

**SYNAPTIC INPUT TO CHEMICALLY IDENTIFIED BASAL
FOREBRAIN NEURONS IN THE RAT**

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PUBLICATIONS

- I. **Hajszán T**, Zaborszky L, Direct Catecholaminergic-Cholinergic Interactions in the Basal Forebrain. III. Adrenergic Innervation of Choline Acetyltransferase-Containing Neurons in the Rat. *J Comp Neurol*, 449(2):141-157, 2002. *Impact factor: 3.848; times cited: 2*

- II. Wu M, **Hajszan T**, Leranath C, Alreja M, Nicotine Recruits a Local Glutamatergic Circuit to Excite Septohippocampal GABAergic Neurons. *Eur J Neurosci*, 18:1155-1168, 2003. *Impact factor: 4.163*

- III. **Hajszan T**, Alreja M, Leranath C, Intrinsic Vesicular Glutamate Transporter 2-Immunoreactive Input to Septo-hippocampal Parvalbumin-Containing Neurons: Novel Glutamatergic Local Circuit Cells. *Hippocampus*, *in press*, 2003. *Impact factor: 3.421*

INTRODUCTION

The basal forebrain corticopetal system

Since the classic experiments of Giuseppe Moruzzi and Horace Magoun in the late 1940's, it has been well known that both sleep and wakefulness are actively induced, actively maintained, and highly organized states of the brain. An extensive network of neurons that is designated as the ascending arousal system (AAS) orchestrates the characteristic forebrain activities during sleep and wakefulness. In addition to being the effector in the sleep/wake-inducing circuitry, the AAS plays a crucial role in regulating consciousness, cognitive and visceral functions, as well as motor responses by means of controlling the responsiveness of cortical and thalamic neurons to incoming stimuli. The AAS comprises of several widely distributed groups of neurons that can be characterized by their neurotransmitter content. They include the monoaminergic and cholinergic neurons in the brainstem and hypothalamus, the perifornical orexin-containing cells, the cholinergic and GABAergic neurons in the basal forebrain (BF), as well as the glutamatergic neurons in the thalamic intralaminar nuclei.

The basal forebrain corticopetal system plays a prominent role in the AAS. Since the discovery in the late '70s that Alzheimer's disease is characterized by a severe decline of cholinergic activity in the forebrain, the BF cholinergic system has been in the center of scientific attention. It also has become widely accepted that besides Alzheimer's disease, the derailment of the BF cholinergic system can play a role in the pathomechanism of other neurodegenerative and neuropsychiatric disorders such as Parkinson's disease, or schizophrenia. During the past decades, several studies have provided evidence that BF neurons are implicated in the regulation of sleep/wakefulness, sensory processing, attention, learning and memory, and motivation. The rat cholinergic BF comprises several regions closely associated to the medial and basal surfaces of the cerebral hemispheres. These areas include the medial septum, vertical and horizontal limbs of the diagonal band of Broca, substantia innominata, bed nucleus of stria terminalis, as well as the pallidal regions including the globus pallidus and ventral pallidum. These regions are richly populated by cholinergic, GABAergic, and peptidergic neurons that are intermingled with fiber bundles of several ascending

and descending neuronal pathways. BF neurons serve either as projection cells innervating the entire cortical mantle, the amygdala, and hippocampus, and/or interneurons forming the complicated circuits underlying local information processing.

Recent discoveries in neuroscience have raised new important functional and morphological aspects of the basal forebrain corticopetal system. These new discoveries prompted the series of experiments that are detailed in the present thesis. Using neuroanatomical examination techniques, our goal was to address the following specific issues.

Do basal forebrain cholinergic neurons receive autonomic input?

There is accumulating evidence that the BF cholinergic system participates in autonomic regulation, especially in cardiovascular control. It is still unresolved, however, how the viscerosensory information is conveyed to the BF, and how BF cholinergic neurons may affect autonomic regulation. Based on these data, we hypothesized that BF cholinergic cells receive neuronal input from known autonomic centers of the brain. To test the validity of our hypothesis, we studied the morphological aspects of a possible adrenergic/cholinergic link in the BF. The central adrenergic system participates almost exclusively in autonomic and/or neuroendocrine regulation and, therefore, the possible adrenergic innervation of BF cholinergic neurons may transmit only autonomic information. The adrenergic neurons are dispersed in the brainstem reticular formation and are arranged in three loose cell groups designated as C1, C2 and C3. Earlier morphological studies have repeatedly highlighted the widespread existence of phenylethanolamine N-methyltransferase (PNMT)-containing fibers in BF areas both at light and electron microscopic levels. It is still unresolved, however, whether the central adrenergic system has any direct physiological influence on the BF. To address the morphological aspect of this issue, we performed a series of light and electron microscopic double-immunolabeling studies in our first set of experiments.

What is the morphological foundation of glutamatergic effect on memory?

The recently discovered vesicular glutamate transporter (VGLUT) molecules have been proven to be highly specific markers for neurons that utilize glutamate as

neurotransmitter. In situ hybridization and immunohistochemical demonstration of VGLUTs enabled scientists to study the extensive glutamatergic circuitry in the central nervous system. It is well known that glutamate elicits strong functional responses in BF neurons. However, due to the lack of specific methods to visualize glutamatergic neuronal structures, the details of the BF glutamatergic circuitry are still unresolved. The rostral portion of the BF corticopetal system, the medial septum diagonal band complex (MSDB) is known to be involved in the generation of hippocampal theta rhythm and plays a key role in memory and cognitive functions. GABAergic neurons of the septo-hippocampal pathway selectively inhibit specific populations of GABA cells in the hippocampus. Via this connection, the septo-hippocampal GABA neurons exert a powerful disinhibitory influence on hippocampal pyramidal cells. It has been repeatedly suggested that tonic impulse flow in this septo-hippocampal GABAergic pathway may be critical for the generation of theta rhythm and normal cognitive functions. During the past decade, several authors have reported that intraseptal perfusion of glutamatergic drugs is able to powerfully influence the hippocampal theta rhythm and memory processes, supposedly by controlling the activity of septo-hippocampal neurons. Based on these data, we hypothesized that glutamatergic axons innervate septo-hippocampal GABAergic cells. To clarify whether the glutamatergic input to the MSDB comes from local sources and/or from extraseptal origin, we surgically separated the septum from part of its afferents by septal undercut and fimbria/fornix transection in our second set of experiments. Subsequently, simultaneous immunohistochemical visualization of the recently discovered specific glutamatergic marker VGLUTs and parvalbumin enabled us to test the validity of our hypotheses.

MATERIALS AND METHODS

Animals and surgical procedures

Adult male Sprague-Dawley rats (250-300 g) were group-housed and maintained on a 12/12 h light/dark cycle and provided with unlimited access to water and rat chow. All surgical interventions and perfusions were carried out using a ketamine-based anesthetic [ketamine (25 mg/ml), xylazine (1.2 mg/ml), and acepromazine (0.03 mg/ml) in saline; 3 ml/kg, i.m.]. In order to isolate the MSDB from all of its ventral afferents coming from the right side, unilateral septal undercut (SX) was carried out in a stereotaxic apparatus. Fimbria/fornix transection (FFX) was done to separate the MSDB from all afferents coming from the hippocampus via the right fimbria/fornix. Following SX and FFX, the rats were allowed to survive for five or ten days. In order to visualize VGLUT2-immunoreactive (IR) cell bodies in the septum, the axonal-transport blocker, colchicine was stereotaxically injected into the lateral cerebral ventricle to enhance the accumulation of VGLUT2 in the perikarya. Following the colchicine injection, rats were allowed to survive for 40 hours.

Tissue processing for light microscopy

Rats were transcardially perfused with fixative and the brains were removed and postfixed overnight. Then, coronal Vibratome sections (50 μ m) were cut from the BF.

Experiment I. In order to visualize adrenergic and cholinergic neuronal structures, PNMT and choline acetyltransferase (ChAT), the synthesizing enzymes for adrenaline and acetylcholine, respectively, were detected as markers using the appropriate antibodies. PNMT- and ChAT-IR elements were visualized using the black silver-gold intensified nickel-diaminobenzidine and the brown diaminobenzidine chromogens, respectively.

Experiment II. In order to visualize glutamatergic and septo-hippocampal GABAergic neuronal structures, VGLUT1 and VGLUT2 were detected as markers for glutamatergic elements and parvalbumin for the septo-hippocampal GABAergic neurons. VGLUT- and parvalbumin-IR elements were visualized using the nickel-diaminobenzidine and diaminobenzidine chromogens, respectively.

The sections were mounted on gelatin-coated slides, air-dried, cleared in xylenes, coverslipped with Permount and analyzed under an Olympus BX60 microscope equipped with a Zeiss AxioCam digital camera.

Tissue processing for electron microscopy

Sections were double-immunostained for PNMT/ChAT and VGLUT2/parvalbumin as described above and flat embedded in Araldite. Putative synaptic contacts were selected under the light microscope. Serial ultrathin sections were then cut and collected on single-slot Formvar-coated grids and examined in a Tecnai 12 transmission electron microscope equipped with an AMT Advantage 4.00 HR/HR-B CCD camera system.

Quantitative analysis and digital imaging

Mapping and quantitative analysis of adrenergic/cholinergic interactions in the BF. For the mapping and quantitative analysis of putative contact sites between adrenergic boutons and cholinergic neuronal profiles, a computer-controlled Zeiss Axioskop microscope equipped with the NeuroLucida software was used. Screening with the 100x oil-immersion lens, ChAT-IR cell bodies and adrenergic/cholinergic appositions were mapped and counted. Appositions were mapped and counted only if they qualified for electron microscopic analysis. Profiles mapped and counted as cholinergic cell bodies were ChAT-IR perikarya with clearly recognizable cell nucleus. In order to find out whether there are any statistical differences in the distribution patterns of adrenergic/cholinergic appositions across BF territories, a hypothetical distribution pattern of adrenergic/cholinergic appositions was established based on the null hypothesis that the appositions are distributed homogeneously on BF cholinergic neurons across the entire extent of the BF. The Student's t-test was finally used to determine whether there are any significant differences between the hypothetical homogeneous and the actual distribution patterns.

VGLUT2 bouton density analysis in the MSDB. In order to quantitatively analyze the relative changes in VGLUT2 bouton density caused by the deafferentations, VGLUT2-immunopositive varicosities were counted within 400 μm^2 unit areas from septal regions, such as the medial septum/lateral septum border zone (MS/LS) both ipsi-

and contralateral to the lesion, and from the middle septal area (between the right and left MS/LS, along the midline). Bouton densities obtained this way were statistically compared between ipsi- and contralateral MS/LS in SX and FFX animals, as well as between the same areas of SX versus intact and FFX versus intact rats.

Quantitative analysis of VGLUT2 input to parvalbumin-positive neurons in the MSDB. To reveal whether SX has any influence on the VGLUT2 innervation of MSDB parvalbumin neurons, parvalbumin cells in the MSDB were counted and sorted into two groups. Parvalbumin neurons were sorted into the first group if they were contacted by VGLUT2 boutons according to the following criteria. Parvalbumin cells were considered as targets of VGLUT2-IR boutons, if at least one VGLUT2 varicosity was found in close apposition either to the cell body or to a dendrite being continuous with the perikaryon. Parvalbumin cells that did not fulfill these criteria were sorted into the second group. These very same criteria were applied for both the electron microscopic studies and the quantitative analysis. Thus, the validity of the light microscopic quantitative analysis of VGLUT2 input to parvalbumin-positive neurons is backed by the ultrastructural findings. Finally, the amount of parvalbumin neurons contacted by VGLUT2 boutons (first group) was expressed as the percentage of all parvalbumin cells in the MSDB (first group + second group). These data obtained from SX animals were then statistically compared to those from intact rats.

RESULTS

Experiment I

Distribution and quantitative analysis of PNMT/ChAT appositions. The adrenergic/cholinergic appositions showed an uneven distribution across different BF structures. Preferential distribution sites were in the ventral part of horizontal limb of the diagonal band (HDB), within the cholinergic cell cluster of the substantia innominata, and in a narrow band bordering the substantia innominata from the globus pallidus and internal capsule. The majority of appositions were found in the substantia innominata (51%) followed by the HDB (30%). The remaining BF structures together contained 19% of all appositions.

By comparing a hypothetical homogeneous distribution of PNMT/ChAT appositions with the actual pattern, the analysis demonstrated that the number of adrenergic appositions was significantly higher in the substantia innominata than it should be in case of homogeneous distribution. On the other hand, the septal complex contained significantly fewer appositions. The numbers of appositions in all of the remaining areas were not significantly different from those of the hypothetical homogeneous distribution.

Ultrastructural characteristics of PNMT/ChAT relations. A total of 16 individual PNMT-IR varicosities, closely associated to cholinergic profiles, were randomly selected for ultrastructural analysis. Ten appositions were classified as axodendritic involving either proximal or distal cholinergic dendrites. During the ultrastructural analysis, eight of them were confirmed as synaptic. All of these axodendritic synapses were of the asymmetric type with clear and prominent postsynaptic densities. The remaining 6 selected boutons were located adjacent to cholinergic cell bodies, however only one of them was confirmed as synaptic.

Experiment II

VGLUT2-immunoreactive boutons synapse with parvalbumin-containing neurons in the MSDB. VGLUT2-positive varicosities gathered mainly in the medial septum/lateral septum border zone and along the boundaries of the vertical limb of the diagonal band. Along the midline, relatively fewer boutons were observed.

Parvalbumin-containing neurons were dispersed in this rich network of VGLUT2-IR axons and several close associations were observed both with cell bodies and dendrites. In some cases, parvalbumin-positive cell bodies were completely surrounded by VGLUT2-containing varicosities that resembled pericellular baskets. A total number of 24 VGLUT2/parvalbumin close associations were ultrastructurally analyzed both in intact and SX animals. Ten of the 13 VGLUT2-IR boutons closely associated to parvalbumin cell bodies were revealed to establish synaptic contacts. Furthermore, of the 11 VGLUT2/parvalbumin axo-dendritic close associations, eight were found to form synapses. All synapses were of the asymmetric type with prominent postsynaptic densities.

The majority of VGLUT2-containing varicosities in the MSDB remained intact following septal deafferentation. Despite the lack of recognizable changes under the light microscope, the quantitative analysis of the density of VGLUT2-containing boutons in the MSDB revealed significant alterations. Comparing the density values of VGLUT2-IR boutons between the ipsi- and contralateral MS/LS, there were significant decreases in the ipsilateral side both in SX and FFX animals by 24% and 12%, respectively. Taking into account the possibility that VGLUT2-positive axons may cross over, the density data from SX and FFX animals were compared to those from intact rats. In this paradigm, SX resulted in a significant decrease in the density of VGLUT2-containing boutons of the ipsilateral MS/LS and of the middle area of the MSDB by 28% and 13%, respectively. Following FFX, a significant decrease in the density of VGLUT2-IR boutons was observed in both the ipsi- (21%) and contralateral (10%) MS/LS.

The quantitative light microscopic analysis also showed alterations in the innervation of MSDB parvalbumin-positive cells by VGLUT2-containing axons, following SX. In intact animals, about 68% of parvalbumin neurons were found to be contacted by at least one VGLUT2-containing bouton on their perikarya and/or proximal dendrites, at all three levels of the MSDB. In SX rats, this percentage decreased significantly by 24% at the rostral level of the MSDB and by 22% at the middle level. The SX caused no change in this percentage at the caudal level.

VGLUT2-immunoreactive neurons exist in the septum. Light microscopic observation of the septum of colchicine-treated rats revealed a large number of

VGLUT2-IR cell bodies distributed throughout the MSDB. The majority of these cells occupied mainly the MS/LS and relatively few were seen along the midline. A very dense population of VGLUT2-containing neurons was found in the caudal part of the septal complex, along the midline and within the fiber bundles of the fornix. In the septofimbrial and triangular nuclei, as well as in the caudal end of the lateral septal division, a large number of VGLUT2-IR perikarya gathered to form clusters and bundles of cell bodies.

DISCUSSION

The adrenergic/cholinergic link in the basal forebrain

Participation of the basal forebrain PNMT/ChAT interaction in autonomic control. The central adrenergic system has been well known for decades to be one of the key components of autonomic regulatory mechanisms. Adrenergic axons from the medullary C1-C2 cell groups may transfer viscerosensory-related information directly or via the brainstem parabrachial relay to cholinergic neurons in the BF. Recently, it has been shown that lesioning of the rat BF cholinergic system results in the abolition of β -carboline induced cardiovascular responses, suggesting the involvement of the BF cholinergic system in autonomic regulation. Our quantitative analysis of the distribution of PNMT/ChAT appositions showed that the adrenergic input is biased towards cholinergic neurons in the substantia innominata. This region contains the majority of cholinergic neurons that are known to project to cortical areas involved in autonomic control, including the insular cortex and medial prefrontal cortex. Furthermore, the BF cholinergic effects on autonomic functions may be mediated via central amygdaloid cells since the amygdala and BF cholinergic neurons are in reciprocal connection. In addition to influencing specific cortical and amygdaloid areas, the adrenergic/cholinergic link in the BF may participate in autonomic control by modulating hypothalamic neuroendocrine and autonomic networks.

Role of the basal forebrain PNMT/ChAT interaction in cortical activation.

Recently, it has been shown that β -receptor stimulation in the medial septum, the shell of the nucleus accumbens, ventral pallidum, and the rostral part of the substantia innominata elicits robust activation of both cortical and hippocampal EEG in rats. Atropine pretreatment was sufficient to abolish this EEG activation, indicating that it is mediated via muscarinic cholinergic mechanisms. On the other hand, infusion of isoproterenol, a β -receptor agonist into the substantia innominata, an area that is heavily innervated by PNMT-IR fibers, failed to evoke any changes in cortical EEG. In another study, however, it has been reported that infusion of noradrenaline into the substantia innominata facilitates γ -EEG activity and elicits waking. This, together with previous electrophysiological results from guinea pig BF slices, suggests that mainly α_1 receptors mediate the arousal-enhancing effects of both noradrenaline and adrenaline in the

substantia innominata. These physiological data are in line with our results to suggest that the central adrenergic system can support cortical activation via α_1 and β adrenoceptors located on cholinergic corticopetal neurons in the BF.

Glutamate in the septo-hippocampal system

Implications of the deafferentation experiments. Changes in VGLUT2-positive bouton densities caused by the surgical interventions suggest that about 28% of these fibers in the MS/LS and 13% in the middle septal area have an extraseptal origin and arrive via the ventral pathway, since SX caused a VGLUT2 bouton density depletion of this magnitude. Another 31% of VGLUT2-containing boutons in the MS/LS are from axons traversing the fimbria/fornix of hippocampal origin (21% via the ipsilateral + 10% via the contralateral fimbria/fornix; data obtained from FFX animals). All of the remaining boutons, i.e., 41% in the MS/LS and 87% in the middle septal area may originate within the septum. Indeed, in colchicine-treated rats, we detected VGLUT2-immunopositive neurons throughout the septum. The distribution of these cell bodies was generally the same as previously determined by in situ hybridization experiments. However, previous studies have not shown data about the large posterior septal population of glutamatergic neurons described in the present study. Furthermore, we elaborated a new immunohistochemical approach that enables the visualization of VGLUT proteins in cell bodies. This new method can boost future studies on the organization and physiology of the glutamatergic neuronal systems in the brain.

Functional considerations. It has been reported that perfusing the septum with an N-methyl-D-aspartate (NMDA)-receptor antagonist results in a decreased septal GABA outflow suggesting a tonic glutamatergic activation of septal GABA neurons. Furthermore, intraseptal infusions of glutamate, NMDA and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) increased hippocampal theta frequency, while infusion of an NMDA-receptor antagonist caused an opposite effect. Since septo-hippocampal cholinergic neurons regulate primarily theta amplitude, changes in theta frequency elicited by intraseptal administration of glutamatergic agents are probably mediated by septo-hippocampal GABA neurons. In behaving animals tested in different memory tasks, several authors have reported that memory processes and long-term potentiation are blocked at the time of their initiation by intraseptal infusions of both

NMDA- and metabotropic glutamate-receptor antagonists, while infusion of glutamate causes memory facilitation. All of these experiments lend support to the view that the glutamatergic influence upon septo-hippocampal GABA neurons may be a crucial and powerful driver of theta rhythm and hippocampus-associated memory processes. Our present data provide a morphological explanation for these strong glutamatergic effects.

To date, glutamatergic excitatory neurons of the central nervous system have been considered as projection cells. On the other hand, our results support the possibility that they may function as local circuit neurons, as well, and raise a new functional aspect of the extensive glutamatergic neuronal network in the brain. Recently, using electrophysiological recordings in a rat brain slice preparation, we discovered that rapid applications of nicotine excited 90% of retrogradely labeled septo-hippocampal GABA-type neurons and increased the frequency of spontaneously occurring fast GABAergic and glutamatergic synaptic currents via the $\alpha 4\beta 2$ -nicotinic receptor. Interestingly, tetrodotoxin blocked all effects of nicotine on septo-hippocampal GABAergic neurons, suggesting involvement of indirect mechanisms. We demonstrated that these effects of nicotine involve the recruitment of a novel, local glutamatergic circuitry as (1) group I metabotropic glutamatergic receptor antagonists reduced the effects of nicotine; (2) the number of nicotine-responsive neurons was significantly reduced in recordings from slices that had been trimmed so as to reduce the number of glutamate-containing neurons within the slice preparation. These findings coupled with the knowledge of strong local glutamatergic input to septo-hippocampal GABA cells reveals intraseptal glutamatergic neurons as new members of the local information processing circuitry in the MSDB. This glutamatergic excitatory influence on the activity of septo-hippocampal parvalbumin-IR neurons seems to be as powerful as the action of its GABAergic inhibitory counterpart.

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