Role of synaptic and nonsynaptic nicotinic acetylcholine receptors in dendritic integration: a two-photon microscope study

Doctoral thesis

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1. Introduction

It's been known for almost fifty years that *hippocampus* is a main player in memory and learning processes. This part of the brain is directly connected to certain cognitive functions, it forms associative memory traces underlying long term memory from incoming sensory information. It has been observed that in smokers *nicotine* boosts cognitive functions. The assumption offers itself that *nicotinic acetylcholine receptors* (nAChR) of the hippocampus underlie this phenomenon.
The role of combining dendritic backpropagating action potentials (bAPs) and coincident synaptic inputs in plasticity has widely been accepted. One can assume that highly Ca^{2+} permeable nAChRs may alter the function of this complex structure. Studying intracellular Ca^{2+} dynamics is a proper way of following neuronal activity. Present study uses high tech imaging techniques to reveal how synaptic and nonsynaptic nAChRs modify Ca^{2+} dynamics in dendrites of hippocampal pyramidal cells and interneurons as well as its impact on the complex system of information processing and storage.
Despite the fact that nAChRs play a key role in *Alzheimer's Disease (AD), Parkinson's Disease, epilepsy, pain* and of course *nicotine addiction* we have limited information on their functions. This by all means justifies the need for further studies with the final goal of nAChRs becoming therapeutic targets in the above diseases.
Research discussed in this thesis has been focused on hippocampal CA1 pyramidal cells and stratum radiatum interneurons. Low incidence of nicotinic synapses in the central nervous system favors the primarily nonsynaptic nature of nicotinic transmission both in case of interneurons and pyramidal cells.

2. Goals

The function of the hippocampus is – similarly to other parts of the brain – based on the interplay between excitatory and inhibitory neurons. Their ability to process information is modulated by neurotransmitters released from cells of or tracts projecting to the hippocampus. An example of this is the effect of ACh on nAChRs that is of known importance although the underlying cellular mechanisms are mostly unknown.
Further investigation is needed to clarify how nAChRs described on pyramidal cells and interneurons of the hippocampus as well as on innervating terminals modify information processing in hippocampal neurons. The goals of our work:

2.1. Developing a method for pharmacological studies of nAChRs using two-photon microscopy combined with electrophysiology

Subcellular processes underlie the phenomena to be investigated. The used optical imaging method requires movement-free tracking of changes in the function of dendrites and spines. This is a challenge for drug administration devices. We set an aim of developing a protocol that allows us to model the effect of both slow and stable as well as fast and localized drug evoked processes.

2.2. Reveal effects of nAChRs on the integrative functions of hippocampal pyramidal neurons

There is some inconsistency in the literature regarding the existence of functional nAChRs on hippocampal pyramidal cells. Although several studies support the presence of postsynaptic nAChRs on pyramidal cells, it's still unclear how they modify the cell's function when activated or inhibited. We aim to prove the effect of nicotine on pyramidal neurons and investigate whether the effects are pre- or postsynaptic?

2.3. Importance of direct nicotinic effects on Ca^{2+} dynamics of interneurons in network functions

Is has been known that almost all interneurons in stratum radiatum and lacunosum moleculare give a positive response to nicotinic stimulation. In hippocampal interneurons nAChRs are able to induce intracellular Ca^{2+} accumulation at least as big as attributed to NMDA receptors. Spatial and temporal dynamics of Ca^{2+} transients following activation of extrasynaptic nAChRs is yet unknown so we aimed to find how intracellular Ca^{2+} levels are modulated by interacting synaptic and extrasynaptic nAChRs.

3. Methods

3.1. Acute brain slice preparation

Transverse slices (300 µm) containing the hippocampus from 16- to 19–day-old Wistar rats were dissected using a vibratome as described previously. After dissecting the brain, slices were placed in ACSF containing 126 mM NaCl, 26 mM $NaHCO_3$, 10 mM D-glucose, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 1.26 mM NaH_2PO_4, and incubated for 30 min at 32°C. Slices were allowed to recover at room temperature for 30 min then they were placed onto a water immersion microscope (Olympus BX50WI) and continuously perfused with ACSF equilibrated with 95% O_2 and 5% CO_2.

3.2. Electrophysiology

Pyramidal neurons in the CA1 subfield of the hippocampus were visualized under video IR-DIC. Patch pipettes were pulled from borosilicate glass (1.2 mm OD, Harvard Instruments). For current clamp recordings, 3–6 MΩ electrodes were filled with 125 mM K-gluconate, 20 mM KCl, 10 mM HEPES, 10 mM di-Tris-salt phosphocreatine, 0.3 mM Na-GTP, 4 mM Mg-ATP, 10 mM NaCl, and 112 µM Oregon Green BAPTA-1. Cells with an initial resting membrane potential more negative than -50 mV were used. Whole-cell recordings were performed using an Axopatch 2D amplifier (Axon Instruments, Union City, CA). Somatic current injections were applied (90– 170 pA, duration: 900 µs) to induce 20 APs at 22 Hz. Data acquisition and analysis were performed using pClamp 8.1 (Axon Instruments).

3.2.1. Two-Photon microscopy

Ca^{2+} transients were measured using a custom-made two-photon laser scanning system consisting of a modified confocal microscope (Olympus Fluoview FV300) and a

titanium-sapphire laser providing 150 fs pulses at 80 Hz at 820-nm wavelength (Millenia/Tsunami, SpectraPhysics, USA). Fluorescence was detected in external, whole-field detection mode with photomultiplier tubes (R3896, Hamamatsu). In order to minimize photo damage, the excitation laser intensity was always kept at a minimum.

3.2.2. Following fast action potentials *in vitro*

High-time-resolution fluorescence measurements were obtained in line-scan mode (6,144 μs per line) after zooming onto a dendritic section. Data recording was started 15–30 min after break-in. To study nicotinic modulatory activity, dendritic Ca^{2+} transients were evoked by trains of 20 action potentials. The fluorescence response had a plateau that allowed the precise estimation of the amplitude by averaging 100 data points, with the highest DF/F value in the center. Three consecutive trains at a 0.5-Hz intertrain frequency were evoked and Ca^{2+} responses were averaged to decrease the noise of the optical traces. Variations in amplitudes of the three Ca^{2+} responses were then analyzed to exclude unstable responses. Responses were considered unstable, where the standard deviation (SD) of the three consecutive Ca^{2+} traces in the control or in the drug-treated period was above the average. In some cases, drugs were administered using a modified DAD-12 pressure ejection device (ALA Science, NY) through a glass pipette with a tip diameter of 100 μm. We used a pressure of 13.3 kPa to eject nicotinic agonists onto the cells. Duration of puffs was set to 5 s. We selected cells relatively close to the surface of the slice and positioned the drug ejection pipette 30–40 μm from the cell. At the end of each experiment, a focus series of the entire neuron was taken. Image data were analyzed offline using a custom-made program written in Matlab. Fluorescence traces are expressed as relative fluorescence changes:

$$\frac{\Delta F}{F} = \frac{(F - bg) - (F_0 - bg)}{F_0 - bg} = \frac{F - F_0}{F_0 - bg},$$

where F_0 is the prestimulus base fluorescence and bg is the background noise.

3.3. Drug administration techniques

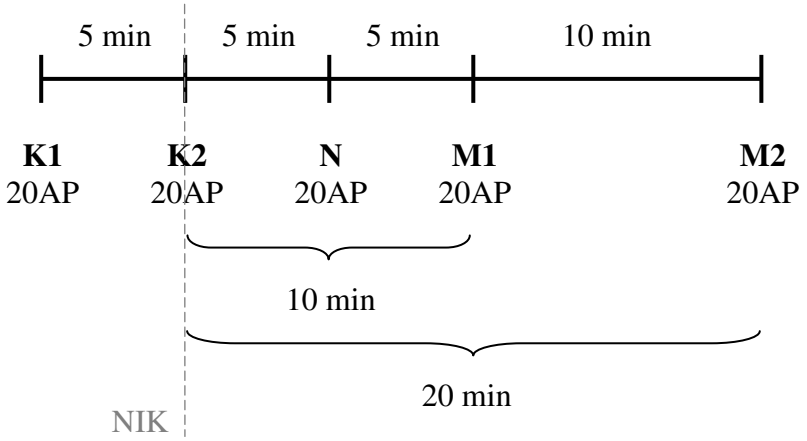
We used two different approaches to apply drugs in our experiments.
One possibility is to **use pressure** to rapidly puff the drug (depending on the placement of the pipette relative to the cell even less than 1 sec) onto the cell. We used a 12 channel computer controlled system (DAD-12, ALA Scientific, USA).
The other possibility is to simply put the substances into the perfusion fluid. In this case the time required by the drugs to reach the cells is slower compared to the previous method: it is in the range of minutes.

4. Results

4.1. Nicotine induced modulation of backpropagating action potential evoked Ca^{2+} transients in basal dendrites of pyramidal cells

To explore the modulatory effects of nicotine we set somatic current injection parameters to reliably generate 22 Hz bursts containing 20 action potentials (APs). We

used following protocol to study effects of low dose nicotine observed in smokers (~1 µm):
- two controls with a 5 minutes interval (K1 and K2);
- nicotine is added to the perfusion (N) in 1 or 20 µM. Another 5 minutes later transients are recorded again;
- after washing out nicotine, another two measurements 10 and 20 minutes after the beginning of nicotine administration (NIK) respectively (M1 and M2).



1 µM of nicotine clearly enhanced Ca^{2+} transients, especially in spines. The Ca^{2+} buffering effect of the dye can influence the measurement. To avoid this we calculated ratios from the amplitude maximums of the consequent measurements (K2/K1, N/K2, M1/N). This way the ratio of the two controls (K2/K1) served as "the control" and we compared this to the ratio of nicotinic response and the second control (N/K2). This method revealed a significant excitatory effect of 1 µM nicotine on Ca^{2+} transients in the spines (p=0.002, paired t-test). Surprisingly 1 µM nicotine did not have any significant effect in dendrites (P = 0,507, n = 25). In 20 µM though it increased the amplitudes of bAP evokes Ca^{2+} transients both in spines (P = 0,002, n = 24) and dendrites (P = 0,032, n = 15).

The next step was to examine whether the observed modulation is attributable to pre- or postsynaptic nAChRs. We blocked fast synaptic transmission with an antagonist cocktail (*bicuculline*, 10 µM; *d-2-amino-5-phosphonovalerianic acid (AP-5)*, 50 µM; *6-cyano-7-nitroquinoxalin-2,3-dion (CNQX)*, 10 µM, ko) administered to the perfusion that almost completely vanished any effect of presynaptic nAChRs. In the presence of these antagonists the excitatory effect of nicotine disappeared (N/ko vs. ko/K2, P = 0,188 in spines, P = 0,258 in dendrites, paired t-test, n = 13-17).

4.2. Nicotine induced firing and Ca^{2+} accumulation in dendritic spines of CA1 pyramidal cells

Besides the above presented facilitation we wanted to further support our results so we studied the direct effects of nicotine on CA1 pyramidal cells in a new set of experiments. We used pressure to puff nicotine in high concentrations (250-500 µM) *directly to the basal dendrites of the cells.* Using this method we could avoid desensitization modeling a physiological situation when ACh is released from a nearby bouton and reaches its receptors in high concentrations. To exclude any movement artifacts caused by pressure we have taken frame images (XYt scans) instead of linescans.

High dose nicotinic agonists evoked dendritic Ca^{2+} transients only in a fraction of the cells (7 out of 27 cells examined). In these cases nicotinic agonists caused firing that made it impossible to study bAP evoked Ca^{2+} transients. The start of the Ca^{2+} transients perfectly matched to the first bAP consequently the observed Ca^{2+} transients were attributable to the bAPs. Pressure applied lower dose nicotine (10 μM) did not evoke APs nor Ca^{2+} transients.

We were also interested in the subtype composition of nAChRs responsible for firing. Choline, an α7 nAChR specific agonist evoked identical Ca^{2+} transients on basal dendrites (n = 15 spines, P = 0,643). After nicotine or choline administration we observed a cell level "learning effect": time elapsed between drug administration and start of responses became shorter while the amplitudes of evoked Ca^{2+} transients became larger (R^2 = 0,41, P = 0,08).
We further examined the subtype selectivity of the evoked responses: nAChR antagonists have been administered to the perfusion to block responses. Using *voltage-clamp* we measured nicotinic currents induced by pressure applied agonists. In voltage-clamp APs do not disturb the measurement. Nicotine was able to evoke currents in all examined cells (n=18). These currents clearly fell into two categories based on their amplitudes: this suggests the existence of a more and a less nicotine sensitive group of cells. This may be attributable to the different density of nAChRs and different subtype composition. Nicotine (500 μM, 5s) also enhanced the amplitudes and frequency of *EPSC*s.

Again the question arises regarding the pre- or postsynaptic nature of the phenomenon. To exclude the action of excitatory inputs we administered blockers of fast synaptic transmission (see above). Using these blockers nicotine failed to evoke Ca^{2+} transients or firing under our experimental conditions that proved our presynaptic action theory.
We could also determine the nAChR subtype behind the observed effect: the subtype nonselective antagonist *mecamilamyne* (50 μM) inhibited nicotinic currents in pyramidal cells (P<0,003, n=9). The inhibitory effect (P<0,05, n=4) of α7 nAChR selective antagonist *methyllycaconitine* (MLA, 50 nM) was less explicit (35% as opposed to 85% with mecamilamyne). This points to the dominance of non α7 nAChRs in the observed effect.

4.3. Direct effect of nAChRs on Ca^{2+} dynamics of hippocampal interneurons

Previous studies revealed that – especially α7 – nAChRs are able to produce intracellular Ca^{2+} accumulation in cell bodies of interneurons that is similarly high as that caused by NMDA receptors. However temporal dynamics of Ca^{2+} transients following nAChR stimulation has yet remained unknown. Our experiments aimed to shed some light on this using pressure applied nicotine stimulus. We have shown before that the concentration of pressure applied drugs is highly decreasing from the surface of the slice inward. Since the observed cells were always under the surface (~25-75 μm), we assumed that we activated high affinity nAChRs at extrasynaptic locations that can be activated with low concentration of drugs.

4.4. Direct Ca^{2+} transients mediated by nAChRs in dendrites of hippocampal interneurons

We were first to report that activation of nAChRs on dendrites of CA1 stratum radiatum interneurons leads to appearance of Ca^{2+} transients or modulates bAP evoked responses. Alike to our previous experiments we loaded cells with Oregon Green BAPTA-1 through a patch pipette used for electrophysiology and performed two-photon experiments to explore the Ca^{2+} dynamics of dendrites. We administered 500 µM nicotine for 5 seconds.
In accordance with earlier reports nicotine evoked several action potentials in stratum radiatum interneurons of the hippocampal CA1 region under current clamp. Because backpropagating action potential-evoked Ca^{2+} signals interfere with the local Ca^{2+} entry produced by nAChRs we blocked membrane Na^{+} channels by QX-314 in order to exclude the Ca^{2+} influx initiated by Na^{+} channel dependent action potentials. In current clamp recordings depolarization made by nicotine puffs showed large variability in the presence of QX-314 (n = 16). These responses illustrate how nonsynaptic actions may combine with fast synaptic responses in the absence of action potentials: a relatively slower rise in dendritic Ca^{2+} levels suddenly corroborated by coincident activity of a nearby synapse. Dendritic origin of nAChR activation is further supported: we detected Ca^{2+} responses in dendrites of interneurons in a considerable portion of dendrites (67%, n= 16 cells). Amplitudes of Ca^{2+} responses exhibited a relatively large variation: some dendrites exhibited very large local Ca^{2+} responses. There were dendrites, which showed Ca^{2+} responses in spite of the lack of depolarization at the soma. This suggests the existence of dendritic hot-spots of nicotinic activation in interneurons.
The α7 selective choline evoked similar transients that suggest the role of this subtype in the observed phenomenon.

4.5. Interplay between extrasynaptic nAChRs and synaptic activity in interneurons

Almost every interneurons in stratum radiatum and lacunosum moleculare is responsive to nicotinic stimulation. One of the most important features of nAChRs is the high Ca^{2+} throughput. Ca^{2+} influx can be different for synaptic and non-synaptic receptors based on their different roles. We provided examples how these two receptor types interact and influence intracellular Ca^{2+} levels. Ca^{2+} transients could be clearly separated into subgroups based on time-to-peak values: we observed a response with a fast rising phase (39 ± 4 ms, n = 3), and a slower Ca^{2+} response (time-to-peak, 7.0 ± 1.4 s, n = 11) demonstrating the large variability of Ca^{2+} dynamics of interneuron dendrites. Pressure applied nicotine mainly activates extrasynaptic high affinity nAChRs. In contrast, fast transients represent facilitation of synaptic transmission. A closely located synapse strengthens the relatively slower rise of the dendritic Ca^{2+} level.

Experimentally, a few msec-long perfusion with 10–100 nM nicotine cannot elicit detectable Ca^{2+} responses using current technologies. However, there is evidence that low nicotine concentrations may induce physiologically important but small changes in Ca^{2+} dynamics of cells. They combine at systems level resulting in a shift in network activity. Stratum radiatum interneurons mediate primarily feed forward inhibition because they receive inputs from fibers entering the CA1. Therefore, nicotinic stimulation can shift the balance of inhibition/excitation in a slower timescale. The nonsynaptic nicotinic input can also synchronize interneurons by elevating Ca^{2+} levels

of dendrites of different cells. Overall after nicotine administration inhibition may come to the front that may be an important part of neural mechanisms observed in smokers.

5. Discussion

5.1. Nicotinic effects on pyramidal cells

Our experiments have proven the effect of nAChRs on pyramidal neurons. Activation of presynaptic nAChRs (mainly on glutamatergic axons) evokes APs in the innervated postsynaptic pyramidal cell that propagates back to the spines and triggers Ca^{2+} influx. Although earlier studies established the existence of nAChRs on the cell bodies and dendrites of hippocampal pyramidal neurons, we failed to resolve their direct action on dendritic Ca^{2+} dynamics when pressure applied to cells. Low concentration nicotine also acted on nAChRs of presynaptic glutamatergic terminals. This effect did not lead to firing or direct Ca^{2+} accumulation. Even this low concentration (1 μM) was enough to produce modulatory effects in basal dendrites: bAP evoked Ca^{2+} responses were facilitated by nicotine.
Based on our results, it is assumable that the activation of presynaptic nAChRs on the rich glutamatergic innervation of pyramidal neurons produced the observed excitatory effects: presynaptic nAChRs, expressed on terminals of the recurrent axon collaterals of CA1 pyramidal neurons, induced synaptic activity at the level of spines in a coincidence with postsynaptic APs generated by current injection. These collaterals come from the neighboring cells, which innervate other pyramidal neurons of the CA1 at the basal dendrite. Our results confirmed these data by showing an increase in the frequency of EPSCs by nicotine in CA1 pyramidal neurons.
Our presynaptic site of action theory is proven by the fact that synaptic blocker abolished both the effect of low dose nicotine on bAP evoked Ca^{2+} responses of CA1 pyramidal cells as well as firing caused by high dose nicotine.
Most likely glutamate receptors were responsible for the enhancement by low-dose nicotine, because in the presence of AP-5, an NMDA receptor antagonist, it was completely blocked. This small amount of Ca^{2+} entering the spine might be invisible for the direct optical recording, but it can supralinearly summate with bAP evoked Ca^{2+} transients enabling the detection of nicotinic modulation in spines. Bath application of nicotine simulates central nicotine delivery. During smoking, the diffusing nicotine may not induce large and easily detectable responses because of its low concentration, but it rather modifies backreporting by activating presynaptic nAChRs of axons innervating pyramidal neurons. Taking together the fact that nicotine is able to improve cognition in humans and animals at the system level and the well-known amplifying effect of bAPs on the computational capacity of dendrites we can conclude that nAChR-mediated plastic processes may partly be attributable to the effect of nicotine on bAP-evoked dendritic responses and contribute to the positive cognitive effects of smoked nicotine.

One can speculate that the infrequent high-amplitude currents evoked by nicotine, which were observed in a fraction of pyramidal neurons in our study, are responsible for the firing and subsequently for the Ca^{2+} accumulation in dendritic spines that we saw at high-dose nicotine application in current clamp. Corroborating this assumption, the proportion of large currents (44% of cells) was similar to the proportion of cells (26%) showing firing upon pressure application of nicotine.

The presynaptic locus of high-dose nicotine to induce firing also indicates that the same mechanism, that we described for low-concentration nicotine (1 μM), can operate not

only to modulate the bAP-evoked Ca^{2+} transients but also change the Ca^{2+} levels in dendritic spines in an indirect way; by inducing action potentials.
What could be the functional importance of the presynaptic nicotinic enhancement of dendritic responses? Firing of a high-frequency train of action potentials helps distal dendrites receive the soma message consequently modifies dendritic backreporting. the finding that only a fraction of pyramidal neurons could produce firing by nicotine puffs raises the possibility that different spatial patterns of nicotinic activity can organize dendrites into ‘‘nicotine-sensitive’’ workgroups. These new organizations may act as extra storage capacities within the hippocampal neural network.

5.2. Nicotinic effects on interneurons

In this report we demonstrated the Ca2+ response patterns in dendrites of stratum radiatum interneurons following activation of nAChRs. Puff ejection of drugs and the difficulty to find nicotinic synaptic function in interneurons all indicate that extrasynaptic nAChRs produced the responses we observed in the dendrites of these cells. During nicotine puffs random Ca^{2+} transients appeared in dendrites of interneurons. The block of voltage-sensitive Na^{+} channels enabled us to study dendritic Ca^{2+} responses in isolation from somatic action potentials. In addition to earlier findings, our data revealed that activation of nAChRs is able to organize into sections of neural processes. The finding that dendritic Ca^{2+} response may appear even in the absence of somatic depolarization strongly indicates functionally separated dendrites during nicotinic actions. Furthermore, it indicates the autonomy of dendritic nAChRs from the somatic events. Another important observation was in our study that nicotine could variably interfere with synaptic inputs. The integral of Ca^{2+} under the coincident synaptic (EPSPs) and extrasynaptic (nAChR-evoked response) events greatly varies according to their patterns and relative timing. This feature of the nicotinic system should increase the computational capacity of interneuron dendrites.
Stratum radiatum interneurons mediate primarily feed forward inhibition, because they receive inputs from fibers entering the CA1. Therefore, nicotinic stimulation can shift the balance of inhibition/excitation in a slower timescale.
These differences may play important role in the higher level functions of synaptic and nonsynaptic nAChRs.

6. Summary and Conclusions

I. Developing a method for pharmacological studies of nAChRs using two-photon microscopy combined with electrophysiology

1. We developed a method to pharmacological stimulation of fast desensitizing receptors to be used in combination with two-photon microscopy on any species.
2. We described our method in detail compared to the conventional drug administration to the perfusion.
3. We empirically chosen the right fluorescent probe for following fine modulation and have proven its efficacy.

II. Reveal effects of nAChRs on the integrative functions of hippocampal pyramidal neurons

1. We have shown examples to facilitation of bAPs attributable to functional nAChRs.
2. We were first to prove that this facilitation is of presynaptic nature, mainly originating from activation nAChRs on glutamatergic axons.
3. We have shown that elevated Ca^{2+} levels following nAChR activation facilitate bAP evoked Ca^{2+} transients.
4. We have proven that high concentration of nicotine is able to generate APs that produce Ca^{2+} transients in spines.
5. We were also first to show that dendrites and even pyramidal cells have a "nicotine sensitive" population that is able to extend the computational and storage capacity of neural networks.
6. Modulation of dendritic function that is considered fundamental in synaptic plasticity is already present at low concentrations of nicotine that is similar to concentrations observed in smokers. This further supports that system level positive cognitive effects in human and animal experiments may be at least partly attributable to the facilitation of dendritic backreporting in pyramidal cells.

III. Importance of direct nicotinic effects on Ca^{2+} dynamics of interneurons in network functions

1. We have shown examples of how diverse nAChR activation evoked Ca^{2+} transients can be in dendrites of interneurons.
2. We were first to show that slow nonsynaptic cholinergic input evoked dendritic Ca^{2+} accumulation can combine in many different ways with the fast synaptic responses of the same region. The function of this is most likely to extend the input processing capacity of interneurons.
3. Our data showed that dendrites are consisted of different functional units based on their activation patterns.
4. These differences combine at the system level and may play a major role in high level functions attributed to synaptic and nonsynaptic nAChRs.
5. We can also declare that nonsynaptic nicotinic activity can synchronize cooperation of interneurons in the network through raising the Ca^{2+} levels of dendrites.

7. List of Publications

Publications connected to the thesis:

Lendvai B, Szabo SI, Barth AI, Zelles T & Vizi ES. (2006)
Application of two-photon microscopy to the study of cellular pharmacology of central neurons. ***Advanced Drug Delivery Reviews*** **58,** 841-849.

Szabo SI, Zelles T & Lendvai B. (2008)
Intracellular Ca2+ dynamics of hippocampal interneurons following nicotinic acetylcholine receptor activation. ***Neurochemistry International*** **52,** 135-141.

Szabo SI, Zelles T, Vizi ES & Lendvai B. (2008)
The effect of nicotine on spiking activity and Ca2+ dynamics of dendritic spines in rat CA1 pyramidal neurons. ***Hippocampus*** **18,** 376-385.

Publications not connected to the thesis:

Makara JK, Mor M, Fegley D, Szabo SI, Kathuria S, Astarita G, Duranti A, Tontini A, Tarzia G, Rivara S, Freund TF & Piomelli D. (2005)
Selective inhibition of 2-AG hydrolysis enhances endocannabinoid signaling in hippocampus. ***Nature Neuroscience*** **8,** 1139-1141.

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