Role of neurogliaform cells in cortical microcircuits

Ph.D. thesis

Szabolcs Oláh

Supervisor:
Gábor Tamás, Ph.D., D.Sc.
Department of Physiology, Anatomy and Neuroscience
University of Szeged

2009
Szeged

INTRODUCTION AND AIMS

Neocortex is the most developed structure of the cellular organization in the mammalian brain. Two main divisions of the cortical cells are pyramidal cells and the so called non-pyramidal cells or interneurons. Pyramidal cells release excitatory amino acid as neurotransmitter, and innervate their postsynaptic target cells by glutamatergic synapses. They are organized into layers according to their projections. Their axoncollaterals could be in connection with contralateral and ipsylateral cortical areas, moreover, they establish connections with subcortical areas like the spinal cord or the thalamus.

In contrast, the members of the latter division release γ-amino-butirate (GABA) as inhibitory neurotransmitter. They innervate their postsynaptic targets within the cortex, therefore their axons remain in the same cortical region. GABAerg interneurons show great morphological diversity regarding their dendritic and axonal arborization as we might have seen in Golgi-stained samples. Every fifth cell in the neocortex is GABAergic cell. GABAergic cells with different firing patterns could be classified into different physiological classes which could correlate with certain morphological cell types described in the frontal cortex. Moreover, cortical interneurons could be classified using morphological, molecular and physiological features, but it should not be forgotten that there is only one unitary reality behind each classification. These heterogeneous GABAergic interneurons are typically present in different cortical areas of the brain. Great diversity of these cells is also reflected in their complex synaptic relations and their efferent connectivity. On the basis of this observation interneurons could be classified not only by their various cell-type preferences, but by their spatially selective innervation of the surface of the postsynaptic cells. Regarding to this, basket cells are innervate cellbodies, axo-axonic cells innervate exclusively the axon initial segments of their postsynaptic pyramidal cells, Martinotti cells and regular spiking cells innervate dendritic shafts and spines. Synaptically active GABA is essential for keeping the cortical excitatory processes in a certain level and for specifying the receptive field characteristics of sensory-cortex neurons. 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. Postsynaptic effects of the GABA, which consist of the transitional hyperpolarization of the postsynaptic membrane and the generation of the inhibitory postsynaptic potential (IPSP) in the postsynaptic cell, are mediated by two type of receptors. $GABA_A$-receptors are chlorine-channels with fast

activation and the $GABA_B$-receptors are activating potassium conductance in a slower, G-protein mediated process. It is generally accepted, that $GABA_A$-receptors are mainly activated around the soma, while dendritic iontoforetic application of the GABA evokes multiphasic process. GABA release in synaptic places mainly comes from the local cortical neurons. Although dual recordings revealed several classes of interneurons evoking fast, $GABA_A$ receptors 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. To find the origin of neocortical slow inhibition we investigated the kinetics, pharmacology and postsynaptic localisation of inhibitory inputs evoked by distinct type of interneurons in pyramidal cells. During the experiments we faced the neurogliaform cell, a distinct member of the GABAergic interneuron family. Involved in this project my tasks were the drawing of the three dimensional reconstructions of the cell pairs, describing their exact morphology and mapping the possible chemical synaptic places between them.

Besides chemical synapses electrical connections also play a crucial role in the cell-to-cell communication. 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. 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 current flow across the gap junction is virtually instantaneous, communication can occur without delay that is characteristic of chemical synapses. Gap junctions were theoretically and experimentally proved to contribute to synchronous activity of interneuron 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. There are rising evidences for homologous electrical connections between neurogliaform cells, but the

exact anatomy of this kind of connection have not been identified yet. This was the reason why I chose the identification of the homologous and heterologous electrical connections of the neurogliaform cells.

METHODS

All procedures were performed according to the Declaration of Helsinki 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. 300-320 μm slices were obtained from Whistar rats (P19-35) and 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. Whole-cell patch clamp recordings were carried out at ~35 °C from concomitantly recorded pairs, triplets or quadruplets of layers 2-3 putative interneurons and/or pyramidal cells as detailed previously. The cells were chosen by their shape with the help of the infrared differential interference contrast microscope (IR-DIC). Further identification of the GABAergic neurons were carried out by their physiological properties. Micropipettes (5-7 MΩ) were filled with (in mM) 126 K-gluconate, 4 KCl, 4 ATP-Mg, 0.3 GTP-Na2, 10 HEPES, 10 creatine-phosphate and 8 biocytin (pH 725; 300 mOsm). Signals were recorded with HEKA EPC9/2 amplifiers in fast current clamp or whole cell mode and were filtered at 5 kHz, digitized at 10 kHz and analyzed. Mann-Whitney U-test was used to compare datasets, differences were accepted as significant if $p < 0.05$.

Presynaptic cells were stimulated with brief (2 ms) pulses to reliably evoke action potential when investigating synaptic connections. The short-term dynamics of a given synapses were measured with 60 ms interval paired pulse protocols. During subthreshold paradigms, the membrane potential of postsynaptic cells were usually held at -51 ± 4 mV to separate the chloride driven IPSPs (reversal potential at -72 mV) and sodium or calcium driven EPSPs. Unless specified, traces shown are averages of 10-30 episodes. The amplitude of postsynaptic events was defined as the difference between the peak amplitude and the baseline value measured prior to the PSP onset. All traces were offseted to align their baselines for the period from -20 to 0 ms prior to the onset of current injections into the presynaptic neuron. Data for analysis were used only from epochs in which the postsynaptic response remained stationary, i.e. the mean amplitude of 10 consecutive events remained within ±10 % of the mean of the first 10 events of the epoch. Presynaptic neurogliaform 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. SPSS for Windows software package was used for statistical analysis. The significance level for all comparisons was set at $p < 0.05$. Data are given as mean ± s. d. Mann-Whitney U-test, Wilcoxon-test and Friedman-test was used to compare datasets.

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 for at least 12 hours. Three-dimensional (3D) light microscopic reconstructions of recovered cells were carried out using Neurolucida. For the statistical analyses parametric and non-parametric tests were carried out with the SPSS 11.0 and the ORIGIN 6.0 softwares. Differences were accepted as significant if $p < 0.05$. Blocks containing the cells were cut out from the sections and reembeded. 65 nm serial sections were cut with an ultramicrotome, mounted on Pyeloform-coated copper grids and stained with lead citrate. Light microscopically detected presumed synapses were checked under electron microscope.

RESULTS AND DISCUSSION

1. Gap junctional connections established by neurogliaform cells

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. We have recorded from pairs, triplets and quadruplets of cortical neurons of rat somatosensory cortex. We tested these connections with injection of action potentials and/or hyperpolarization current pulses. Based on previous experiments showing widespread evidence for electrical coupling between similar GABAergic cells, we started by characterizing homologous electrical connections between neurogliaform cells. Of the 16 pairs of neighboring neurogliaform cells tested for electrical coupling, we confirmed electrical connections between neurogliaform cells in eight cases, indicating a 50% rate for coupling. All electrical connections between ngf cells were reciprocal.

Apart from homologous electrical connections between neurogliaform cells, we detected heterologous gap junctions linking neurogliaform cells and various other types of interneurons. From the 32 connections tested between closely spaced neurogliaform and fast

spiking basket cells and axoaxonic cells, we confirmed reciprocal electrical coupling in seven pairs (of which one was neurogliaform-axoaxonic cellpair), indicating a 22% rate for interaction. When testing connections between pairs (n = 30) of neurogliaform and regular spiking nonpyramidal cells, we detected heterologous coupling in six cases, indicating a 20% rate for electrical synapses between these cell populations. All electrical connections between neurogliaform and regular spiking nonpyramidal cells cells were mutual and all six neurogliaform cells involved in electrical coupling elicited IPSPs on the postsynaptic regular spiking nonpyramidal cells.

My collegaues managed to identify the gap junctions between the tested cells with correlated electronmicroscopy. Electrical interactions were mediated by one or two electron microscopically verified gap junctions linking the somatodendritic domain of the coupled cells. Electrical connections were more frequent between neurogliaform cells (50%), than in neurogliaform cell – other type of GABAergic interneuron relations (~20%).

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.

2. Output of neurogliaform cells to various neuron types in the human and rat cerebral cortex

As we have already mentioned, neurogliaform cells in the rat elicit combined GABAA and GABAB receptor-mediated postsynaptic responses on cortical pyramidal cells and establish electrical synapses with various interneuron types. However, the involvement of GABAB receptors in postsynaptic effects of neurogliaform cells on other GABAergic interneurons is not clear. We measured the postsynaptic effects of neurogliaform cells *in vitro* applying simultaneous whole-cell recordings in human and rat cortex.

We recorded 13 connections in layers 2/3 in slices derived from human association cortices in which the presynaptic neurons were neurogliaform cells. Human neurogliaform cells evoked long-lasting inhibition in postsynaptic cells. These cells were pyramidal cells (n = 4) and various interneurons (n = 7). When comparing IPSPs arriving to postsynaptic

pyramidal cells (not shown) and interneurons in the human sample, we could not detect significant differences in 10–90% rise times and half-widths (20.1±9.8 ms and 12.7±6.8; 230.4±64.6 ms and 148.6±108.4, respectively). We applied pharmacological blockers to dissect the contribution of the two GABA receptor subtypes to the inhibition evoked by human neurogliaform cells. Addition of the GABAB receptor blocker CGP35348 (60 μM) into the bath solution could shorten the duration of slow IPSPs evoked by neurogliaform cells (n = 2; postsynaptic cells were interneurons). When testing the connections by applying the GABAA receptor blocker gabazine (10 μM), we could isolate the slow component of the IPSPs (n = 2; postsynaptic cells were interneurons). In addition to chemical neurotransmission elicited by human neurogliaform cells, we detected homologous electrical connections from neurogliaform cells to other neurogliaform cells (n = 2, not shown) and a heterologous electrical connection between a neurogliaform cell and a different type of interneuron which was combined with a slow IPSP triggered by the neurogliaform cell. Electrical synapses were detected as spikelets in the postsynaptic cells in response to presynaptic action potentials. When injecting hyperpolarizing current pulses into either of the connected cells, the other cell responded with an electrical coupling potential of similar polarity confirming electrical interaction.

Following our findings in the human cortex based on a relatively limited sample, we performed supporting experiments in slices taken from the rat cerebral cortex. We recorded 48 pairs of neurons in which neurogliaform cells evoked slow inhibition in various types of interneuron (neurogliaform cells, regular spiking cells, fast spiking basket cells, fast spiking axo-axonic cells, and unclassified interneurons).

The first simultaneous multiple recordings of human neurogliaform cells and their postsynaptic targets revealed that single spikes in neurogliaform cells elicit long-lasting unitary IPSPs composed of GABAA and GABAB receptor-mediated components in various types of interneuron. We confirmed these results in the rat cortex and, moreover, our experiments showed that human neurogliaform cells, similar to those tested in the rat, evoke long-lasting IPSPs in pyramidal cells. Besides, our experiments represent the first electrical synapses recorded electrophysiologically in the human cerebral cortex. Neurogliaform cells represent a unique element in human and rat microcircuits of the cerebral cortex. Embedded into an extensive network of homologous and heterologous electrical synapses linking several interneuron classes, neurogliaform cells are able to monitor the sub- and suprathreshold activity of coupled neurons and can transform this activity to long-lasting chemical signaling through metabotropic $GABA_B$ receptors on multiple neuron populations.

3. Single interneuron driven GABAergic volume transmission in the cerebral cortex

A distinctive feature of neurogliaform cells among cortical interneurons is the very dense axonal arborization in which presynaptic boutons on the same or neighboring collaterals can be found a couple micrometers from one another. GABA can activate receptors located up to several micrometers from the release site. We hypothesized that the high density of neurogliaform axons could help in counteracting transmitter reuptake mechanisms and release of the transmitter from neurogliaform cells might reach the majority of GABA receptors expressed in the tissue intermingled by the axonal cloud.

Ultrastructural analysis of GABAergic neurogliaform cell to pyramidal cell interactions in the rat cortex showed synaptic junctions in two pairs, but identified no synapses in three other pairs tested. Potentially nonsynaptic communication suggests a very high rate of functional coupling between neurogliaform cells and neighboring neurons. Indeed, when searching our database containing 183 simultaneously recorded pairs of neurogliaform cells and other neurons with somata located <100 μm apart, we detected hyperpolarizing effects of neurogliaform cells in 157 (86 %) of tested cells, a ratio unprecedented in paired recordings of cortical neurons.

We asked whether GABA, released from neurogliaform cells, modulates axon terminals which do not receive synaptic junctions in the cerebral cortex but frequently express GABAB receptors. First, we tested if neocortical neurogliaform cells could modulate their own axon terminals via GABAB receptors similar to hippocampal neurogliaform cells. The paired pulse ratio of slow IPSPs elicited by neurogliaform cells in their target cells could be reversibly altered with the GABAB receptor blocker CGP35348 (40 μM; n=8; 0.17±0.06, 0.45±0.17 and 0.16±0.10 in control, CGP35348 and wash, respectively, $p<0.001$). Thus, GABA has homosynaptic or autocrine action on neurogliaform axon terminals involving presynaptic GABAB receptors which contribute to the massive downregulation of neurogliaform output lasting for more than a minute. Modulatory action of neurogliaform cells was not limited to homosynaptic downregulation of axon terminals. Heterosynaptic or paracrine effects of neurogliaform cells on axons were also suggested by experiments in which we simultaneously recorded from three neurons consisting of a pyramidal cell to an interneuron connection (test EPSPs) and a neighboring neurogliaform cell activated 60 and 120 ms before the first and second test EPSP, respectively. Switching on the action potential in the neurogliaform cells 60 ms before the spike in the pyramidal cell could not change the amplitude of the first test

EPSPs (n=5; 98±4 %) relative to control, i.e. when the spike in the neurogliaform cell was not elicited. This indicates that tonic inhibition through GABAA receptors potentially activated by neurogliaform cells did not interfere significantly with test EPSPs apart from contributing to input resistance changes. However, the neurogliaform cells were effective in decreasing the amplitude of the second test EPSPs timed 120 ms after the spike in the neurogliaform cells to 77±5 % ($p<0.03$) of control. Moreover, neurogliaform cells, activated 120 ms prior to test IPSPs triggered by other neurogliaform cells, successfully suppressed the amplitude of test IPSPs to 74±4 % (n=10; $p<0.02$) of control.

We investigated whether preceding activation of neurogliaform cells could modulate the test connections in the presence of the GABAB receptor blocker CGP35348 (40 μM). The amplitudes of test EPSPs (n=3) or test IPSPs (n=10) under these conditions remained unchanged (101±4% and 98±8%, respectively) by switching on and off the spike in the neurogliaform cells showing that GABAB receptors were required for the heterosynaptic modulatory action of neurogliaform cells.

GABAB receptors are located on axon terminals as well as dendritic compartments of cortical neurons, thus we investigated whether GABA released by neurogliaform cells acts on GABAB receptors located pre- or postsynaptically. We introduced the G-protein uncoupler N-ethyl-maleimide (100 μM) into the intracellular solution used for postsynaptic cells of test IPSPs in order to block the effect of GABAB receptors postsynaptically but not presynaptically. In these cases, neurogliaform cells remained effective in suppressing the amplitude of test IPSPs to 77±6 % of control (n=6; $p<0.04$).

The experiments presented above suggest that single neurogliaform cells release GABA in ways which overcome the capacity of GABA reuptake mechanisms available. For further testing of this idea, we blocked the major neocortical plasma membrane GABA transporter (GAT-1) known to regulate the spillover of synaptically released GABA to see if the modulatory effect of neurogliaform cells could be enhanced. Adding the GAT-1 blocker NO711 (40 μM) into the extracellular solution produced a profound modulatory effect of neurogliaform cells on test IPSPs (n=8; 59±19 % of control). The heterosynaptic suppression of test connections during GABA reuptake blockade was significantly ($p<0.025$) enhanced compared to recordings without NO711 confirming our hypothesis that the amount of extrasynaptically available GABA released from neurogliaform cells contributes to the modulatory effect.

Unlike the rest of interneuron types characterized to date which form highly specific circuits in placing their synapses on somatodendritic compartments and the axon initial

segment of postsynaptic cells, neurogliaform cells follow a different strategy of spatial unspecificity and provide nonsynaptic input to the entire surface of target cells in addition to conventional synaptic junctions. Neurogliaform cells are capable of flooding the volume of their axonal field with GABA in effective concentrations and target the overwhelming majority of nearby neurons which, in turn, selectively express receptors sensitive to low concentrations of the neurotransmitter on their various compartments. Solitary spikes in a single neurogliaform cell might replace the concerted action potentials of interneuron populations in modulating presynaptic terminals and postsynaptic domains expressing GABA receptors at certain operational states of the microcircuit.

PUBLICATIONS

Anna Simon, **Szabolcs Oláh**, Gábor Molnár, János Szabadics, Gábor Tamás: Gap-junctional coupling between neurogliaform cells and various interneuron types in the neocortex. Journal of Neuroscience 2005 Jul 6;25(27):6278-85.
IF: 7.49

János Szabadics, Csaba Varga, Gábor Molnár, **Szabolcs Oláh**, Pál Barzó, Gábor Tamás: Excitatory effect of GABAergic axo-axonic cells in cortical microcircuits. Science 2006 Jan 13;311(5758):233-5.
IF: 26.37

Szabolcs Oláh, Gergely Komlósi, János Szabadics, Csaba Varga, Éva Tóth, Pál Barzó, Gábor Tamás: Output of neurogliaform cells to various neuron types in the human and rat cerebral cortex. Frontiers in Neural Circuits 2007 Nov 2;1(1)

Gábor Molnár, **Szabolcs Oláh**, Gergely Komlósi, János Szabadics, Csaba Varga, Pál Barzó and Gábor Tamás: Single pyramidal cells activate feed-forward networks in the human cerebral cortex. Submitted to PLOS Biology, 2008 Sept 2; 6(9)
IF: 13.5

Szabolcs Oláh, Gergely Komlósi, Pál Barzó and Gábor Tamás: Single interneuron driven GABAergic volume transmission in the cerebral cortex, under reviewing.

Accumulated impact factor: 47.4
Number of references: 93

CONFERENCE POSTERS

Anna Simon, **Szabolcs Oláh**, Gábor Molnár, János Szabadics, Gábor Tamás: Gap junctional coupling between neurogliaform cells and various interneuron types in the neocortex. 3rd Inmed/TINS Conference, La Ciotat, France, 2004

Anna Simon, **Szabolcs Oláh**, Gábor Molnár, János Szabadics, Gábor Tamás: Gap junctional coupling between gabaergic interneurons in the neocortex. XI. Conference of Hungarian Neuroscience Society, Pécs, Hungary, 2005

Szabolcs Oláh, Anna Simon, János Szabadics, Csaba Varga, Gábor Molnár, Gábor Tamás: Connection specific output of neurogliafornm cells. Internetional IBRO Workshop, Budapest, 2006

Szabolcs Oláh, Anna Simon, János Szabadics, Gergely Komlósi, Gábor Molnár, Csaba Varga, Éva Tóth, Pál Barzó, Gábor Tamás: Axonal and dendritic effects of neurogliaform cells in rat and human neocortex. Gordon Research Conferences, Mechanism of Epilepsy & Neuronal Synchronization, Colby College, USA, 2006

Szabolcs Oláh, Gergely Komlósi, Éva Tóth, Pál Barzó, Gábor Tamás: Presynaptic Effects of Neurogliaform Cells: Unitary GABAergic Volume Transmission. IBRO World Congress of Neuroscience, Melbourne, Australia, 2007

Szabolcs Oláh, Gergely Komlósi, Éva Tóth, Pál Barzó, Gábor Tamás: Presynaptic Effects of Neurogliaform Cells: Unitary GABAergic Volume Transmission. FENS Forum, Geneva, Switzerland, 2008

Szabolcs Oláh, Gergely Komlósi, Éva Tóth, Pál Barzó, Gábor Tamás:Unitary, GABAergic volume transmission: presynaptic effects of single neurogliaform cells in the neocortex. Annual Meeting of Society of Neuroscience, Washington, USA, 2008

Szabolcs Oláh, Gergely Komlósi, Éva Tóth, Pál Barzó, Gábor Tamás:Unitary, GABAergic volume transmission: presynaptic effects of single neurogliaform cells in the neocortex. International IBRO Workshop, Budapest, 2009

LECTURES

Szabolcs Oláh, Gábor Molnár, Pál Barzó, Gábor Tamás: Intrinsic properties of identified human neurons following increased intracranial pressure, 4th Pannonian Symposium on Central Nervous System Injury, Conference of Hungarian Neurosurgical Society, Pécs, Hungary, 2008