP produced by trains was larger than with a single
stimulus and that the GABA\textsubscript{B}R-dIPSPs produced by a train or after subtracting the contribution of the first stimulus were not significantly different. In addition, ANOVA analysis indicated that the increase in the mean hyperpolarization post-dIPSP and post-dlIPSP trains by NO711 did not reach significance $[F(1,60) = 3.096, P = 0.083]$. However, post hoc tests showed a significant effect of NO711 for IPS1 and IPS3 trains ($P < 0.05$).

**D:** representative experiment showing that shortly after application of the GABA\textsubscript{A}R antagonist gabazine (10 $\mu$M), suppression of the GABA\textsubscript{A}R-dIPSPs revealed a hyperpolarizing GABA\textsubscript{B}R-dIPSP component. **E:** plotting the $\Delta V_m$ values measured in control and NO711 conditions revealed a significant correlation for dIPSPs ($r = 0.4289$, $P < 0.02$, $n = 30$) and an absence of correlation for psIPSPs ($r = 0.1109$, $P = 0.443$, $n = 50$).

Due to their different reversal potentials in our experimental conditions, the GABA\textsubscript{A}R- and GABA\textsubscript{B}R-mediated currents activated by stimulation of dendrite-targeting inputs should produce opposite effects on the decay of the pyramidal cell membrane potential at the end of dIPSP trains. Indeed blockade of GABA\textsubscript{B}Rs produced a depolarizing shift in the decay of membrane potential after the dIPSP trains (Fig. 5A) and application of GABA\textsubscript{A}R antagonists produced a hyperpolarizing shift (Fig. 5D). The opposing effects of the GABA\textsubscript{A} and GABA\textsubscript{B} currents suggest that the heterogeneity in the effects of NO711 on the decay of dIPSP trains (Fig. 4, D and E) could be due to variability in the relative amplitudes of the GABA\textsubscript{A}R- and GABA\textsubscript{B}R-IPSPs produced before NO711 application. Consistent with this interpretation, a significant correlation was found ($r = 0.4289$, $P < 0.02$, $n = 30$) between the dIPSP $\Delta V_m$ control and $\Delta V_m$ NO711 values (Fig. 5E). This correlation indicated that GAT1 block enhanced both the GABA\textsubscript{A}R- and GABA\textsubscript{B}R-dIPSPs, the increase in the GABA\textsubscript{B}R-mediated component predominating if its amplitude in control conditions was more negative than approximately $-0.5$ mV (Fig. 5E). In contrast to the dIPSPs, no correlation was found ($r = 0.1109$, $P = 0.443$, $n = 50$) between $\Delta V_m$ control and $\Delta V_m$ NO711 values for psIPSPs, and NO711 induced a depolarizing change in $\Delta V_m$ in most experiments (Fig. 5E). These results suggest that the presence of the GABA\textsubscript{B}R-IPSP with stimulation of dendrite-targeting inputs interfered with the assessment of the effects of GAT1 block on the dendritic GABA\textsubscript{A}R-IPSPs. Because the GABA\textsubscript{B}R-IPSP could be elicited by low-frequency stimulation (Fig. 5C1), it is likely that its presence precluded visualization of the NO711-induced prolongation of the GABA\textsubscript{A}R-IPSP, including in cases when a GABA\textsubscript{B}R-IPSP could not be readily detected or produced a very small hyperpolarization that could be enhanced by NO711 (Fig. 4B).

**GAT1 block prolongs the GABA\textsubscript{A}R-IPSPs elicited by stimulation of dendrite-targeting inputs**

If the lack of significant prolongation of dIPSPs by NO711 (Fig. 4, B–E) is indeed due to shunting or hyperpolarizing effects of the GABA\textsubscript{B}R-dIPSP, then the GABA\textsubscript{A}R-dIPSP should be consistently prolonged by applying NO711 after GABA\textsubscript{B}Rs are blocked. To test this prediction, we recorded dIPSPs in the presence of the GABA\textsubscript{B}R antagonist CGP35348 (Fig. 6A) and found that NO711 significantly prolonged the duration of the GABA\textsubscript{A}R-dIPSPs evoked at 0.1 Hz (Fig. 6B; GABA\textsubscript{A}R-dIPSP decay tau CGP35348: $52.7 \pm 7.5$ ms;
GABA<sub>AR</sub>-dIPSP decay tau CGP35348+NO711: 125.0 ± 31.3 ms; paired samples t-test, t = 2.466, P < 0.05, n = 16). As in the case of GABA<sub>AR</sub>-psIPSPs, in some experiments, the GABA<sub>AR</sub>-dIPSPs recorded in the presence of CGP35348 were NO711-insensitive. The proportion of NO711-insensitive GABA<sub>AR</sub>-dIPSPs (31.2%, 5 of 16) was not significantly different (Pearson's χ<sup>2</sup> test, P = 0.794) from the proportion of NO711-insensitive GABA<sub>AR</sub>-psIPSPs (26.7%, 12 of 45). If NO711-insensitive GABA<sub>AR</sub>-IPSPs reflect cases with low propensity for GABA spillover, these results suggest that the likelihood of GABA spillover is similar for psIPSPs and dIPSPs. The decay time constant of the GABA<sub>AR</sub>-dIPSPs before NO711 application was not different between NO711-sensitive (48.8 ± 7.45 ms, n = 11) and NO711-insensitive (61.3 ± 18.5 ms, n = 5; independent samples t-test: t = 0.759, P = 0.459) responses. These data suggest that, as for psIPSPs, GAT1 effectively prevents the effects of GABA spillover on dIPSP decay time.

dIPSPs evoked by repetitive stimulation after GABA<sub>B</sub>R blockade with CGP35348 did not display a posttrain hyperpolarizing potential (Fig. 6C). Furthermore, subsequent NO711 application strongly prolonged the decay of the membrane potential at the end of the depolarizing GABA<sub>AR</sub>-dIPSP train (Fig. 6C; ΔVm CGP35348: 0.283 ± 0.055 mV; ΔVm CGP35348+NO711: 2.400 ± 0.678; paired samples t-test: t = 3.333; P < 0.005, n = 16). These results indicate that when the shunting or hyperpolarizing effects of the GABA<sub>AR</sub>-IPSPs were pharmacologically blocked, the NO711 effect was similar for dIPSPs and psIPSPs. With GABA<sub>B</sub>Rs blocked, NO711 increased significantly the posttrain ΔVm for inputs that produced NO711-insensitive single dIPSPs (ΔVm CGP35348: 0.17 ± 0.14 mV; ΔVm CGP35348+NO711: 1.27 ± 0.27 mV, P < 0.05, n = 5), although this effect was smaller than for inputs producing NO711-sensitive single dIPSPs (ΔVm CGP35348: 0.86 ± 0.43 mV; ΔVm CGP35348+NO711: 4.48 ± 1.32 mV, P < 0.005, n = 11). Thus similar to psIPSPs, for dendritic-targeting inputs GABA spillover may be negligible during low frequency activity but may become significant during repetitive activation of the same set of inputs.

In the absence of GABA<sub>B</sub>R antagonists, NO711 decreased the amplitude of GABA<sub>AR</sub>-dIPSPs and GABA<sub>AR</sub>-psIPSPs (Figs. 3B and 4B). Interestingly, in the presence of CGP35348, the peak amplitude of the GABA<sub>AR</sub>-dIPSPs was not decreased by NO711 application (Fig. 6B; dIPSP amplitude CGP35348: 2.06 ± 0.44 mV; dIPSP amplitude CGP35348+NO711: 2.20 ± 0.49 mV; paired samples t-test: t = 0.8082, P = 0.438, n = 17). Similarly, in the presence of CGP35348 NO711 did not affect the GABA<sub>AR</sub>-psIPSP amplitude (psIPSP amplitude CGP35348: 3.72 ± 1.26 mV; psIPSP amplitude CGP35348+NO711: 4.65 ± 0.86 mV; paired samples t-test: t = 0.7631, P = 0.2501, n = 4), although in the same neurons NO711 significantly increased the posttrain ΔVm (ΔVm CGP35348: −0.082 ± 0.031 mV; ΔVm CGP35348+NO711: 2.801 ± 0.977 mV, 1-tail paired samples t-test: t = 2.9986, P < 0.05, n = 4). These data show that the decrease in GABA<sub>AR</sub>-IPSP amplitude by NO711 is GABA<sub>B</sub>R-dependent.
GAT1 prevents spillover at GABAergic synapses

Figure 6. Effects of GAT1 blockade on dIPSPs in the presence of the GABABR antagonist CGP35348. A: scheme showing the arrangement of the recording and stimulation electrodes. B: an example recording and summary graphs illustrating the prolongation of dIPSPs by NO711 (20 μM) in the presence of CGP35348 (50 μM). Shown are averages of 10 consecutive sweeps. The bar graphs display the time constants of single-exponential decay functions fit to the decay of the dIPSPs recorded from each neuron in the presence of CGP35348 and in the presence of CGP35348+NO711. C: an example recording and summary graphs illustrating the effects of NO711 (20 μM) on dIPSP trains recorded in the presence of CGP35348 (50 μM). The effect on dIPSP trains was measured as the difference ΔV_m between the membrane potential measured 10 ms pre-IPSP train and at 300 ms after the onset of the stimulus train.

One possibility is that the GABABR-dependent reduction in GABA_A-IPSP amplitude is mediated by presynaptic GABABRs that negatively control GABA release as shown in rat hippocampus (Buhl et al., 1995; Hefft et al., 2002; Neu et al., 2007; Price et al., 2008). GAT1 block may increase the transmitter available to activate such presynaptic GABABRs (Lei and McBain, 2003). Whereas in certain synapses, presynaptic GABABRs are tonically
activated by ambient GABA with uptake intact (Buhl et al., 1995; Lei and McBain, 2003; Price et al., 2008), in other synapses, such tonic presynaptic receptor activation is not observed (Neu et al., 2007). Here we found that blockade of GABA_BRs in the absence of NO711 did not affect the amplitude of dIPSPs (dIPSP amplitude control: 1.16 ± 0.43 mV; dIPSP amplitude CGP35348: 1.26 ± 0.35 mV, t = 0.657, P = 0.539, paired samples t-test, n = 6). These data suggest that in monkey DLPFC GABA_BR-mediated regulation of GABA_AR-IPSP amplitude, presumably via presynaptic mechanisms, is secondary to an increase in extracellular GABA levels by GAT1 block.

DISCUSSION

We determined the role of the GABA transporter GAT1 in regulating phasic GABA transmission in monkey DLPFC. We found that GAT1 block did not enhance miniature (single-synapse) GABA transmission but prolonged, most likely by increasing GABA spillover, IPSPs evoked by activating multiple synapses with extracellular axonal stimulation. Dendritic (but not perisomatic) stimulation produced a GABA_BR-IPSP that was enhanced by GAT1-mediated uptake. The GABA_AR-IPSPs evoked by stimulation of perisomatic and dendritic GABA synapses were similarly prolonged by GAT1 block. In some experiments with either perisomatic or dendritic stimulation, the IPSPs were NO711-insensitive, suggesting a low propensity for GABA spillover. Because the proportion of NO711-insensitive IPSPs was similar for perisomatic and dendritic synapses, we conclude that at least in primate cortical circuits the propensity for spillover is similar for inputs onto proximal and distal compartments of the pyramidal cell membrane. Whether a similar situation is found in rat neocortex is not clear because no studies examined the role of GAT1-mediated uptake at dendrite-targeting inputs onto neocortical pyramidal neurons in rat brain. Finally, we found that GAT1 blockade produced a reduction of the GABA_AR-IPSP amplitude that was abolished by application of a GABA_BR antagonist, which possibly blocked presynaptic GABA_BRs that negatively control GABA release.

Effects of GAT1 activity on miniature GABA transmission

Our results with mIPSP recordings argue against the idea that GABA uptake normally downregulates the IPSP amplitude or shortens the IPSP duration during GABA_AR-mediated transmission at isolated synapses in monkey DLPFC. These data are consistent with findings from rat hippocampus showing that blockade of GABA uptake does not increase the amplitude nor the duration of single-synapse mIPSCs (Thompson and Gahwiler, 1992; Isaacson et al., 1993; Overstreet and Westbrook, 2003). Furthermore, our findings are unlikely to represent incomplete GAT1 block because NO711, a potent inhibitor with high efficiency at human and rat GAT1 homologs (Borden, 1996), effectively blocks GAT1-mediated transport of endogenous GABA (Wu et al., 2007) we used a saturating NO711 concentration; and mIPSCs are also unchanged in
GAT1 knock-out mice (Jensen et al., 2003; Bragina et al., 2008). Indeed we found that NO711 application slightly accelerated the mIPSP decay as well as the cells' membrane time constant (see results). These effects were strongly correlated, as expected if GAT1 blockade enhanced a tonic membrane conductance by increasing the ambient GABA levels that activate extrasynaptic GABA_{A}Rs, as suggested elsewhere (Farrant and Nusser, 2005). Investigating the control by GAT1 of GABA_{A}R-mediated tonic currents was beyond the scope of the present study.

The absence of GAT1 effects on single-synapse transmission may be due to the predominantly extrasynaptic localization of this transporter (Vitellaro-Zuccarello et al., 2003). In addition, the kinetics of GABA transport by GAT1 is slow relative to the rapid kinetics of transmitter-receptor binding and channel gating (Bicho and Grewer, 2005), probably contributing to the absence of regulation of single-synapse transmission. It is also possible that the synapses mediating mIPSPs by action potential-independent spontaneous GABA release lack GAT1 and thus differ from those mediating action potential-evoked release. However, previous studies ruled out this possibility by showing that IPSCs elicited by action potential-evoked release from single synapses are similarly NO711-insensitive (Overstreet and Westbrook, 2003).

**GAT1-mediated regulation of perisomatic versus dendritic IPSPs**

In contrast to the absence of GAT1-mediated regulation of single-synapse transmission, blocking GAT1 typically prolonged GABA_{A}R-IPSPs evoked by focal extracellular stimulation. Because the axons of GABA neurons typically make multiple synaptic contacts onto individual pyramidal cells, action potential-evoked IPSPs result from multiple-synapse stimulation, suggesting that IPSP prolongation produced by GAT1 blockade is due to between-synapse GABA spillover. Consistent with this interpretation, in GAT1 knock-out mice, IPSCs evoked by axonal stimulation exhibit significant prolongation without an increase in amplitude (Bragina et al., 2008). That GABA spillover slows the IPSC decay but does not increase the IPSC amplitude may be explained by the fact that the IPSC amplitude is determined by within-synapse GABA diffusion, which activates GABA_{A}Rs much earlier than GABA diffusing between synapses (Barbour, 2001).

We found that in some experiments, GABA_{A}R-IPSPs evoked by extracellular axonal stimulation were NO711-insensitive, a finding also consistent with an absence of regulation of within-synapse GABA_{A}R activation by GAT1. Our data suggest that such NO711-insensitive GABA_{A}R-IPSPs were not due to stimulation of synapses lacking GAT1 because NO711 had significant effects when the probability of spillover was increased by repetitive stimulation of the same inputs. These findings may be explained if the NO711-insensitive IPSPs are mediated by distant synapses, and thus GABA spillover currents become significant only when, during repetitive stimulation, large amounts of GABA diffuse between synapses and can reach GABA_{A}Rs more distant from the transmitter release sites.
Although GABA synapse density in the neuropil is thought to be lower in primate than rodent neocortex (DeFelipe, 2002), our data show that in monkey DLPFC GABA synapse density appears to be sufficient to produce significant spillover after GAT1 block. In addition, we found a similar proportion of NO711-insensitive pIPSPs and dIPSPs, suggesting a similar probability of spillover onto perisomatic and dendritic synapses. These data suggest that GABA synapse density in the neuropil is a more significant determinant of the probability of spillover than synapse density in the dendritic versus somatic membrane of individual pyramidal cells. Interestingly, prior to GAT1 block, NO711-sensitive GABA$_A$R-IPSPs had similar duration than NO711-insensitive GABA$_A$R-IPSPs, suggesting that GAT1 activity effectively prevents spillover.

We found that blocking GAT1 produced some differential effects at perisomatic versus dendritic GABA synaptic inputs. Specifically, stimulation of distal (but not proximal) synapses elicited, after the GABA$_A$R-IPSP, a GABA$_B$R-IPSP that was enhanced by GAT1 block. That GABA$_B$R-IPSPs were preferentially evoked by distal synapse stimulation may be explained by the subcellular distribution of the GABA$_B$Rs and K$^+$ channels mediating the GABA$_B$R-IPSPs. Compared with the perisomatic compartment, distal pyramidal cell dendrites have higher density of GABA$_B$R subunits and Kir3.2 K$^+$ channels (López-Bendito et al., 2002b; Kulik et al., 2006). Kir3.2 channels and GABA$_B$R subunits are co-clustered in the extrasynaptic membrane near dendritic GABA synapses (Kulik et al., 2006). An extrasynaptic localization suggests that GABA$_B$R activation requires GABA diffusion over a distance from the release sites. Consistent with this possibility, we found that GABA$_B$R-IPSPs were larger during repetitive activity and were enhanced by GAT1 blockade.

In rat neocortex, most interneuron subtypes elicit exclusively GABA$_A$R-IPSPs. Similarly, we reported previously that in monkey DLPFC, perisomatic-targeting fast-spiking basket and chandelier neurons elicit in pyramidal cells IPSPs that are exclusively GABA$_A$R-mediated (Gonzalez-Burgos et al., 2005a). Cells in a third subtype of interneuron furnishing perisomatic inhibition, the nonfast-spiking basket neurons, signal exclusively through GABA$_A$Rs, suggesting that perisomatic inhibition postsynaptically is purely GABA$_A$R-mediated (Freund and Katona, 2007). In contrast, rat neurogliaform cells (NGFCs) elicit dual IPSPs mediated by both GABA$_A$Rs and GABA$_B$Rs (Tamas et al., 2003; Szabadi et al., 2007). In human neocortex, GABA$_B$R-IPSPs were described previously (McCormick, 1989) and recently identified to be mediated by NGFCs (Olah et al., 2007) as in the rat neocortex. Therefore the GABA$_B$R-dIPSPs recorded here may have been mediated by stimulating axons of NGFCs, which are present in monkey DLPFC (Povysheva, 2007; Zaitsev et al., 2009) and are known to target almost exclusively pyramidal cell dendritic spines and shafts (Vida et al., 1998; Tamas et al., 2003).

Some data, however, are not consistent with this interpretation. First, in rat neocortex, NGFCs make relatively proximal synapses (Tamas et al., 2003;
Szabadics et al., 2007), which can be activated by perisomatic extracellular stimulation. Interestingly, however, in human neocortex NGFCs preferentially contact distal dendrites (Kisvarday et al., 1990). Second, in both rat and human neocortex, NGFC synapses display strong activity-dependent depression of GABA release with an extremely slow rate of recovery (Tamas et al., 2003; Olah et al., 2007). Thus in this study, NGFC-IPSPs should have been substantially or completely depressed by baseline stimulation. However, we found that GABA$_B$R-dIPSPs were stronger or were exclusively observed with repetitive stimulation, suggesting that some of the underlying inputs did not show significant depression. One possibility is that the GABA$_B$R-IPSPs evoked in this study were mediated by stimulating axons of NGFC subtypes. For instance, in rat hippocampus, NGFCs elicit GABA$_B$R-dIPSPs with strong depression (Price et al., 2008), but other interneuron subtypes produce GABA$_B$R-IPSPs that facilitate with repetitive stimulation (Thomson and Destexhe, 1999). It is also possible that during repetitive stimulation and GAT1 blockade, GABA$_B$Rs usually activated by NGFCs are activated by GABA released from other interneuron subtypes.

**Functional implications**

Our results suggest that in monkey DLPFC GAT1-mediated uptake restricts transmitter spillover at both perisomatic and dendritic GABA inputs onto pyramidal neurons. We also demonstrated that the effects of spillover induced by GAT1 blockade are sufficient to cause IPSP prolongation in addition to the IPSC prolongation found in previous studies. GAT1-mediated control of IPSP duration could be critical to the timing of GABA-mediated inhibition during network oscillations when interneurons of a given subtype show synchronized firing locked to a particular phase of the oscillation cycle (Klausberger and Somogyi, 2008). Synchronous firing of multiple interneurons of the same subtype during oscillations would produce pooling of GABA released from multiple synapses, increasing the probability of spillover. Because the IPSP duration may be critical for the oscillation frequency (Whittington et al., 1995; Traub et al., 1996; Kramer et al., 2008), a deficit in GAT1 activity could alter the oscillation period, as shown in computational modeling studies (Vierling-Claassen et al., 2008). Moreover, IPSP prolongation may perturb the relation between inhibitory inputs from different interneuron subtypes during the oscillation cycle. For instance, IPSP prolongation may lead to overlapping effects of hyperpolarizing and depolarizing IPSPs that with GAT1 activity intact would have independent effects. Preserving IPSP duration from spillover-induced prolongation may thus be critical for independent cell type-specific inhibition during oscillations and therefore for cognitive functions that may depend on signal propagation based on oscillatory synchrony in neural circuits.

We found that GAT1 activity regulates the strength and duration of GABA$_B$R-IPSPs. Dendritic GABA$_B$R-IPSPs powerfully inhibit dendritic Ca$^{2+}$ spikes (Perez-Garci et al., 2006) and spike backpropagation into dendrites (Leung and Peloquin, 2006). Activation of the predominantly extrasynaptic
dendritic GABA$_{\beta}$Rs (Kulik et al., 2006), may be tightly regulated by GAT1-controlled GABA diffusion. Thus GAT1 activity modulation may be critical for the control by GABA of dendritic excitability, the timing of dendritic Ca$^{2+}$ spike initiation and therefore of computations performed at pyramidal neuron dendrites to induce plasticity at glutamate synapses (Kampa et al., 2007). Interestingly, GAT1 activity can be regulated without changing the levels of GAT1 protein (Ortinski et al., 2006), for instance by phosphorylation-dependent internalization (Quick et al., 2004).

The effects of GAT1 block reported here were not different between psIPSPs recorded from neurons of pre- and postadolescent monkeys. However, because psIPSPs were evoked by stimulating axons of unknown source, some inputs may have been underrepresented, in particular the connections from chandelier neurons onto pyramidal cells, which display an adolescence-related decrease in GAT1 levels (Cruz et al., 2003). In our experimental conditions (EGABA$_{h}$ = 0 mV), the GABA$_{A}$R-IPSPs produced by chandelier cell connections would strongly depolarize the pyramidal cell axon near the spike initiation zone (Khirug et al., 2008) readily eliciting firing (Szabadics et al., 2006). Suprathreshold psIPSPs as those expected from chandelier cell axon stimulation were observed in some experiments (data not shown) but were not suitable to assess the effects of GAT1 block. Because chandelier cell synapses were most likely excluded from analysis, the absence of difference in the effects of NO711 on IPSPs from pre- and postadolescent animals is consistent with data showing that the overall density of GAT1-containing axon terminals does not change through adolescence in monkey DLPFC (Erickson and Lewis, 2002). Furthermore, in monkey neocortex, the neuropil density of inhibitory synapses, which probably determines the propensity for spillover, appears to reach stable adult-like values early in development, well before adolescence begins (Rakic et al., 1986). Our findings thus support the idea that the decline in GAT1 levels seen during adolescence in chandelier cell axon cartridges (Cruz et al., 2003) is not observed at synapses from other GABA neurons in monkey DLPFC. The functional consequences of such a chandelier cell-specific adolescence-related decrease in GAT1 remain to be determined.

In the DLPFC of subjects with schizophrenia, GAT1 levels are decreased (Hashimoto and Sakai, 2002) in a subset of GABA neurons (Volk et al., 2001). GAT1 reduction may increase spillover, disrupting GABA signaling and contributing to cortical circuit dysfunction in the illness (Vierling-Claassen et al., 2008). However, decreased GAT1 expression in schizophrenia co-occurs with a decrease in the mRNA for the GABA synthesis enzyme GAD67 (Hashimoto et al., 2007). GAD67 deficiency may reduce the concentration of GABA inside synaptic vesicles (Jin et al., 2003), decreasing the amount of GABA released and inhibitory synaptic strength but also decreasing the likelihood of spillover. Because long-term decreases in extracellular GABA reduce GAT1 expression (Bernstein and Quick, 1999) GAT1 downregulation in schizophrenia may be a compensatory response (Lewis et al., 2005). Although GAT1 does not regulate within-synapse transmission when GABA release is
normal, decreased GAT1 activity may be beneficial by helping restore synaptic strength if the amount of GABA released is reduced. To assess whether reduced GAT1 expression in schizophrenia is deleterious or beneficial, the role of GAT1 must be tested under conditions of decreased GABA synthesis and release.
Chapter 5

Functional properties of inhibitory synaptic inputs onto interneurons in the monkey dorsolateral prefrontal cortex

DC Rotaru, NV Povysheva, AV Zaitsev, DA Lewis, G Gonzalez-Burgos
Translational Neuroscience Program, Department of Psychiatry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, USA

GABA neurons critically affect neuronal network dynamics via GABAergic inputs onto particular postsynaptic targets. Because of their morphological, neurochemical, and physiological heterogeneity, it is important to understand the type-specific synaptic interactions within interneuron networks. For example, in rodents GABA neurons receive substantial GABA<sub>α</sub> receptor mediated inhibitory inputs and the kinetics of inhibitory inputs onto GABA neurons appears to be distinctive of interneuron subtype. To determine if inhibitory inputs onto interneurons in the primate neocortex also have cell type-specific properties, we studied miniature GABA<sub>α</sub> transmission in layer 3 interneurons recorded from the macaque monkey dorsolateral prefrontal cortex. Interneurons were classified according to their electrical properties as either Fast Spiking (FS) or Non-Fast Spiking (NFS). Comparison of miniature IPSP (mIPSP) properties showed that single-synapse responses were significantly smaller and faster in FS than NFS neurons and were strongly influenced by the membrane properties of the two cell types. Comparison of miniature GABA<sub>α</sub> currents (mIPSCs) showed similar amplitude but significantly faster decay kinetics for the FS group. To test if the faster kinetics of mIPSCs in FS cells was due to higher expression of α1 containing subunits GABA<sub>α</sub> receptors (which determine a faster IPSC decay than other α subunits), we examined the effects of zolpidem, an α1-selective modulator. mIPSP amplitude and decay increased with zolpidem, but the increase was significantly larger in FS versus NFS neurons. This findings are consistent with a higher level of synaptic α1 subunits in FS cells. The fast kinetics of inhibitory inputs onto FS neurons in DLPFC is consistent with the involvement of these cells in high frequency (gamma) oscillations.
Interneurons in the brain are extremely diverse (Ascoli, 2008) and it was suggested that the division of labor might be the main reason for their diversity (Freund and Katona, 2007; Klausberger and Somogyi, 2008). Activity of GABAergic cells is crucial for normal cortical function because they provide cortical feedforward and feedback inhibition (Porter et al., 2001), entrain and shape several types of cortical oscillations (Tamás et al., 2004) underlying various brain functions (Steriade, 2001a), prevent development of hyperexcitability and epileptiform activity, and modulate plastic changes at glutamatergic synapses (Markram et al., 2004). Dysfunction of inhibitory neurotransmission is implicated in psychiatric pathology such as schizophrenia, epilepsy, sleep and anxiety disorders (Akbarian et al., 1995; DeFelipe, 1999; Knable, 1999; Gleeson and Walsh, 2000; Lewis and Levitt, 2002; Powell et al., 2003; Baraban and Tallent, 2004; Krimer et al., 2005). One central issue is to understand how interneuronal networks are organized to perform these multiple functions, in particular whether they are organized according to specific rules.

GABAergic interneurons impose their fast phasic inhibition onto postsynaptic targets via synapses containing GABA_A receptors which are pentameric chloride-permeable channels. The subunit composition of GABA_A receptors confers its electrophysiological and pharmacologic properties (Macdonald and Olsen, 1994). A variety of subunits have been describe such as (α1-6, β1-3, γ1-3, δ, ε, θ, and ρ1-3) (Cherubini and Conti, 2001; Rudolph et al., 2001; Vicini, 2001) but the most common stoichiometry of GABA_A receptors is 2α2βγ (Farrar et al., 1999; Cherubini and Conti, 2001).

Postsynaptic targets of interneurons include both pyramidal cells and interneurons (DeFelipe, 2002). Inhibition onto pyramidal cells has been extensively studied as compared to interneuron to interneuron signaling (Markram et al., 2004). One interesting aspect of the inhibitory neurotransmission onto pyramidal cells is that the postsynaptic GABA_A receptors appear to be specific to the presynaptic interneuron type (Markram et al., 2004). For example, two different types of interneurons, Fast Spiking (FS) and Cholecistokine (CCK) basket cells have respectively α1 and α2 containing GABA_A subunits, at their synapses onto pyramidal cells. (Klausberger et al., 2002; Freund and Katona, 2007). It is thus possible that in the cortex there are separate signaling sub-networks of neurons. Interneuron to interneuron paired recordings showed for example that FS interneurons have a higher tendency to connect with other FS interneurons but NFS synapse more often onto other NFS interneurons. (Beierlein et al., 2000) Moreover, the kinetics of GABA_A responses were also distinctive of the cell type (Beierlein et al., 2000; Vida et al., 2006; Bartos et al., 2007). Imunoelectron microscopy studies showed that Parvalbumin (PV) and Calretenin (CR) containing interneurons tend to form independent networks (Gulyas et al., 1999). Taken together these findings suggest that distinct interneurons form reciprocally interconnected networks
such as: FS prefer synapsing onto other FS interneurons while NFS prefer other NFS interneurons.

Several studies in rodents showed that there are significant differences among inhibitory transmission onto different classes of interneurons (Bacci et al., 2003b; Cossart et al., 2006; Dumitriu et al., 2007). Here we asked whether this is true also for the monkey dorsolateral prefrontal cortex (DLPFC).

In primate cortex few studies have investigated the functional aspects of GABAergic neurotransmission and while the basic types of interneurons have been also described in primates (Lund et al., 1988a), still several differences exist when compared with rodents. For example, the percentage of cortical neurons that are GABAergic is larger (Gabbott and Bacon, 1996; Gabbott et al., 1997), the developmental origin of at least some interneurons appears to differ (Letinic et al., 2002; Molyneaux et al., 2007) and the relative proportions of chemically identified subtypes of interneurons are dissimilar (Condè et al., 1994; Kawaguchi and Kubota, 1997). Thus, the canonical cortical circuit differs in at least some aspects of its constituent elements across species.

To better understand the role of subclasses of interneurons in controlling cortical excitability and sustaining different cortical network activities, we analyzed the properties of inhibitory inputs onto interneurons from monkey DLPFC.

MATERIAL AND METHODS

Brain slice preparation

Brain slices were prepared from rhesus monkeys like previously explained in chapter 4, and (Gonzalez-Burgos et al., 2004) Cortical slices (350 μm thick) were cut in the coronal plane using a vibrating microtome (VT1000S, Leica Microsystems, Nussloch, Germany) in ice-cold ACSF. Immediately after cutting, slices were transferred to an incubation chamber maintained at room temperature and filled with a solution containing (in mM) 126.0 NaCl, 2.0 KCl, 1.2 Na2HPO4, 10.0 glucose, 25.0 NaHCO3, 6.0 MgCl2, and 1.0 CaCl2, pH 7.3–7.4 when bubbled with 95% O2-5% CO2.

Electrophysiological recordings

For recording, slices were submerged in a chamber superfused at a rate of 2–3 ml/min with a solution containing (in mM) 126.0 NaCl, 2.5 KCl, 1.2 Na2HPO4, 25.0 Na2HCO3, 10.0 glucose, 2.0 CaCl2, 1.0 MgCl2, 0.02 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX), d,l-amino-5-phosphonopentanoic acid (AP5) 0.1, bubbled with 95% O2-5% CO2, and maintained at 30–32°C. Whole cell recordings were obtained from visually identified neurons in layer 3 of DLPFC areas 9 and 46 using infrared differential interference contrast video microscopy in Olympus BX51 and BX61 microscopes (Olympus), or Zeiss FS Axioskop microscopes (Zeiss). Recording micropipettes pulled from
borosilicate glass had a resistance of 3–5 MΩ when filled with a solution containing (in mM) 120.0 KCl, 10.0 NaCl, 0.2 EGTA, 10.0 HEPES, 4.0 MgATP, 0.3 NaGTP, 14.0 NaPhosphocreatine, and biocytin 0.5% (pH adjusted to 7.2–7.3). Recordings were done using a Multiclamp 200B amplifiers (Axon Instruments, Union City, CA) operating in current-clamp (bridge) mode or voltage clamp. Signals were low-pass filtered at 4 kHz, digitized at 10 or 20 kHz, and stored on disk for off-line analysis. Data acquisition was performed using Power 1401 data-acquisition interface boards (Cambridge Electronic Design, Cambridge, UK) and Signal 3 software (Cambridge Electronic Design). Throughout the experiments, the series resistance was monitored, and if it exceeded 20 MΩ, recordings were excluded from data analysis.

**Recording and analysis of mIPSPs**

Synaptic miniature inhibitory events, mIPSPs or mIPSCs were recorded from layer 3 interneurons. To block action potentials thus focusing on IPSPs resulting from spontaneous GABA release at single synapses, the voltage-dependent Na+ channel blocker tetrodotoxin (1 μM) was added to a bath solution that otherwise had the same composition as that used for slice perfusion. The cells were recorded at −80 mV for 20 min and then Zolpidem (1 μM) was applied for 15 min. For each cell, the mIPSPs were detected using MiniAnalysis (Synaptosoft, Decatur, GA). At least 100 nonoverlapping events were included to automatically generate an average mIPSP or mIPSC for each cell in control conditions and at the last 5 min of the zolpidem application. The amplitude and the decay time constant of an exponential function fit to the 10–90% decay phase were determined for the average mIPSPs or mIPSCs obtained for each cell.

**Morphological analysis**

Biocytin-filled neurons were visualized using the Vectastain Elite ABC kit (Vector Laboratories) and their axonal and dendritic trees reconstructed using the Neurolucida Tracing System (Microbrightfield Bioscience) as described previously (Povysheva et al., 2008; Zaitsev et al., 2009).

**Pharmacological compounds**

Fast glutamate transmission was blocked with continuous bath application of 100 μM of d,l-AP5 and 20 μM CNQX, to block, respectively, NMDA and AMPA receptors. To block voltage-dependent sodium channels during mIPSP or mIPSC recordings we used tetrodotoxin (1 μM).

**Statistical data analysis**

Data are expressed as means ± SE unless indicated otherwise. The statistical significance of the difference between group means was assessed using independent samples t-test, paired-samples t-test or two-way ANOVA, as indicated in each case.
RESULTS

To determine the properties of inhibitory inputs onto monkey DLPFC interneurons, we grouped the recorded cells into different subtypes. In several studies, we used cluster analysis to define specific subtypes of interneurons, and found that monkey DLPFC interneurons are divided into two main clusters, namely fast-spiking (FS) and Non-Fast Spiking (NFS) cells (Krimer et al., 2005; Zaitsev et al., 2009). FS interneurons have spikes with short duration and fire at almost constant frequency during sustained current injection. In contrast, NFS cells have spikes with longer duration and their firing frequency is not constant during sustained excitation.

The data sample in this study included 25 FS neurons and 32 NFS neurons (Fig. 1A). FS interneurons recorded herein had homogeneous electrical properties (Fig. 1B) and morphological features corresponding to chandelier or basket neurons (Fig. 1C), as described previously (Krimer et al., 2005; Gonzalez-Burgos et al., 2005a; Povysheva et al., 2008). Interneurons in the NFS group had heterogeneous electrical properties and were divided into three subtypes, based on differences in their firing patterns. The first subtype, called adapting cells, or regular spiking (RS) showed progressive spike frequency adaptation (Fig. 1B). The second subtype, intrinsic bursting (BS) cells, displayed a burst of high frequency spikes at the onset of stimulation, followed by spike frequency adaptation (Fig. 1B). In the third subtype, irregular spiking (IS) cells, the firing frequency was variable and without clear spike frequency adaptation (Fig. 1B). The majority of the NFS cells were adapting (20/32, 62.50 %), whereas intrinsic bursting and irregular spiking cells constituted smaller fractions of the total (6/32; 18.75 % and 6/32; 18.75 %, respectively).

Figure 1C shows examples of the morphological properties of the NFS cells. Although all recorded cells included in this study were confirmed to be interneurons by their morphology, in many of the NFS interneurons the morphological properties could not be characterized in detail. Thus, we could not reliably determine differences and similarities between the morphology of adapting, intrinsically bursting, and irregular spiking neurons. In our previous studies of monkey DLPFC interneurons, intrinsically bursting and irregular spiking neurons were rare or absent (Krimer et al., 2005; Gonzalez-Burgos et al., 2005a). Although the actual causes of this discrepancy are not clear, one possibility is that the differences in the interneuron firing pattern were due to the chemical composition of the pipette solution, as highlighted elsewhere (Ascoli, 2008). Here, we used a KCl-based pipette solution to improve the detection of IPSPs and IPSCs. In contrast, previously we used a KGluconate-based solution which, relative to KCl, may alter the ionic mechanisms of intrinsic bursting (Velumian et al., 1996; Jarsky et al., 2008).
Properties of mIPSPs in FS and NFS interneurons

First we recorded mIPSPs to determine if there are differences in the properties of inhibitory inputs onto interneuron subtypes. After injecting current steps to
determine the membrane properties and firing pattern of each neuron, tetrodotoxin (1 µM) was applied to block action potential firing and to record mIPSPs. At least 100 GABA$_A$R-mediated mIPSPs were recorded from each neuron to obtain an average mIPSP (see Methods).

**Figure 2 Properties of mIPSPs in FS and NFS interneurons** (A) Examples of mIPSPs recorded from FS and NFS interneurons. (B) Averages of the mIPSPs events recorded from the FS and NFS interneuron showing the smaller amplitude and faster decay for FS compared with NFS cell. (C), Bar graphs summarizing the differences in the amplitude and (D) decay time constant of average mIPSPs. Color coded bars show averages for FS and the 3 groups of NFS (RS, BS, IS). Letters represent significant differences (ANOVA,
followed by planet comparison, p < 0.05). Groups sharing letters are not different*. (E, F)
Cumulative distribution including all the analyzed events.

We found that the properties of inhibitory inputs, as determined through the average mIPSP, were markedly different between the FS and NFS neuron groups. The average mIPSPs had smaller amplitude and faster decay time in FS than in NFS neurons (Fig. 2B,C). An analysis of the cumulative probability distribution also revealed significant differences between FS and the NFS neurons subgroups (Fig. 2E,F). Among the NFS subgroup of cells, the RS had the highest amplitude of events while the BS had the longest decay.

Together with the synaptic inputs, the cells’ membrane properties may determine the features of GABA\textsubscript{A}R-mediated IPSPs, by either obscuring the effects of different IPSC properties, or by producing different IPSPs when IPSC properties are similar. Therefore, the different input resistance and membrane time constant of FS and different NFS cell subgroups (Table 1) could underlie the observed differences in mIPSP shape.

Table 1. Summary of electrophysiological intrinsic membrane properties of subclasses of interneurons in layers 2/3 of monkey DLPFC

<table>
<thead>
<tr>
<th></th>
<th>Membrane input resistance (Mohm)</th>
<th>Membrane time constant (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>mIPSP Amplitude (mV)</strong></td>
<td>0.44 ± 0.01</td>
<td>1.01 ± 0.02</td>
</tr>
<tr>
<td><strong>mIPSP Time Constant (ms)</strong></td>
<td>2.04 ± 0.14</td>
<td>4.17 ± 0.07</td>
</tr>
</tbody>
</table>

The statistical significance of the differences between group means was assessed by one way ANOVA followed by planned comparisons (p<0.05). In each column, groups sharing a letter are not significantly different.

Figure 3. Effects of membrane properties on mIPSPs. (A) Cells input resistance correlated significantly with average mIPSP amplitude both in FS (N=25, R=0.45 p<0.04) and NFS cells (N=32, R=0.31, p<0.04). (B) The membrane time constant correlated significantly with mIPSP decay time in both FS (R=0.59, p<0.0001) and NFS (R=0.58, p<0.0001) cells.

Consistent with a contribution of membrane properties, we found a significant correlation between the cells’ membrane time constant and the
mIPSP decay time (Fig. 3B). Similarly, the cells’ input resistance was correlated with the mIPSP amplitude (Fig. 3A).

These data suggest that the slower mIPSP decay and larger mIPSP amplitude in the NFS cell group may be due, at least in part, to their longer membrane time constant and higher input resistance. However, although statistically significant, the observed correlations were relatively weak suggesting that the effects of membrane time constant and input resistance cannot fully explain the differences in mIPSP properties.

### Properties of mIPSCs in FS and NFS interneurons

Since the influence of membrane time constant and resistance might not fully explain the differences in mIPSP properties, we determined if properties of the IPSCs also contribute to the differences between cell groups. In a series of experiments, we recorded from neurons in current clamp mode to characterize their membrane properties, and then voltage clamp recordings were used to record mIPSCs (Figure 4A). Voltage clamping neurons with intact dendritic trees produces severe space clamp errors (Spruston et al., ; Williams and Mitchell). However, somatic voltage clamp has good control of the membrane potential at the soma (Williams and Mitchell), thus preventing the influence of intrinsic properties on mIPSCs of the proximal membrane compartments.

We recorded mIPSCs from 10 FS neurons and 11 NFS neurons. Based on their firing pattern, 9/11 NFS neurons were classified as adapting cells and 2/11 as irregular spiking. During recordings from individual FS or NFS, fast- and slow-rising mIPSCs were observed. The ranges of 10-90% rise times for mIPSCs recorded from FS or adapting NFS neurons overlapped extensively, going from ~ 0.1 to ~ 3 ms in either cell subtype. Fast and slow rising mIPSCs were observed in an alternated fashion, frequently within a few ms (Fig. 4A), suggesting that the wide range of mIPSC rise times was not due to changes in recording conditions but most likely to differences in synapse location or individual synaptic properties. The mIPSC amplitude distributions were very similar (Fig. 4C) and the mean mIPSC amplitude did not differ between FS and adapting NFS neurons (Fig. 4D). These results suggest that the larger mIPSPs in NFS neurons may be due to the higher input resistance of these cells. On the other hand, similar to the mIPSPs, the mIPSCs decayed significantly faster in FS neurons than in adapting NFS neurons (Fig. 4E, F).
Effects of Zolpidem on FS and NFS mIPSPs

The faster mIPSC decay in FS neurons compared to NFS cells might be due to differential properties of the GABA_ARs mediating the IPSCs in FS neurons. Since α1 subunit-containing GABA_ARs (α1-GABA_ARs) produce currents with faster decay than other GABA_AR subtypes, we tested the effects of zolpidem, a benzodiazepine site ligand that binds preferentially to α1-GABA_ARs. The effects of zolpidem (1 μM) were tested in 15 FS neurons, 7 adapting neurons, 3 intrinsically bursting and in one irregular spiking cell. By increasing the GABA binding affinity at GABA_ARs, benzodiazepine site agonists generally increase the amplitude and or duration of the response to synaptically released GABA.
Inhibitory synaptic inputs onto interneurons

Figure 5. Effects of Zolpidem on FS and NFS mIPSPs. (A) Example recordings from FS and NFS cells during control and zolpidem. (B) Bar graph summarizing the significant differences (similar letters represent no significant differences between group) in the effects of Zolpidem. (Anova)

In monkey DLPFC interneurons, zolpidem increased both the mIPSP amplitude and the mIPSP decay (Fig. 5A). Thus, to compare the effects of zolpidem between cell subtypes we measured the changes in mIPSP area, which reflects the increase in mIPSP amplitude and decay time constant. As shown in (Fig. 5 B), the effect of zolpidem on the average mIPSP area was stronger in FS neurons than in other cell subtypes. These results, together with the voltage clamp data, suggest that there are significant differences in the properties of GABA synapses onto FS and NFS neurons. Moreover, the data suggest that such differences are due at least in part to a more significant contribution of α1-GABA<sub>A</sub>Rs in FS than NFS cell synapses.

DISCUSSION

In this study we recorded inhibitory synaptic responses from FS and NFS interneurons from layer 3 of monkey DLPFC. We found significantly different kinetics of the GABAergic inputs onto the two types of interneurons which are at least partially underlined by a different contribution of α1 subunits to these synapses. The amplitude and duration of miniature inhibitory events were significantly different in FS compared with NFS cells suggesting thus important functional consequences for the network activity in primate DLPFC. These differences are similar with the ones found in rodent cortex. Thus we conclude that monkey DLPFC contains at least two separate subnetworks of interneurons. More sensitive methods are necessary to determine whether subtle differences exist between primate and rodent cortex in this aspect.

Classification of GABAergic interneurons is a well known problem (Ascoli, 2008) and presently there is no clear scheme that can allow a clear distinction between different subtypes. Our previous studies, attempting to describe the different classes of interneurons in monkey DLPFC, showed that electrophysiological criteria lead to only two separate clusters interneurons, the FS and NFS cells (Krimer et al., 2005; Zaitsev et al., 2005; Zaitsev et al., 2009).
Although multiple subtypes of interneurons might be comprised within the NFS group, they are not easily distinguishable when the patch clamp method is used. Here, the recorded interneurons were clearly separated into two distinct groups, FS and NFS interneurons, with membrane and firing properties similar to those previously described in monkey PFC (Krimer et al., 2005; Zaitsev et al., 2005; Zaitsev et al., 2009).

**Major differences in GABAergic synaptic transmission onto two general classes of monkey cortical interneurons**

We recorded both voltages and currents mediated by the spontaneous activation of the GABA_\text{A} receptors onto the two types of interneurons. The decay kinetics of both mIPSP and mIPSC were faster in FS than in NFS cells. The amplitude of mIPSP was smaller in FS compared with NFS whereas mIPSC amplitude did not show significant differences between the two cell types. Two major factors contributed to the integration of the postsynaptic GABA_\text{A} mediated transmission onto interneurons. On one hand the membrane properties were significantly different between FS and NFS and they in turn, significantly modulated the integration of mIPSCs. Thus a smaller input resistance in FS cells can account for smaller mIPSP amplitude even though the peak IPSC amplitude was similar with the NFS group. On the other hand, a stronger effect of Zolpidem for the FS cells, suggests a higher contribution of \( \alpha_1 \) subunits to synapses onto these cell type. Therefore during in vivo activity the inhibition of FS cells is weaker and more transitory compared with the NFS cells.

Our results can be explained by a combination of morphological and functional factors that contribute strongly to signaling properties of synapses (Johnston, 2002). Thus synaptic location or dendritic structure together with electrical cable properties, and molecular factors shape the postsynaptic GABAergic responses onto interneurons.

**Morphological aspects**

Regardless of the significant differences between the membrane properties in these two cells classes we found that the decay kinetics of the GABAergic synaptic currents was also different. A combination of morphological factors can account for the observed difference in IPSC kinetic. Morphology of the synapse such as the size of the synaptic cleft can strongly affect the kinetics of synaptic currents (Nusser et al., 2001). Thus larger synaptic cleft could generate slower kinetics due to an increased time of GABA diffusion from the pre to the postsynaptic site (Tamas et al., 1998; Nusser et al., 2001). A second morphological aspect refers to the location of synapses along the dendritic tree such that synapses closer to the somatic compartment are less filtered during somatic recording and thus they appear as having faster kinetics as compared with distal ones. Various interneuron classes generate IPSCs with different rise times in pyramidal neurons of both the hippocampus (Banks et al., 1998; Maccaferri et al., 2000) and neocortex (Xiang et al., 2002) indicating differential location of synaptic contacts onto pyramidal cells. Therefore one
can speculate that synapses onto NFS cells are located on distal dendrites as compared to the ones onto FS cells. A morphological analysis of the inhibitory inputs onto different types of interneurons from the CA1 area of hippocampus showed that inhibition is concentrated in the perisomatic region and on proximal dendrites on most of the interneurons (Gulyas et al., 1999) Whether this is the case for cortical interneurons it is not entirely clear, although some studies suggest that interneurons can target specific domain of other interneurons (Tamas et al., 1998; Markram et al., 2004) Therefore it might be possible that differences in the IPSC kinetics are partially due to differences in synapse location onto these subtypes of interneurons in monkey DLPFC. If indeed there is a domain specific GABAergic inhibition on interneurons, just like it is found on pyramidal cells, it could add to even more levels of complexity to the connectivity scheme in the network. Selective targeting of inhibitory axons to different dendritic domains can arise during development under the guidance of the GABA_A subunit composition of the postsynaptic receptors expressed by the postsynaptic target (Bozza et al., 2002). Alternatively, the target of the axon might determine the subunit expression or receptor trafficking of the presynaptic interneuron through a retrograde signaling process (Christie et al., 2002; Hennou et al., 2002; Markus et al., 2002)

**Functional aspects**

The cable parameters, specific membrane resistance (Rm), membrane capacitance (Cm), and intracellular resistivity (Ri), will shape neuronal signaling (Johnston and Wu, 1994; Spruston, 2008) For example, a low value of Rm, which may be generated by a high density of leak channels (Torborg et al., 2006) would accelerate the decay of EPSPs by speeding up the membrane time constant. Functionally low vs high Rm values for different interneurons could be related to their different purposes in the network dynamics. In FS cells, low Rm will fasten the decay of IPSPs facilitating thus fast recovery from synaptic inhibition and rendering the cell ready to integrate incoming excitatory inputs which transfer to the network as feedforward of feedback inhibition. In contrast a higher Rm in the NFS cell will prolong the decay of IPSPs promoting inhibition of these cells for longer periods of time and thus remove them from the circuit dynamics for these periods of time. Removal of NFS from network dynamics allows pyramidal cells to receive and integrate incoming inputs.

Apart from the membrane properties, synaptic factors could also underlie the differences between two cell types. On one hand, differences in the presynaptic properties of GABAergic synapses such as the amount of vesicular GABA, and the life time of GABA in the synaptic cleft could be reflected on the kinetics of IPSC (Hill et al., 1998; Nusser et al., 2001; Overstreet and Westbrook, 2003).On the other hand, postsynaptic mechanism including post-translational modification of GABA_A receptors such as endogenous phosphorylation (Nusser et al., 1999; Brandon et al., 2000), clustering of GABA_A receptors at the synapse (Christie et al., 2002) or GABA_A receptor subunit composition (Bacci et al., 2003b). Here we investigated the role of
GABA\textsubscript{A} receptor subunits on the mIPSP kinetics. Different GABA\textsubscript{A} receptor subunit assemblies will likely generate different IPSC kinetics.

**The importance of GABA\textsubscript{A} subunit composition on integration of postsynaptic inhibitory inputs onto interneurons**

A switch from $\alpha2$ to $\alpha1$ subunits in the GABA\textsubscript{A} receptors, during development of the primate cortex was associated with a decrease in the decay of GABA mediated miniature events in pyramidal cells of the DLPFC (Hashimoto et al., 2009). The same phenomenon has been described in rodents (Hollrigel and Soltesz, 1997; Dunning et al., 1999; Okada et al., 2000) suggesting a strong association between GABA\textsubscript{A} subunit composition and IPSC kinetic properties (Farrant and Nusser, 2005). We used the benzodiazepine-like drug zolpidem, which acts primarily onto $\alpha1$-containing and, is less potent for, $\alpha2$- and $\alpha3$-containing GABA\textsubscript{A} receptors (Rudolph et al., 2001). Zolpidem, dramatically increased the IPSP area in FS interneurons with a much smaller, although significant, effect on NFS IPSPs. These data indicate that NFS cells may have a lower expression of functional $\alpha1$-containing GABA\textsubscript{A} receptors, suggesting that $\alpha1$ expression may be an important factor in shaping IPSC decays in FS interneurons. Our findings are in agreement with previously described effects. For example (Bacci et al., 2003b) showed differential effects of zolpidem in FS and Low Threshold interneurons, a subtype of NFS cells. Also immunoelectron microscopy studies showed that FS PV cells tend to act mostly via $\alpha1$ subunits as compared with other GABAergic cells (Klausberger et al., 2002). Together these studies suggest that certain subtypes of NFS interneurons have almost no $\alpha1$ subunits in their GABA\textsubscript{A} receptors. The $\alpha1$ subunit plays a key role in rapid activation and deactivation of GABA\textsubscript{A} receptors (Lavoie et al., 1997) becoming thus a very important factor in accelerating the time course of GABAergic currents. Here we reinforce the findings showing that FS cells receive brief inhibitory inputs mediated by $\alpha1$ subunits (Fritschy and Mohler, 1995; Nusser et al., 1995; Klausberger et al., 2002). It remains to be answered which $\alpha$ subunits are present in Non FS cells?

**Conclusion**

In hippocampus it was shown that rapidly decaying IPSCs between interneurons are crucial for network oscillation and frequency control (Bartos et al., 2001; Bartos et al., 2002). Different GABAergic neurotransmission kinetics in distinct interneuronal networks might be responsible for entraining cortical oscillations at different frequencies. Interneuron to interneurons signaling, received increased interest recently, due to its hypothesized role in oscillatory rhythms (Bartos et al., 2007) and thus in cognitive function (Buzsaki and Draguhn, 2004b). The kinetics of inhibitory responses have an important role in the oscillatory period (Bartos et al., 2007). Changing the properties of synaptic GABA\textsubscript{A} receptors has a strong effect on the frequency of oscillations both in slices and in computational models (Whittington et al., 1995). Thus, different GABA\textsubscript{A} receptor subunit assemblies will likely generate different IPSC kinetics.
and have an effect on the oscillatory rhythm. For example faster IPSC kinetics could tune the oscillatory cycle towards faster oscillations such gamma oscillations. The fast spiking subtype of interneurons was shown to receive fast GABA_A inputs due to their high content of alpha_1 subunits (Bartos et al., 2002; Bacci et al., 2003b). The firing of the FS subtype is also coupled with the gamma cycle in hippocampus (Klausberger and Somogyi, 2008).
Chapter 6
General Discussion

The combined activity of different interneuron classes in the neocortex regulates pyramidal cell firing pattern leading that underlies cortical network function and cognitive abilities such as memory and thought. The main purpose of the studies presented here was to add to the understanding of how synaptic communication of interneurons is organized in superficial layers, in particular layer 2-3, of prefrontal cortex (PFC) circuit of rodents and primate. We described several synaptic properties that are important for feedforward inhibition and oscillations which are neural processes implicated in sensory processing and cognitive function.

RODENT AND PRIMATE PFC DIFFERENCES AND SIMILARITIES

The data presented here was collected from the prefrontal cortex of rodents, (including rats and mice) brains of primates. Bellow I will discuss a few aspects regarding species differences in PFC functions.

Although there is still great debate about the extent to which the PFC is represented in rats (Uylings and van Eden, 1990; Preuss, 1995; Heidbreder and Groenewegen, 2003; Uylings et al., 2003), the fact that there are certain similarities between primates and rodents, including cytoarchitectonic patterns, functional properties, presence of specific neurotransmitters and receptors, and embryological development, has made people consider that at least some parts of the frontal lobe in rodents can be considered as PFC (Uylings et al., 2003), although some authors disagree (Wise, 2008) and support the idea that the largest part of the primate frontal cortex does not have an equivalent in other mammals. As suggested by Warren and Kolb (Warren and Kolb, 1978), the cognitive functions can be classified as either class-common behaviors, when they refer to general capacities present in all mammals, or as species-typical behaviors, if the functions evolved in order to promote survival for that species. Early lesion studies that involved the rostral part of the frontal lobe in rodents produced behavioral manifestations that were similar to those observed in monkeys with lesions of the dorsolateral and orbitofrontal cortices (Kolb, 1984, 1990). Moreover, different divisions of the rat PFC seem to be implicated in different aspects of cognitive behavior. Uylings (Uylings et al., 2003) argues, in one of his reviews, for the presence of PFC in rats, based on some of the
functional impairments following medial PFC lesions. For example, such lesions induce cognitive deficits belonging to class-common behaviors like acquisition and retention of working memory tasks such as delayed response (Kolb, 1974c) or delayed alternation (Divac, 1971; Wikmark et al., 1973), attention deficits (Muir et al., 1996), and strategy formation (Kolb et al., 1994). Other studies showed that medial PFC lesions also generate impairment of species-typical behaviors like sequenced behavior for food hoarding, nest building, or latch opening (Kolb, 1974a, 1974b; Kolb and Whishaw, 1981).

Another criterion that has been used to argue for the presence of PFC in rodents is the mediodorsal thalamus (MD) projection pattern (Uylings and van Eden, 1990). MD represents the main thalamic nucleus that provides inputs to the PFC. Moreover, the connections between these two areas are reciprocal and highly topographically organized (Krettek and Price, 1977; Groenewegen, 1988; Kuroda et al., 1998a). In the late 1940s, Broadman’s view that the granular frontal region is unique to primates was challenged, and a new criterion based on projection of the MD was introduced to help delineate this area in different species (Rose and Woolsey, 1948). In primates, the medial (magnocellular) division of the MD targets the orbital cortex, the lateral (parvocellular) division sends inputs to the dorsolateral region and the frontal eye field receives projections from the most lateral MD, the paralamellar division (Preuss, 1995). In rats, the medial and central segments of the MD together are homologous to the medial, magnocellular subnucleus of the primate MD, given that this medial MD segment in both rats and monkeys receives inputs from limbic, olfactory and ventral pallidal structures (Groenewegen, 1988). However, the projection patterns of these two subdivisions in rats are rather different in that the medial segment is reciprocally connected with the infralimbic, prelimbic, ventral anterior cingulate, and dorsal agranular insular areas, whereas the central segment is interconnected with ventral agranular insular and lateral orbital areas. The lateral segment of the rat MD has reciprocal connections with the dorsal part of the cingulate area and to a lesser extent with the ventral orbital area (Groenewegen, 1988). The rat MD subdivision is not so strongly developed as in primates, but it does receive similar inputs from brain stem structures. The comparison of the anatomical projection patterns involving PFC in rats and primate, has lead people to consider that the ventral part of the medial PFC and the orbitofrontal areas of the monkey correspond in rats to the prelimbic area in rats (Preuss, 1995).

If functional aspects are considered, some authors argue in favor of a dorsolateral-like PFC in rats. These features would be present in area Fr2 and the anterior cingulate, and the prelimbic area also seems to be implicated in some dosolateral-like features (Uylings et al., 2003). With respect to this definition of the PFC in rats, we focused our study on the medial PFC, specifically the prelimbic and infralimbic subdivisions, being generally accepted that this PFC area shares at least some of the higher cognitive functions encountered in primates. It is also known that some of these functions are segregated within the rodent medial PFC. The ventral region encompassing
the ventral prelimbic and infralimbic subregions appears to be involved in an animal’s ability to adapt behavior to new spatial cues and, via connections with autonomic centers, to integrate the internal physiological state of the animal with environmental cues. The dorsal region that includes the dorsal anterior cingulate and dorsal prelimbic subregions is responsible for temporal shifting in behavioral sequences.

The way that the reciprocal interactions between the PFC and MD affect cognitive behavior is not fully understood. Behavioral studies in rats subjected to MD lesions or electrophysiological recordings from MD showed that a series of cognitive functions, including object recognition (Mumby et al., 1993), working memory (Freeman et al., 1996), planning and prospective coding (Daum and Ackermann, 1994), depended on intact inputs from the MD. Floresco et al. (Floresco et al., 1999) showed that the interactions between these two structures are important in the executive processing that is involved when previously acquired trial-unique information must be used to guide memory-based behavior after a delay. Other similar studies suggest that MD impairments generate behaviors that resemble those observed after PFC lesions (Hunt and Aggleton, 1998a, 1998b). Therefore, specific evidence suggests the interdependence of the PFC and MD with working memory functions but also with other cognitive aspects of behavior.

**FINDINGS SUMMARY**

In the first two research chapter presented in the thesis we analyzed the glutamatergic inputs onto interneurons. We explored ways of recruiting inhibition in Chapter 2, by performing the first ultrastructural analysis of the MD inputs onto different classes of interneurons in layer 3 of the rat PFC. We showed that there is a preference of the MD terminals for the Parvalbumine (PV) subclass of interneurons. Additionally, MD axons also synapse on other interneurons but less frequently. Nevertheless, the vast majority of inputs from MD contact spines of the pyramidal cells. We thus expanded the studies investigating ways of activating interneurons specifically in PFC. Although MD axons synapsed onto other subclasses of interneurons as well, it can be expected that the feedforward inhibition via MD synapses onto PV cells is more efficient, because of these cells strong influence onto pyramidal neurons via their contacts with proximal dendrites, cell bodies, and axon initial segments (Williams et al., 1992; Melchitzky and Lewis, 2003). MD pattern of excitation onto different types of interneurons appears to be similar with other sources of input onto these cells, reinforcing the conclusion that PV cells are in general more targeted by glutamatergic inputs then the rest of the interneurons. This could reflect a specific rule stating how different sources of inhibition are recruited in network dynamics.

In the next chapter, we analyzed the receptor subtypes that mediate excitation onto PV FS interneurons and pyramidal cells, in the mouse PFC. Additionally, we compared how NMDA receptors contribute to the recruitment
of the two cell types. We showed that FS cells had faster decay excitatory postsynaptic currents (EPSCs) due to a weaker contribution of NMDA receptor currents then pyramidal cells. Thus, NMDA receptors contribute much more to the recruitment of pyramidal cells compared with FS interneurons. The axons of the excitatory inputs analyzed in this study can have diverse origins. Because the study was performed in brain slices we cannot specify their origin and we assume they include both local inputs arriving from the pyramidal cells within the medial PFC and projecting inputs from different sources that project to middle layer of PFC. This finding has important consequences for a recently developed hypotheses concerning Schizophrenia. An attractive hypotheses, it suggests that the well known cognitive deficits of schizophrenia (Gold, 2004) derive from an impairment of gamma band synchrony which is caused by alterations in PV neuron-mediated inhibition (Gonzalez-Burgos et al., 2010; Lewis, 2010; Lewis et al., 2011). The causes leading to impairment of PV neurotransmission in schizophrenia are still under investigation (Lewis, 2010). Interestingly, NMDA receptors antagonism results in schizophrenia-like symptoms in healthy adults (Javitt and Zukin, 1991; Javitt, 2009). Additionally, systemic NMDA antagonist administration increases PFC pyramidal cell firing (Homayoun and Moghaddam, 2007), apparently by producing disinhibition, and repeated exposure to NMDA antagonists leads to changes in the GABAergic markers that mimic the impairments found in schizophrenia (Behrens et al., 2007; Lisman et al., 2008; Behrens and Sejnowski, 2009). Based on these findings, PV neuron deficits in schizophrenia have been proposed to be secondary to NMDA receptors hypofunction at glutamatergic synapses onto these cells (Coyle, 2006; Lisman et al., 2008). However, NMDARs generate long-lasting postsynaptic currents that result in prolonged depolarization of the postsynaptic cells, a property inconsistent with the role of PV cells in network dynamics. We present evidence leading to the conclusion that cortical disinhibition and GABAergic impairment produced by NMDA receptor antagonists are unlikely to be mediated via NMDA receptors at glutamatergic synapses onto mature cortical PV neurons.

Our results raise interesting questions about the mechanisms that might link NMDA receptor hypofunction to alterations of FS neurons in schizophrenia. Although here we showed that on average, the level of NMDA receptors is low at glutamatergic inputs onto FS cells there could be important differences between individual glutamatergic synapses. Here, we could not assess the level of NMDA receptors for specific inputs onto FS cells. For example, there might be different levels of NMDA receptors at MD inputs received by FS cell then at the inputs arising from local collaterals of layer 3 pyramidal cells synapsing on FS cells. Thus one question is how MD feedforward inhibition compares with local pyramidal neuron feedback inhibition. Another question that remains to be answered is how the subunit composition of the few glutamatergic inputs onto NFS cells contributes to the network dynamics. Moreover, several subclasses of NFS interneurons have
strong synaptic NMDAR currents (Behrens et al., 2007; Wang and Gao, 2009) making them good candidates for generating disinhibition.

In the second part of the thesis including chapter 4 and 5, we switched the focus from the mechanism responsible for recruitment of inhibition, towards the postsynaptic target of interneurons. Thus, in chapter 4 we analyzed the inhibitory inputs onto somatic and dendritic compartments of pyramidal cell. Specifically, we studied how phasic GABA transmission is regulated by the GABA transporter GAT1, along the dendritic tree of the pyramidal cell in monkey dorsolateral prefrontal cortex (DLPFC). Somatic inhibition is provided mostly by the PV FS interneurons while the dendritic inhibition is arising from the NFS subtypes. We showed that dendritic but not somatic, GABA_B mediated transmission is strongly regulated by GAT1 while both perisomatic and dendritic GABA_A synapses are similarly regulated by GAT1 activity. A major functional role for GAT1 in primate cortical circuits is to prevent the effects of GABA spillover when multiple synapses are simultaneously active. We showed that GAT1, critically controls the spatiotemporal specificity of inhibitory inputs onto proximal or distal compartments of the pyramidal cell membrane.

In chapter 5, we assessed the interneuron to interneuron- transmission. We studied the kinetics of GABA_A mediated currents onto FS and NFS interneurons to understand how inhibition controls its own activity in a network. We determined that GABAergic inputs onto interneurons are cell type-specific in macaque monkey DLPFC similar with finding in rodent cortex (Bacci et al., 2003b; Cossart et al., 2006; Dumitriu et al., 2007). After classifying the interneurons according to their electrical properties as either FS or NFS, we showed that FS cells received fast kinetic inhibition while the NFS cells had slow kinetic inhibition. The faster kinetics of the inhibitory inputs onto FS cells is at least partially determined by a higher level of α1 subunits at the GABA_A receptors. Thus FS cells are part of a network of interneurons connected via fast kinetic GABA_A receptors, while the opposite is true for the NFS class of interneurons. The functional importance of our findings is related to the role of interneurons in oscillatory rhythms in the cortex (Bartos et al., 2007). While it is now well established that fast oscillations such as gamma rhythms (30-80 Hz) are strongly dependent on FS interneurons (Klausberger and Somogyi, 2008; Cardin et al., 2009) it is still unknown at this point how NFS cells regulate oscillatory activity in general. Newly developed methods (Taniguchi et al., 2011) will soon generate an answer to this important question since it is already established that the activity of NFS cells is present in several oscillatory rhythms in cortex (Hartwich et al., 2009; Klausberger, 2009).
Figure 7.1 Summary of the findings in this thesis. (Chapter 2) Inputs from the mediodorsal thalamus synapse heavily onto pyramidal cells but also onto interneurons. Thus inputs from MD make more synapses onto FS interneurons as compared with NFS interneurons. (Chapter 3) The glutamatergic inputs at FS cells contain less NMDA receptors as compared with the glutamatergic inputs onto pyramidal cells. (Chapter 4) The inhibitory inputs onto the somatic compartment of pyramidal cells, provided mostly by FS interneurons, contain exclusively GABA_\text{A} while the inhibitory inputs onto distal dendrites, provided mostly by NFS interneurons contain both GABA_\text{A} and GABA_\text{B} receptors. The GAT1 transporter (which remove released GABA from the extracellular space) regulates the activation of all these receptors with a stronger effect on the distal GABA_\text{B} receptors they. (Chapter 5) FS interneurons receive fast kinetics inhibitory inputs, mostly via \(\alpha_1\) containing GABA_\text{A}, putatively from other FS interneurons, whereas the NFS interneurons receive slow kinetics inhibitory inputs that contain perhaps different GABA_\text{A} receptor (here we speculate that GABA_\text{A} receptor at this receptors contain predominantly \(\alpha_2\) subunits). Additionally, the NFS cells might receive their inhibitory inputs form other NFS cells.

In summary, our findings reinforced the idea that FS cells are preferentially targeted by different sources of excitation via fast, predominantly AMPA containing glutamatergic inputs. These mechanisms of activation show that these cells are intricately implicated in the activity of pyramidal cells and we could speculate that pyramidal soma and the inputs from several FS cells work as a unit. Via divergent inputs onto several pyramidal cells, FS cells connect other somatic compartments with each other while at the same time interconnecting with the rest of the interneurons via fast \(\alpha_1\) subunit GABA_\text{A}.
receptors. This microcircuit might be of great importance in generating and maintaining rhythmic activity. Thus somatic compartments of pyramidal cells together with their FS interneurons might be a subnetwork that continuously receives and further transmits the already integrated dendritic inputs. Distal or dendritic regions might be independent of the somatic compartment and “free” to receive and integrate incoming inputs. Their regulation by NFS cells can be brain state (Gilbert and Sigman, 2007) dependent since these cells are less responsive to the fast timed glutamatergic inputs, but more sensitive to the activity of different neuromodulators. During specific sensory processing, the activity of NFS cells can be regulated by strong and slow decaying GABA_A inputs which hyperpolarize these cells and in turn relieve the pyramidal dendrites of inhibition. In addition all the GABAAergic inputs are tightly regulated by mechanism of GABA removal after intense firing and release of neurotransmitters in the brain.

THE ROLE OF INTERNEURONS IN PFC NETWORK DYNAMICS

The prefrontal cortex is involved in the cognitive control of working memory, planning and decision-making (Miller, 2000; Jones, 2002; Dalley et al., 2004; Vertes, 2006; Fuster, 2009). It is suggested that PFC exerts a top-down control over the flow of information leading to selection of relevant stimuli (Miller and Cohen, 2001; Fries, 2009). Signals from several brain areas are received and integrated mostly at the superficial layers of the PFC which in turn feed an output of the processed information to deeper layers which will further input into the regions of the cortex involved in sensory processing and executive functions. The models of top–down attention selection and cognitive control consider that there is a cross-systems interactions (Desimone and Duncan, 1995; Frith and Dolan, 1997; Miller, 2000; Miller and Cohen, 2001; Schall, 2001; Fuster, 2009) besides a single processing stream that would involve only primary somatosensory areas. Functional imaging (Frith and Dolan, 1997; Fuster, 2009) and cellular data (Desimone and Duncan, 1995; Miller, 2000; Miller and Cohen, 2001; Schall, 2001) indicate that top–down influences originate in prefrontal and parietal cortical areas. It is proposed that assemblies of neurons that represent action goals in the prefrontal cortex provide modulatory “bias signals” (Miller, 2000; Miller and Cohen, 2001) to sensory-motor circuits that have to carry out response selection. Thus, prefrontal signals are assumed to exert top–down control over the routing of information flow through specific sensory-motor loops. Reward signals (Schultz, 2000) are thought to gate learning processes that optimize functional connections between prefrontal and lower-order sensory-motor assemblies.

A theoretical model that can explain how top-down influences from PFC can be implemented at cortical level is the so called temporal binding models (Engel et al., 1992; Von der Malsburg, 1994; Roelfsema et al., 1996; Singer, 1999; Engel and Singer, 2001). This model assumes that neural
Synchrony with precision in the millisecond range is crucial for object representation, response selection, attention and sensory-motor integration. Synchronized or correlated neuronal discharge produces a much stronger impact on the neurons in the target areas (Von der Malsburg, 1994) as opposed to the temporally disorganized ones that tend to fail to elicit a significant response (Abeles, 1982; Alonso et al., 1996; Konig et al., 1996). As a consequence, synchrony can enhance response saliency and can select and group subsets of neuronal responses for further joint processing. Therefore, synchronized assemblies of neurons in association cortices would carry an abstract value (such as ordering the sensory representation according to their importance for the task that has to be performed and selecting only the ones that have the highest rank, or value, for further processing) of the representations from primary sensory areas with importance in guiding selective feature representation. Thus, in a goal oriented task, as a result of continuous large-scale interactions (Engel and Singer, 2001; Varela et al., 2001; Von Stein and Sarnthein, 2001) between higher- and lower-order cortical areas only the specific sensory features important for the specific goal will be extracted and further used.

Synchrony may help the integration of information that arises from distributed networks in the brain (Buzsaki et al., 2004c). It is proposed that synchrony can arise via strong common inputs such as feedforward mechanism or via oscillatory mechanism. Both mechanisms are important in sensory processing but the later is the most energy-efficient physical mechanism for temporal coordination (Mirollo and Strogatz, 1990). In cortical networks, a way to implement reliable synchrony is by selectively recruiting inhibition in the network dynamics. Inhibition precisely controls the firing of pyramidal cells and also contributes to rhythmic cortical activity at different oscillatory frequencies (Buzsaki and Draguhn, 2004b) supporting the transfer and processing of information within and between cortical structures (Engel and Singer, 2001).

**FEED FORWARD MECHANISMS**

Computationally there are two ways in which inhibition participate in a network, the feedback or the feedforward configuration. In a feedback inhibitory circuit, increased firing of the pyramidal cell can increase the interneuron firing rate which, in turn, via a feedback connection may decrease the pyramidal cell output. In this way the firing of the pyramidal cells can regulate its own output. In a feed-forward inhibitory configuration, the interneuron and the pyramidal cell receives common input from a different sources. Increased firing of the interneuron, results in the decreased activity of the pyramidal cell and this can substantially increase the temporal precision of firing (Buzsaki, 1984) of the pyramidal cells. Thus the activity of the pyramidal cell initiated by the excitatory input, is reduced quickly by the repolarising or
shunting effect of feed-forward inhibition, thus narrowing the temporal window of discharge probability (Pouille and Scanziani, 2001).

The PFC receives monosynaptic glutamatergic inputs from many brain structures including the mediodorsal thalamus (Krettek and Price, 1977; Giguere and Goldman-Rakic, 1988) and the CA1 area and subiculum of the hippocampus (Jay and Witter, 1991; Hoover and Vertes, 2007) innervating both pyramidal cells and interneurons (Gabbott et al., 2002; Kuroda et al., 2004; Tierney et al., 2004). The synaptic integration depends on the precise spatial and temporal characteristics of synaptic inputs changing with the state of the cortical network (Ho and Destexhe, 2000). Cortical and subcortical inputs communicate differently with excitatory and inhibitory cells in the cortex, providing much stronger and faster excitation to inhibitory interneurons. In chapter 3 and 4 of the thesis we provide several evidences revealing the synaptic mechanisms underling these differences.

Thalamocortical circuits of the primary somatosensory cortex are responsible for the early stages of sensory processing. One important step in achieving this function is coordinating excitation with inhibition which is achieved by a disynaptic feedforward inhibitory circuit in which excitatory fibers diverge onto both interneurons and principal cells (Agmon and Connors, 1991; Swadlow and Gusev, 2001; Wehr and Zador, 2003; Gabernet et al., 2005; Wilent and Contreras, 2005). In primary sensory areas, the feedforward inhibition provided by FS cells is a critical functional property of the thalamocortical microcircuit. As a response to sensory stimuli, thalamic afferents recruit FS cells with high probability, disynaptically inhibiting the pyramidal cells (Agmon and Connors, 1991; Swadlow and Gusev, 2001) and leading to a restriction of the time window for pyramidal cells to integrate EPSPs (Gabernet et al., 2005). In turn this allows high temporal fidelity responses to different stimuli (Pinto et al., 2000; Petersen and Sakmann, 2001; Wilent and Contreras, 2004). Our study found a similar property of the MD inputs into PFC network. Although MD is not activated by sensory stimulation but rather by inputs from higher cortical regions (Guillery and Sherman, 2002; S. Murray and V.A. Casagrande, 2005) the basic synaptic properties of the MD to PFC connections might be similar with those of the primary sensory areas circuits.

There are several elements contributing to the reliable feedforward inhibition of pyramidal cells in response to excitatory synaptic activity, of a disynaptic circuit. Thus, as we show in Chapter 2, excitatory afferents converge onto FS cells with a higher probability than onto other interneurons subtypes (Inoue and Imoto, 2006). Each FS cell also targets a large fraction of the neighboring pyramidal cells (Swadlow et al., 2002; Gabernet et al., 2005; Inoue and Imoto, 2006) where they elicit large unitary inhibitory conductances (Gabernet et al., 2005; Sun et al., 2006). Perhaps more importantly though, single thalamocortical afferents provide much stronger and faster monosynaptic excitation to FS cells than to NFS cells or pyramidal cells (Gibson et al., 1999; Gabernet et al., 2005; Inoue and Imoto, 2006; Cruikshank et al., 2007). Thus a
stimulus of sufficient strength to activate pyramidal cell is also likely to rapidly recruit FS cells, thereby resulting in fast, precisely-timed feedforward inhibition.

The role of receptors mediating excitation is extremely important in controlling and filtering information, thus we further studied and compared excitatory inputs onto electrophysiologically defined FS and pyramidal cells. In addition to their well-known role in synaptic plasticity and excitotoxicity (Choi, 1987; Tymianski et al., 1993; Malinow and Malenka, 2002; Hardingham and Bading, 2010), NMDARs may differentially contribute to postsynaptic integration. In pyramidal cells, synaptic NMDAR-mediated currents prolong EPSPs (Hestrin et al., 1990; Cull-Candy and Leszkiewicz, 2004) specifically at depolarized potentials due to the decrease of the voltage-dependent Mg$^{2+}$ block of the NMDAR channel (Thomson and West, 1986; Forsythe et al., 1988; Jones and Baughman, 1988; Thomson et al., 1988; Thomson, 1997). Together with NMDARs, voltage-dependent conductances act at depolarized potentials, to shape the EPSP kinetics allowing integration of inputs over prolonged time windows in pyramidal cells (Stuart and Sakmann, 1995; Magee, 1998; Fricker and Miles, 2000; Gonzalez-Burgos and Barrionuevo, 2001; Galarreta and Hestrin, 2001a; Rotaru et al., 2007) or in interneurons (Fricker and Miles, 2000; Maccaferri and Dingledine, 2002).

In chapter 3 we revealed that larger and faster responses mediated predominantly by AMPARs account for the excitation of FS cells. Furthermore, although glutamatergic inputs activate NMDARs at both FS and pyramidal cells, the NMDARs at synapses onto pyramidal cells pass more current at physiological potentials. Importantly, significant differences have been described in the synaptic inputs onto FS versus NFS interneurons (Gulyas et al., 1999; Gupta et al., 2000; Porter et al., 2001; Markram et al., 2004; Mátyás et al., 2004) leading to the conclusion that recruitment of FS and NFS is differentially dependent on the network activity level. FS neurons display a remarkably fast synaptic activation (Hu et al., 2010), that precisely follows the presynaptic pyramidal cell activity pattern favoring the integration of coincident inputs (Galarreta and Hestrin, 2001a). This requires short-lasting EPSCs, because long-lasting EPSCs produce spikes during prolonged time windows (Fricker and Miles, 2000; Maccaferri and Dingledine, 2002). Thus a low level of NMDA onto FS cells is consistent with the fast synaptic activation of these cells, because compared with AMPAR-EPSCs, NMDAR-EPSCs typically are long-lasting (Hestrin et al., 1990; Cull-Candy and Leszkiewicz, 2004). As a direct consequence of this differential excitation of FS and pyramidal cells, one can speculate that AMPARs selectively mediate cortical feedforward inhibition, while both AMPARs and NMDARs play a critical role in cortical recurrent excitation.

Although the number of excitatory inputs onto NFS cells is lower then FS cells it has been shown that several subclasses of NFS interneurons have strong synaptic NMDAR currents (Lu et al., 2007; Wang and Gao, 2009). These
cells summate and integrate EPSPs over much greater time windows (Glickfeld and Scanziani, 2006), favoring temporal integration of synaptic inputs.

**OSCILLATORY MECHANISMS**

Oscillations in cortical structures provide temporal windows that could bind synchronously active neuronal assemblies with importance for the representation, processing, storage, and retrieval of information (Buzsaki and Chrobak, 1995; Traub, 1998; Traub et al., 1999; Engel and Singer, 2001). Different classes of oscillations emerge from the dynamic interplay between intrinsic cellular and circuit properties (Llinás, 1988; Hutcheon and Yarom, 2000; Steriade, 2001a; Destexhe and Sejnowski, 2003; Wang et al., 2003; Whittington and Traub, 2003). Whereas the spiking of single cortical principal neurons typically displays Poisson statistics (Bair et al., 1994), their assembly behavior is often characterized by oscillatory properties (Gray et al., 1989b; Destexhe and Sejnowski, 2003; Wang et al., 2003). The cortex uses the inhibitory interneuron “clocking” networks as a specialized mechanism for the grouping of principal cells into temporal coalitions (Buzsaki and Chrobak, 1995; Buzsaki and Draguhn, 2004b). In many systems, electrical coupling by gap junctions assists chemical synaptic signaling in oscillatory synchronization (Draguhn et al., 1998; Gibson et al., 1999; Whittington and Traub, 2003). In the cortex and hippocampus, rhythmic firing is an emergent property of interactions between excitatory cells and inhibitory interneurons (Whittington et al., 2000). Activation of excitatory cells leads to excitation of inhibitory interneurons which then act to inhibit further excitation. As inhibition wears off excitatory cells are free to fire again (Whittington et al., 2000). Thus, inhibitory neurons firing synchronously are effective in defining a window in which the excitatory cells can fire. Excitatory cells and inhibitory interneurons are both important for the stable rhythmic firing patterns (Whittington et al., 2000). Variability in excitatory drive results in variability in the firing rate of pyramidal cells and inhibitory interneurons act together to stabilize a network with firing at variable time windows and rates.

Among the synchronized rhythms in the brain, gamma-band (30-80 Hz) oscillations are present in behavioral states ranging from simple sensory stimulation (Gray et al., 1989b) to attention selection (Fries et al., 2001a; Fries et al., 2001b; Bichot et al., 2005; Womelsdorf et al., 2006) and working memory maintenance (Pesaran et al., 2002). Although the specific gamma-band synchronization can emerge in any network of excitatory and inhibitory neurons, certain basic prerequisites must be fulfilled (Tiesinga et al., 2001; Börgers et al., 2005). Among these requirements, the time constant of synaptic currents mediating the excitation – inhibition neurotransmission is one of the most important determinants of the gamma rhythms (Vida et al., 2006; Bartos et al., 2007). The fast period of the gamma cycle implies the presence of a short window of opportunity for the excitatory neurons to fire when inhibition wears off and the next inhibitory input arrives (Hasenstaub, 2005). In order to reliably
generate this window of opportunity, the phasic excitation of interneurons is thought to be required for gamma oscillations (Hájos and Paulsen, 2009; Whittington et al., 2011). Excitatory inputs must be strong enough to drive inhibitory cells to fire with high precision and low variability which can be achieved by producing a reliable EPSP-spike coupling mechanism implemented via strong predominantly AMPA receptor-mediated glutamatergic inputs onto inhibitory cells. The strongly driven inhibitory cell will provide inhibition to numerous postsynaptic targets including both pyramidal cells and other interneurons cells. The synaptic input from the interneuron to the excitatory neurons must strongly inhibit pyramidal cells, but only for a short period of time, and thus are mediated by GABA_A receptors with fast kinetics as determined by their specific subunit composition (Bartos et al., 2007).

Such “clocking” networks can be brought about by the FS cells specifically because of their anatomical and functional connections with pyramidal cells and other interneurons. Here we describe synaptic properties of the inputs onto FS cells that are consistent with their implication in fast gamma oscillations. Such properties include strong AMPA mediated glutamatergic inputs together with fast GABA_A mediated inhibition. Local synchronization between PFC and the rest of the cortex can be achieved by a small fraction of long-range connections (Braitenberg and Schuz, 1998), which effectively reduces the synaptic path lengths between distant cell assemblies (Buzsaki, 2004a).

Thus we suggest that FS and NFS cells serve significantly different functions. Due to their synaptic properties FS interneurons play an important role in generating and maintaining fast synchronous activity at the level of PFC network. On the other hand, the synaptic properties of the NFS cells allow them to participate in brain functions that require integration of stimuli over longer time periods.

Another possibility is within the FS population there different subsets of FS cells serving different functions like feedforward or feedback inhibition or setting a specific oscillatory rhythm of the large gamma band that includes frequencies between 40 to 80 Hz. Layer specificity might also play an important role in this, such that layer 5 FS cells might be more important in oscillatory rhythms and layers 3 FS cells in feedforward inhibition.

THE ROLE OF GABAERGIC NETWORKS

An intriguing question is why do interneurons need to inhibit their own activity and how is this done? Interestingly, the NFS cells receive a much lower number of excitatory synapses as compared with FS. When we turn our attention to inhibitory inputs we find that NFS and FS cells receive almost identical numbers of inhibitory synapses. This suggests that the ratio of inhibitory to excitatory inputs is much higher in NFS cells compared with FS and therefore we could speculate that the dynamics of FS and NFS cells are different when
they participate in the network activity. The role of interneuron to interneuron signaling has been hypothesized to be important in mediating specific oscillatory activity. Computational models concluded that an interneuron network model based on fast, strong and shunting synapses as well as synaptic delays is an efficient gamma frequency oscillator (Bartos et al., 2007). Experiments showed that FS to FS synapses fulfill all these requirements (Vida et al., 2006) and thus the natural conclusion is that networks of FS are the main oscillator in cortex. The kinetics of GABA_A mediated inhibition is extremely important for this purpose. Thus α1 subunit containing GABA_A receptor mediate fast kinetic and high amplitude Chloride currents that could impose the gamma cycle (Bartos et al., 2007) at the level of FS networks. The question remains whether different GABAergic neurotransmission kinetics in distinct interneuronal networks might be responsible for entraining cortical oscillations at different frequencies. Our data from monkey dorsolateral PFC confirms the fact that fast GABA_A synapses are found onto FS cells as opposed to other physiological subtypes.

If indeed different interconnected networks of interneurons are responsible for imposing specific oscillatory rhythm then based on the fact that different interneurons subtypes synapse on specific dendritic domains one hypotheses is that pyramidal cells compartments have different oscillatory rhythms depending on their functions. Thus the somatic region could be mostly set on gamma frequencies whereas distal ones on slower frequencies. Maintaining this specificity might be very important for the network functions.

**IMPORTANCE OF REGULATING THE AMBIENT LEVELS OF GABA**

As previously discussed, the activity of pyramidal cells inevitably triggers activity of GABAergic cells leading to release of GABA at synaptic cleft which often diffuses in the extracellular space. Ambient levels of GABA activate GABA_A and GABA_B receptors at extrasynaptic sites leading to tonic inhibition (Mody et al., 1994) or at other synapses leading to unspecific phasic activation. When unspecific activation of synapses happens it can lead to changes in network states which in turn affect sensory processing (Gilbert and Sigman, 2007; Petersen, 2007). Neurons and glia have mechanisms of removing synaptically released neurotransmitters in order to decrease unspecific activation of receptors in the brain. For example, synchronous firing of multiple interneurons of the same subtype might happen during specific oscillatory frequencies, resulting into accumulation of GABA from that specific class of interneurons. For example, ambient GABA resulting from firing of FS cells can have different dynamics compared with the one from different NFS cells. The effects of FS cells should be restricted to the somatic compartment of pyramidal cells. On the contrary, in the NFS group, Martinotti cells for example synapse on distal dendrites only. With increased activity that recruits more and more interneurons into the network the compartmentalization of pyramidal cells
might be lost if “cleaning” mechanisms supposed to remove GABA from the extracellular space are not in action. For example, it is possible that GABA released by FS cells to activate GABA receptor not only somatically but also indirectly distally. This is specially the case in the layered cortical regions (as opposed to hippocampus for example) where distal dendrites of cells from deep layers are nearby somatic compartment of cells from outer layers.

Our results suggest that in monkey DLPFC uptake mechanisms mediated by the GAT1-transporter are in place at both proximal and distal compartments of the pyramidal cells. Spillover induced prolongation of IPSP and because the IPSP duration may be critical for the oscillation frequency (Whittington et al., 1995; Traub et al., 1996; Klausberger and Somogyi, 2008; Kramer et al., 2008), a deficit in GAT1 activity could alter the oscillation period, as shown in computational modeling studies (Vierling-Claassen et al., 2008). At distal synapses we found that GAT1 activity regulates the strength and duration of GABA_B receptor mediated IPSPs. Dendritic GABA_B-IPSPs powerfully inhibit dendritic Ca^{2+} spikes (Perez-Garci et al., 2006) and spike back propagation into dendrites (Leung and Peloquin, 2006). Therefore, restricting the actions of GABA to the sites where they should be is extremely important for plasticity also (Kampa et al., 2007).

In the DLPFC of subjects with schizophrenia, GAT1 levels are decreased (Gonzalez-Burgos et al., 2008a) in a subset of GABA neurons (Volk et al., 2001). We can speculate that some of the cognitive deficits present in schizophrenia can thus be due to unspecific activation of GABA receptors (Vierling-Claassen et al., 2008). GAT1 down-regulation in schizophrenia may be a compensatory response (Lewis et al., 2005) following a primary decrease in GABAergic neurotransmission in these patients.

To investigate global brain functions such as behavioral states both simplified preparations and normally operating networks are needed (Steriade, 2001a). One important goal is to apply the information obtained from studies of isolated neurons and simple networks within the context of an intact brain.
References


Coyle JT (2004a) The GABA-glutamate connection in schizophrenia: which is the proximate cause? Biochem Pharmacol 68:1507-1514.


Diamond ME, Armstrong-James M, Budway MJ, Ebner FF (1992) Somatic sensory responses in the rostral sector of the posterior group (POm) and in the ventral posterior medial nucleus (VPM) of the rat thalamus: Dependence on the barrel field cortex. The Journal of Comparative Neurology 319:66-84.


References


Hashimoto T, Volk DW, Lewis DA (2004) GABA Neurons in the Human Prefrontal Cortex. Am J Psychiatry 161:1764-.


References


Lewis DA, Hashimoto T, Volk DW (2005) Cortical inhibitory neurons and schizophrenia. Nat Rev Neurosci 6:312-324.


Liang F, Jones EG (1997) Differential and Time-Dependent Changes in Gene Expression for Type II Calcium/Calmodulin-Dependent Protein Kinase, 67 kDa Glutamic Acid Decarboxylase, and Glutamate Receptor Subunits in Tetanus Toxin-Induced Focal Epilepsy. The Journal of Neuroscience 17:2168-2180.


References


Schmolesky M (2005) The Primary Visual Cortex
In: The Organization of the Retina and Visual System [Internet]. (Kolb H FE, Nelson R, ed): Salt Lake City (UT): University of Utah Health Sciences Center.


Sherman SM, Guillery RW (1998) On the actions that one nerve cell can have on another: Distinguishing the drivers from the modulators. Proceedings of the National Academy of Sciences 95:7121-7126.


Uylings H, van Eden C (1990) Qualitative and quantitative comparison of the prefrontal cortex in rat and in primates, including humans. Prog Brain Res 85:3-17.


Von der Malsburg C (1994) Models of Neural Networks II. In, pp 95-119.


References


Young KA, Manaye KF, Liang C, Hicks PB, German DC (2000) Reduced number of mediodorsal and anterior thalamic neurons in schizophrenia. Biol Psychiatry 47:944-953.


Summary

Prefrontal cortex has important roles in the coordination of incoming sensory and motor information with representations of internal goals and rules. This coordination facilitates a context-dependent behavioral response important for cognitive function like planning, or decision making. Being connected with multiple brain areas prefrontal cortex is capable to gather and process complex information but also to send output electrical signals. These electrical outputs can affect the activity pattern of several brain regions implicated in sensory and motor processing, thus influencing the processing properties of the cells within these areas. To understand how prefrontal cortex contributes to cognitive behavior it is important to understand how different neuronal networks are organized within this brain area. For example, important information converge within layer 3 of prefrontal cortex and is being processed locally before an output is sent out to other regions of the cortex or to deeper layers of prefrontal cortex. Thus the activity pattern of middle layers can reflect computations with roles in cognition.

The activity pattern of neuronal networks strongly depends on two fundamentally different classes of neurons, the glutamatergic pyramidal cells and GABAergic interneurons. Pyramidal neurons represent the majority of neurons and mediate the communication between brain areas, but their activity is controlled by GABAergic interneurons. How interneurons carry out this function within the superficial layers of the prefrontal cortex constitutes the main topic of this thesis.

The temporal regulation of the activity of pyramidal cells is achieved via division of labor among a rich diversity of GABAergic interneuron subtypes. The specific subgroup of interneurons called the fast-spiking (FS) cells, are reliably differentiated from other interneuron subtypes (Non-Fast Spiking; NFS) based on their electrical properties and the presence of the calcium binding protein parvalbumin (PV) which is not contained in any of the NFS interneuron subtypes. The FS PV cells innervate the somatic region of the target pyramidal neurons. Via activation of GABA _A_ receptors, FS cells can establish a “break” on the electrical activity of pyramidal cells. Thus FS cells are capable to exercise precise control of the neuronal output by dictating whether already processed information from the entire dendritic tree of the pyramidal cells can be transmitted to other neurons. Moreover, PV basket cells contact a vast number of postsynaptic cells and are able to temporally regulate the firing of large groups of neurons leading to the establishment of neural oscillations and synchrony within cortical networks with important roles in cognition. On the other hand, many of the NFS interneuron subtypes synapse in close contact to excitatory inputs arriving along the pyramidal dendritic tree, a
location which enables them to regulate synaptic plasticity or integration of inputs from specific brain regions.

In this thesis four specific questions have been addressed, in order to better understand the functions of GABAergic interneurons in neuronal circuits of middle layer PFC. We used electron microscopy and electrophysiology approaches to assess the properties of synaptic inputs onto and from GABAergic interneurons. Thus we investigated the glutamatergic inputs onto interneurons, in the second and the third chapter. In the fourth and the fifth chapter we focused on GABAergic inputs onto pyramidal cells and interneurons. We described several synaptic properties that are important for feedforward inhibition and oscillations which are neural processes implicated in sensory processing and thus in cognitive function.

Glutamatergic inputs that arise from excitatory pyramidal cells in different brain areas are the main ways of depolarizing neurons in a temporally controlled manner. In the second chapter we showed that the mediodorsal thalamus which is the main subcortical area projecting to prefrontal cortex recruits PV interneurons with a higher probability than the other subtypes. Thus the FS cells are the main subtype of interneurons implicated in feedforward inhibition from mediodorsal thalamus. The next chapter focused on the glutamatergic receptors of the excitatory synapses onto FS cells. Glutamatergic synapses are complex structures containing both AMPA and NMDA receptors with different roles in mediating excitatory transmission. We showed that FS cells have a low amount of NMDA receptors at their synapses and thus these cells are recruited via synapses containing mostly AMPA receptors that confer fast activation kinetics.

Activation of GABAergic synapses containing GABA_A and GABA_B receptors is important for controlling the firing pattern of the postsynaptic targets. These postsynaptic targets include both pyramidal cells and interneurons. Intense firing of GABAergic cells generates release of GABA in the extracellular space that may lead to unspecific activation of GABA receptors in the brain. The membrane GABA transporters 1 (GAT1) contributes to the removal of released GABA and thus helps maintaining synapse independence. In the fourth chapter we assessed the role of GAT1 in controlling somatic and dendritic inhibition onto pyramidal cells. We showed that GAT1 is equally important in regulating GABA_A transmission both at somatic and dendritic sites. Moreover, GAT1 has important roles in removing extracellular GABA from dendritic sites where we found a strong GABA_B mediated transmission.

The fifth chapter focused on interneurons to interneuron signaling. It was suggested that networks of interneurons are important in the maintenance of oscillatory rhythms. Moreover, the oscillatory frequency appeared to be influenced by the kinetics of GABA_A mediated transmission between these networks of interneurons. We showed FS cells had faster GABAergic currents...
then NFS cells and this was at least partially dependent on a higher level of α1
GABA_A subunits at their synapses.

Our results reinforced previous conclusions suggesting that within the
large group of interneurons the FS cells are intricately implicated in the activity
of pyramidal cells and other interneurons. These cells are preferentially targeted
by different sources of excitation via fast, predominantly AMPA containing
glutamatergic inputs. Moreover FS cells are interconnected with the rest of the
interneurons via fast α1 subunit GABA_A receptors. This microcircuit made up
of the somatic compartments of pyramidal cells together with their FS
interneurons in which receptors with fast kinetics predominate for both
excitatory and inhibitory synapses might be of great importance in generating
and maintaining fast rhythmic activity such as gamma oscillations with
importance in cognitive behavior. As opposed to FS cells, the NFS subgroup
participates in networks with slower kinetics that may involve computations at
distal dendrites, which currently are less understood.
De prefrontale cortex (PFC) is een hersengebied betrokken bij cognitieve functies als planning, attentie en het maken van beslissingen. In de PFC vindt daarom een afweging plaats van inkomende sensorische en motorische informatie met representaties van interne doelen. De PFC staat in contact met diverse andere hersengebieden, en is daarom in staat om verschillende vormen van informatie te verzamelen en te verwerken, maar ook om output naar deze gebieden te verzenden in de vorm van elektrische signalen. Deze elektrische signalen kunnen activiteitspatronen van hersengebieden die betrokken zijn bij de verwerking van sensorische en motorische informatie beïnvloeden en zo veranderingen teweeg brengen in het gedrag van neuronen in deze gebieden. Om te begrijpen hoe de PFC bijdraagt aan cognitie is het belangrijk een begrip te hebben van hoe de verschillende neuronale netwerken in dit hersengebied zijn georganiseerd. Zo komt er in laag 3 van de PFC bijvoorbeeld belangrijke informatie van verschillende bronnen samen. Deze informatie wordt eerst lokaal verwerkt voordat er output wordt verstuurd naar andere lagen van de PFC of andere corticale gebieden. Activiteitspatronen van deze middelste lagen kunnen daarom een weerspiegeling zijn van computaties die ten grondslag liggen aan of betrokken zijn bij cognitie.

Activiteitspatronen van neuronale netwerken zijn sterk afhankelijk van twee fundamenteel verschillende typen neuronen; de glutamaterge pyramidaalcellen en GABAerge interneuronen. Pyramidaalcellen vormen de meerderheid van de neuronen in ons brein en verzorgen de communicatie tussen hersengebieden, maar hun activiteit wordt gereguleerd door GABAerge interneuronen. Hoe interneuronen in oppervlakkige lagen van de PFC deze controllerende rol ten uitvoer brengen staat centraal in dit proefschrift.

Temporele regulatie van de activiteit van pyramidaalcellen wordt bewerkstelligd door een samenwerking van een rijke diversiteit aan GABAerge interneuron subtypes. De zogeheten “fast-spiking” (FS) cellen vormen een specifieke subgroep van interneuronen die gemakkelijk te onderscheiden zijn van andere, “non-fast spiking” (NFS) subtypes op grond van hun elektrische eigenschappen en de expressie van het calcium-bindende eiwit ‘parvalbumine’ (PV), dat niet aanwezig is in NFS interneuronen. Deze FS cellen synapsen op gebieden rond het soma van pyramidaalcellen. Door GABA<sub>A</sub>-receptoren te activeren kunnen FS cellen de elektrische activiteit van pyramidaalcellen onderbreken. FS cellen zijn dus in staat een sterke controle uit te oefenen over
neuronale output door te bepalen of de informatie die over de hele dendritische boom van pyramidaalcellen binnenkomt wordt doorgestuurd naar andere neuronen. FS cellen hebben bovendien vele postsynaptische targets, waardoor zij in staat zijn het vuren van grote neuronale populaties te reguleren. Er wordt gedacht dat dit de grondslag vormt voor het ontstaan van neuronale oscillaties en synchronie binnen corticale netwerken, wat erg belangrijk is voor cognitie. In tegenstelling tot FS cellen hebben veel NFS interneuron subtypes hun synapsen dicht in de buurt van excitatoire synapsen verspreid door de dendritische boom van pyramidaalcellen, wat hun in staat stelt onder andere synaptische plasticiteit te reguleren en de integratie van inputs van specifieke hersengebieden te beïnvloeden.

In dit proefschrift worden vier specifieke vraagstukken behandeld, die tot doel hebben de rol van GABAerge interneuronen in neuronale circuits van de middelste lagen van de PFC te verduidelijken. Hiertoe maken wij gebruik van electronenmicroscopie en electrofysiologische technieken om de eigenschappen van synaptische inputs naar en van GABAerge interneuronen te bestuderen. Op deze manier hebben wij onderzoek gedaan naar glutamaterge inputs op interneuronen, waarvan de resultaten in hoofdstuk twee en drie worden gepresenteerd. In het vierde en vijfde hoofdstuk richten wij ons op GABAerge inputs naar pyramidaalcellen en interneuronen. Wij beschrijven enkele synaptische kenmerken die belangrijk zijn voor ‘feed-forward’ inhibitie en oscillaties, wat beide neuronale processen zijn die zijn voorgesteld als belangrijk voor het verwerken van sensorische informatie en dus in de uitvoer van cognitieve functies.

Vooral glutamaterge inputs van excitatoire pyramidaalcellen uit verschillende hersengebieden zijn verantwoordelijk voor de wijze waarop neuronen gedepolariseerd kunnen worden op een temporeel gecontroleerde manier. In het tweede hoofdstuk laten wij zien dat het belangrijkste subcorticale hersengebied dat naar PFC projecteert, de mediodorsale thalamus, voornamelijk FS cellen rekruteert. FS cellen vertegenwoordigen daarom de voornaamste bron van feed-forward inhibitie vanuit de mediodorsale thalamus. Het daaropvolgende hoofdstuk richt zich op de glutamaatreceptoren van excitatoire synapsen op FS cellen. Glutamaterge synapsen zijn complexe structuren die zowel AMPA- als NMDA-receptoren bevatten, die beiden een verschillende rol hebben in signaaloverdracht. Wij tonen aan dat FS cellen een relatief kleine hoeveelheid NMDA-receptoren tot expressie brengen, wat betekent dat deze cellen worden gerekruiteerd door synapsen met voornamelijk AMPA-receptoren die een snelle activatie kinetiek hebben.

Door activatie van GABAerge synapsen die GABA_A- en GABA_B-receptoren bevatten kunnen vuurpatronen van postsynaptische targets, zoals pyramidaalcellen en interneuronen, worden gereguleerd. Intensief vuurgedrag van GABAerge cellen leidt tot GABA afgifte in de extracellulaire ruimte en kan zorgen voor specifieke activatie van GABA-receptoren in het brein. De membraan GABA transporter 1 (GAT1) draagt bij aan het verwijderen van vrijgekomen GABA en helpt zo synaptische onafhankelijkheid te waarborgen.
In het vierde hoofdstuk onderzoeken wij de rol van GAT1 in het controleren van somatische en dendritische inhibitie in pyramidaalcellen. Wij tonen aan dat GAT1 even belangrijk is voor het reguleren van GABA_A transmissie in gebieden rondom het soma als in de dendrieten. Bovendien heeft GAT1 een belangrijke rol in het verwijderen van extracellulair GABA van dendritische locaties waar wij een sterke GABA_B-receptor afhankelijke synaptische transmissie vonden.

Het vijfde hoofdstuk richt zich op de communicatie tussen interneuronen. Netwerken van interneuronen lijken belangrijk te zijn voor het onderhouden van oscillatie patronen. Oscillatie frequenties blijken tevens onder invloed te staan van de kinetiek van GABA_A receptor afhankelijke transmissie binnen deze netwerken van interneuronen. Wij tonen aan dat FS cellen snellere GABA-erge stromen vertonen dan NFS cellen, wat voor een deel is toe te schrijven aan een hogere mate van α1 GABA_A-subunit expressie in deze synapsen.

Onze resultaten ondersteunen eerder getrokken conclusies die suggereren dat FS cellen sterk betrokken zijn bij de regulatie van de activiteit van pyramidaalcellen en andere interneuronen. Deze neuronen worden voornamelijk aangestuurd door verschillende bronnen van excitatie via snelle AMPA-receptor gedomineerde glutamaterge inputs. Daarnaast staan FS cellen over en weer in contact met de rest van de interneuronen via snelle α1 subunit bevattende GABA_A-receptoren. Dit microcircuit, bestaande uit de somatische regio’s van pyramidaalcellen en FS cellen met hun receptoren met snelle kinetiek in zowel excitatoire als inhibitoire synapsen, zou van groot belang kunnen zijn voor het genereren en het onderhouden van snelle ritmische activiteit zoals de voor cognitie zo belangrijke gamma oscillaties. In tegenstelling tot FS cellen participeert de NFS subgroep in netwerken met een langzamere kinetiek. NFS cellen zijn waarschijnlijk betrokken bij de tot op heden nog niet goed begrepen computaties die plaatsvinden in distale dendrieten.
I would like to thank to my supervisors, Dr. Susan R. Sesack, Dr. Guillermo Gonzalez-Burgos and Dr. Huibert Mansvelder for offering me excellent training in neuroscience and being inspiring mentors. They graciously guided, my transition from a medical doctor to a doctor of philosophy. Working with each one of them and becoming familiar with their very different personalities as mentors has been a rich experience for me. I value this experience and the relationship with them as much as I value all the life changing events that happened to me so far. My scientific skills and my understanding of science has been strongly shaped by Susan, Guillermo and Huib. I remember a few advises they briefly mentioned to me, like, “your interests in neuroscience are not yet clear to you! Spend more time on this.” or, “when you want to prove something, somehow your experiments will be biased towards finding exactly that. Be careful and critical to your own results!” and, “if you manage to have a good scientific story, you will be worshiped!”

I would also like to thank to those I worked and collaborated with, in Pittsburgh and in Amsterdam. This is a long list of scientists with fascinating characters without whom neuroscience would have never been so interesting.

The research presented here was performed at the University of Pittsburgh, USA and funded by various NIH grants. Many thanks to these two institutions for supporting research and scientists.

Lastly, I should thank Mihai, my life companion, for constantly reminding me about the importance of being “easy-going”.

Acknowledgments
List of publications

Mediodorsal thalamic afferents to layer III of the rat prefrontal cortex: synaptic relationships to subclasses of interneurons. Published in J Comp Neurol. 2005 Sep 26;490(3):220-38. Rotaru DC, Barrionuevo G, Sesack SR. Department of Neuroscience, University of Pittsburgh, Pittsburgh, PA 15260, USA.

Glutamate receptor subtypes mediating synaptic activation of prefrontal cortex neurons: relevance for schizophrenia. Published in J Neurosci. 2011 Jan 5;31(1):142-56. Rotaru DC, Yoshino H, Lewis DA, Ermentrout GB, Gonzalez-Burgos G. Translational Neuroscience Program, Department of Psychiatry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, USA.

GABA transporter GAT1 prevents spillover at proximal and distal GABA synapses onto primate prefrontal cortex neurons. Published, J Neurophysiol. 2009 Feb;101(2):533-47. Epub 2008 Dec 10. Gonzalez-Burgos G, Rotaru DC, Zaitsev AV, Povysheva NV, Lewis DA. Translational Neuroscience Program, Department of Psychiatry, University of Pittsburgh School of Medicine, W1651 Biomedical Science Tower, 200 Lothrop St., Pittsburgh, PA 15261, USA. gburgos@pitt.edu

Functional properties of inhibitory synaptic inputs onto interneurons in the monkey dorsolateral prefrontal cortex
In submission
DC Rotaru, NV Povysheva, AV Zaitsev, DA Lewis, G Gonzalez-Burgos
Translational Neuroscience Program, Department of Psychiatry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, USA

PUBLICATIONS NOT PRESENTED IN THE THESIS

Protracted developmental trajectories of GABAA receptor alpha1 and alpha2 subunit expression in primate prefrontal cortex.
Hashimoto T, Nguyen QL, Rotaru D, Keenan T, Arion D, Beneyto M, Gonzalez-Burgos G, Lewis DA
Department of Psychiatry, University of Pittsburgh, Pittsburgh, Pennsylvania 15213, USA

Interneuron diversity in layers 2-3 of monkey prefrontal cortex.
Zaitsev AV, Povysheva NV, Gonzalez-Burgos G, Rotaru D, Fish KN, Krimer LS, Lewis DA.
Department of Psychiatry, University of Pittsburgh, Pittsburgh, Pennsylvania 15213, USA

Parvalbumin-positive basket interneurons in monkey and rat prefrontal cortex.
Povysheva NV, Zaitsev AV, Rotaru DC, Gonzalez-Burgos G, Lewis DA, Krimer LS.
Department of Psychiatry, University of Pittsburgh, Pittsburgh, Pennsylvania 15213, USA

Dopamine D1 receptor activation regulates sodium channel-dependent EPSP amplification in rat prefrontal cortex pyramidal neurons.
Rotaru DC, Lewis DA, Gonzalez-Burgos G.
Department of Psychiatry, University of Pittsburgh, Pittsburgh, Pennsylvania 15213, USA

Electrophysiological differences between neurogliaform cells from monkey and rat prefrontal cortex.
Povysheva NV, Zaitsev AV, Kröner S, Krimer OA, Rotaru DC, Gonzalez-Burgos G, Lewis DA, Krimer LS.
Department of Psychiatry, University of Pittsburgh, Pittsburgh, Pennsylvania 15213, USA