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THE ROLE OF GABAERGIC INTERNEURONS IN MICROCIRCUITS OF THE CAT VISUAL CORTEX

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Introduction and aims

On average, every fifth neuron and every sixth presynaptic terminal in the neocortex synthesizes and presumably releases gamma aminobutyric acid (GABA). Synaptically released GABA is essential for limiting excitability within the cortex to operational levels, to maintain the receptive field properties of neurons in sensory cortex and it plays a critical role in the timing of both subthreshold events and the firing of cortical cells. Postsynaptic effects of GABA in cortex are mediated by fast activating anion channels, the $GABA_A$ receptors, and by $GABA_B$ receptors which activate a potassium conductance via a slower G-protein mediated mechanism. There is general agreement that near the soma mainly $GABA_A$ receptors are activated, whereas GABA iontophoretically applied to the dendrites evokes complex multiphasic responses.

Most of the synaptically released GABA in cortex originates from intrinsic cortical neurons, which differentiated into distinct cell types that appear to target different compartments of the postsynaptic neuron with their terminals. Although very few GABAergic neurons have been characterised quantitatively for their postsynaptic targets, it appears that for example, cortical basket cells innervate the soma and proximal dendrites, and are therefore likely to act through $GABA_A$ receptors. The postsynaptic effect of neurons that innervate the dendritic domain of neocortical cells is unknown, although it has been suggested, mainly in analogy with results on hippocampal neurons, that synaptically activated $GABA_B$ receptors are located in the dendritic region. The action of GABA in the dendritic region is of interest because in this domain of the neuron GABAergic mechanisms probably interact with other receptor mediated events and voltage dependent conductances specific to the dendritic region. Recently, microapplication of glutamate was used to evoke GABAergic responses from different sites in the cortex *in vitro* and the results suggested that $GABA_A$ and $GABA_B$ receptor mediated responses can be evoked from different sites, which was interpreted as indicating that separate neurons activate $GABA_A$ and $GABA_B$ receptors, but the cells were

not identified. Thus, although there is some information about the neuronal classes that release GABA and two major mechanisms of postsynaptic responses have been found in cortical cells, only indirect experimental data link some presynaptic cell types and postsynaptic mechanisms.

The identification of cortical neurons evoking particular postsynaptic effects can be made by recording simultaneously from both the presynaptic and postsynaptic neurons followed by the microscopic visualisation of the pre- and/or postsynaptic cells. Combining this method with subsequent electron microscopic verification of the synaptic junctions mediating synaptic interactions provides an opportunity for the rigorous assessment of differences and similarities in synaptic interactions. In the neocortex, to our knowledge, only one identified and visualised sparsely spiny interneuron has been reported to evoke fast IPSPs in a pyramidal cell, although the sites of interaction between the two cells were not determined.

When addressing experimental questions regarding the functional properties of identified GABAergic neurons, cat visual cortex appears to be suitable for two major reasons. First, detailed work has been done to investigate GABAergic local-circuit neurons whereas in the rat the equivalent cell types remain considerably less well-defined. Second, three decades of intense research have elucidated many of the physiological properties of cat visual cortex. Several of these studies have already highlighted the importance of GABAergic circuits in shaping the functional characteristics of cortical neurons and numerous specific models and wiring diagrams have been put forward, although the majority of them remain to be tested experimentally.

In order to evaluate quantitative differences in the termination patterns of cortical GABAergic cells and to establish the precise placement of synapses that mediate identified postsynaptic effects we used paired intracellular recordings and subsequent light- and electron microscopic analysis of the connections in the first series of our experiments. We examined how the postsynaptic effect of identified GABAergic cells is influenced by the number of synapses between pairs of neurons and the location of synaptic release sites on the target cells.

After the identification of postsynaptic responses evoked by interneurons we examined the activation of cortical GABAergic cells using similar experimental procedures. It is generally accepted that locally activated interneurons are essential to control cortical excitation and circumscript classes of interneurons may subserve distinct functional roles. However, the GABAergic cell types, targeted by recurrent axon collaterals of pyramidal and spiny stellate cells, are unknown. In the hippocampus, association of GABAergic axons with subsets of excitatory afferents strongly suggests a pathway-specific modulatory function. Not surprisingly, this apparent division of labour is probably paralleled by differences in the excitatory inputs and activation of GABAergic neurons. Due to the geometry of their dendrites certain types of interneurons in the hippocampal molecular layer may only be activated in feedforward manner. Others, such as the somatostatin and mGluR1α-positive interneurons, receive predominantly, if not exclusively recurrent excitatory input, whereas some cell classes, such as basket neurons, are presumably involved in both types of circuit.

Thus, we addressed the following questions in the second set of our experiments: Which types of interneurons receive local excitatory feedback? Is the recurrent input targeted to a particular domain of the postsynaptic somato-dendritic surface? What are the properties of recurrent unitary EPSPs? What is the strength of individual connections? Which factors contribute to the variability of postsynaptic responses?

Neocortical interneurons receive input from pyramidal and nonpyramidal cells and also from subcortical afferents. In the experiments mentioned above, we labeled synaptically coupled neuron pairs from slices of the cat visual cortex. During the anatomical analysis of such biocytin filled cell pairs, we found, to our surprise, that a significant portion of the light- and electron microscopically detected connections was formed by axons originating undoubtedly from the parent cell. We detected the same result also from preparations containing only one filled neuron.

In 1972, VAN DER LOOS and GLASER proposed the word autapse to describe a synapse between a neuron and its own axon. In addition to their original Golgi study in rabbit neocortex, possible autaptic contacts have been observed in dog and rat cerebral cortex, monkey neostriatum and cat spinal cord with the classical method. Several groups detected such self-innervating connections with intracellular markers from various brain regions, like substantia nigra, striatum and spinal cord. All of these studies were based on light microscopical observations except PETERS and PROSKAUER'S work, which verified an autapse on a multipolar stellate cell and a fraction of recent data by LÜBKE *et al.* on layer V pyramids from developing cortex. Autapses of cell cultures have been used as model for synaptic interactions in several physiological experiments, but a few study proposed in vivo functional significance for inhibitory autaptic innervation in the rat neostriatum and in *Aplysia* buccal ganglia.

Using intracellular biocytin labeling and correlated light- and electron microscopy, we determined the exact number of autapses on several neocortical cell types. We found that different cortical cell types exhibit various degree of self-innervation with a subcellular location reflecting the postsynaptic target preference of the parent cells. Moreover, filling synaptically coupled cell pairs it became feasible to compare the number and position of synaptic junctions on a given postsynaptic cell with that of autapses established by the same presynaptic neuron.

Materials and methods

Brain slices (400 µm thick) were obtained from areas 17 and 18 of adult cat visual cortex. The slices were transferred to a recording chamber, where they were maintained at 34-35 °C at the interface between oxygenated artificial cerebrospinal fluid (ACSF) and a humidified atmosphere saturated with 95% O_2 / 5% CO_2. Recording electrodes were filled with 2% biocytin. Putative GABAergic neurons were identified due to their physiological characteristics, such as short-duration action potentials followed by large amplitude fast afterhyperpolarizing potentials (fAHP). Once a stable recording had been obtained a search was made for cells displaying the electrophysiological properties of pyramidal and spiny stellate neurons. Synaptic coupling was tested using on-line spike-triggered averaging whilst eliciting firing in the interneuron with either depolarizing current pulses or constant DC current injections.

In most of the cases, diffusion, presumably aided by depolarizing (0.1-0.5 nA) current pulses employed during recording resulted in an adequate filling of neurons by biocytin. Slices were fixed and re-

sectioned at 60 μm thickness and the biocytin filled cells were visualised by the avidin-biotinylated horseradish peroxidase (ABC) method with diaminobenzidine as chromogen.

Recovered cells were reconstructed from the serial 60 μm thick sections of the entire slice under a light microscope using a drawing tube. The total number of axonal varicosities in the slice, some of them shown by subsequent electron microscopy to correspond to synaptic boutons, was counted during the drawing procedure. The tissue volume containing the axonal arbour was calculated for each interneuron. The tissue shrinkage factor was determined by stereotaxic injection of wheat germ agglutinin (WGA) followed by the WGA's visualization identical to that of biocytin. In each case, the location of the pre- and postsynaptic cell in the central portion of the slice enabled us to perform nearly full reconstructions of the dendritic arbours. The entire somatodendritic surface of both recorded cells was tested for close appositions with filled axons, each of which were traced back to the parent soma.

Following light microscopic analysis, axon-rich areas were re-embedded for ultrathin sectioning. The sections were scanned in the electron microscope and all biocytin-filled axonal profiles were followed until they formed synaptic contacts. Since all profiles were followed and the plane of the section randomly cuts through the axonal branches, the above procedure ensured a random sample of postsynaptic targets. Each presynaptic terminal that was studied was completely examined in serial sections to establish the number of synapses it formed. Subsequently, *all* light microscopically detected sites of close appositions between filled axons and labeled somata, dendrites or spines were tested in serial electron microscopic sections. Moreover, *all* filled somata were serially sectioned completely for electron microscopic analysis to check for the presence of axonal branches which may have been obscured by the opaque cell bodies.

We applied *a priori* and *a posteriori* cluster analysis using the postsynaptic elements as variables to determine the interneuron cell classes. FISHER'S exact test for heterogeneity was used to compare the frequency of postsynaptic elements amongst the targets of different presynaptic cells. The non-parametric MANN-WHITNEY U-test was applied to compare the properties of the different cell types.

Results and conclusions

Fast IPSPs elicited via multiple synaptic release sites by different types of GABAergic neuron in the cat visual cortex

All smooth dendritic cells established type II synapses previously shown to be made by terminals containing GABA, therefore the studied cells are very likely GABAergic. Three classes of presynaptic cell could be defined, based on their efferent synaptic target preference determined from random samples of unlabeled postsynaptic cells. *a) Basket cells* (n = 6) innervated mainly somata (49.9 ± 13.8%) and dendritic shafts (45.2 ± 10.7%) and, to a lesser extent, dendritic spines (4.9 ± 4.6%). *b) Dendrite-targeting cells* (n = 5) established synapses predominantly on dendritic shafts (84.3 ± 9.4%) and less frequently on dendritic spines (11.2 ± 6.7%) or somata (4.5 ± 4.7%). *c) Double bouquet cells* (n = 4) preferred dendritic spines (69.2 ± 4.2%) to dendritic shafts (30.8 ± 4.2%) as postsynaptic elements and avoided somata.

Interneurons formed 5240 ± 1600 (range 2830-9690) synaptic junctions in the slices. Based on the density of synapses made by single interneurons and the volume density of GABAergic synapses, it was calculated that an average interneuron provides 0.45 ± 0.13 % of GABAergic synapses in its axonal field.

The location of synaptic junctions on individual identified postsynaptic cells reflected the overall postsynaptic target distribution of the same GABAergic neuron. The number of synaptic junctions between pairs of neurons could not be predicted from light microscopic examination, the number of electron microscopically verified synaptic sites were generally smaller for the dendritic domain and larger for the somatic domain,

than expected from light microscopy. All presynaptic cells established multiple synaptic junctions on their postsynaptic target cells. A basket cell innervated a pyramidal cell via 15 release sites, the number of synapses formed by 3 dendrite-targeting cells on pyramidal cells were 17 and 8 respectively, and 3 on a spiny stellate cell; the interaction between a double bouquet cell and a postsynaptic pyramidal cell was mediated by 10 synaptic junctions.

All three types of interneuron (n = 6; 2 for each type of cell) elicited short-latency IPSPs with fast rise time (10 – 90 %; 2.59 ± 1.02 ms) and short duration (at half-amplitude 15.82 ± 5.24 ms), similar to those mediated by $GABA_A$ receptors.

Average amplitudes of unitary IPSPs (n = 6) were 845 ± 796 mV ranging from 134– 2265 mV. Variability of IPSP amplitude was moderate, the average ratio of IPSP and baseline noise variance was 1.54 ± 0.96. High frequency activation of single presynaptic dendrite targeting cells led to an initial summation followed by use-dependent depression of the averaged postsynaptic response. Double bouquet cell evoked IPSPs, recorded in the soma, had a smaller amplitude than those evoked by the other two cell types. In all connections, transmission failures were rare or absent, particularly when mediated by a high number of release sites.

The results demonstrate that different types of neocortical GABAergic neurons innervate distinct domains on the surface of their postsynaptic target cells. Nevertheless, all three types of cell studied here elicit fast IPSPs and provide GABAergic input through multiple synaptic release sites with few, if any failures of transmission.

Effect, number and location of synapses made by single pyramidal cells onto aspiny interneurons of cat visual cortex

Pyramidal neurons in layers II/III elicited monosynaptic EPSPs in three distinct categories of smooth dendritic local-circuit neurons, namely basket cells (n = 3), a dendrite-targeting cell (n = 1) and a double bouquet cell (n = 1). Unitary EPSPs in basket cells were mediated by 1, 2, and 2 synaptic junctions, whereas the pyramid-to-dendrite-targeting cell and pyramid-to-double bouquet cell interaction were mediated by 5 and 7 synaptic junctions, respectively. Synaptic junctions were found on all somato-dendritic compartments, with a tendency to be clustered in individual connections. Two pairs were reciprocally connected.

Unitary EPSPs had an average amplitude of 1,005 ± 518 mV, fast rise times (10–90 %; 0.67 ± 0.25 ms) and were of short duration (at half-amplitude 4.7 ± 1.0 ms). Their decay was monoexponential (τ = 7.8 ± 4.3 ms) and, at hyperpolarised membrane potentials, appeared to be shaped by passive membrane properties (τ = 9.2 ± 8.5 ms). All parameters of concomitantly recorded spontaneous EPSPs (mean rise time = 0.68 ± 0.18 ms; mean duration = 4.7 ± 1.7 ms; mean amplitude = 981 ± 433 mV) were remarkably similar.

By the analysis performed by OLE PAULSEN and CHRISTIAN STRICKER, the amplitude fluctuations of the EPSPs could be accounted for by a quantal model of transmitter release. Without quantal variance, however, the minimum number of components in the model, excluding the failures, exceeded the number of electron microscopically determined synaptic junctions for all five connections. In contrast,

incorporating quantal variance gave a minimum number of components which was compatible with the number of synaptic junctions, and which fitted the data equally well as models incorporating additional components but no quantal variance.

In conclusion, at least three distinct interneuron classes receive local excitatory pyramidal cell input which they relay to different compartments on their postsynaptic target neurons. Unitary EPSPs show fluctuations that can be accounted for by a quantal model of transmitter release, when incorporating quantal variance. The reliability of transmission is high, while the fast time-course of the EPSPs constrains their temporal summation and predisposes these classes of interneurons to act as coincidence detectors.

Massive autaptic self-innervation established by neocortical interneurons

Self-innervation was light microscopically tested on 10 pyramidal, 7 spiny stellate cells and on 39 smooth dendritic interneurons from cortical layers II-V. Putative autapses (n = 171) could be observed on each smooth interneuron and on 7 pyramidal cells, but not on spiny stellate cells. After the electron microscopic evaluation of all putative sites, 134 autapses could be verified, but the correctness of light microscopic estimation varied between cell types.

Pyramidal cells showed rare (10 %) and relatively weak self-innervation as only one pyramidal cell innervated itself with two autapses on the same dendritic spine, in spite of the examination of ultrathin sections from all (n = 25) predicted sites on the pyramids.

After the classification by the postsynaptic target distribution, all putative autapses of 6 basket (n = 68), 3 dendrite targeting (n = 58) and 2 double bouquet cells (n = 20) were scrutinised. Self-innervating junctions could be verified on each basket (n = 66; 11 ± 7 per cell) and dendrite targeting (n = 66; 22 ± 12 per cell) cells, but none of the double bouquet cells formed autapses. Although autapses were identified on all parts of the somatodendritic domain on both cell classes, basket cell autapses were significantly closer (13.3 ± 23.2 mm) to the soma, than autapses established by the dendrite targeting cells (51.8 ± 49.9 mm).

In summary, neocortical cell types establish various degree of self-innervation. Unlike on spiny cells, autapses are abundant on basket and dendrite targeting interneurons with similar subcellular target preference to that of synapses of the parent cell. The extensive self-innervation may modulate local dendritic information processing and intrinsic excitability via inhibitory feedback.