A distinct GABAerg cell type: studies on the neurogliaform cell in the cerebral cortex

Ph.D. thesis

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Cytoarchitectonics of the neocortex

The human cerebral cortex, the most elaborated living structure, is responsible for higher order brain functions such as perception, cognition and consciousness. It consists of a shell of cells, about 2 mm thick and contains an extraordinary number (approximately $28 \cdot 10^9$) of neurons (Braendgaard et al., 1990; Pakkenberg, 1992), the basic units of the brain, and several times as many neuroglial cells (Pakkenberg et al., 2003). Conventionally it is divided into 6 layers, but in many regions, for example in the visual cortex, there is evidence for more than 6 laminae (Peters, 1984). The study of the laminar organization of these cells in the neocortex began in the early part of the 20th century and became known as cytoarchitectonics. The origin of this field goes back to Meynert (Meynert, 1867), who recognized the difference between the white and the gray matter. Cytoarchitectonic maps were first created by Campbell in England (Campbell, 1905) and by Vogt and Brodmann in Germany (Brodmann, 1903; Vogt, 1903). They divide the neocortex to about 20 different regions. Although many more areas have since been identified, there are three major cytoarchitectural divisions of the neocortex. The coniocortex, or granular cortex of the sensory areas, contains small, densely packed neurons in the middle layers. These small neurons are mainly absent in the agranular cortex of the motor and premotor cortical areas. The third type of cortex has varying populations of granule cells and is called eulaminate or homotypical cortex. It includes much of “association cortex”.

Since the advent of functional imaging studies such as positron emission tomography (PET), functional magnetic resonance imaging (fMRI), and electroencephalography (EEG), there has been a rapid increase in our knowledge of the functional and anatomical map of the human cortex. However, these techniques do not identify the mechanism or neural circuits responsible for these functions. The main challenge is to discover what is actually happening when different regions of the cortex are activated under different sensory or behavioral tasks. Therefore, understanding of the structure and function of the microcircuits of the neocortex and their components with modern electrophysiological and anatomical methods, like applying the patch clamp technique “in vitro” or “in vivo” or using correlated light- and electronmicroscopy for morphological characterization is an important approach.

The Neuron Doctrine

It is difficult to ascertain who first saw a neuron in the cerebral cortex. The early observations on the cortex were made on tissue that was unfixed and poorly stained (Remak, 1841; VonGerlach, 1872). The first effective technique to examine the overall morphology of neurons was applied by Camillo Golgi in 1873 (Golgi, 1873). This particular silver impregnation technique became to be known as "black reaction". Thanks to this method, he was able to provide a thorough and precise description of nerve cells, clearly distinguishing the axon from the dendrites. He supposed that the neurons are connected through cytoplasmic connections (continuity theory) (Golgi, 1886). At the end of the 1880's, Ramon y Cajal was using Golgi's microscopic technique, and began to elaborate his neuron doctrine, stating that individual neurons communicate by contact (contiguity theory). Cajal published the histology of the nervous system with enormous number of reconstruction of several cell types of different brain areas and the dynamic polarization of the neuron. He also suggested the (input)-dendrite-soma-axon-synapse-(output) direction of information flow (Ramon y Cajal, 1894). Golgi, however, did not accept this theory, and a controversy arose between the two scientists that was not put to rest even after the rivals were both awarded the Nobel Prize in 1906 (Berciano et al., 2001). Any lingering doubt was finally resolved with the advent of electron microscopy in the 1950s. The high-magnification, high-resolution pictures obtained with the electron microscope clearly established that nerve cells are indeed functionally independent units communicating with each other through synapses. Modern electrophysiological studies have also confirmed his theory about the spreading of synaptic potentials from the dendrites to the axonal terminals and verify that basic functions of nerve cells are 1: reception of synaptic inputs (mostly in dendrites; to some extent in cell bodies; in some cases on the axons); 2: generation of intrinsic activity at any given site on the neuron through voltage-gated membrane properties and internal second-messenger mechanisms; 3: integration of synaptic responses with intrinsic membrane activity; 4: generation of patterns of impulse discharges primerily in the axon, encoding outputs from cell; 5: distribution of synaptic outputs (mostly from axon arborizations; in some cases from cell bodies and dendrites) (Zigmond et al., 1999).

Classification of the neocortical neurons

Since then – still partly on the basis of Golgi's method - a number of attempts have been made to classify the neurons of the cerebral cortex, but none of the classification schemes has gained wide acceptance. It is generally agreed that morphological separation of cortical neurons into pyramidal and nonpyramidal types is acceptable, but the main problem is how to classify the cell types that are not pyramids, and what names to assign them. Initially they are frequently referred to as stellate cells (Sholl, 1956), but at the end of the XX. century they were preferably called as nonpyramidal cells or interneurons (Jones, 1984; DeFelipe and Farinas, 1992). Nowadays most of the research teams have characterized cortical interneurons using physiological, morphological and molecular features. Ideally, the characterization of a neuron would be complete when information regarding all these three criteria is considered. At the same time, neurons do not have a separate physiology, morphology or molecular biology, these are features of their multidimensional existence created by detection methods. Each investigator may choose a particular method to characterize a cell, but it should not be forgotten that there is only one unitary reality behind it all. In 2005 there was a meeting devoted to this topic in Ramón y Cajal's birthplace, Petilla de Aragón, where the most important laboratories try to standardize the nomenclature of different features of neocortical interneurons (Alonso-Nanclares et al., 2005). They had classified the neurons with numbered terms describing their utmost parameters. These terms are a set of labels which are not exclusive. More than one label from each category can be applied to describe a given neuron. This nomenclature can pave the way for a future classification of neuron types of the cerebral cortex.

Neuronal elements of the neocortex

The principal cells of the neocortex are the pyramidal cells (Zigmond et al., 1999). They are also the numerically dominant cell type of the cortex. In the association cortex pyramidal cells form about 80% of the neurons (Sloper et al., 1979; Winfield et al., 1980). They can be found in all cortical layers except layer I. Their common characteristic features are the spiny dendrites. A typical pyramidal cell has a triangular cell body, a thick, radially oriented apical dendrite, forming a terminal tuft in the most superficial cortical layer and a set of basal dendrites. Despite all of these, the term "pyramidal cells" is referred to a diverse cell group, where these principal cells differ from each other not only by their appearance but by their connectivity as well. Their variability in cell size, dendritic arborization and the presence

of their axonal collaterals depend on the laminar localization of the neuron. Different types of pyramidal neurons with a precise laminar distribution project to different regions of the brain (Jones, 1984). Small sized layer II and layer III pyramidal cells establish corticocortical connections, giant pyramidal cells of the layer V establish connections to subcortical centres as the spinal cord, striatum, thalamus, basal ganglia or the pons. Layer VI pyramidal cells make connections with the thalamus and the claustrum. In addition to the projection to subcortical or other cortical areas, each pyramidal cell has an axon collateral network that forms part of the local circuitry. Layer II pyramidal cells project to layer I-II and layer V. Medium sized, deep layer III pyramidal cells project to layer III and layer V, layer V pyramidal cells project to layer VI and finally, layer VI pyramidal neurons mainly project to layer IV (Zigmond et al., 1999). Using glutamate as neurotransmitter, they are responsible for the majority of excitatory postsynaptic potentials (EPSP) evoked in their postsynaptic cell-pairs. Pyramidal cells mainly innervate dendritic spines of other pyramidal cells and dendritic shafts of aspiny interneurons through asymmetric synapses. No excitatory synapses are made on the somata of pyramidal cells.

Spiny stellate cells cells share many characteristics with pyramidal cells (…in having spiny projections on their dendrites, in release of excitatory neurotransmitter etc.) despite the absence of an apical dendrite. They are located mainly in the fourth cortical layer and they are often the principal target of the thalamic axons. Axons of the spiny stellate neurons are largely intrinsic in their projections establishing connections above and below the lamina of origin (Gilbert and Wiesel, 1979; Tarczy-Hornoch et al., 1999).

Interneurons participate mainly in inhibitory processes and they are responsible for inhibitory postsynaptic potentials (IPSP) evoked on their postsynaptic target cells. They are heterogeneous in many aspects, but have a common feature compared to pyramidal cells, that their axons remain inside the cortex, making short-range projections to pyramidal cells and other interneurons (Buhl et al., 1994; Kawaguchi, 1995; Freund and Buzsaki, 1996). Every fifth cell in the cerebral cortex release γ-aminobutiric acid (GABA) as inhibitory neurotransmitter (Somogyi and Hodgson, 1985), therefore they are commonly referred as GABAergic cells. GABAergic cells have influenced both the origin and the backpropagation of the action potentials, and play an important role in the synchronisation of the activity of different cell-populations.

GABAergic interneurons constitute only a minor fraction of the total number of neurons in the neocortex (15–25%) (Fairen et al., 1984; Peters and Kara, 1987), but are crucial for normal brain function (Berman et al., 1992; McBain and Fisahn, 2001). Despite

their small numbers, these interneurons are extremely diverse in their morphological, electrophysiological and molecular properties (Fairen et al., 1984; White et al., 1989; DeFelipe, 1993; Cauli et al., 1997; Kawaguchi and Kubota, 1997; Gupta et al., 2000), therefore it is possible to classify between them on the basis of these characteristics. Functional characterisation is based on action potential firing patterns (Kawaguchi and Kubota, 1996, 1997; Gupta et al., 2000) and the facilitating or depressing nature of postsynaptic potentials evoked by short trains of presynaptic action potentials (Reyes et al., 1998). Physiological classes could be correlated with certain morphological cell types described in the frontal cortex (figure 1.1) (Kawaguchi, 1993; Kawaguchi and Kubota, 1993, 1996, 1997). Great variety of these cells are also reflected in their complex synaptic relations and their efferent connectivity (Gulyas et al., 1993; Buhl et al., 1994). On the basis of these facts the interneurons could be classified not only by their various cell-type preference (Miles et al., 1996; Cobb et al., 1997), but by their spatially selective innervations of the surface of the postsynaptic cell (Halasy et al., 1996). Another useful characterization of these interneuron subtypes is based on their neurochemical content. Many kinds of calcium-binding proteins exhibit various cell-type preference, like parvalbumin, calretinin and calbindin. The chemical heterogeneity is much more distinct among nonpyramidal cells than pyramidal cells(DeFelipe, 1993; Jones, 1993). In addition, GABAergic cells are characterized by a diversity of morphological forms (Jones, 1975; Fairen et al., 1984; Somogyi, 1989). However, the morphologies of chemically defined subclasses of interneurons have been well identified in only a few cases (Houser et al., 1983; DeFelipe, 1993). Immunoreactivity for neuropeptides, like somatostatin, cholecystokinine, neuropeptide-Y, vasoactive intestinal polypeptide, is also mostly detected in GABAergic cells (Hendry et al., 1984; Somogyi et al., 1984; Rogers, 1992; DeFelipe, 1993).

Diversity of GABAergic neurons

Typical members of the GABAergic cells are the basket cells. The name was given by Ramon y Cajal to a distinct type of cerebellar neurons (Ramon y Cajal, 1911). Their axonterminals surround the somata of the Purkinje cells, creating „baskets" around the cellbodies. Interneurons that form a high fraction of axo-somatic synapses have therefore traditionally been classified as basket cells (Somogyi et al., 1983; Hendry and Jones, 1985).

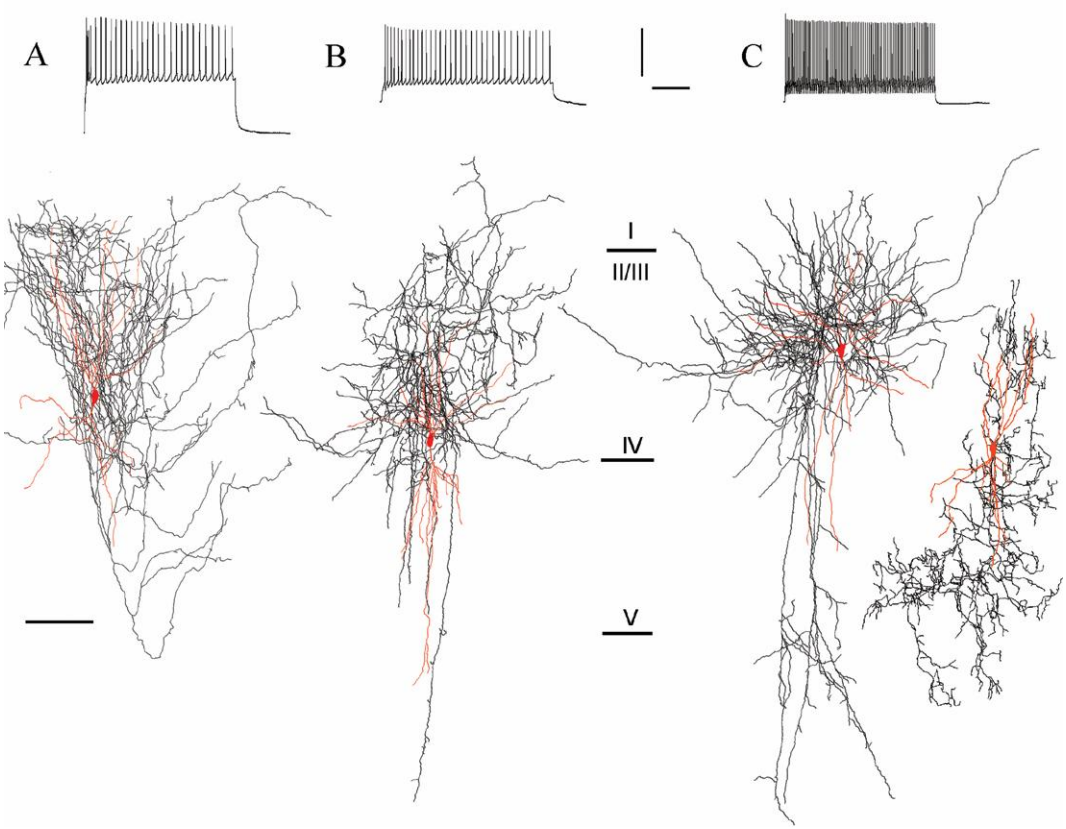

Figure 1.1. **Firing- and anatomical properties of some GABAergic interneurons**
A, Firing properties of a regular spiking/adopting cell. Descending firing frequency reflected by the widening interspike intervals. Underneath the firing properties the morphology of the regular spiking cell can be seen. B, Firing- and anatomical details of a Martinotti cell. The firing propery is similar to the regular spiking cell. C, Large basket cell (left) and axo-axonic cell (right) have high frequency fast spiking firing properties with no acomodation. Scalebar for the anatomical reconstructions: 80 µm. Scalebar for the electrophysiology: 50 mV, 200 ms.

This name in the cerebral cortex denotes a heterogeneous cell population, its members have different firing properties, different axonal arborizations and different neurochemical characteristics (Kawaguchi and Kubota, 1993; Kawaguchi, 1995; Kawaguchi and Kubota, 1997; Kawaguchi and Shindou, 1998). Two unambiguous subclasses of basket cells are the classical large basket cells and small basket cells. In the neocortex, large basket cells are generally large, aspiny multipolar neurons that place ~20–40% of their synapses on target cell somata (Somogyi et al., 1983; Kisvarday et al., 1993). Their axons usually originate from the pial aspect of the soma, and typically ascend to give rise to many long horizontally and vertically projecting axon collaterals that traverse neighbouring columns (generic ~300 µm diameter columns are used in this study to refer to the local micro-circuitry) and can extend through all cortical layers and might mediate the GABAergic effects within the columns (Tamas et al., 1997). Smaller side branches terminate in pericellular baskets around somata and proximal dendrites of neurons (Somogyi et al., 1983; Hendry and Jones, 1985; Kisvarday

et al., 1993). Small basket cells are also aspiny multipolar cells that place 20–30% of their synapses on target cell somata (Fairen et al., 1984; Kisvarday et al., 1985). Their axonal arbours, composed of frequent short, curvy axonal branches, tend to be near their somata and within the same layer. Multipolar neurons with radiating axonal collaterals that are not typical of either the large basket cell or small basket cell morphologies have been noted by several authors (Jones, 1975; Feldman and Peters, 1978; DeFelipe and Fairen, 1982; Lund and Lewis, 1993). These ‘atypical' cells were identified as basket cells because they targeted somata in synaptically coupled pairs, but were considered a separate class of basket cell because some morphological features differed from large basket cells and small basket cells, which led to the name “nest basket cell” (Gupta et al., 2000).

Another special cell type in the cortex is the axo-axonic cell, which exclusively innervates the axon initial segments of the pyramidal cells. The axon has the lowest threshold for action potential generation in neurons; thus, axo-axonic cells are considered to be strategically placed inhibitory neurons controlling neuronal output. 4-8 axo-axonic cells innervate a single pyramidal cell and roughly 300 pyramidal cells are innervated by a single axo-axonic cell (Somogyi, 1977; Somogyi et al., 1982). This special cell type is unique among the GABAergic cells eliciting action potentials on pyramidal cells despite of releasing GABA (Szabadics et al., 2006). Axo-axonic cells can depolarize pyramidal cells and can initiate stereotyped series of synaptic events in cortical networks because of a depolarized reversal potential for axonal relative to perisomatic GABAergic inputs. In addition, there is also emerging recognition of the involvement of this unique cell type in several neurological diseases including epilepsy and schizophrenia (Howard et al., 2005).

Axonal terminals of the so-called dendrite targeting cells or regular spiking/adapting cells mostly end up in dendritic shafts (Tamas et al., 1997), while double bouquet cells form synaptic contacts mainly on dendritic spines. These cells can display bipolar or bitufted dendritic morphology and their radial axons span all layers (Ramon y Cajal, 1904; Peters and Sethares, 1997; Tamas et al., 1998). Martinotti cells establish their synapses on the proximal dendrites of the postsynaptic cells (Tamas et al., 1998).

Discovering the neurogliaform cell

Neurogliaform cells were first described by Ramon Y Cajal at the end of the XIX. Century, in 1899 as *cellule neurogliaforme* (Ramón y Cajal, 1899). He observed small neuroglial cell-like neurons in both the striatum and the II layer of the human cerebral cortex,

though they are found in all layers and are especially common in the deeper layers. He also described them in human visual and auditory cortex and in cat auditory cortex as well (figure 1.2. and figure 1.3.). According to Ramón y Cajal, the neurogliaform cell is very small, even "minuscule" with a "feeble" cell body and a large number of fine, radiating dendrites that are short, varicose, and rarely branched. The axon is extremely thin and shortly after its origin it brakes up into a very dense, highly ramified arborization composed of delicate branches. Later, in his description of the visual cortex, he emphasizes, that the axonal arborization tends to remain within the territory of the dendrites. The short length and lack of lateral branches on the dendrites, coupled with the difficulty of identifying an axon, was what in Ramón y Cajal's eyes caused these neurons to resemble neuroglial cells. "The cells," he says, "are less common in the dog and cat than in men but in those species are remarkable for their larger size and extreme richness of their axonal arborizations" (Peters, 1984). He called these cells as *spiderweb cells* (cellule araneiforme) and *dwarf cells* as synonyms. Following Cajal's last account it seems doubtful that neurogliaform cells were clearly identified for more than half a century. Lorente de Nó, O'Leary and Bishop in the 1940s might drew neurogliaform cells (Lorente de Nó, 1922; O'Leary, 1938; O'Leary, 1941; Lorente de Nó, 1949), but the drawings of the axonal arborizations remained incomplete. The next author who draw attention to these cells was Valvedere, who had likened the axons to a ball of thread and, by analogy, refers to them as *clewed cells* (Valverde, 1971). His description was from the visual cortex of the monkey. In 1975 Jones identified spiderweb cells from the somatosensory and motor cortex of monkey (Jones, 1975). He called them *type 5 cells.*

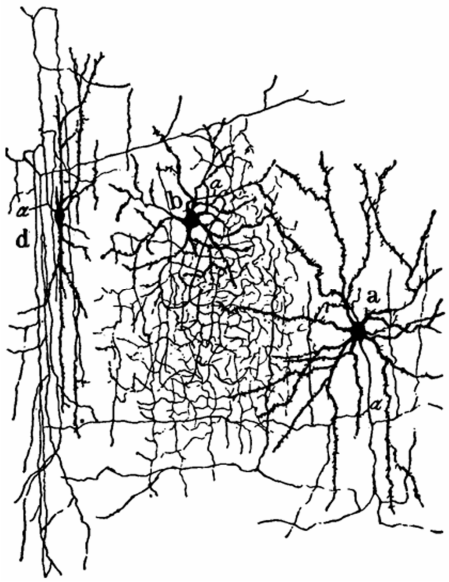

Figure 1.2. **Short-axon neurons drawn by Ramón y Cajal (1911)**
Cell b is called a „dwarf cell" in the legend. This is one of Ramón y Cajal's synonyms for a neurogliaform or spiderweb cell. (Peters, 1984)

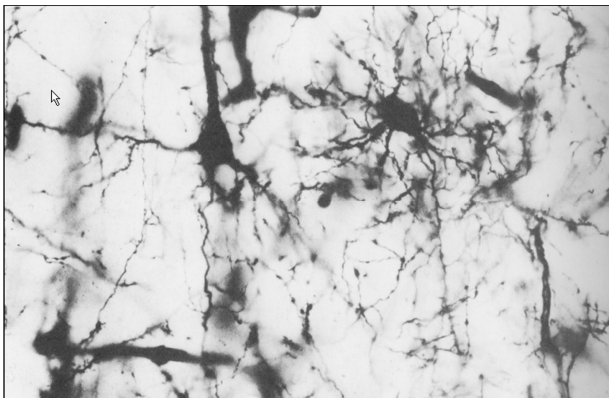

Figure 1.3 **Photomicrograph from one of Cajal's preparations.**
We can see the occipital pole of an eighteen-day-old cat, showing the soma of a pyramidal cell (left) and a neurogliaform cell (right). x 550. (DeFelipe and Jones, 1988)

In the sequel - based on their morphology – neurogliaform cells were identified from several areas of the nervous system: for example from basolateral amygdala of the opossum (Didelphis virginiana) (McDonald and Culberson, 1981), rat visual cortex (Hedlich and Werner, 1986; Hestrin and Armstrong, 1996), rat frontal cortex (Kawaguchi and Kubota, 1997), medial part of the rat prefrontal cortex (Gabbott et al., 1997), rat striatum (Sancesario et al., 1998), guinea-pig (Cavia porcellus) visual cortex (Hedlich and Werner, 1986), cat visual cortex (Naegele and Katz, 1990), primates prefrontal cortex (Gabbott and Bacon, 1996), primate striatum (Fox and Rafols, 1971), and human neocortex (Marin-Pallida, 1985; Kisvarday et al., 1990; Kalinichenko et al., 2006).

As the electrophysiological methods got more sophisticated, more and more cells have been labeled by intracellular dye injections. Under whole cell patch clamp electrophysiology cell types could be separated from each other by the detailed analysis of their membrane- and firing properties (Kawaguchi, 1995). Therefore the electrophysiological properties of the neurogliaform cells could also be examined more easily nowadays (Kisvarday et al., 1990; Kawaguchi, 1993, 1995; Hestrin and Armstrong, 1996; Vida et al., 1998; Tamas et al., 2003; Markram et al., 2004; Krimer et al., 2005; Price et al., 2005; Zsiros and Maccaferri, 2005; Ashwell and Phillips, 2006).

Several efforts have been made towards the neurochemical characterization of the neurogliaform cell. Their GABAergic phenotype were confirmed by several studies. They are shown to be immunoreactive for neuropeptide-Y and partly for neuronal nitric-oxide synthase and cholechystokinin. Moreover much of the neurogliaform cells coexpress an actin binding protein α-actinin 2 (Conde et al., 1994; Kawaguchi and Kubota, 1996; Uylings and Delalle, 1997; Price et al., 2005).

Communication between cortical neurons

To enable communication among at least 28 billion neurons highly sophisticated and efficient mechanisms are needed. Cortical neurons are connected to each other and to subcortical areas by a vast number of synapses (~10^{12}). These chemical synapses[1] (Gray, 1959; Colonnier, 1968) transform all-or-none action potentials of the presynaptic axon into graded responses in postsynaptic neurons. Cortical neurons, therefore, integrate and process signals arriving from lower brain areas and other cortical structures within the framework of their connections. The complexity of cortical operations, therefore, depends both on the specialized characteristics of individual neurons and also on their precisely organized connections. The synaptic connectivity of the neocortex is extremely complex. Each neuron receives 5,000–60,000 synapses (Cragg, 1967; DeFelipe and Farinas, 1992), 70 % of which originate from other cortical areas (Gruner et al., 1974; Ahmed et al., 1994; Gulyas et al., 1999; Megias et al., 2001; Martin, 2002). Neurotransmitters activate receptors composed from various subunits in the postsynaptic membranes. These subunits determine the different effects, kinetics, pharmacological sensitivity of receptors and are expressed in cell-type specific and subcellular domain selective patterns (Hollmann and Heinemann, 1994; Barnard et al., 1998).

At first glance cortical circuits are composed of huge numbers of similarly functioning neurons (Mountcastle, 1997). The classical view considers cortical neurons as integrate-and-fire devices. This allows the temporal summation of synaptic events over temporally extended intervals and the model implies that inputs contribute to output generation regardless of their timing. Information is carried by the rate at which neurons discharge action potentials. This view is supported by in vivo observations showing that the spikes of single pyramidal cells from the motor cortex can evoke whisker movements independent from the ongoing local activity in the cortex. The evoked movement depends on the number and frequency of initiated action potential trains (Manns et al., 2004). The alternative concept on the operation of single neurons in the network is the so-called coincidence detection theory, stating that only synchronous signals contribute effectively to output generation, therefore the number of relevant inputs is small compared to the total number of inputs arriving to a neuron. This model emphasizes the critical role of input timing

[1] Excitatory axon terminals establish Type I. or asymmetric synapses on dendritic spines and shafts, with the characteristic feature of large postsynaptic density. Synaptic vesicles in case of Type I. synapse are tend to be spherical. GABAergic interneurons make Type II. or symmetric synapses primarily on dendritic shafts and somata according to their target specificity. In this case the synaptic vesicles are mostly elongated.

and synchronous oscillatory patterns in cortical information flow (Konig et al., 1996; Stevens and Zador, 1998; Salinas and Sejnowski, 2001). Coincidence detection could be a particularly effective way of enhancing response saliency. Supralinear boosting of input summation by synchronous activity could effectively enhance transmission without raising discharge rates. Thus, synchronization can express unambiguous relations among neurons because it selectively amplifies the impact of synchronous responses (Singer and Gray, 1995). Modeling and experimental results show the existence of stereotyped firing sequences (Prut et al., 1998; Ikegaya et al., 2004) and the ability of cortical networks to propagate such sequences at high temporal fidelity (Reyes, 2003).

Information flow in neuronal networks

Characteristic types of neural population dynamics occur in behaving animals. Neuronal networks in the mammalian forebrain show oscillations in several bands covering frequencies from approximately 0.05 Hz to 500 Hz (Buzsaki and Draguhn, 2004). Different cortical rhythms can be linked to particular behavioral patterns: theta oscillations (4-12 Hz) were recorded in the hippocampal formation of mammals and humans during sensory-motor integration (Buzsaki, 2002; Howard et al., 2003) and in neocortex during memory tasks (Caplan et al., 2001); beta oscillations (15-25 Hz) are related to simple motor tasks (Salmelin et al., 1995; Howard et al., 2003); gamma oscillations (40-80 Hz) occur during processes related to perception, REM sleep and working memory (Bragin et al., 1995; Howard et al., 2003) and 200 Hz sharp-wave ripples can be observed during quiet sleep, consummatory behavior and immobility (Buzsaki, 1986). In general, firing of single neurons is related to the rhythm of the cortical networks, however they fire usually at a lower frequency (Fisahn et al., 1998; Fries et al., 2001). In vivo recordings show that oscillatory activity in the neocortex is associated with rhythmic IPSPs (Steriade et al., 1993; Ylinen et al., 1995). The rhythmic activity of inhibitory networks was proposed to have a crucial role in generating gamma activity in the cortex (Whittington et al., 1995). Single inhibitory inputs are able to generate rhythmic activity by interacting with intrinsic conductances and excitatory inputs in their postsynaptic target cells. Activation of individual GABAergic cells are sufficient to synchronize the firing of their postsynaptic cells providing a temporal reference which could be followed by hundreds of neurons (Cobb et al., 1995). In hippocampal slices, pharmacologically evoked 40 Hz oscillations have been shown to depend on the interaction of neuronal excitatory network and inhibition mechanisms (Fisahn et al., 1998). Recent results

provide another mechanism through which inhibitory cell discharges might be synchronized (Galarreta and Hestrin, 1999; Gibson et al., 1999; Tamas et al., 2000).

GABAergic transmission

Although mechanisms linking oscillating brain states and cellular mechanisms are not clear, recent studies emphasize the importance of GABAergic processes originating from local networks. Both in cases of identified interneuron - interneuron connections and interneuron – principal cell connections the inhibition mediating interneuron groups divide the surface of the postsynaptic target cells differentially (Buhl et al., 1994; Miles et al., 1996; Cobb et al., 1997; Somogyi et al., 1998). Synaptically active GABA is essential for keeping the cortical excitatory processes at a certain level and for specifying the receptive field characteristics of a sensory-cortex neurons. Postsynaptic effects of GABA, which consist of a transitional hyperpolarization of the postsynaptic membrane and the generation of the IPSP in the postsynaptic cell, are mediated by two types of receptors. $GABA_A$-receptors are chloride- and bicarbonate channels with fast activation and decay kinetics. $GABA_B$-receptors activate a potassium conductance in a slower, G-protein mediated process[2]. It is generally accepted, that $GABA_A$-receptors are mainly activated around the soma, while dendritic iontoforetic application of the GABA evokes a multiphasic process (Misgeld et al., 1995). Experiments on hippocampal neurons suggest dendritic positions of the synaptically activated $GABA_B$-receptors, while in the cat visual cortex the dendritic synapses evoked $GABA_A$-receptor mediated IPSPs (Tamas et al., 1997). Responses mediated by $GABA_A$-receptors are not homogenous at all, due to the different combinations of receptor subunits.

Functional impact of different GABAergic synapses depends on the structure and type of postsynaptic GABA receptors. Presynaptic cell type specific variability in subunit composition of postsynaptic $GABA_A$ receptors has been shown previously (Nusser et al.,

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[2] $GABA_A$ receptors are heteropentameric, composed from distinct subunit classes including: α.(1-6), β(1-3),. γ(1-3), π, δ, ε, and θ. The five subunits can combine in different ways to form $GABA_A$ channels, but the most common type in the brain has two α's, two β's, and a γ. The receptor binds two GABA molecules, between an α and a β subunit. The ion channel associated with the receptor is selective for Cl^- anions. When the Cl^- enters into the cell, the influx of chloride ions according to their electrochemical gradient result in either de- or hyper-polarization of the postsynaptic membrane.
$GABA_B$ receptors are G-protein-coupled – metabotropic - receptors that can produce a wide variety of effects including inhibition of adenylate cyclase, inhibition of voltage-gated calcium channels, and activation of inwardly rectifying potassium channels (Kir.3.1/3.2). $GABA_B$ receptors can hyperpolarize neurons postsynaptically, and modulate the release of several neurotransmitters. They are heterodimers consisting of two subunits R1 and R2. Both subunits are required to form fully functional receptors at the cell surface. GBR1 carries the ligand-binding site and GBR2 couples to the G-protein. $GABA_B$ receptors are present both pre- and postsynaptically.

1996; Nyiri et al., 2001). Although postsynaptic $GABA_A$ and $GABA_B$ receptor mediated responses can be evoked in the cerebral cortex by extracellular stimulation or glutamate application (Benardo, 1994), GABAergic interneurons have only been shown to act on postsynaptic $GABA_A$ receptors. The sources of postsynaptic $GABA_B$ receptor mediated responses are still unknown. This issue will be discussed in chapter 5.1.

Gap junctions between neurons

Electrical synapses also establish functional contacts between cortical neurons[3] (Sloper and Powell, 1978; Peters et al., 1991). Electrical coupling by gap junctions is an important form of cell-to-cell communication in early brain development. Whereas glial cells remain electrically coupled at postnatal stages, adult vertebrate neurons were thought to communicate mainly via chemical synapses. There is accumulating evidence that in certain neuronal cell populations the capacity for electrical signalling by gap junction channels is still present in the adult. Connexins (Cx) are the gap junction-forming proteins of vertebrates, and the multigene family comprises at least 14 different Cx (Bennett and Verselis, 1992; Goliger et al., 1996). Of the seven Cx known to be present in the brain, Cx26, Cx32, Cx43 (Dermietzel, 1998), and the recently discovered Cx36 (Condorelli et al., 1998; Sohl et al., 1998) are the most abundantly expressed. The expression analysis of Cx26, Cx32, and Cx43 revealed their differential localization and their unique temporal expression profiles, which correlate with important developmental events such as cell proliferation, migration, and neuronal circuit formation (Nadarajah et al., 1997; Dermietzel, 1998). Compelling evidence for a role for neuronal gap junctions in the mature brain is indicated by the persistent expression of Cx36 in many structures in the adult brain (Condorelli et al., 1998; Sohl et al., 1998; Chang et al., 1999; Priest et al., 2001; Cruikshank et al., 2005). Recent studies indicated that in the somatosensory cortex approximately 40% of parvalbumin immunoreactive neurons are expressed either Cx32 or Cx43, and approximately 50% of parvalbumin immunoreactive neurons express Cx36 (Priest et al., 2001; Fukuda et al., 2006).

[3] Electrical synapses permit direct, passive flow of electrical current from one neuron to another. The membranes of the two communicating neurons come extremely close at the synapse and are actually linked together by an intercellular specialization called a gap junction. Gap junctions connect the cytoplasm of two cells through lot's of precisely aligned, paired hemichannels, the connexons. The elements of these pore forming proteins are the connexins which come together of hexameric complexes. The closely apposed membranes, providing a narrow, 2-3 nm wide cleft. This arrangement has a number of interesting consequences. One is that transmission can be bidirectional; that is, current can flow in either direction across the gap junction, depending on which member of the coupled pair is invaded by an action potential. Another important feature of the electrical synapse is that transmission is extraordinarily fast: because passive currentflow across the gap junction is virtually instantaneous, communication can occur without delay.

The functional significance of neuronal gap junctions in the adult brain still have unresolved questions. In addition to mediating the exchange of metabolic factors, gap junctions may provide means for temporal coordination of neuronal activity (Christie et al., 1989; Mann-Metzer and Yarom, 1999; Yang and Michelson, 2001). Electrical synapses might play a role in neuronal synchrony (Christie et al., 1989; Draguhn et al., 1998) and gap junctional coupling can promote synchronous activity in connections of cortical interneurons (Galarreta and Hestrin, 1999; Gibson et al., 1999; Koos and Tepper, 1999; Tamas et al., 2000; Venance et al., 2000; Cruikshank et al., 2005).

More recently it was found that firing of distinct types of hippocampal interneurons is coupled to stereotyped phases of oscillations (Klausberger et al., 2003; Hajos et al., 2004; Klausberger et al., 2004) suggesting that distinct GABAergic pathways might be involved in the execution of particular tasks of spatiotemporal signal processing. Although the presence of gap junctions (Sloper and Powell, 1978) and different types of connexins (Condorelli et al., 2000; Rozental et al., 2000; Rouach et al., 2002) were shown in the cerebral cortex, direct anatomical evidence for electrical coupling between interneurons has been provided only between parvalbumin immunreactive basket cells in hippocampus (Fukuda and Kosaka, 2000) and neocortex (Tamas et al., 2000; Szabadics et al., 2001; Fukuda and Kosaka, 2003). Direct physiological and anatomical evidence will be provided for dendro-dendritic and somato-dendritic gap junctions between neurogliaform cells and between neurogliaform cell and distinct types of GABAergic interneurons in chapter 5.2.

This thesis studies the role of a special type of GABAergic interneuron, the neurogliaform cell in the mammalian neocortex. Although detailed morphological and electrophysiological properties of neurogliaform cells have been revealed earlier, the function of this cell type in the neocortical microcircuits remained unclear.

Several studies adressed the chemical connections between GABAergic interneurons and pyramidal cells showing that interneurons might evoke fast or/and slow inhibitory postsynaptic potentials from their postsynaptic target cells. The anatomical background of the fast inhibition have been widely examined but the sources of the slow inhibition remained elusive.

Accordingly, our first major aim is the following:

1. What is the role of the neurogliaform cells in the slow neocortical inhibition?

Electrical synapses between neocortical interneurons is firmly established by anatomical studies and electrophysiological experiments. Electrical coupling between neocortical interneurons shows high specificity: GABAergic neurons establish electrical coupling almost exclusively with interneurons of the same type, but the position of the neurogliaform cell is not clear in gap junctionally linked networks.

This second part of our experiments focused on the following question:

2. Do neurogliaform cells establish electrical synapses with other types of GABAergic interneurons?

Whole-cell patch-clamp recordings and electrophysiological data analysis were performed by other members of the group: János Szabadics, Szabolcs Oláh, Gábor Molnár and Gábor Tamás. For chapter 5.1 János Szabadics and Gábor Tamás performed the electrophysiological experiments and Andrea Lörincz made the correlated electron microscopy. I performed the three dimensional light microscopic reconstructions and the synaptic mapping of the neurogliaform cells. I also contribued to the random sampling of postsynaptic targets of neurogliaform cells. For chapter 5.2 I performed the three-dimensional reconstructions – both under light microscope and on the basis of electron microscopic serial sections – of interneurons and the correlated light- and electron microscopic analysis of GABAergic synapses and gap junctions.

Electrophysiology

All procedures were performed with the approval of the University of Szeged and in accordance with the National Institutes of Health Guide to the Care and Use of Laboratory Animals. Young (P16–P35) Wistar rats were anaesthetized by i.p. injection of ketamine (30 mg per kg) and xylazine (10 mg per kg), and following decapitation coronal slices (350 μm) were prepared from their somatosensory cortex. Slices were incubated at room temperature for 1 h in a solution composed of 130 mM $NaCl_2$, 3.5 mM KCl, 1 mM NaH_2PO_4, 24 mM $NaHCO_3$, 1 mM CaCl, 3 mM $MgSO_4$, 10 D(+)-glucose, saturated with 95% O_2 and 5% CO_2. The solution used during recordings differed only in that it contained 3 mM $CaCl_2$ and 1.5 mM $MgSO_4$. Whole cell patch clamp recordings were obtained at ~32°C from concomitantly recorded pairs triplets or quadruplets of layer II/II putative interneurons and/or pyramidal cells visualized by infrared differential interference contrast (DIC) videomicrospcopy (Olympus BX60WI microscope, Hamamatsu CCD camera). Micropipettes (5–7 MΩ) were filled with 126 mM K-gluconate, 4 mM KCl, 4 mM ATP-Mg, 0.3 mM GTP-Na_2, 10 mM HEPES, 10 mM creatine phosphate and 8 mM biocytin at pH 7.25 and 300 mOsm. Signals were recorded in fast current clamp or whole cell mode (Luigs & Neumann Infrapatch set-up and two HEKA EPC 10/double patch-clamp amplifiers) and were filtered at 5 kHz, digitised at 10 kHz and analysed with PULSE software (HEKA, Lambrech/Pfalz, Germany). Presynaptic cells were stimulated with brief (2 ms) suprathreshold pulses at > 90 s intervals to avoid exhaustion of transmission, other cell types were stimulated at 0.1 Hz. We applied the same paradigm throughout the study for consistency. Postsynaptic cells were held at –51 ± 4 mV membrane potential. Unless specified, traces shown are averages of 30-200 episodes. The amplitude of postsynaptic events was defined as the difference between the peak amplitude and the baseline value measured prior to the postsynaptic potential onset. Firing-probability plots were constructed from 50–100 consecutive trials as follows: within a given cycle (interval separating two presynaptic action potentials), postsynaptic spike latencies were measured from the peak of the preceding presynaptic action potential. Subsequently, unless otherwise specified, the data were pooled regardless of the position in the presynaptic spike train. Controls were collected before the onset of the presynaptic spike train using an identical

cycle length. Data are given as mean ± s. d. Mann-Whitney U-test and Friedman-test was used to compare datasets, differences were accepted as significant if $p \leq 0.05$.

Testing the electrical synapse

Electrical synapses were tested by spike transmission or by injection of hyperpolarizing pulse (100 pA) into the presynaptic cells. Those methods were carried out in both direction and coupling ratio was determined dividing the amplitude of the postsynaptic voltage changes by the amplitude of the presynaptic voltage responses. Neurogliaform cells are very sensitive to frequent stimulation; therefore they were stimulated only in every 2 minutes.

Histological processing of biocytin filled cells

Histology and anatomical evaluation were performed as described in Tamas et al. 1997. Depolarizing current pulses employed during recording resulted in an adequate filling of neurons by biocytin. Slices were sandwiched between two Millipore filters to avoid deformations and fixed in 4% paraformaldehyde, 1.25 % glutaraldehyde and 15 % picric acid in 0.1 M phosphate buffer (PB) (pH 7.4) for at least 12 hours. After several washes in 0.1 M PB, slices were cryoprotected in 10%, then 20 % sucrose in 0.1 M PB. Slices were freeze-thawed in liquid nitrogen, then embedded in 10 % gelatine. 300 µm thick slices embedded in gelatine blocks were resectioned at 60 µm thicknesses. Sections were incubated in avidin-biotin-horseradish peroxidase (ABC; Vector Labs) complex made in TBS (1:100, pH 7.4) at 4° C overnight. The enzyme reaction was revealed by 3'3-diaminobenzidine tetrahydrochloride (0.05 %) as chromogen and 0.01% H_2O_2 as oxidant. Sections were postfixed with 1 % OsO_4 in 0.1 M PB. After several washes in distilled water, sections were stained in 1 % uranyl acetate, dehydrated in ascending series of ethanol. Sections were infiltrated with epoxy resin (Durcupan, Fluka) overnight and embedded on glass slices.

Anatomical evaluation of recovered cells

Light microscopic investigations

Three-dimensional light microscopic reconstructions of recovered cells were carried out using Neurolucida (MicroBrightfield, Colchester, VT) with 100x objective from the 60 µm thick serial sections. Dendrogram constructions, synaptic distance measurements were aided by Neuroexplorer (MicroBrightfield) software. Synaptic distances were measured along

the dendrites of the postsynaptic cell. In case of the dendrograms, the perpendicular line symbolize the postsynaptic cell body and the horizontal lines represents the dendrites emerging from the postsynaptic soma. Close appositions were marked with triangles. The entire somatodendritic surface of recorded cells was tested for close appositions with filled axons or filled dendrites, each of which was traced back to the parent soma. Representative reconstructions were made from the fully recovered neurogliaform cells.

Electron microscopic investigations

Since the resolution of light microscope (200 nm) is not sufficient for the precise identification of synaptic connection, all of the mapped close appositions were tested in the electron microscope. Light micrographs taken from each close apposition in different focal depth were used for the exact identification of these presumed synaptic connections under electron microscope. Blocks containing the cells were cut out from the sections on slides and reembedded. 70 nm serial sections were cut with an ultramicrotome (Leica Ultracut R; Leica Microsystems, Vienna, Austria and RMC MTXL; Boeckeler Instruments, Tucson, Arizona) and mounted on Pyeloform-coated copper grids and stained with lead citrate (EM Stain; Leica Microsystems). Light microscopically detected presumed synapses were checked on the ultrathin sections in electron microscope (TEM, Philips).

We used the following criteria for the identification of chemical synapses: 1, accumulation of synaptic vesicles in the presynaptic terminal; 2, rigid membrane apposition between the pre- and postsynaptic membranes, with the characteristic widening of extracellular space (synaptic cleft). Direct membrane appositions alone didn't predict the presence of synaptic junction.

Gap junctions were identified as follows: membranes of the coupled and stained cells had to be arranged in parallel. The distance between the two electron-lucent stripes was 7-9 nm. 3D electron microscopic reconstructions were made with the help of Reconstruct software which was created with funding, in part, from the National Institutes of Health and the Human Brain Project (Fiala, 2005).

Random sampling of postsynaptic targets

Axon rich areas, including all layers covered by the axonal field, were cut out from sections on the slide and re-embedded for ultrathin sectioning. 70 nm serial sections were scanned under electron microscope and biocytin filled axon profiles were followed until they established synaptic connections with unlabelled postsynaptic profiles. Since all profiles were

followed and the plane of the section randomly cuts through axonal branches, the above procedure ensured a random sample of postsynaptic targets. Tracing of serial sections were also used for the identification of postsynaptic target. Dendritic spines contain smooth endoplasmic reticulum or as called spine apparatus, but lack in the most cases mitochondria. Postsynaptic profiles containing mitochondria and/or microtubules were identified as dendritic shafts.

5.1. Identified sources and targets of slow inhibition in the neocortex

Introduction

Gamma aminobutyric acid is the major inhibitory transmitter in the cerebral cortex (Krnjevic, 1967). Extracellular stimulation of afferent cortical fibers elicits biphasic IPSPs in cortical cells. The early phase is due to the activation of $GABA_A$ receptors producing a Cl^- conductance, the late phase is mediated by K^+ channels linked to $GABA_B$ receptors through G-proteins (Dutar and Nicoll, 1988; Mody et al., 1994; Misgeld et al., 1995; Barnard et al., 1998; Bowery et al., 2002). Although dual recordings revealed several classes of interneurons evoking fast, $GABA_A$ receptor mediated responses in the postsynaptic cells, it is not clear whether distinct groups of inhibitory cells are responsible for activating $GABA_A$ and $GABA_B$ receptors. GABAergic neurons terminate on separate subcellular domains of target cells (Freund and Buzsaki, 1996; Somogyi et al., 1998), and several studies suggested that dendritic inhibition is mediated by $GABA_B$ receptors and possibly by a discrete group of interneurons (Lacaille and Schwartzkroin, 1988; Benardo, 1994) that can modulate dendritic excitability (Larkum et al., 1999). IPSPs with similar kinetics to $GABA_B$ receptor mediated responses were produced by interneurons possibly targeting the dendritic regions in the hippocampus (Lacaille and Schwartzkroin, 1988), but other experiments provided evidence for pure $GABA_A$ responses evoked on dendrites (Buhl et al., 1994; Miles et al., 1996; Thomson et al., 1996b; Gupta et al., 2000). Moreover, it was suggested that repetitive firing of interneurons and/or cooperation of several interneurons is necessary for the activation of $GABA_B$ receptors (Mody et al., 1994; Thomson et al., 1996b; Kim et al., 1997; Thomson and Destexhe, 1999) possibly by producing extracellular accumulation of GABA to levels sufficient to activate extrasynaptic receptors (Dutar and Nicoll, 1988; Isaacson et al., 1993; Destexhe and Sejnowski, 1995; Thomson et al., 1996b; Kim et al., 1997; Thomson and Destexhe, 1999). Here we report that single action potentials evoked in a class of cortical interneuron are sufficient to elicit combined $GABA_A$ and $GABA_B$ receptor mediated responses and determine the site of postsynaptic action as dendritic spines and shafts.

Results

Whole cell recordings with biocytin filling from synaptically coupled pairs of three types of presynaptic interneuron and postsynaptic pyramidal cells, combined with correlated light and electron microscopy, were performed (Tamas et al., 2000). Studies on the localization of $GABA_B$ receptors indicated a gradient-like immunoreactivity for $GABA_B$ receptors with stronger labelling in the upper layers (Lopez-Bendito et al., 2002), therefore we tried to identify the sources of slow inhibition in layers 2-3 of rat somatosensory cortex. Neurogliaform cells (NGFCs, n = 67) were identified based on late spiking firing pattern and their axonal and dendritic morphology (Ramon y Cajal, 1904; Valverde, 1971; Jones, 1975; Kisvarday et al., 1990; Hestrin and Armstrong, 1996; Kawaguchi and Kubota, 1997). (figure 5.1.1. A, D and figure 5.1.2)

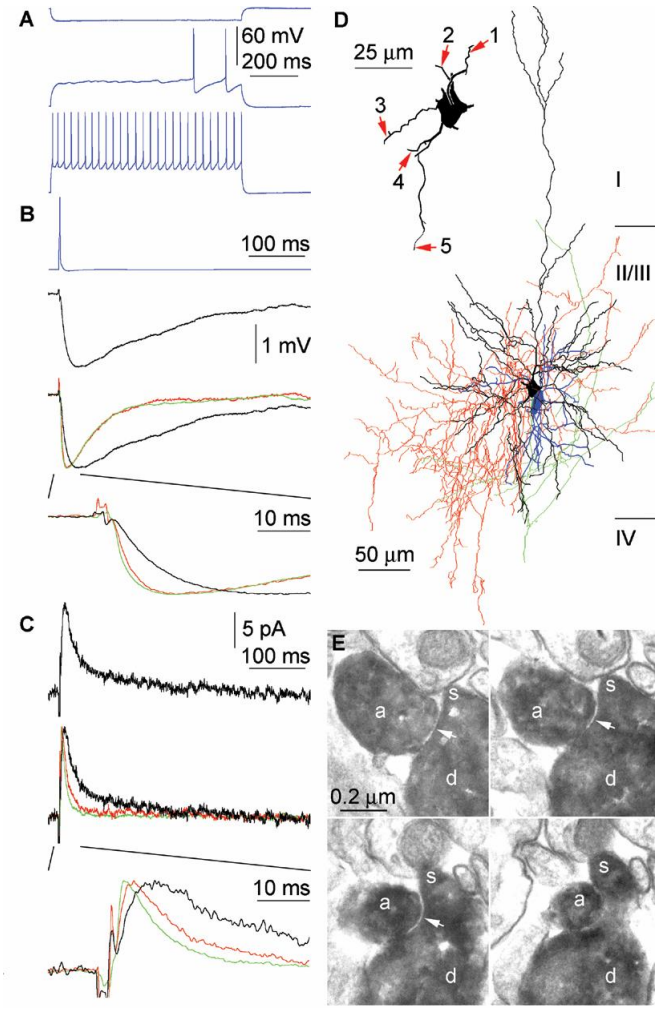

Figure 5.1.1. **Electrophysiology and anatomy of neurogliaform cell to pyramidal cell connections.**

A, Responses of a neurogliaform cell to hyperpolarizing (top) and depolarizing (middle and bottom) current steps. **B, C**, A single action potential (blue) elicited in a neurogliaform cell evokes IPSPs (black) in the postsynaptic pyramidal cells (average of n = 54 paired recordings). Superimposed traces comparing fast and slow IPSPs (B) and IPSCs (C) from basket cell to pyramid (green, average of n = 19 pairs) and Martinotti cell to pyramid (red, average of n = 15 pairs) with the neurogliaform to pyramid connections at –50 mV membrane potential. The expanded timescale of the bottom panels reveals the differences in activation kinetics. **D**, Reconstruction of a neurogliaform cell (soma and dendrites, blue, axon red) to pyramid cell (soma and dendrites, black, axon green) connection. Inset, number and position of electron microscopically verified synapses mediating the connection. Cortical layers are indicated on the right. **E**, Serial electron microscopic sections of a synaptic junction (arrow, corresponding to number 1 on panel D) established by the axon of the neurogliaform cell (a) targeting the base of a spine (s) emerging from a dendritic shaft (d) of the pyramidal cell.

Basket cells (n = 19) showed fast spiking firing pattern, received depressing unitary EPSPs arriving from pyramidal cells (n = 5), immunoreactivity for parvalbumin (n = 4 out of 4 tested) and they preferentially innervated postsynaptic somata (31 %), dendritic shafts (66 %)

and occasionally spines (3 %). Martinotti cells (n = 15) responded to depolarizing current pulses with a so-called low-threshold spiking firing pattern (Kawaguchi and Kubota, 1997; Reyes et al., 1998), received facilitating EPSPs from neighboring pyramidal cells (n = 3), placed their synapses onto dendritic shafts and spines (74 %; 26 %; n = 45), and, when tested, contained somatostatin (n = 4). Postsynaptic potentials in pyramidal neurons elicited by NGFCs showed slower ($p < 0.001$, Mann-Whitney test) 10-90 % rise times (23.4 ± 9.87 ms, n = 38) when compared to IPSPs due to basket (5.8 ± 2.0 ms, n = 19) or Martinotti cell (6.5 ± 1.7 ms, n = 15) activation (figure 5.1.1. B). The decay of NGFC to pyramid IPSPs could not be fitted with single or double exponential functions, therefore we have measured the half-width of IPSPs for statistical comparison and found that NGFC to pyramid IPSPs were significantly longer ($p < 0.001$; 183.9 ± 82.5 ms, 61.3 ± 16.3 ms, and 58.9 ± 17.9 ms for NGFC, basket and Martinotti to pyramid connections, respectively). Repeating the experiments holding the postsynaptic pyramidal cells in voltage clamp mode confirmed the conclusions of voltage recordings (figure 5.1.1. C).

Somata of the NGFCs (n=24) are small and often spherical, diameter: 13 ± 2.8 µm, (figure 5.1.2).

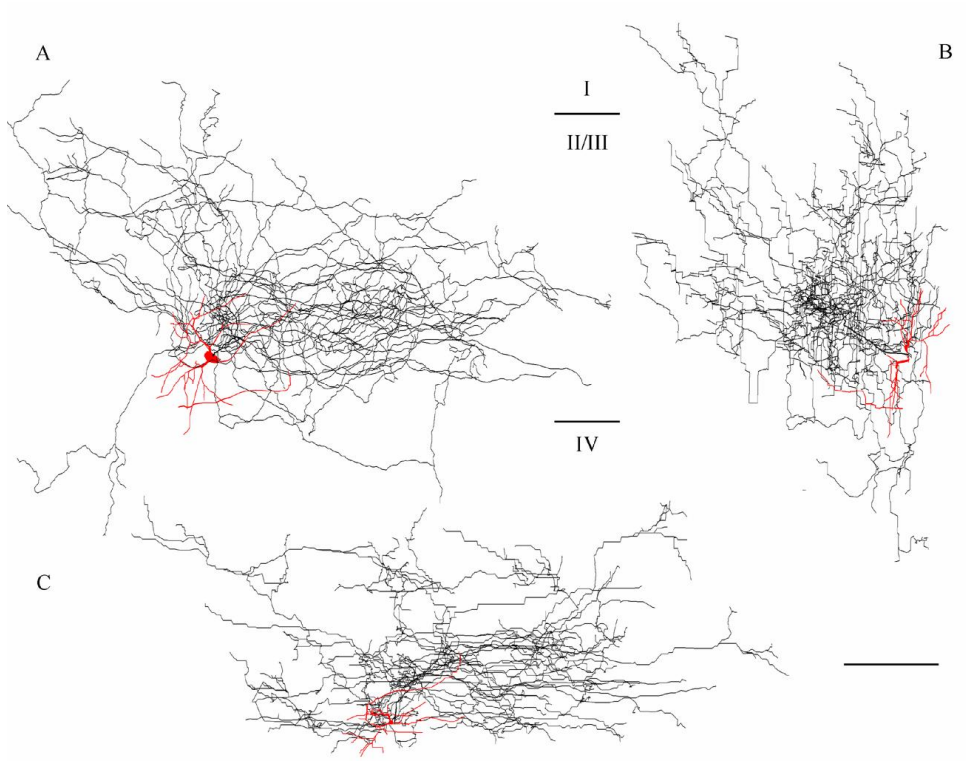

Figure 5.1.2. **Three dimensional light microscopic reconstruction of a NGFC from the rat somatosensory cortex.**
A. Coronal view of the NGFC. Soma and dendrites are red, the axon is grey. **B**. NGFC from sagital view. **C**. NGFC from the pial surface. Neocortical layers are indicated in the middle. Scale bar: 50 µm.

Seven or nine thin dendrites radiate out from the soma forming an almost symmetrical spherical dendritic field (86 ± 24 µm in diameter). Dendrites are usually beaded and rarely borne spines. The axon may arise from any part of the soma and, after the third or fourth ramification, it branches out into extremely thin and dense axonal collaterals enmeshing the space around the soma. The branches suffer very little reduction in diameter so that the plexus has a homogenous appearance. The axonal cloud, 360 ± 39 µm in diameter, in almost every case remains in the same layer as the neurogliaform soma and rarely extend over the neighbouring layers. The neurogliaform axon collaterals are densely packed with small boutons compared to the large axonal terminals of a basket- or regular spiking (adapting) cells (figure 5.1.3.). Three dimensional reconstructions were made on NGFC axonal segments on the basis of electronmicroscopic serial sections. By the measurements on these reconstructions the *en passant* boutons were 400-800 nm in diameter and they followed each other on the axon collaterals in 3 – 8 µm.

Figure 5.1.3. **Comparison of the axons of several types of GABAergic interneurons.** Left: basket cell axon with large boutons; middle: neurogliaform axon with very small boutons; right: axons of regular spiking (adapting) cells with rarely placed middle sized boutons. Scale bar: 5 µm.

Random electron microscopic sampling of postsynaptic targets showed that NGFCs predominantly innervated dendritic spines (71 %) and shafts (29 %, n = 65 target profiles; figure 5.1.4) (Kisvarday et al., 1990; Kawaguchi and Kubota, 1997). Three dimensional light microscopic mapping of NGFC to pyramid connections (n = 8) confirmed the results of

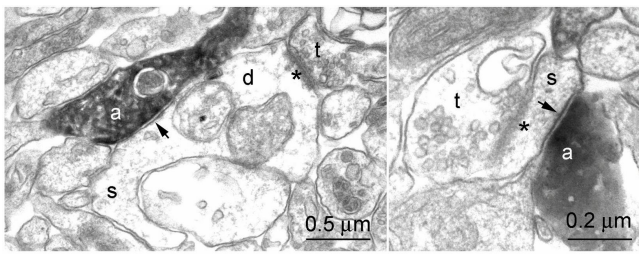

Figure 5.1.4. **Two examples of random electronmicroscopic samples of targets postsynaptic to NGFCs.** Labeled neurogliaform axons (a) form synapsis (arrows) on a dendrite (d) at the base of a spine (s, left) and on a spine head (s, right). Asterisks mark asymmetrical synapses established by unidentified terminals (t).

random electron microscopic analysis of targets postsynaptic to NGFCs and predicted 10 ± 6 synapses on dendritic spines and shafts of pyramidal cells at distances 62 ± 28 µm from the somata. Full electron microscopic analysis of all light microscopically mapped synapses was

performed on a randomly selected pair and revealed one synapse on a dendritic spine neck, three on spine heads and one on a dendritic shaft 63 µm ± 27 µm (25-92 µm) from the soma (figure 5.1.1. D, E).

Pharmacological analysis of NGFC to pyramid interactions revealed that these IPSPs were composed of two components (n = 18, figure 5.1.5 A-B). The early component could be blocked by bicuculline (10 µM, n = 10) indicating the involvement of $GABA_A$ receptors in the transmission (figure 5.1.5 A). Bicuculline blockade alone never abolished the response completely and revealed a residual slow component of neurogliaform IPSPs. This late component could be blocked by further addition of the $GABA_B$ receptor antagonist CGP35348 (60 µM) indicating the involvement of $GABA_B$ receptors in the postsynaptic response. The presence of a postsynaptic $GABA_B$ receptor mediated slow component was confirmed by experiments in which the decay of NGFC to pyramid IPSPs was reversibly shortened by CGP35348 (n = 8, figure 5.1.5 B). Furthermore, the early component was absent at -72 ± 1 mV (n = 8), the calculated reversal potential for chloride ions (figure 5.1.5. C), therefore chloride passage through $GABA_A$ receptors was responsible for the early phase in agreement with the bicuculline blockade.

The compound IPSPs were highly sensitive to the firing rate of the presynaptic neurones and this could explain why the sources for slow inhibition remained obscure up to date. Temporal dynamics of the neurogliaform IPSPs were first tested by activating the presynaptic cells with single action potentials delivered at various intervals and stable amplitude of postsynaptic responses could only be achieved if the interval between presynaptic spikes was more than 1.5 minutes. Accordingly, all single action potential evoked responses for kinetics, pharmacology and reversal potentials detailed above were collected at especially low presynaptic firing rates (one spike in 100-120 s). When activating the presynaptic NGFCs with trains of action potentials at 40 Hz, the amplitude of postsynaptic responses decreased rapidly (figure 5.1.5 D). Even at a train interval of 4 minutes (n = 7), postsynaptic responses showed rapid decrease in amplitude resulting in complete loss of response after five to eight presynaptic spike trains. After total exhaustion, recovery of IPSP amplitude was tested with a single presynaptic spike in every 15 minutes and showed recovery in all cases. The recovery was initially detectable after 15-45 minutes and reached 31-79 % of control amplitude as measured after 90 minutes of exhaustion indicating that the synapses remained functional.

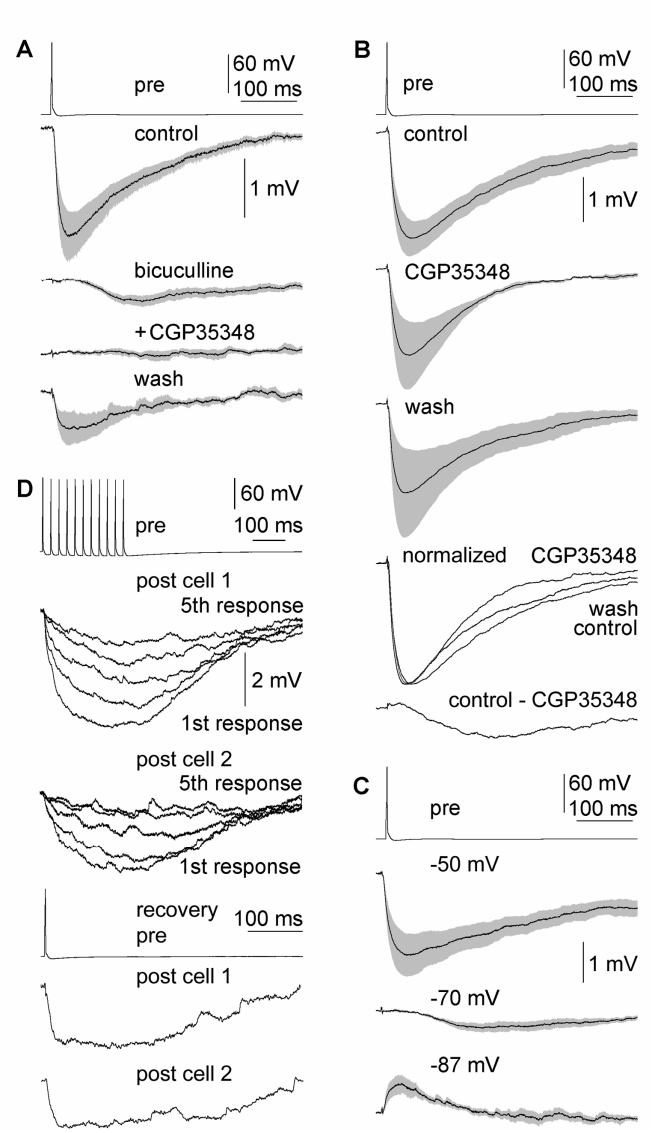

Figure 5.1.5. **Pharmacology of neurogliaform cell to pyramidal cell connections.**

Traces show averages ± s.e.m. /gray/ of several pairs. **A**, The initial component of control IPSPs (n = 10) elicited by single presynaptic action potentials (top) was blocked by bicuculline (10 µM) and the late phase of IPSP was abolished by the subsequent addition of CGP35348 (60 µM). The IPSPs showed recovery after 30 minutes of washout. **B**, The decay of the IPSPs (n = 8) evoked by single spikes in neurogliaform cells (top) could be shortened by application of CGP35348 (60 µM) and this effect could be partially reversed by returning to the control solution. Superimposed traces are shown normalized to the amplitude of control IPSPs (bottom). **C**, Voltage dependency of the unitary neurogliaform to pyramid IPSPs (n = 6) recorded at –50 and –72 mV membrane potential. The early phase shows a reversal potential of –72 mV, but the late phase remains identifiable. **D**, Rapid, use-dependent exhaustion of NGFC to pyramid connections demonstrated by a triple recording with a single presynaptic and two postsynaptic cells. Top, the first five consecutive postsynaptic responses (single sweeps) to presynaptic spike trains elicited once in four minutes. Bottom, single postsynaptic responses to a presynaptic spike after an inactive period of 30 minutes.

Discussion

Our results provide evidence that slow, $GABA_B$ receptor-mediated IPSPs arrive from unitary sources in cortical networks. We identify the first cell type, NGFCs, which consistently recruit postsynaptic $GABA_B$ receptors in addition to $GABA_A$ channels. Synapses of neurogliaform cells appear specialized for sparse temporal operation tuned for long-lasting metabotropic effects. Although it was suggested that in some interneuron to pyramidal cell connections, repeated presynaptic activation might be necessary to recruit slow inhibition (Thomson et al., 1996a; Thomson and Destexhe, 1999), single action potentials at very low firing rates are sufficient to elicit the metabotropic $GABA_B$ component. GABA uptake mechanisms powerfully remove the transmitter from the extracellular space within a distance

restricted to about a micrometer from the release sites (Overstreet et al., 2000), therefore our results suggest that postsynaptic $GABA_B$ receptors could be spatially associated with the synapses formed by NGFCs. Electron microscopic studies could reveal extrasynaptically placed $GABA_B$ receptors on dendritic spines and shafts (Fritschy et al., 1999; Li et al., 2001; Kulik et al., 2002; Lopez-Bendito et al., 2002), but a possible synaptic enrichment of these receptors remains to be determined.

We show that action of NGFCs is predominantly targeted to site of the anatomically defined biochemical and functional compartments of pyramidal cells, the dendritic spines. The slow rise times of NGFC to pyramidal cell IPSPs and IPSCs might also support spines as targets reflecting the filtering effect of the spine necks but, alternatively, $GABA_A$ receptor subunit composition could also influence activation kinetics (Pearce, 1993; Banks et al., 1998). Although we cannot rule out that neurogliaform synapses on dendritic shafts and spines act through different receptors, data from the cerebellum suggest that $GABA_B$ receptors could be placed on spines (Fritschy et al., 1999; Lopez-Bendito et al., 2002). Spines also receive the majority of excitatory input and simulations showed that if inhibitory synapses found on cortical spines are effective, then they should be mediated through $GABA_B$ receptors providing powerful hyperpolarizing inhibition reducing the excitatory synaptic potentials on the same spine (Qian and Sejnowski, 1990). In addition to hyperpolarizing inhibitory effects, the diffusion barrier provided by the targeted postsynaptic spines can locally enhance metabotropic changes following $GABA_B$ receptor activation, therefore even sparse temporal operation of NGFCs could result in sustained modulation of excitability.

5.2. Gap junctional coupling between neurogliaform cells and various interneuron types in the neocortex

Introduction

Electrical coupling between neocortical interneurons is firmly established by anatomical studies and electrophysiological experiments (Galarreta and Hestrin, 2001; Bennett and Zukin, 2004; Connors and Long, 2004; Sohl et al., 2005). Dendro-dendritic or dendro-somatic gap junctions or gap junction like structures were shown between morphologically identified interneurons (Sloper, 1972; Tamas et al., 2000; Szabadics et al., 2001; Fukuda and Kosaka, 2003) and actual measurements of electrical coupling were performed between neighbouring GABAergic interneurons (Galarreta and Hestrin, 1999; Gibson et al., 1999; Tamas et al., 2000; Venance et al., 2000; Szabadics et al., 2001; Blatow et al., 2003; Chu et al., 2003). Electrical coupling between neocortical interneurons appears to be highly specific: GABAergic neurons establish electrical coupling almost exclusively with interneurons of the same type forming several distinct networks linking homologous interneurons (Galarreta and Hestrin, 2001; Bennett and Zukin, 2004; Connors and Long, 2004).

The most prominent function demonstrated so far for electrical coupling is the increased synchrony of firing between the coupled cells (Galarreta and Hestrin, 2001; Bennett and Zukin, 2004; Connors and Long, 2004). Synchronization within networks of particular interneurons is then propagated downstream in cortical microcircuits by the concerted action of GABAergic synapses entraining postsynaptic firing (Tamas et al., 2000; Szabadics et al., 2001; Blatow et al., 2003; Buhl et al., 2003). Several GABAergic cell types, homologously interconnected by gap junctions, are also effective in timing postsynaptic action potentials suggesting that multiple pathways of synchronization operate in parallel within the cortical circuit (Cobb et al., 1995; Tamas et al., 2004). However, distinct cell types in neural circuits are known to establish electrical coupling and could perform special functions. For example, electrical coupling between rods and cones in the retina allows summation of inputs to different cells and can improve resolution by reducing noise (Guldenagel et al., 2001; Demb

and Pugh, 2002; Sohl et al., 2005). Sporadic evidence for heterologous electrical coupling in the neocortex shows coupling of spiny stellate cells and fast spiking GABAergic interneurons and occasional coupling between different GABAergic cells (Venance et al., 2000; Chu et al., 2003), but the position of several known cortical cell types is not clear in gap junctionally linked networks.

Neurogliaform interneurons have a so far unique position among cortical interneurons as they are capable of eliciting slow IPSPs in postsynaptic pyramidal cells through a combined recruitment of $GABA_A$ and $GABA_B$ receptors (Tamas et al., 2003). Here we investigate the electrical synapses of this cell type and identify a multitude of interneuron types gap junctionally coupled to neurogliaform cells using a combination of simultaneous patch clamp recordings and correlated light and electron microscopy. Analysis of chemical synapses established by neurogliaform cells on various interneuron types requires a separate study.

Results

Electrical coupling between neurogliaform cells

Neurogliaform cells (n=94) were identified during simultaneous dual, triple and quadruple recordings of neurons in layers 2-3 of rat somatosensory cortex based on their firing characteristics followed by light microscopic evaluation of their dendritic and axonal morphology. Similarly to neurogliaform cells identified by earlier studies (Hestrin and Armstrong, 1996; Kawaguchi and Kubota, 1997; Tamas et al., 2003), neurogliaform interneurons in our sample responded to long (800 ms), just above threshold current injections with late spiking firing pattern (figures 5.2.1A, 5.2.2B, 5.2.4B) or with an accelerating train of spikes (figures 5.2.1A, 5.2.2B, 5.2.3B) when activated from resting membrane potential (-64 ± 3 mV). This firing behavior was changed to a decelerating pattern when applying larger current pulses (figure 5.2.2B). All neurogliaform cells showed similar axonal features: small boutons frequently occurred along the thin axonal collaterals forming a dense meshwork within the compact axonal field (figures 5.2.2-4).

When looking for connections to neurons closely located (< 100 µm) to the presynaptic neurogliaform cells, we sampled pyramidal cells as well as several types of interneuron. According to our earlier studies showing strong frequency sensitivity of postsynaptic responses elicited by neurogliaform cells, we triggered single action potentials with an interval of 90 s to avoid the loss of responses. Simultaneous triple recordings showed

that neurogliaform cells formed divergent connections which were transmitted by electrical and chemical synapses (figure 5.2.1). Neurogliaform cells eliciting slow, presumably GABAA and GABAB receptor mediated IPSPs on pyramidal neurons (n=7) also triggered spikelets on other neurogliaform cells (n=3, figure 5.2.1) and other types of interneuron (n=4) which could be followed by long lasting IPSPs (n=2 on neurogliaform cells and n=2 on other interneurons). Electrical coupling indicated by spikelets in response to presynaptic action potentials was confirmed by the passage of hyperpolarizing signals between the coupled neurons (figure 5.2.1). These observations encouraged us to search for electrically coupled pairs of neurogliaform cells and interneurons representing various types.

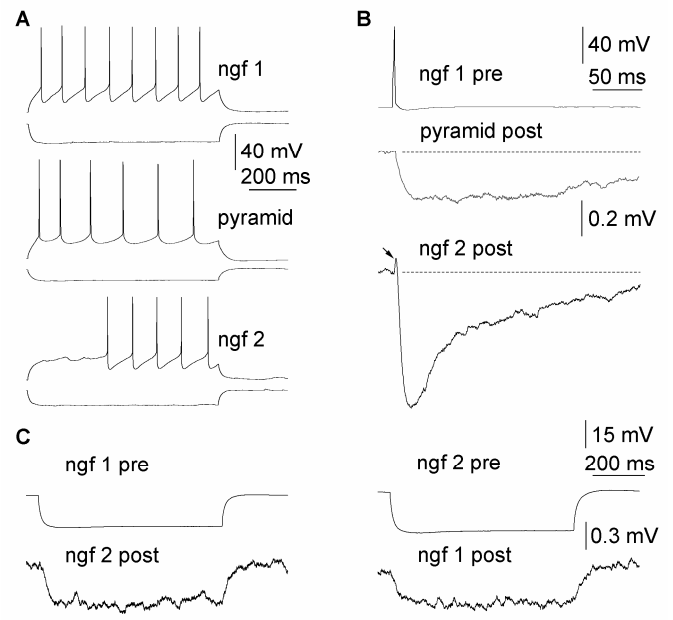

Figure 5.2.1.**NGFCs eliciting slow IPSPs establish homologous electrical synapses.**

A. Firing pattern of two NGFCs (NGF 1 and 2) and a pyramidal cell (pyramid) recorded in the same slice. **B**. Single action potentials in the presynaptic neurogliaform cell (ngf 1) elicited a slow IPSP in the pyramidal cell and spikelet (arrow) followed by a long-lasting IPSP in the other neurogliaform cell. Note that the IPSPs do not return to baseline (dashed line) 250 ms after the presynaptic spike. **C**, Hyperpolarizing current injections into either neurogliaform cells were transmitted to the other cell, confirming electrical coupling. From the 16 NGF–NGF cell connections tested, we confirmed electrical coupling in eight pairs (50%). pre, presynaptic; post, postsynaptic.

Based on previous experiments showing widespread evidence for electrical coupling between similar GABAergic cells (Galarreta and Hestrin, 2001; Bennett and Zukin, 2004; Connors and Long, 2004), we proceeded by characterizing homologous electrical connections between neurogliaform cells. Out of the 16 pairs of neighboring neurogliaform cells tested for electrical coupling, we confirmed electrical connections between neurogliaform cells in 8 cases indicating a 50 % rate for coupling (figures 5.2.1. and 5.2.2.). All electrical connections between NGF cells were reciprocal. Electrical coupling potentials in response to presynaptic spikes showed a relatively wide range in amplitude (0.09 - 2.08 mV; 0.62 ± 0.79 mV) at –50 ± 2 mV membrane potential. They followed presynaptic action potentials with a delay of 0.34 ± 0.19 ms, measured as the period spanning the maximal rates of rise of the presynaptic action potential and the electrical coupling potential, respectively. The average amplitude ratio (coupling coefficient) for spikelets and presynaptic potentials was 0.17 ± 0.06 % and 1.89 ± 0.77 % when eliciting action potentials and applying long current steps (-200 pA, 200 ms

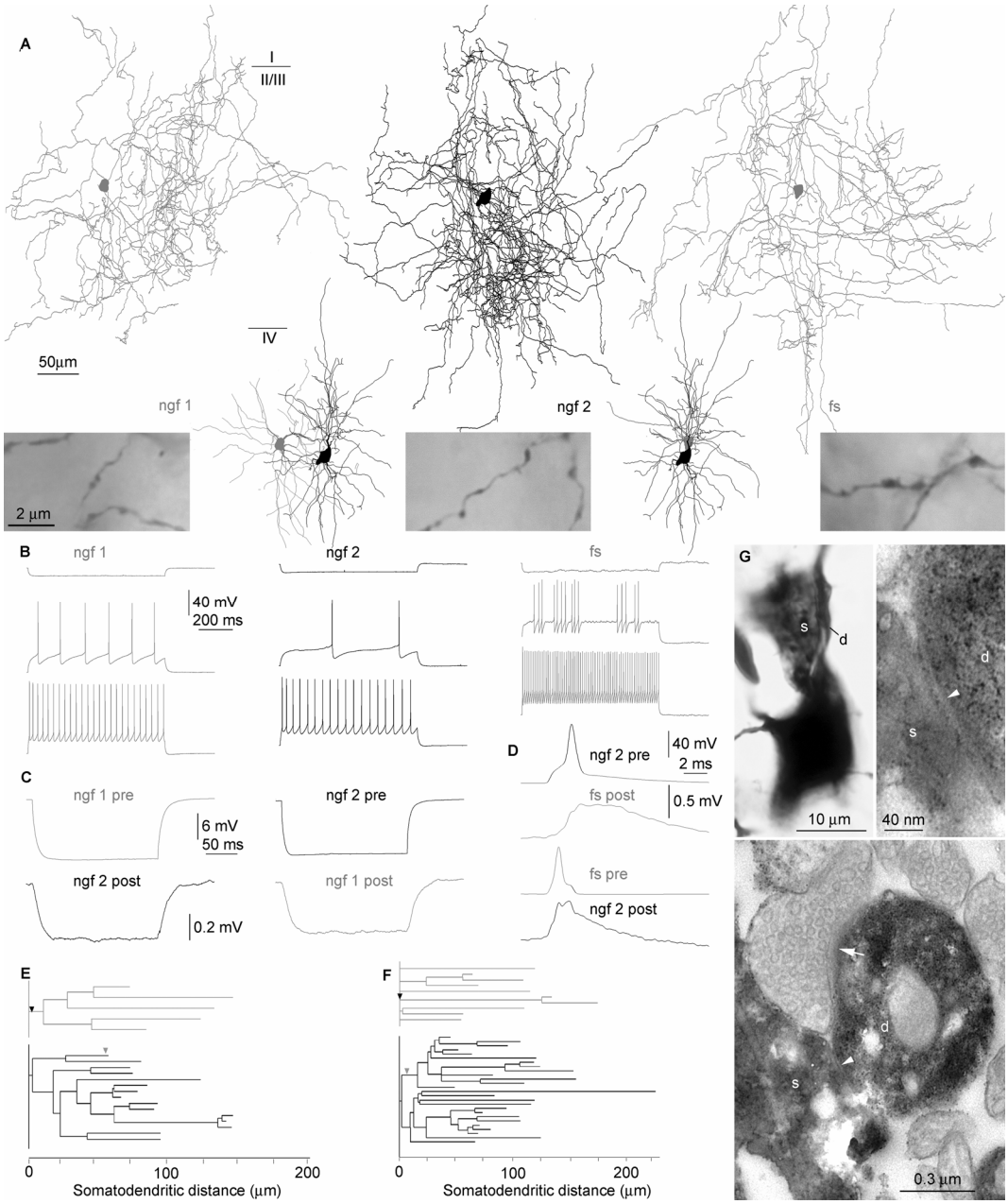

Figure 5.2.2. **Heterologous gap junctions link the network of neurogliaform cells and fast-spiking basket cells. A**, Reconstructions of two neurogliaform cells (ngf 1, gray; ngf 2, black) and a fast-spiking basket cell (gray). Axonal and dendritic arborizations are shown separately for clarity. The position of cells is shown relative to the dendrites of ngf 2, and the laminar position is shown relative to the axons. Light micrographs illustrate morphological differences between axon terminals. **B**, Responses of the three cells to hyperpolarizing (top) and depolarizing current pulses (middle and bottom). **C**, Transmission of hyperpolarizing current injections into one of the cells confirmed electrical coupling between the neurogliaform cells. **D**, Action potentials elicited in neurogliaform cell 2 triggered spikelets in the fast spiking cell and vice versa. The rising phase of the spikelets corresponded to the rise of action potentials, indicating electrical coupling. From the 31 NGF–FS cell connections tested, we confirmed electrical coupling in six pairs (19%). **E**, **F**, Dendrograms representing three-dimensional distances measured from the somata to the presumed gap junctions (arrowheads) mediating the interactions between the neurogliaform cells (**E**) and between neurogliaform cell 2 and the fast-spiking cell. **G**, Correlated light and electron microscopy identified a single gap junction as the site of interaction between the soma (s) of the fast-spiking cell and a proximal dendrite (d) of the neurogliaform cell 2. The dendrite of the neurogliaform cell also receives a synaptic junction (arrow) from an unidentified terminal (t). pre, presynaptic; post, postsynaptic.

duration) in the first neuron to elicit a response in the second neuron, respectively. Coupling strength was similar in both directions and did not correlate with the distance between the recorded cell bodies (39 ± 13 μm). From the 16 presynaptic NGF cells, 11 triggered spikelets which were followed by long lasting IPSPs (figure 5.2.1B); these IPSPs will be characterized in a separate paper. Three dimensional light microscopic reconstruction and mapping of an electrically connected pair of neurogliaform cells indicated one close apposition between a proximal and a distal dendrite (4 and 57 μm from the somata, figure 5.2.2E) suggesting a single place for electrical communication.

Gap junctional coupling between neurogliaform and fast spiking basket cells

Apart from homologous electrical connections between NGF cells, we detected heterologous gap junctions linking NGF cells and fast spiking (FS) basket cells. Fast spiking basket cells were identified according to their firing characteristics followed by light microscopic evaluation of their dendritic and axonal morphology. Similarly to basket cells identified by earlier studies (Tamas et al., 1997; Galarreta and Hestrin, 1999; Gibson et al., 1999; Gupta et al., 2000), FS basket interneurons responded to just above threshold current injections with "stuttering" groups of spikes (figure 5.2.2B, refs) and with a nonaccomodating train of spikes when applying increased current steps (figure 5.2.2B). Membrane time constants of FS cells were also similar to values published earlier (Kawaguchi, 1995; Tamas et al., 2003). All FS basket cells showed characteristic axonal branches frequently surrounding somata with large boutons relative to those of NGF cells (figure 5.2.2A). Electron microscopic sampling of postsynaptic targets of randomly selected FS cells (n=3) confirmed the identity of these neurons as basket cells (Somogyi, 1989) showing a target preference towards somata (28 ± 5 %) and dendritic shafts (68 ± 7 %) and occasionally innervating dendritic spines (3 ± 3 %).

From the 31 connections tested between closely spaced NGF and FS cells, we confirmed electrical coupling in 6 pairs indicating a 19 % rate for interaction (figure 5.2.2). All electrical connections between NGF and FS cells were reciprocal and in three pairs NGF cells elicited IPSPs on postsynaptic FS cells. Electrical coupling potentials in response to presynaptic spikes showed amplitudes of 0.52 ± 0.23 mV (range, 0.17 – 0.87 mV) at –50 ± 3 mV membrane potential. The duration of electrical coupling potentials measured at half amplitude was longer in FS cells (n=3) than in NGF (n = 6) cells (9.11± 2.95 ms vs. 3.80 ± 1.23 ms, $p < 0.02$ Mann-Whitney U-test), presumably due to the faster afterhyperpolarization

in FS cells (1.33 ± 0.13 ms vs. 8.49 ± 0.68 ms measured from the peak of action potential to the maximum of afterhyperpolarization). Spikelets followed presynaptic action potentials with a latency of 0.28 ± 0.12 ms. Amplitude ratios of pre- and postsynaptic potentials did not show direction selectivity. Coupling coefficients for spikelets and presynaptic potentials were 0.39 ± 0.23 % and 3.76 ± 1.42 % when eliciting action potentials and applying long current steps in the first neuron to elicit a response in the second neuron, respectively. Thus, electrical coupling appears stronger in our limited sample of NGF-FS than in NGF-NGF connections measured by transferring relatively long lasting hyperpolarization ($p < 0.002$ Mann-Whitney U-test). The strength of coupling did not correlate with the distance measured between the somata of connected cells (31 ± 16 µm). Three dimensional light microscopic reconstructions of two electrically connected NGF-FS pairs suggested one close apposition in each connection between the somata of the FS cells and proximal dendrites of the NGF cells (9 µm and 40.2 µm from the somata). Subsequent electron microscopy confirmed a single gap junction as the morphological correlate of electrical communication in both connections (figure 5.2.2F-G).

Gap junctional coupling between neurogliaform and regular spiking cells

Finding heterologous gap junctions between NGF and basket cells propelled us to see whether NGF cells are electrically coupled to a multitude of interneuron types. We detected regular spiking nonpyramidal (RS) cells as the second type of interneuron forming heterologous gap junctions with NGF cells. Similar to RS cells identified earlier (Cauli et al., 1997; Kawaguchi and Kubota, 1997; Cauli et al., 2000; Szabadics et al., 2001; Tamas et al., 2004) they responded to suprathreshold current injections with a regular spiking firing pattern (figure 5.2.3B, refs). The morphology of sparsely spiny dendrites and individual axonal branches of all RS cells appeared similar showing undulating axon collaterals with relatively large, bead-like boutons (figure 5.2.3A). The overall axonal arborization of RS cells was restricted to a part of layers 2/3 (figure 5.2.3A) or in addition to the arborization around the soma, RS cells sent a loose bundle of radially oriented branches towards layer 6 (Szabadics et al., 2001). Confirming previous results (Szabadics et al., 2001), electron microscopic samples of postsynaptic targets of randomly selected RS cells (n=3) showed a target preference towards dendritic shafts (53 ±13 %) and dendritic spines (47 ± 13 %) and did not target postsynaptic somata.

When testing connections between pairs (n=30) of NGF and regular spiking nonpyramidal (RS) cells, we detected heterologous coupling in 6 cases indicating a 20 % rate

for electrical synapses between these cell populations. All electrical connections between NGF and RS cells were mutual and all 6 NGF cells involved in electrical coupling elicited IPSPs on the postsynaptic RS cells.

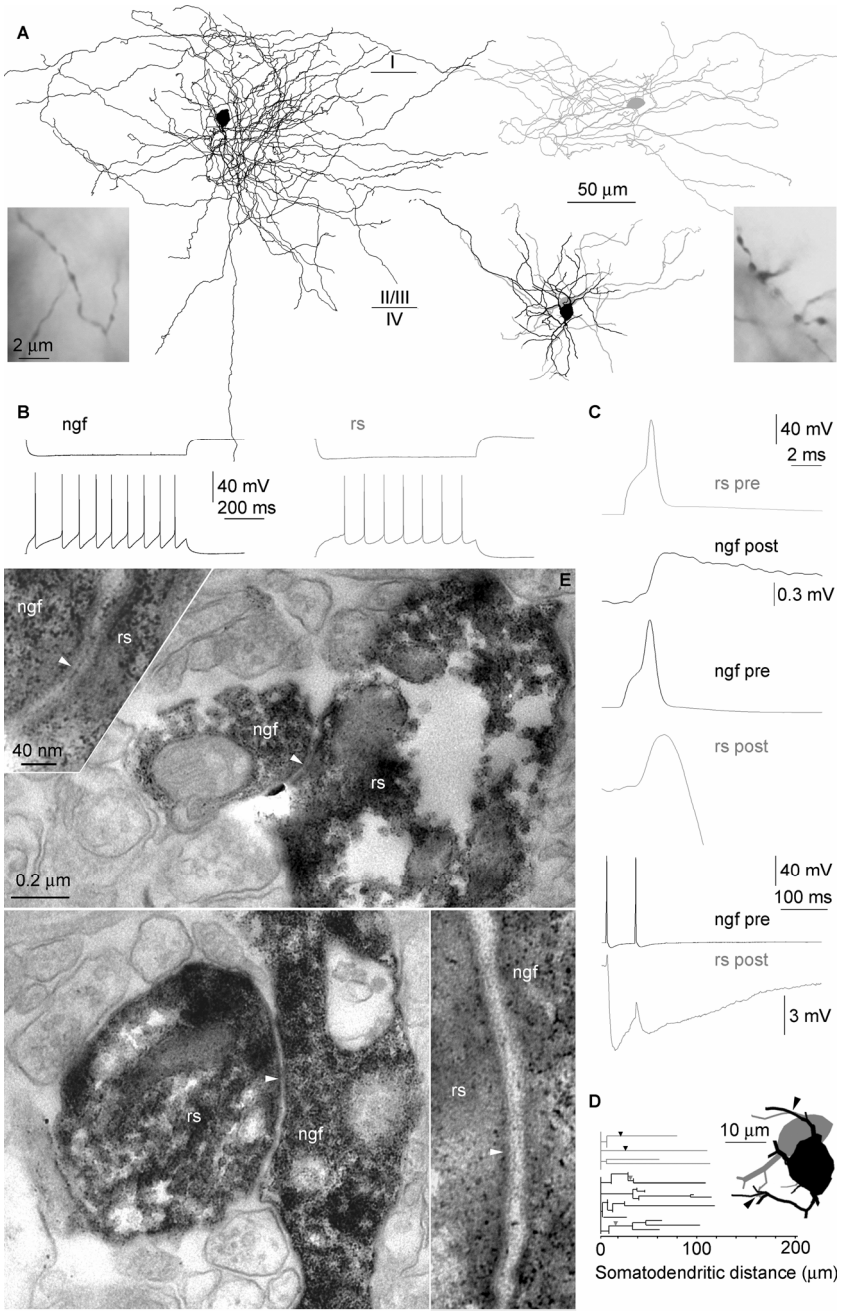

Figure 5.2.3. Heterologous gap junctions connect the network of neurogliaform cells with regular-spiking cells. **A**, Reconstructions of the neurogliaform cell (black) and the regular-spiking cell (gray). Axonal and dendritic arborizations are shown separately for clarity. Dendritic reconstructions show the relative position of the cells. The laminar position is shown relative to the axons. Light micrographs illustrate morphological differences between axon terminals. **B**, Responses of the cells to hyperpolarizing (top) and depolarizing current pulses (bottom). **C**, Action potentials elicited in the regular-spiking cell elicited gap-junctional potentials or spikelets postsynaptically (top). Presynaptic spikes in the neurogliaform cell triggered a spikelet (middle) followed by a longlasting IPSP (bottom). Of the 30 NGF–RS cell connections tested, we confirmed electrical coupling in six pairs (20%). **D**, Dendrograms representing three dimensional distances measured from the somata to the gap junctions (arrowheads) mediating the interactions between the neurogliaform cell (black) and the regular-spiking cell (gray). Inset, The route of dendrites from the somata to the gap junctions (arrowheads). ***E***, Electron microscopic verification of the two gap junctions (arrowheads) between proximal dendrites of the NGF and RS cells. pre, presynaptic; post, postsynaptic.

Electrical coupling potentials in response to presynaptic spikes showed amplitudes of 0.45 ± 0.38 mV (range, 0.18 – 0.72 mV) at –50 ± 3 mV membrane potential. Spikelets followed presynaptic action potentials with a latency of 0.32 ± 0.12 ms. Electrical coupling strength was symmetrical between NGF and RS cells. Coupling coefficients for spikelets and presynaptic potentials were 1.01 ± 0.60 % and 4.59 ± 3.98 % when eliciting spikes and applying hyperpolarizing current steps in the first neuron to elicit a response in the second neuron, respectively. The strength of coupling was not correlated with the distance measured between the somata of connected interneurons (28 ± 19 μm). Three dimensional light microscopic mapping and subsequent electron microscopy of an electrically connected NGF-RS pair determined two gap junctions mediating the interaction between proximal dendrites equidistant (31.025 ± 3.83 μm) from the somata (figure 5.2.3D-E).

Neurogliaform cell – axo-axonic cell electrical coupling

In search for electrically coupled partners for NGF cells, we recorded a single NGF cell – axo-axonic (AA) cell pair and this cell pair was electrically coupled (figure 5.2.4). Although this was a single example, the unique output specificity of AA cells among cortical interneurons and the relatively limited number of AA cell connections studied so far encouraged us to present the relevant data. Only the somata and axons were recovered from the cell pair, and we determined the identity of the AA cell based on the presence of characteristic cartridges or candles formed by axonal boutons (Szentagothai and Arbib, 1974; Somogyi, 1977) (figure 5.2.4A). Moreover, the AA cell innervated a pyramidal cell which was also recorded in the same slice exclusively on the axon initial segment through five electron microscopically verified synaptic junctions (figure 5.2.4B). The AA cell had a firing pattern similar to FS cells (figure 5.2.4B). Electrical coupling between the AA and NGF cell was tested with presynaptic action potentials only; postsynaptic spikelets with onsets during the rising phase of presynaptic action potentials, the matching polarity of pre- and postsynaptic signals and the reciprocity of connection ensured electrical coupling (figure 5.2.4C). The spikelet triggered by NGF cell was followed by an IPSP which was not characterized pharmacologically (figure 5.2.4D).

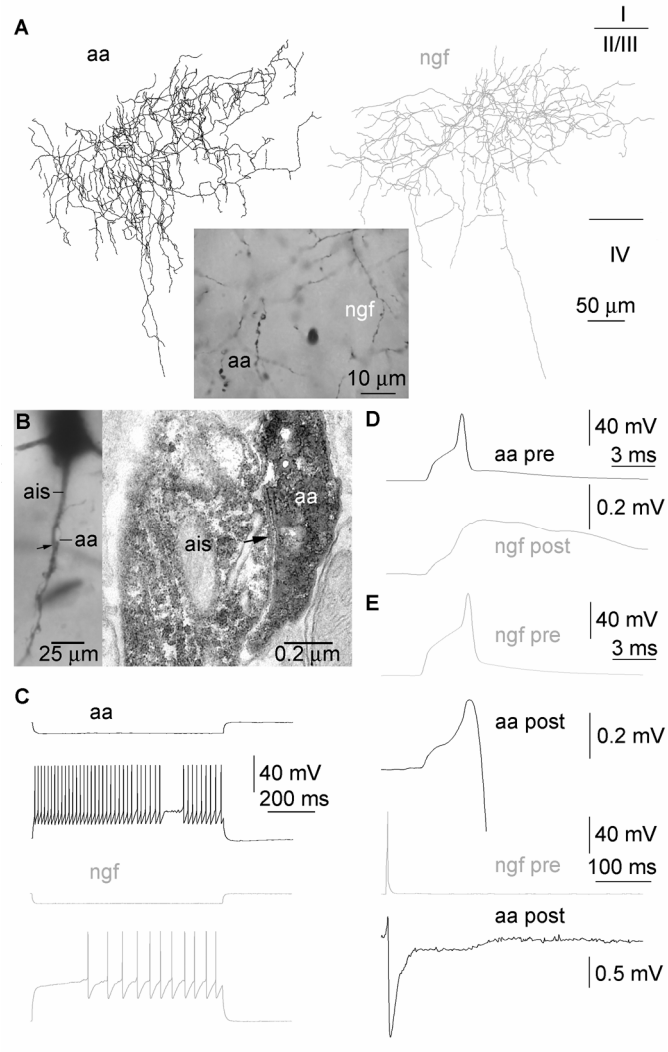

Figure 5.2.4. **Heterologous electrical coupling between a neurogliaform cell and an axoaxonic cell.**
A, Reconstructions of the NGF cell (gray) and the AA cell (black). Only axons were recovered from both cells. The light micrographs show morphological features of AA and NGF axon terminals.
B, Innervation of a pyramidal cell by the axoaxonic cell. Left, The axon of the axoaxonic cell (aa) forms a cartridge around the axon initial segment (ais) of the pyramidal cell. Right, Example of one of the five electron microscopically verified synaptic junctions (arrow) established by the axoaxonic cell on the axon initial segment of the pyramidal cell.
C, Responses of the cells to hyperpolarizing (top) and depolarizing current pulses (bottom).
D, Action potentials elicited in the axoaxonic cell elicited gap-junctional potentials or spikelets postsynaptically.
E, Presynaptic spikes in the neurogliaform cell triggered a spikelet (top, expanded timescale) followed by an IPSP (bottom). pre, presynaptic; post, postsynaptic.

Electrical synapses between neurogliaform cells and other interneurons

Electrical coupling between NGF and interneurons with firing properties and morphological characteristics distinct from NGF, FS, RS and AA cells was tested in 26 pairs (data not shown). In 13 pairs the interneurons recorded simultaneously with NGF cells responded to depolarizing current pulses with a so-called low-threshold spiking firing pattern and showed dendritic and axonal features of Martinotti cells (Gibson et al., 1999; Tamas et al., 2003; Tamas et al., 2004). We identified 2 NGF - Martinotti cell pairs and 2 NGF cell - unidentified interneuron pairs connected by electrical synapses indicating an average coupling rate of 15 %. Apart from the spikelets evoked by the electrically coupled interneurons, an NGF cell elicited IPSPS in a Martinotti cell and an unidentified interneuron evoked an IPSP in an NGF cell (figure 5.2.5.).

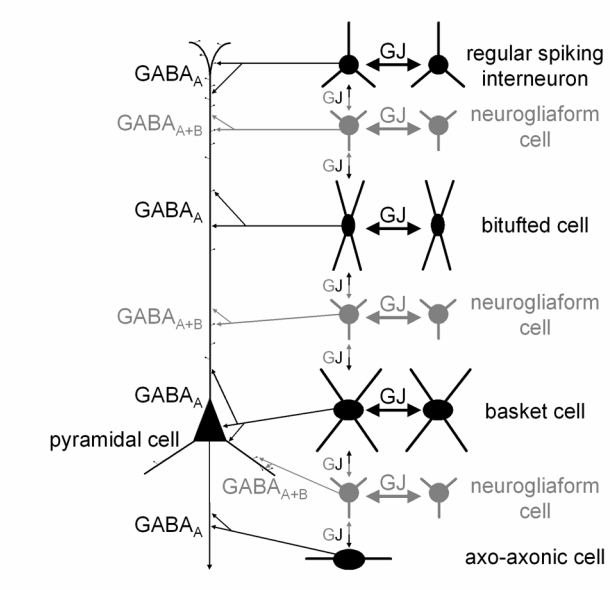

Figure 5.4.5. **Neurogliaform cells have a unique position in the cortical microcircuit.** To date, neurogliaform cells are the only interneuron type capable of eliciting slow $GABA_A$ and $GABA_B$ IPSPs on pyramidal cells. Several types of interneurons eliciting $GABA_A$ receptor-mediated postsynaptic responses are known to form electrically coupled networks with interneurons of the same class. We found that neurogliaform cells are electrically coupled to other neurogliaform cells (gray), but, unlike other interneurons, neurogliaform cells also establish heterologous gap junctions (at a lower rate of coupling) with regular-spiking nonpyramidal cells, Martinotti cells, basket cells, and axoaxonic cells. Thus, heterologous electrical synapses of neurogliaform cells link multiple networks formed by gap junctions restricted to a particular class of interneuron. Widespread electrical connections might enable neurogliaform cells to monitor the activity of different interneurons acting on $GABA_A$ receptors at various regions of target cells. GJ, gap junction.

Discussion

We showed that NGF cells eliciting slow IPSPs on pyramidal cells also triggered divergent electrical coupling potentials on interneurons. In line with earlier results showing widespread electrical coupling between interneurons of the same type, NGF cells formed homologous electrical synapses with other neurogliaform cells. The major point of these findings is that NGF cells established heterologous electrical coupling with several types of interneuron. We also identified the sites of electrical interaction between different cell types and provided ultrastructural evidence for gap junctions linking somatodendritic sites of the coupled cells.

Our earlier results on homologous electrical coupling between interneurons representing the same class identified the site of coupling ultrastructurally as gap junctions between the somata and/or dendrites of electrophysiologically recorded cells (Tamas et al., 2000; Szabadics et al., 2001). The possible axo-axonal electrical coupling between hippocampal pyramidal cells suggests that the formation of gap junctions could follow cell type or connection specific subcellular patterns. However, heterologous electrical coupling identified here with combined recordings and electron microscopy also operates via somatodendritically placed gap junctions. Thus, it appears to be a generalized feature of cortical circuits that individual cells in electrically coupled networks of cortical interneurons are located within the dendritic arborization of each other and communicate through the

dendrites. This could limit the size of electrically coupled networks, but the relatively uniform and moderate number of gap junctions identified within a single connection so far allows the formation of widespread electrically interconnected circuits (Tamas et al., 2000; Deans et al., 2001; Szabadics et al., 2001; Bennett and Zukin, 2004). Ultrastructural identification of gap junctions between coupled cells recorded electrophysiologically also suggest that interneurons of the cerebral cortex are not coupled by cytoplasmic continuity which can result from artifacts induced by slice cutting. This is also supported by previously documented absence of dye coupling between cortical interneurons known to form electrically coupled microcircuits (Galarreta and Hestrin, 2001; Bennett and Zukin, 2004; Connors and Long, 2004). The lack of dye coupling between interneurons of the same type and between NGF cells and other interneurons suggest that permeability properties and possibly the molecular composition of homologous and heterologous gap junctions of interneurons might be similar and involve connexin 36 (Venance et al., 2000). It should be added, however, that NGF cell like dwarf neurons in the striatum of young rats can be dye-coupled with neurons of different classes (Sancesario et al., 1998) but dye coupling may not be a reliable measure of gap junction coupling among postnatal cortical neurons (Gutnick and Prince, 1981; Knowles et al., 1982; Connors et al., 1984; Roerig and Feller, 2000).

Several types of interneuron eliciting $GABA_A$ receptor mediated postsynaptic responses are known to form electrically coupled networks with interneurons of the same class (Galarreta and Hestrin, 1999; Gibson et al., 1999; Tamas et al., 2000; Venance et al., 2000; Szabadics et al., 2001; Blatow et al., 2003; Chu et al., 2003; Bennett and Zukin, 2004). Fast IPSPs triggered by members of these networks are elicited on separate subcellular domains. It appears that the somatodendritic domain of postsynaptic cells is targeted by several separate networks basket or basket-like cells intrinsically linked by gap junctions (Galarreta and Hestrin, 1999; Gibson et al., 1999; Tamas et al., 2000). Similarly, the dendritic region of postsynaptic neurons also receives a multitude of inputs from a number cell populations (RS cells, Martinotti or low threshold spiking cells, layer I interneurons) which were shown to form electrical synapses restricted to the same type of interneuron (Gibson et al., 1999; Venance et al., 2000; Szabadics et al., 2001; Chu et al., 2003). In spite of targeting the dendritic domain of postsynaptic neurons and forming electrical synapses within the cell class, NGF cells could have an exceptional position in the cortical architecture. From the multitude of cortical GABAergic interneurons, NGF cells represent the first cell type capable of eliciting slow IPSPs composed of GABAA and GABAB receptor mediated postsynaptic responses with single presynaptic spikes (Tamas et al., 2003). Moreover, neurogliaform cells

establish electrical synapses and link multiple networks formed by gap junctions restricted to a particular class of interneuron.

In depth analysis of the function of electrical synapses established by NGF cells awaits further experiments. Homologous electrical synapses within a multitude of cell populations were found to promote synchronization of the coupled cells (Hestrin and Armstrong, 1996; Bennett and Zukin, 2004; Connors and Long, 2004; Sohl et al., 2005). Unlike other known types of interneuron, NGF cells elicit GABAA and GABAB receptor mediated postsynaptic potentials on pyramidal cells and synchronous release of GABA from several presynaptic interneuron was suggested to be involved in the activation of postsynaptic GABAB receptors (Mody et al., 1994; Thomson et al., 1996b; Kim et al., 1997; Thomson and Destexhe, 1999). Although single spikes in NGF cells are sufficient in eliciting the GABAB receptor mediated response, we did not detect spike transmission through gap junctions linking NGF cells in agreement with earlier reports showing that synchronization is promoted by electrical coupling but is usually too weak for spike to spike coupling (Tamas et al., 1997; Galarreta and Hestrin, 1999; Gibson et al., 1999; Szabadics et al., 2001; Blatow et al., 2003). However, unlike other known networks of electrically coupled interneurons, NGF cells are embedded into a widespread mesh of electrical synapses linking multiple interneuron classes. Our results suggest that NGF cells form electrical connections with half of the neurogliaform cells and with every fifth interneuron representing at least three distinct types within the range of their relatively compact dendritic field. The strength of coupling is similar in homologous and heterologous electrical synapses established by NGF cells and is comparable to values previously published for homologous coupling in networks of other interneurons (Galarreta and Hestrin, 1999; Gibson et al., 1999). Whether heterologous electrical coupling of neurogliaform cells has a specific function similarly to retinal gap junctions between dissimilar components of the circuit remains to be seen (Guldenagel et al., 2001; Demb and Pugh, 2002; Lee et al., 2003; Hornstein et al., 2004; Sohl et al., 2005). Theoretical studies suggest that electrical synapses could stabilize synchronization by compensating network heterogeneity (Sherman and Rinzel, 1992; Chow and Kopell, 2000; Kopell and Ermentrout, 2004). Thus, it is conceivable that heterologous coupling between NGF cells and other interneuron types could contribute to operational state dependent and simultaneous recruitment of NGF cells which can boost their efficacy in reaching GABAB receptors. On the other hand, widespread homologous and heterologous electrical coupling counteracts the spike triggering effectiveness of excitatory inputs arriving to NGF cells by siphoning current into coupled cells. It was estimated that approximately 1/3 to 1/2 of input conductance of

neurons involved in electrical coupling occurs via gap junctions to neighboring cells (Amitai et al., 2002; Long et al., 2002). Such “postsynaptic” conductance load could contribute to the late spiking firing characteristics of NGF cells and might regulate firing behavior during network operations favoring sporadical and delayed firing of NGF neurons paralleled by the prominent frequency sensitivity of NGF output (Tamas et al., 2003). Thus, neurogliaform cells eliciting slow IPSPs and forming homologous and heterologous gap junctional connections appear well positioned to monitor the activity of different interneurons acting on $GABA_A$ receptors at various regions of target cells.

The human cerebral cortex is composed of precisely connected neurons which are responsible for perception, cognition and consciousness. There are two main types of neuron in the mammalian cerebral cortex, excitatory pyramidal cells and inhibitory non-pyramidal cells. The principal pyramidal cells use glutamate as an excitatory neurotransmitter and send its axon to neighbouring and distal cortical and subcortical areas. Non-pyramidal cells use γ-aminobutyric acid (GABA) to exert their effect on other neurons therefore they are referred as GABAergic interneurons. GABAergic interneurons are heterogeneous regarding their morphological, molecular and physiological properties and they typically present in different cortical areas of the brain. Great diversity of these cells are also reflected in their complex synaptic relations and their efferent connectivity. The interneurons could be classified by their spatially selective innervation of the surface of the postsynaptic cell. Structural and functional diversity of GABAergic interneurons has become increasingly important in our understanding of the elementary steps of information processing in the brain. One of the main tasks of today's neuroscience is to find out what is the function of the inhibitory interneurons in the neuronal microcircuits.

This thesis studies the role of a special type of GABAergic interneuron, the neurogliaform cell in the mammalian neocortex. Although detailed morphological and electrophysiological properties of the neurogliaform cell have been revealed previously, the exact function of this cell type in the neocortical microcircuits remained unclear. Our results identify the chemical and electrical connections of neurogliaform cells in the cerebral cortex.

Neurogliaform cells were first described by Ramon Y Cajal as *cellules neurogliforme*. He first observed them in the human visual area in 1899. His most extensive description of the cells comes in his general account of layer II of the cortex, though he notes that they are found in all layers and are especially common in the deeper layers. Although electrophysiological properties of this cell were not studied until the end of the 20th century, neurogliaform cells were identified from several areas of the nervous system on the basis of their morphology. In comparing to other GABAergic cells neurogliaform cell is smaller. Its round soma is radially surrounded by 6-9 short dendrites which are usually beaded and occasionally have spines. On these short dendrites, spiderweb cells may receive spatially selective inputs from cortical and

subcortical glutamate afferents and from local GABAergic interneurons. The axon may arise from any part of the soma and after the third or fourth ramification it branches out into extremely thin and dense axonal collaterals enmeshing the space around the soma. The homogenous axonal cloud is densely packed with small boutons, has a 360 ± 39 μm in diameter and in almost every case remains in the same layer as the soma and rarely extend into the neighbouring layers.

There are two types of inhibitory postsynaptic potentials in the cerebral cortex. Fast inhibition is mediated by ionotropic γ-aminobutyric acid type A ($GABA_A$) receptors which are chloride channels, and slow inhibition is due to metabotropic, G protein coupled $GABA_B$ receptors. Several types of GABAergic neurons (basket cells, Martinotti cells, regular spiking cells) elicit inhibitory postsynaptic potentials through $GABA_A$ receptors, but possible distinct sources of slow inhibition remain unknown. Combination of biocytin filling during whole-cell patch-clamp recordings of cell pairs with their anatomical analysis following the visualization of biocytin provided a useful tool in identifying a class of GABAergic interneurons, the neurogliaform cells, that, in contrast to other GABA-releasing cells, elicited combined $GABA_A$ and $GABA_B$ receptor–mediated slow inhibition. A single action potential is enough in the presynaptic cell to evoke the complex inhibition on the postsynaptic pyramidal cell. Neurogliaform cells also have target preferency, they selectively innervate the dendritic spines (71%) and distal dendritic shafts (29%) of pyramidal neurons. Modelling experiments shows that GABAergic synapses targeting dendritic spines, especially the bases of the spine necks could be substantial in controlling the excitatory inputs, which predominantly arrive the spine heads. Inhibitory chloride conductance ($GABA_A$) terminated at the basis of the spine neck could shunt the excitatory inputs effectively and the inhibitory potassium conductance ($GABA_B$) might powerfully reduce the effects of the EPSPs through hyperpolarization. These predictions overlap with our results, therefore we suppose, that neurogliaform cells may play an important role in controlling excitatory inputs arriving to the same spine. Slow inhibition evoked by a distinct interneuron in spatially restricted postsynaptic compartments could locally and selectively modulate cortical excitability.

Electrical synapses could also contribute to the selective modulation of the cortical excitability as well as the generation of synchronous activity in neuronal networks. Several types of cortical GABAergic neurons acting via postsynaptic $GABA_A$ receptors also form electrical synapses with interneurons of the same class, suggesting that synchronization through gap junctions could be limited to homogenous interneuron populations. Homogenous gap junctional mesh of basket cells/fast spiking cells play role in generating and maintaining

γ-rhythm, regular spiking cells connected through gap junction are able to generate β- and γ-rhythm and electrical connections among multipolar bursting cells help developing θ-rhythm. Neurogliaform cells elicit combined $GABA_A$ and $GABA_B$ receptor-mediated postsynaptic responses in cortical pyramidal cells, but it is not clear whether neurogliaform cells are involved in networks linked by electrical coupling. Electrophysiological experiments revealed that neurogliaform cells also triggered divergent electrical coupling potentials on interneurons. Neurogliaform cells were electrically coupled to other neurogliaform cells, basket cells, regular-spiking cells, to an axo-axonic cell, and to various unclassified interneurons showing diverse firing patterns and morphology. Neurogliaform cells establish homologous electrical connections with 50% rate of coupling and heterologous electrical connections with basket cells and regular spiking cells with 19-20% rate for interactions. Electrical interactions were mediated by one or two electron microscopically verified gap junctions linking the somatodendritic domain of the coupled cells. Neocortical development of electrical synapses is terminated at the 3-week-old animals, therefore the existence of electrical coupling can not be considered as a transitional developmental state. Moreover, electrically coupled neurogliaform cells are able to inhibit each other reciprocally with slow IPSPs. The complexity of chemical and electrical connections effectively promote the synchronisation therefore we can suppose that the same mechanism is available for the neurogliaform cell.

Our results suggest that neurogliaform cells have a unique position in the cortical circuit. Apart from eliciting combined $GABA_A$ and $GABA_B$ receptor-mediated inhibition on pyramidal cells, neurogliaform cells establish homologous and heterologous electrical synapses and link multiple networks formed by gap junctions restricted to a particular class of interneuron. Widespread electrical connections might enable this special GABAergic cell type to monitor the activity of different interneurons acting on $GABA_A$ receptors at various regions of target cells.

Az emlős agyban a nagyagykéreg a sejtes szerveződés legfejlettebb struktúrája. Bár sejtanatómiai szempontból az agykérget felépítő idegsejtek igen sokfélék, mégis két fő csoportba sorolhatjuk őket: a glutaminsavat felszabadító piramissejtek-, valamint a γ-amino vajsavat felszabadító idegsejtek (GABAerg interneuronok) csoportjába. A távolról sem egységes piramis - vagy principális - sejtek axonjaik révén a szomszédos, illetve a távoli agykérgi és kéregalatti területek serkentéséért felelősek. A GABAerg sejtek - habár a kérgi idegsejtállomány csupán mintegy egyötödét teszik ki - dendrit- és axonarborizációjukat tekintve nagyfokú morfológiai diverzitást mutatnak, ahogy az már a Golgi-festéssel készült metszeteken is látható volt a XIX. század végén. Elkülönítésük alapulhat morfológiai, fiziológiai jellemzőkön, valamint az alapján, hogy milyen neurokémiai markert expresszálnak. A különböző tüzelési mintázatú GABAerg interneuronok különböző fiziológiai osztályokba sorolhatóak. Ezen osztályok jól korrelálnak az egyes morfológiai csoportokkal. A sokféle interneuron típus – egymással is kölcsönhatásban – egyedi neuronhálózatok része, amelyek az érzékelésben, valamint a tanulási, érzelmi, motivációs és mozgató jellegű agyműködésben összetett feladatokat hajtanak végre. A mai idegtudomány egyik nagy kérdése, hogy mi lehet a gátló interneuronok feladata ezekben a hálózatokban.

Ez a tézis egy speciális GABAerg interneuron, a neurogliaform sejt szerepét vizsgálja az emlős agykéregben. Bár a neurogliaform sejt elektrofiziológiai és morfológiai tulajdonságait többen vizsgálták(ják) a sejt szerepe az agykérgi idegsejthálózatokban tisztázatlan maradt. Az itt ismertetett eredmények a neurogliaform sejt agykéregben kiépített kémiai és elektromos kapcsolataival foglalkoznak.

A neurogliaform sejteket elsőként Ramon Y Cajal írta le 1899-ben, „cellule neurogliaforme”-nak nevezve őket a gliasejttel való hasonlatosság miatt. A sejt fiziológiáját a kilencvenes évek második feléig kevesen vizsgálták, morfológiájuk alapján azonban az idegrendszer több területéről is azonosították. Anatómiájukat tekintve ezek a sejtek picinyek a többi GABAerg interneuronhoz viszonyítva. 8-10 fődendritjük gömbszerű dendritfát alakít ki a többnyire kerek sejttest körül. Axonja bárhol eredhet, axonfelhője rendkívül sűrű hálózatot formáz, de legtöbbször abban az agykérgi rétegben marad, ahol a sejttest is helyet foglal. Az

axon megjelenése egyértelműen meghatározza a sejt anatómiáját; vékony, apró kis boutonokkal borított. A neurogliaform sejtek más interneuronokkal való morfológiai összehasonlítása alapján, a neurogliaform sejteket más GABAerg interneuronoktól dendritfájuk kompaktsága alapján is megkülönböztethetjük. Ezen tulajdonságuk arra enged következtetni, hogy a neurogliaform sejtek térben szelektív bemenetet kaphatnak mind az intrakortikális és szubkortikális glutamáterg afferensektől, mind a döntően helyi GABAerg forrásoktól.

Az emlős agykéregben a gátlás két fő formáját különböztetjük meg. A gyors gátlást ionotrop $GABA_A$, illetve $GABA_C$ receptorok közvetítik, - ezek kloridioncsatornát formáló receptorok. A lassú gátlásért pedig metabotrop, G-fehérjéhez kapcsolt $GABA_B$ receptorok felelősek. Az utóbbi évek kutatásai több olyan GABAerg sejtet azonosítottak, amelyek $GABA_A$ receptorokon keresztül hatnak, de a lassú gátló posztszinaptikus potenciálok (IPSP-k) forrásait nem ismertük. Munkatársaimnak elektrofiziológiásan és farmakológiailag sikerült azonosítania páros whole cell patch clamp elvezetéssel lassú kérgi IPSP-ket, amelyek $GABA_B$ receptoron keresztül fejtik ki hatásukat. A neurogliaform sejtben kiváltott akciós potenciál kétkomponensű ($GABA_A$ + $GABA_B$ receptorok közvetítette), lassú IPSP-t hoz létre posztszinaptikus piramissejtekben. A fénymikroszkópos rekonstrukciók szerint a neurogliaform sejtek axonja által alkotott szinapszisok térszelektíven innerválják a posztszinaptikus sejtfelszínt, főként a dendrittüskékre és dendrittörzsekre adva le szinapszisaikat. A dendrittüskéket és különösen a tüskék alapját célzó GABAerg szinapszisok pedig nagyon fontosak lehetnek a denrittüskék fejére érkező serkentő bemenetek kontrolljában. Így az egymáshoz képest szabálytalanul tüzelő agykérgi idegsejtek egyes kisülései szinkronizálódhatnak, ennek megfelelően működésük összerendeződhet. A gátló neuronok tehát pontosan ellenőrizhetik a kérgi oszlopok szinkron működését. A szinkronizáció ily módon néhány sejtes csoportban is létrejöhet annak a veszélye nélkül, hogy a sejtszinkronizáció nagyobb területekre kiterjedjen. Eszerint még egy viszonylag kis amplitúdójú bemenetnek is jelentős hatása lehet a posztszinaptikus sejt akciós potenciáljának időzítésében, ha az térben és időben rendezetten érkezik.

Az agykérgi sejthálózatok szinkron működését az elektromos kapcsolatok is nagymértékben befolyásolhatják. Felnőtt agykéregben több kérgi GABAerg neuron képes a posztszinaptikus $GABA_A$ receptorokon keresztül ható kémiai szinapszisok mellett elektromos szinapszissal is kapcsolódni azonos típusú interneuronhoz. Így a gyorsan tüzelő sejtek homogén, réskapcsolatokkal összefűzött hálózata a γ-ritmus létrehozásában és fenntartásában

játszik szerepet, a szabályosan tüzelő nempiramis sejtek réskapcsolat hálózatában β- és a γ-ritmus alakulhat ki, a multipoláris börsztölő sejtek hálózatában az elektromos kapcsolatok a θ-ritmus kialakulását segítik. Az agykérgi interneuronok réskapcsolat hálózatainak egyik általánosan megfigyelt tulajdonsága az, hogy általában csakis azonos típusú sejtek alakítanak ki ilyen elektromos kapcsolatot egymás között. Ezek az eredmények arra engednek következtetni, hogy az elektromos szinapszisokon keresztüli szinkronizáció csak a homogén interneuron populációkra jellemző.

A fentebb jellemzett, komplex $GABA_A$ + $GABA_B$ választ kiváltó neurogliaform sejtek szintén képesek egymással elektromos szinapszison keresztül kommunikálni. Patkány szomatoszenzoros agykéregben végzett páros, illetve triplet elvezetéseink azt is bizonyítják, hogy a neurogliaform sejtek más típusú GABAerg interneuronokkal is létesítenek elektromos kapcsolatot, mint például gyorsan tüzelő kosársejtekkel, axo-axonikus sejtekkel, vagy szabályosan tüzelő nempiramis sejtekkel is. A neurogliaform sejtek gyakrabban alkotnak elektromos kapcsolatot egymás között, mint más interneuronokkal, ugyanakkor már az a tény is, hogy más interneuronokkal nagy valószínűséggel állhatnak elektromos kapcsolatban, különleges szerepet jósol ezen sejttípusnak. Mivel az elektromos szinapszisokat érintő kérgi fejlődés a vizsgált állatokban a harmadik hét végére lezárul, ezért a fiatal felnőtt elektromos szinapszisok képzése nem tekinthető átmeneti, a fejlődés részét képező, idővel eltűnő jelenségnek. Eredményeink azt mutatják, hogy a neurogliaform sejtek nagyon ritkán kapcsolódnak egymáshoz tisztán elektromos szinapszissal, túlnyomórészt az elektromos szinapszis mellett még reciprok módon gátolják egymást lassú IPSP-vel. Az összetett kémiai és elektromos kapcsolat elég hatásos módja a szinkronizációnak, ezért gyanítható, hogy a neurogliaform sejtek esetében is elősegíti a két sejt szinkron működését ez a mechanizmus. Hasonló funkciója lehet a más típusú interneuronokkal létesített összetett kémiai és elektromos szinapszisoknak. Neuronhálózat-modellek szerint az elektromos szinapszisok szerepe a hálózat heterogenitásának ellensúlyozása a szinkronizációban, ami éppen a szinkronizációt fokozó hatás. Ezek a sejtek minden bizonnyal hozzájárulnak a posztszinaptikus célpontok összehangolt gátlásához, valamint képesek visszatükrözni más interneuron hálózatok aktivitását, és ennek megfelelően gátolni más idegsejteket. A neurogliaform sejtek tehát egyedülálló módon, elektromos és kémiai szinapszisokkal innerválják a különböző interneuron populációkat a felnőtt agykéregben. Így hozzájárulhatnak a kérgi szinkron aktivitás kialakulásához lassú IPSP-kből álló szinkron bemeneteik segítségével, másrészt a különböző interneuronok aktivitásának monitorozásával e szinkron gátlást egyedülállóan tudják hangolni a teljes GABAerg hálózat pillanatnyi állapotához.

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