Caffeine, adenosine and acetycholine and neuronal function in the cortex
The research in this thesis was conducted both at the department of Integrative Neuropsychology of the Centre for Neurogenomics and Cognitive Research, Amsterdam Neuroscience, Vrije Universiteit Amsterdam, the Netherlands, as well as in the Purines group in the Centre for Neuroscience and Cell biology at the University of Coimbra, Portugal. No part of this thesis may be reproduced without prior permission of the author.

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Caffeine, adenosine and acetycholine and neuronal function in the cortex

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General introduction
1. General introduction

1.1 The medial prefrontal cortex is densely connected in a laminar and subtype-specific fashion

The medial prefrontal cortex is characterized by multiple layers and many distinct cell-types that together as a network are responsible for many higher cognitive functions including attention, decision making, impulse control, working memory, behavioral flexibility and goal direction (Passetti et al., 2002; Killcross and Coutureau, 2003; Bissonette et al., 2008; Euston et al., 2013; Funahashi, 2013). The area is densely connected and receives inputs from several brain regions that use different neurotransmitters and neuromodulators to convey information. These projections are both local and long-range, from both subcortical and limbic areas (Sesack et al., 1989; Heidbreder and Groenewegen, 2003; Gabbott et al., 2005; DeNardo et al., 2015). As such, the mPFC receives dopamine from dopaminergic regions such as the VTA, glutamate from thalamic regions, acetylcholine from the basal forebrain, serotonin from raphe nuclei and adrenaline from the locus coeruleus, amongst others (Fig 1.1). The mPFC contains inhibitory and excitatory neurons. The inhibitory GABA is mainly released from local interneurons, while locally signaling excitatory neurons make up only a small fraction of total glutamatergic inputs (Fig 1.1). Neuromodulators in the prefrontal cortex do not uniformly increase or decrease activity. Rather, their effect depends upon the cellular subtype and the long-range targets of this subtype (Aerde and Feldmeyer, 2013; Bloem et al., 2014a; Dembrow and Johnston, 2014). These neuronal subtypes are divided in a laminar structure in the mPFC.

The mPFC has a laminar structure that consists of five main layers, in contrast to the six layers that can be observed in primary sensory cortices. The rodent mPFC lacks a granular layer 4 (L4), the layer in primary sensory areas that receives most thalamic projections (Harris and Shepherd, 2015). L1 of the mPFC is solely made of interneurons that are mostly low-threshold spiking interneurons, that provide feed-forward inhibition to superficial layers in the mPFC (Cruikshank et al., 2012). L2 consist of both inhibitory interneurons and excitatory pyramidal neurons. The pyramidal neurons of L2 possess a unique morphology with wide apical dendritic field spans and a narrow basal field span; together, these superficial cortices receive the bulk of the thalamic glutamatergic input (Riga et al., 2014).
Layer 2/3 contains small pyramidal neurons that trigger bursts of action potentials upon current injection, and these neurons mainly project locally to deeper layer neurons. Pyramidal neurons in L5 of the mPFC can be divided into two subtypes: thick tufted, subcortically projecting cells and thin-tufted, colossally projecting cells (Dembrow and Johnston, 2014). These cells have long-range projections that include the BLA, dorsal striatum, thalamus, brainstem and amygdala (Fig 1.1). L5 pyramidal neurons provide the main output of the mPFC (Aerde and Feldmeyer, 2013; Dembrow and Johnston, 2014; DeNardo et al., 2015). Pyramidal neurons in L6 show a varied morphology with the largest portion of L6 neurons possessing tall apical dendrites that extend into L1 (Aerde and Feldmeyer, 2013). L6 pyramidal neurons preferentially output to the thalamus (Gabbott et al., 2005; Lee et al., 2014). Pyramidal neurons can be mainly subdivided by their morphological and electrophysiological profile, in which more than 10 different pyramidal subtypes can be identified (Aerde and Feldmeyer, 2013). Interneurons are spread over the cortical layers, although the largest portion of interneurons can be found in L1. There are three main types of GABA-ergic interneurons in the rodent neocortex: PV-expressing fast-spiking interneurons (FS-PV), somatostatin-expressing low-threshold spiking interneurons (LTS-SOM) and vasoactive intestinal peptide (VIP) neurons (Tremblay et al., 2016; Yavorska and Wehr, 2016).

In short, mPFC has a laminar structure of which the cells in each layer receive projections from specific brain areas and send laminar-dependent projections out to other brain areas. The effect of neuromodulators is dependent on the cellular subtype and the long-range targets, which will be further described in paragraph 1.3.
1.2 Synaptic transmission and plasticity in the prefrontal cortex

Fast synaptic transmission in the medial prefrontal cortex mainly occurs through glutamatergic excitatory and GABAergic inhibitory signaling. These signals start as an action potential—in a presynaptic neuron within or outside of the prefrontal cortex—that propagates through the afferent axonal fibers. When the signal arrives at the axonal synapses, this will lead to vesicle fusion and the release of the vesicle content.

The postsynaptic cell will receive this signal through its synaptic membrane channels. α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and n-methyl-D-aspartate (NMDA) receptors will respond to signals of glutamate, while gamma-aminobutyric acid (GABA) receptors respond to the neurotransmitter GABA. Upon binding of glutamate, AMPA receptors will open their pore and let ions flow in. NMDA receptors also respond to glutamate, but to open their pore they need both glutamate binding and sufficient depolarization to release the magnesium blockade of the ion channel pore. AMPA and NMDA receptors let positive ions flow in, such as Na+ and Ca2+, leading to short-term depolarized membrane potentials. GABA receptors open their pore in response to GABA neurotransmitter; their pore is capable of letting Cl- flow in, leading to short-term hyperpolarization of the membrane potential.

![Synaptic transmission](image)

**Figure 1.2.** Synaptic transmission is mediated by depressive (A) and facilitatory (B) synapses. Depressive synapses initially release a large pool of vesicles upon action potential firing, leaving a small pool behind that is slowly refilled, while in facilitatory synapses, the release probability is increased due to an action potential that was fired earlier (Blitz et al., 2004)
Upon repeated activation, synapses display transient changes in strength, which can be facilitating, meaning that the signal progressively grows, or depressive, where upon stimulation the signal decays to almost zero. A common feature of pyramidal-to-pyramidal connections is to be depressive (Abbott and Regehr, 2004), while pyramidal-to-interneuron can both be depressing or facilitating, depending on the interneuron type that is projected to (Reyes et al., 1998; Markram et al., 2004). The commonly believed mechanism behind this is that in depressive synapses, initially a large portion of the readily releasable pool is released, leaving behind a smaller pool of vesicles that is then slowly refilled. If the synapse is then reactivated within this time frame, the diminished pool of vesicles available for release results in a lower vesicle release (Fig 1.2). Facilitation of this process relies on presynaptic calcium influx upon activation by an action potential, increasing the release probability of vesicles upon reactivation (Jackman et al., 2016). Both are short-term, because in a few seconds, the responses will be restored to original levels - depending on degree of depression/facilitation and type of synapse.

To store cellular memory for longer term, another mechanism is in place, called long-term synaptic plasticity. Synaptic plasticity occurs in both pyramidal- as well as in interneuron subtypes, although the intracellular processes that they rely on are different.

1.2.1 Long-term plasticity at glutamatergic synapses
Long-term plasticity at glutamatergic synapses is dependent on postsynaptic changes in active AMPA receptor density, ultimately leading to an increased calcium influx into the postsynaptic cell. Initially, glutamate binding to AMPA receptors opens these receptors and the resulting Na$^+$ ions that flows in slightly depolarizes the membrane. When this depolarization is strong enough, Mg$^{2+}$ ions are released from NMDA receptors, allowing not only Na$^+$ to flow in, but also Ca$^{2+}$.

In the case of long-term potentiation (LTP), calcium influx through the NMDA receptor is necessary, in such a way that LTP cannot happen without influx through these receptors (Malenka and Nicoll, 1993). This will lead to signaling cascades that ultimately lead to more active AMPA receptors into the synapse and thus a larger postsynaptic response to a new input (Fig 1.3). In the case of LTD, a signaling cascade leads to the de-activation of synaptic AMPA receptors, upon which these receptors internalize (Fig 1.3). This internalization will in turn cause a reduction of synaptic efficacy. These postsynaptic mechanisms of plasticity are complemented
by presynaptic changes. Presynaptically, calcium entry either through NMDA receptors or through voltage-dependent calcium channels (VDCCs) are necessary for the induction and maintenance of many forms of LTP and LTD (Ferre et al., 2008; Park et al., 2014b).

Neuromodulators can provide a push-pull mechanism onto the induction of either LTD or LTP, meaning that they can either help or abrogate plasticity formation. In the cortex, different G-protein-coupled receptors gate and control the polarity of spike-timing plasticity (STDP) induced LTP: Gs-coupled receptors promote LTP at the expense of LTD and Gq-coupled receptors promote LTD over LTP (Seol et al., 2007; Zhang et al., 2009; Huang et al., 2012). Neuromodulators this way change the shape of the induction window in STDP. Also in plasticity induced by thetaburst stimulation (TBS), several different types of neuromodulators have been shown to either diminish LTP or LTD, while in other situations help the formation of plasticity (Couey et al., 2007; Seol et al., 2007; Pawlak et al., 2010; Huang et al., 2012; Frémaux and Gerstner, 2016; Verhoog et al., 2016). This influence of neuromodulators to be either facilitatory of depressive is region, cell-type, and layer-dependent. For example, the release of acetylcholine in layer 2/3 of the mPFC attenuated LTP, whereas it unveiled LTP in layer 6 of the mPFC (Verhoog et al., 2016). As neuromodulators can give the signal for change in a specific, release-

![Figure 1.3. Long-term plasticity principles. Synapses can undergo two types of plasticity: long-term potentiation (LTP) and long-term depression (LTD). Both are dependent on calcium entry and insertion or deletion of AMPAR. In LTP, the release of glutamate leads to a more efficient membrane depolarization due to the inserted AMPAR. In LTD, the postsynaptic neuron becomes less responsive to the release of glutamate (figure derived from boundless.com - fig-ch35_02_10).](image-url)
site dependent manner, they are thought to represent a selection criterion that allows presynaptic activity and postsynaptic spiking to be associated, both spatially and temporally (Pawlak et al., 2010).

Thus, glutamatergic synapses onto pyramidal neurons can undergo both LTD and LTP. This formation of plasticity is under the influence of neuromodulators. In chapter 3, we investigated whether plasticity in the mPFC is influenced by the neuromodulator adenosine.

1.2.2 Motifs and plasticity of cortical interneurons
The interplay between interneurons and pyramidal neurons defines for a large part the outcome of network activity. Signaling in the cortex relies for a large part on interneurons present in this area, mainly PV-expressing fast-spiking interneurons (FS-PV), somatostatin-expressing low-threshold spiking interneurons (LTS-SOM) and VIP neurons (Tremblay et al., 2016; Yavorska and Wehr, 2016). These interneurons are interconnected with glutamatergic pyramidal neurons and regulate their function (Konstantoudaki et al., 2016). In sensory areas, the receptive fields of interneurons are generally broader than those of principal cells (Poo and Isaacson, 2009; Kerlin et al., 2010). Therefore, the relative strength of their synaptic connections are of importance to the functioning of the network.

Several wiring schemes have been identified by which interneurons shape cortical signal propagation, among which are feed-forward inhibition, feedback inhibition and lateral inhibition (Fino et al., 2013; Tremblay et al., 2016). The pairing of excitation and inhibition can substantially increase the temporal precision of firing (Buzsáki, 1984); depolarization of the principal cell, initiated by the excitatory input, is reduced quickly by the repolarizing or shunting effect of inhibition, thereby narrowing the temporal window of non-zero discharge probability (Pouille and Scanziani, 2001). Lateral inhibition is a form of inhibition generated by activity in local circuits of pyramidal neurons and interneurons, occurring when the activation of a principal cell recruits an interneuron, which in turn suppresses the activity of surrounding principal cells. In this way, an individual neuron can prevent the spiking of surrounding neurons and the output of a network can remain sparse (Silberberg and Markram, 2007). This mechanism is therefore thought to be highly relevant to cortical processing (Silberberg and Markram, 2007). Lateral inhibition can be mediated by parvalbumin (PV)-positive fast spiking interneurons as well as somatostatin (SOM)-positive interneurons (Pouille and Scanziani, 2001; Kapfer et al., 2007; Silberberg and Markram, 2007; Berger et al., 2009). Because of the
profound difference in projection targets on pyramidal neuron dendrites between PV and SOM axons, whereby PV neurons innervate perisomatic regions and SOM neurons generally target distal dendrites, lateral inhibition by PV neurons may be more involved in rapidly silencing action potential firing in neighboring pyramidal neurons, while lateral inhibition through SOM neurons will control synaptic integration, burst firing and dendritic regenerative phenomena (Pouille and Scanziani, 2001; Gentet et al., 2012b; Gidon and Segev, 2012; Tremblay et al., 2016).

The activity of interneurons and pyramidal neurons is very relevant to proper functioning of the cortex in the light of the excitation-inhibition balance (E/I balance) (Haider and McCormick, 2009; Meunier et al., 2017), thought to result from the coordinated activity of excitation and inhibition (Isaacson and Scanziani, 2011). A proper balance between excitatory and inhibitory inputs onto cortical neurons is essential in maintaining the stability of cortical networks in order to perform cognitive functions such as memory and sensory information processing. For instance, it determines proper cortical network rhythms responsible for higher order cognitive functions (Shu et al., 2003; Haider et al., 2006) and it plays an important role in maintaining temporally precise signal integration in local pyramidal neurons (Lu et al., 2000; Pouille and Scanziani, 2001; Kullmann and Lamsa, 2011). Neuromodulators that specifically target either interneuron- or pyramidal neurons can thus influence both E/I balance and the temporal fidelity of the network. For example, in the prefrontal cortex, activation of acetylcholine receptors leads to strong depolarization and induction of action potential firing by SOM-positive neurons (Beierlein et al., 2000; Couey et al., 2007; Fanselow et al., 2008; Poorthuis et al., 2013b; Xu et al., 2013), affecting the induction of long-term potentiation at glutamatergic synapses onto pyramidal neurons (Couey et al., 2007).

The E/I balance can also shift through alterations in plasticity of glutamatergic synapses onto interneurons. At FS-PV interneurons, both LTP and LTD can be generated, although LTP seems to be the dominant form of plasticity in this synaptic connection (Lamsa et al., 2007; Lu et al., 2007; Sarihi et al., 2008; Nissen et al., 2010; Sambandan et al., 2010). Long-term plasticity at glutamatergic synapses onto FS-PV neurons is predominantly independent of NMDAR (Lamsa et al., 2007; Sarihi et al., 2008; Nissen et al., 2010; Sambandan et al., 2010; Huang et al., 2013), although some NMDA-receptor dependent forms of long term plasticity have been observed (Lamsa et al., 2005). In most cases, an essential role for group
I metabotropic glutamate receptors (mGluRs) has been demonstrated in LTP and LTD induction in these FS-PV interneurons (Perez et al., 2001; Lu et al., 2007; Gibson et al., 2008; Sarihi et al., 2008; Galván et al., 2009; Huang et al., 2013). LTP at synapses on SOM interneurons in the sensorimotor cortex appears to be exclusively presynaptic, in that it does not depend on postsynaptic Ca2+ signaling but instead on protein kinase A signaling, a downstream product of G-protein signaling (Chen et al., 2009). LTP could only be induced when a very strong TBS protocol is used, with a total of 600–1000 pulses; otherwise, no plasticity could be observed (Chen et al., 2009). In the somatosensory cortex, a Hebbian form of LTP onto synapses on LTS cells was similarly found to be presynaptically expressed and independent of NMDARs; however, when LTD was induced, this did rely on NMDA receptor activity (Lu et al., 2007). In mouse visual cortex however, no plasticity could be induced in LTS-SOM interneurons, neither with TBS nor with STDP (Sarihi et al., 2008; Huang et al., 2013). These varied and sometimes conflicting results probably reflect the diversity of this group of LTS-SOM interneurons and their synaptic properties, even within the same subtype and brain region (Kullmann and Lamsa, 2007). It may also imply that a certain stimulation pattern yields different forms of plasticity in different subtypes of interneurons. Although evidence of the involvement of neuromodulators onto plasticity of glutamatergic synapses onto interneurons in the cortex is sparse, first explorations into that topic indicate that at least the adrenergic system is involved in the alteration of the direction of LTP: the activation of adrenergic receptors enables the induction of Hebbian bidirectional STDP in FS-PV interneurons and in LTS-SOM interneurons that would otherwise not obtain plasticity (Huang et al., 2013). In chapter 3, we further explore the role of the neuromodulator adenosine on plasticity at glutamatergic synapses of both pyramidal neurons and interneurons.

1.3 The neuromodulators adenosine and acetylcholine play an important role in the mPFC

Acetylcholine and adenosine are abundantly present in the prefrontal cortex. The availability of acetylcholine rises in attention-based tasks, as shown by microdialysis during the 5-choice serial reaction time task (Sarter et al., 2003) and rises similarly in arousal (Van Dort et al., 2009). Adenosine becomes available whenever there is demand of energy in the synapse, and as such is present whenever there is cellular activity (Fredholm et al., 1999; Cunha, 2008a). Psycho-
active drugs such as caffeine and nicotine make use of these systems (Fredholm et al., 1999; Poorthuis and Mansvelder, 2013). These substances can enhance attention and arousal even after moderate use (eg., Lawrence et al., 2002; Wilhelms et al., 2017).

1.3.1. Adenosine as a facilitatory and inhibitory neuromodulator

The neuromodulator adenosine is a special kind of neuromodulator: where other neuromodulators are present selectively in only some projections, adenosine, a byproduct of the cellular source of energy adenosine triphosphate (ATP), is present in every brain region and targets a diversity of neuronal and non-neuronal cell types. This makes that adenosine signaling is involved in a diversity of cognitive behavior, such as psychomotor behavior (Xu et al., 2005), memory (Zhou et al., 2009; Kaster et al., 2015; Pagnussat et al., 2015; Machado et al., 2016) and attention (Prediger et al., 2005; Pandolfo et al., 2013).

Adenosine functions exclusively as a neuromodulator, which means that it does not trigger direct neuronal responses but instead fine-tunes on-going synaptic transmission. Unlike neuromodulators that have a dual role, it is not stored and released from vesicles, but rather is generated by the intra- and extracellular metabolism of ATP, cAMP and AMP through a series of ectonucleotidases (Dunwiddie and Masino, 2001; Wei et al., 2011a). Several processes happen at the same time to ensure an equilibrium in which adenosine levels are estimated to be around 25-250 nanomolar, under which conditions adenosine has mainly an inhibitory effect on basal synaptic transmission (Dunwiddie and Masino, 2001; Pedata et al., 2001). Adenosine release is dependent on the strength of presynaptic stimulation (Cunha et al., 1996; Dunwiddie et al., 1997; Cechova and Venton, 2008; Pajski and Venton, 2010). Released adenosine can bind to four different types of adenosine receptors: A1, A2A, A2B and A3 receptor. Of these, the Gi-coupled A1 and Gs-coupled A2A receptor (A2AR) are most abundantly expressed in the brain. The A1R is expressed throughout the whole brain, while A2AR are present mainly in striatal and (sub)cortical areas (Dunwiddie and Masino, 2001; Fredholm et al., 2005). Low concentrations of adenosine, generated by low or no stimulation, cause A1R-mediated inhibition of synaptic transmission (Dunwiddie and Hoffer, 1980; Sebastião et al., 1990; Ciruela et al., 2006; van Aerde et al., 2013). A1R activation suppresses neuronal activity through pre-synaptic and post-synaptic mechanisms by coupling with Gi to inhibit the AC-cAMP-protein kinase A (PKA) signaling pathway (Calker et al., 1979). In conditions with high levels of
stimulation or in ischemia, in which high concentration levels of adenosine are produced, adenosine facilitates synaptic transmission via A2AR (O’Regan et al., 1992; Sebastião and Ribeiro, 1996; Marcoli et al., 2003; Ciruela et al., 2006). A2AR activation enhances neuronal activity by coupling to Gs to facilitate the AC-cAMP-PKA signaling pathway (Calker et al., 1979), as well as by activating the PLC-PKC signaling pathway (Pinto-Duarte et al., 2005). Both A1R and A2AR are functionally present in the cortex (Qi et al., 2016; Zhang et al., 2015; van Aerde et al., 2015; van Dort et al., 2009; Marchi et al., 2002).

1.3.2 Adenosine in the human brain

Caffeine is the most widely consumed psychoactive substance in the world. Caffeine can easily reach the cortex, as its hydrophobic properties allow passage through all biological membranes and the blood-to-plasma ratio is close to unity (McCall et al., 1982), indicating limited plasma protein binding and free passage into blood cells (Fredholm et al., 1999). Peak plasma caffeine concentration after ingestion of one cup of coffee (50-100 mg) is reached between 15 and 120 min after oral ingestion in humans and equals 5 to 8 mg/kg, estimated to a peak concentration of about 1 to 10 μM (Arnaud and Welsch, 1982; Bonati et al., 1982; Fredholm et al., 1999). From work on rodent brain, the only known biochemical action of caffeine in the concentration range of normal human caffeine consumption is the blockade of adenosine receptors (Fredholm et al., 1999; Ferré, 2008). In the human brain, both A1R and A2AR are present in a similar non-homogenous fashion as in the rodent brain (Svenningsson et al., 1997), with a main expression of A1R in hippocampal area CA1 and of A2AR in striatal areas, thalamus and cerebral cortex (Svenningsson et al., 1997). On the short term, caffeine can affect several aspects of cognitive performance, such as alertness (Hewlett and Smith, 2007), arousal (Barry et al., 2007), attentional task performance (Barry et al., 2007; Wilhelmus et al., 2017) and working memory (Borota et al., 2014), even when applied in doses as low as 60 mg (Wilhelmus et al., 2017). Of note, low doses of caffeine have positive effects while doses above 500 mg are followed by a decreased performance (Kaplan et al., 1997).

The effect of caffeine on synaptic transmission and membrane properties might vary between species, as pyramidal neurons between rodent and human brain differ in several essential properties (Testa-Silva et al., 2014; Mohan et al., 2015). Also, responses in human cortex might differ as A1R have clear inter-species differences, typified by a lower density (Boulenger et al., 1982; Dodd et al., 1986; Fastbom et al., 1987; Svenningsson et al., 1997), a higher affinity for agonists and a
lower affinity for antagonists in human versus rodent cerebral cortex (Murphy and Snyder, 1982; Ferkany et al., 1986; Maemoto et al., 1997). Therefore, we made a first step in elucidating these important mechanisms underlying the human cognitive enhancement effects of caffeine in chapter 2, where we investigated the effect of caffeine on synaptic transmission and intrinsic membrane properties in the human cortex.

1.3.3 Effects of adenosine on synaptic transmission
Most research has focused on elucidating the role of adenosine receptors on cellular processes in brain areas where these receptors are abundantly expressed, such as the striatum and hippocampus, with little emphasis on the function of adenosine in cortical areas, where receptors are expressed in lower magnitudes (Rebola et al., 2003, 2005, 2008). In this thesis, I therefore want to elucidate the role of adenosine receptors on cellular signaling in the prefrontal cortex. I will discuss the literature on the role of adenosine receptors in other brain regions, to ultimately lead back to what we know about adenosine receptors in the prefrontal cortex, and what I want to add in this thesis.

Adenosine can be derived from three main sources: from neuronal firing, in which ATP is used, released into the synaptic cleft and consecutively converted extracellularly by ecto-nucleotidases (Fig 1.4A); from adenosine nucleoside transporters (Fig 1.4B); or from astrocytic activity that can secrete ATP and that way function as a volume transmission mechanism (Fig 1.4C) (Fredholm et al., 2005). At low frequencies of stimulation (Fig 1.4B-C), there is a predominant inhibitory action of adenosine via A1R, while at higher frequencies of nerve stimulation (Fig 1.4A), through the exponential rise in the release of ATP, the contribution of ATP-derived adenosine increases, making it possible to observe an

**Figure 1.4.** The three main sources of adenosine: from neuronal-firing derived ATP (A), through adenosine transporters (B) and from astrocytic-derived ATP (C). ATP is converted extracellularly by ecto-nucleotidases (’5-NT).
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activation of facilitatory A2A R (Cunha, 2001; D’Alcantara et al., 2001; Rebola et al., 2008; Fontinha et al., 2009; Costenla et al., 2011).

As synaptic plasticity occurs only under conditions where synapses are activated either with coinciding timing of activity, or in conditions where there is a high frequency of stimulation, this predicts a predominant role of A2A R in synaptic plasticity; while A1 R would impose a tonic brake mainly on basal excitatory transmission.

Both types of adenosine receptors are primarily synaptically located (Fig 1.5A, Tetzlaff et al., 1987; Rebola et al., 2005) and can be found at both the presynaptic and postsynaptic site (Rebola et al., 2003, 2005, 2008). Adenosine receptors have been found on cell types ranging from dopaminergic (Borycz et al., 2007; Ferré, 2008; Garçao et al., 2013; Pandolfo et al., 2013), GABA-ergic (Cunha and Ribeiro, 2000; Shindou et al., 2003; Rombo et al., 2015), glutamatergic (Rebola et al., 2005) to cholinergic cells (Cunha et al., 1995; Rodrigues et al., 2008). There, it can act through several mechanisms to influence both pre- and postsynaptic processes. First, adenosine has been shown to affect the release of GABA (Kirk and Richardson, 1995; Cunha and Ribeiro, 2000; Wirkner et al., 2004), glutamate (Dunwiddie and Masino, 2001; Lopes et al., 2002; Corsi et al., 2003; Popoli et al., 2003; Rodrigues et al., 2005), acetylcholine (Kirk and Richardson, 1994; Jin and Fredholm, 1996; Rebola et al., 2002) and dopamine (Okada et al., 1996; Ferré, 2008), mainly through actions at the site of the A1 R (Lupica et al., 1990; Fredholm et al., 2005; Rebola et al., 2008; Costenla et al., 2011) Second, adenosine receptors can heteromerize together and with other receptors, such as the dopamine receptor (Fuxe et al., 2005; Rodrigues et al., 2005; Ciruela et al., 2006; Ferré, 2008). In such a heteromeric complex, adenosine can alter the response normally provided by ligand binding: adenosine via A2A R inhibits the dopaminergic binding to the D2 receptor (Fuxe et al., 2005), while the A1 R antagonistically inhibits dopamine binding to the D1 receptor (Ferré et al., 2007). A1 R and A2A R also heteromerize in an antagonistic manner, ensuring that adenosine binding to one receptor type makes the other receptor type less effective (Lopes et al., 2002; Ciruela et al., 2006; Ferré et al., 2007; Zhang et al., 2015). This way, activation of A2A R can facilitate synaptic transmission through a desensitization of the A1 R - mediated neuronal responses (Lopes et al., 2002). If this would not occur, then there would be no means to overcome the potent A1 R inhibition. Last, adenosine receptors can alter signaling through various postsynaptic intracellular signaling cascades. Adenosine A2A R agonists can reduce NMDA currents of striatal neurons
(Gerevich et al., 2002) and increase NMDA receptor currents in the hippocampus (Rebola et al., 2008); while $A_1$R can activate postsynaptic G protein–coupled inwardly rectifying potassium (GIRK) channels, leading to hyperpolarization of the resting membrane potential (RMP) and a decrease in cellular input resistance through the opening of inward rectifying potassium channels on the postsynaptic site resulting in the shunting of synaptic inputs, and a decrease in release probability at the presynaptic site (Rainnie et al., 1994; Cunha, 2001). In addition, adenosine can inhibit hyperpolarization-activated cyclic nucleotide (HCN)-gated channels by decreasing cAMP levels through the adenosine $A_1$R, leading to a further hyperpolarization of the membrane potential (Rainnie et al., 1994; Arrigoni et al., 2005).

Also in the prefrontal cortex, adenosine is shown to affect intrinsic membrane properties and the release of neurotransmitters. In the prefrontal cortex, adenosine receptors affect the release of acetylcholine in vivo, as measured by microdialysis (Van Dort et al., 2009). Also, adenosine is capable of affecting intrinsic membrane properties through actions at the $A_1$R to a different extent depending on the neuronal subtype (Fig 1.5C, van Aerde et al., 2013). The largest hyperpolarizing response to adenosine could be measured on slender tufted layer 5 pyramidal neurons (van Aerde et al., 2013). Also regular spiking neurons in L5, small pyramidal neurons of L6, and some slender tufted L3 pyramidal neurons had a hyperpolarizing response to adenosine (van Aerde et al., 2013). These effects were due to $A_1$R activation. Not all neurons were susceptible to adenosine-induced hyperpolarization: layer 2 neurons and low-threshold and fast-spiking interneurons had no response to adenosine (van Aerde et al., 2013). How this affects synaptic transmission is not clear. However, experiments in the barrel cortex show that adenosine modulates synaptic transmission through the $A_1$R at different synaptic connections in the neuronal microcircuitry of L4 neurons (Fig 1.5B, Qi et al., 2016). Especially at excitatory synapses onto pyramidal neurons (E-E) transmission was reduced by adenosine, while no effects were observed at the inhibitory to interneuron (I-I) connections. Moderate reductions were shown on the two other, excitatory to inhibitory (E-I) and inhibitory to excitatory (I-E) connections, but only on the side of the excitatory synapse. This seems indicatory for the presence of $A_1$R on both presynaptic axonal terminals and postsynaptic somatodendritic compartments of excitatory neurons but not on inhibitory (fast spiking) interneurons (Qi et al., 2016). In an experiment in the visual cortex however, adenosine was shown to be capable of suppressing inhibitory transmission to excitatory pyramidal neurons via presynaptic $A_1$R (Zhang et al.,
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2015). In both barrel cortex and visual cortex, A\textsubscript{2A}R had no to very moderate influence on synaptic transmission (Qi et al., 2016; Zhang et al., 2015). Whether the adenosine receptors in the mPFC are thus involved in synaptic transmission of pyramidal neurons and interneurons, is till date not clear. In this chapter 3 of this thesis, we investigated whether the adenosine A\textsubscript{2A}R is involved in synaptic transmission of glutamatergic synapses onto both pyramidal neurons as well as interneurons.

1.3.4 Adenosine receptors in synaptic plasticity

Due to their synaptic location, both adenosine receptors are at a perfect position to affect plasticity at these synapses. However, due to their engagement in different patterns of cellular activity they might be recruited in different manners during the induction of plasticity. A\textsubscript{2A}R would be predicted to have a predominant role in synaptic plasticity, as these receptors are active when there is high activity in the network, a necessary feature for the induction of long-term plasticity.

![Figure 1.5. Synaptic location and effects of adenosine receptor activity. A. Adenosine A\textsubscript{1} and A\textsubscript{2A}R are present both pre- and postsynaptically. B. Adenosine affects synaptic transmission postsynaptically by decreasing the evoked EPSP amplitude (Qi et al., 2016). C. Adenosine decreases the membrane potential of cortical cells (van Aerde et al., 2013). D. Endogenous adenosine acting at A\textsubscript{2A}R is necessary for the induction of synaptic plasticity (Simões et al., 2016).]
Indeed, antagonists of A$_{2A}$R have been shown to block synaptic plasticity in the hippocampal Schaffer collateral to CA3 pathway (Rebola et al., 2008), in the Schaffer collateral to CA1 pathway (Kessey and Mogul, 1997; Chamberlain et al., 2013; Rombo et al., 2015), in the striatum (Shen et al., 2008b; Flajolet et al., 2009), in nucleus accumbens (D’Alcantara et al., 2001) and in amygdala (Simões et al., 2016). In some cases, the addition of the A$_{2A}$R agonist CGS21680 alone was sufficient to induce long-term facilitation of the recorded potentials (Rebola et al., 2008; Li et al., 2015; Rombo et al., 2015), as this could mimic the adenosine load during or after a high-frequency stimulation (Rombo et al., 2015). On top of that, CGS21680 has the potential to restore disrupted long-term facilitation (Shen et al., 2008b).

Endogenous adenosine can also suppress plasticity through activation of the A$_1$R, while the relief from endogenous adenosine acting at the A$_1$R can increase the magnitude of long-term potentiation (Tanaka et al., 1990; De Mendonça and Ribeiro, 2000; Rex et al., 2005; Costenla et al., 2011). Whether A$_1$R activation is involved in plasticity seems highly dependent on the brain region: A$_1$R antagonists increase LTP in the hippocampal Schaffer collaterals to CA1 pathway and agonists suppress LTP in this region (Arai et al., 1990; Tanaka et al., 1990; Mendonça and Ribeiro, 2000; Rex et al., 2005; Costenla et al., 2011), whereas A$_1$R antagonists and agonists had no effect on the amplitude of synaptic plasticity in synapses of Schaffer collaterals to CA3 (Rebola et al., 2008), amygdala (Simões et al., 2016), nucleus accumbens (D’Alcantara et al., 2001) and visual cortex (Bannon et al., 2016). The adenosine A$_1$R does play a significant role in heterosynaptic plasticity, the process of plasticity at another synapse than the one directly stimulated, a form of plasticity dependent on astrocytic activity (Zhang et al., 2003; Bannon et al., 2016; Manzoni et al., 2016).

However, it is not shown to date whether adenosine can affect synaptic plasticity in the prefrontal cortex. Therefore, in chapter 3 we investigate whether adenosine through actions at A$_{2A}$R can affect cortical synaptic plasticity in the rodent prefrontal cortex.

1.3.5 Acetylcholine modulates mPFC activity

The neuromodulator acetylcholine (ACh) fulfills an important role in attention, motivation, memory and learning-related cognitive processing and behavior (Kilgard and Merzenich, 1998; Hasselmo, 2006; Sarter et al., 2009; Morishita et al., 2010; Poorthuis et al., 2014).
The main cholinergic innervation of the neocortex originates in the basal forebrain (Woolf and Butcher, 2011). Once ACh is released, it can bind to two types of cholinergic receptors: muscarinic acetylcholine receptors (mAChRs) or nicotinic acetylcholine receptors (nAChRs). In a bound state, ACh affects the firing activity and excitability of the target neurons (Dajas-Bailador and Wonnacott, 2004; Gulledge, 2005; Thiele, 2013; Yakel, 2013, Bubser et al., 2012). Activation of the G-protein coupled mAChRs leads to slow and long lasting effects on cellular activity and excitability, while the activation of the ionotropic nAChRs will lead to fast and short effects (Brown, 2010; Thiele, 2013). In the cortex, both mAChR and nAChR are present (Ballinger et al., 2016). The muscarinic receptor has different subtypes, of which mainly the muscarinic receptor types 1(M1), 2 (M2) and 4 (M4) are present in the cortex (Levey et al., 1991). While activation of M1 results in a decreased conductance of potassium channels, M2 and M4 activation increases the conductance of potassium while at the same time decreasing the conductance of calcium channels (Gulledge, 2005; Gulledge et al., 2007, 2009; Thiele, 2013). In contrast, nAChRs open upon activation and let ions, most notably Na\(^+\) and Ca\(^{2+}\), pass through the receptor’s pore, resulting in a depolarization of the membrane (Gotti and Clementi, 2004; Changeux, 2012). nAChRs in the cortex consist of multiple subunits, resulting in different nAChR subtypes that can be either homopentameric with only α7, or heteromeric with a combination of α(2-10) and β(2-4) subunits. Depending on this composition, the different nAChR subtypes have different pharmacological and biophysical properties (Hogg et al., 2003; Gotti and Clementi, 2004). For example, α4β2 nAChRs with an additional α5 subunit show an increased Ca\(^{2+}\) conductance, sensitivity to nicotine and prolonged inwards currents following nicotine application (Ramirez-Latorre et al., 1996; Gotti and Clementi, 2004; Bailey et al., 2012).

Both mAChR and nAChR are expressed in multiple types of pyramidal and interneuron populations in the cortex (Thiele, 2013, Poorthuis et al., 2013). Their expression is dependent on the neuronal subtype, such that some ACh receptor subtypes are exclusively expressed in a specific subset of neurons. For instance, the muscarinic M1 are expressed in both interneurons and pyramidal neurons in layer 2/3 and in pyramidal neurons in layer 5, while M4 are specifically expressed in layer 6 pyramidal neurons (Thiele, 2013) and M2 are mainly expressed on the presynaptic terminals of cholinergic and GABAergic neurons (Thiele, 2013). The expression of the nicotinic AChR in the mPFC is both subtype- as well as layer-specific. The layer-specificity is especially apparent for pyramidal neurons: while pyramidal neurons in layer 6 and a small subgroup of layer 2/3 pyramidal neurons
specifically express nAChRs containing the β2 subunit, layer 5 pyramidal neurons only express nAChRs containing homomeric α7 subunits (Poorthuis et al., 2013a; Verhoog et al., 2016). In contrast, most interneurons do not have a layer-specific nAChR expression (Poorthuis et al., 2013a), while they do have a clear cell-type specific expression, namely, fast-spiking interneurons of layer 2/3 and 5 of the mPFC express α7 nAChRs (Poorthuis et al., 2013a), while SOM-positive interneurons such as Martinotti cells express muscarinic receptors as well as nAChRs composed of β2 subunits (Beierlein et al., 2000; Couey et al., 2007; Fanselow et al., 2008; Poorthuis et al., 2013a; Xu et al., 2013). Martinotti cells are mainly located in layer 2/3 and 5 of the cortex (Silberberg and Markram, 2007).

Activation of AChRs on SOM-interneurons lead to a strong depolarization and firing of action potentials in these neurons (Beierlein et al., 2000; Couey et al., 2007; Fanselow et al., 2008; Poorthuis et al., 2013a; Chen et al., 2015).

In summary, the expression of muscarinic and nicotinic AChRs is cell-type and layer-specific in the mPFC and particularly the activity of interneurons and to a lesser extent the activity of pyramidal neurons is modulated by released ACh.

1.3.6 Modulation of network activity by acetylcholine

The layer specific expression of muscarinic and nicotinic AChRs is reflected by a layer specific modulation of network activity following ACh release (Letzku et al., 2011; Poorthuis et al., 2014; Ballinger et al., 2016). In the superficial layers (L2-3), acetylcholine release leads to a decreased firing rate of pyramidal neurons through an increase in GABAergic inhibition by activating muscarinic and nicotinic AChRs on interneurons (Disney et al., 2012; Alitto and Dan, 2013; Poorthuis et al., 2013a). Moreover, mAChR activation inhibits release from intracortical projections that are important for connecting different columns in a lateral fashion (Kimura and Baughman, 1997). In the deeper layers, excitatory and inhibitory neurons express both muscarinic and nicotinic AChRs (Gulledge et al., 2007; Kassam et al., 2008). Acetylcholine release leads to an increase of GABAergic mediated inhibitory responses in pyramidal neurons of layer 5 (Couey et al., 2007). In contrast, ACh in layer 6 has no effect on the frequency of inhibitory responses (Poorthuis et al., 2013a).

Acetylcholine exerts complex actions on network activity mainly through acting on interneurons (Letzku et al., 2011; Lovett-Barron et al., 2014), while previous theories suggested that the effects of acetylcholine on attentional behavior were likely mediated by rhythmic-bursting layer 5 pyramidal neurons (Metherate et al.,
1992). In specific, acetylcholine acting on LTS-SOM interneurons is crucially involved in coordinating firing patterns of many cortical neurons over a distance of several hundred microns (Beierlein et al., 2000; Fanselow et al., 2008). These LTS-SOM neurons can be found in L2-3 and L5 in the mPFC and project from there to the distal dendrites of L2-3 and L5 pyramidal neurons, where they modulate their firing behavior via lateral inhibition (Silberberg and Markram, 2007). It is therefore thought that specific actions of ACh on lateral inhibition via the activity of LTS-SOM has an important impact on cortical function (Kawaguchi and Kubota, 1997; Fanselow et al., 2008; Hasselmo and Sarter, 2011; Demars and Morishita, 2014). We investigate in chapter 4 the specific mechanism how ACh modulates the activity of Martinotti LTS-SOM cells and how this affects lateral inhibition onto surrounding neurons.

1.4 Aim of this thesis
The aim of this thesis is to better understand the actions of adenosine and acetylcholine and their short- and long-term effects on network signaling in the neocortex of rodents and humans. To reach this aim, we make use of field- and whole-cell electrophysiology to evaluate functional responses both on the network scale and on a single-cell level. We perform our research both in cortical tissue of the rodent prefrontal cortex, as well as on cortical tissue of the human temporal lobe. Using these different methods and tissue from both rodent and human helps us in answering three open questions.

Research question 1:
Does caffeine alter synaptic transmission and intrinsic membrane properties of neurons in the human cortex?

First, although a large body of literature exists on the cognitive enhancement effects of caffeine, we know surprisingly little about the effects of caffeine on cortical signaling in the human brain. From the rodent brain, we know that caffeine has a dual role, as both a signal inhibitor through A₁R, and as a facilitator of synaptic plasticity through A₂A R. We therefore made a first step in elucidating these important mechanisms behind the human cognitive enhancement effects of caffeine in chapter 2. We investigated the effect of caffeine on synaptic transmission and intrinsic membrane properties in the human cortex and sought out to understand better by which fraction and by which receptor this effect was mediated. To that end, we investigated whether the location of the A₁R and the
binding properties of caffeine to A<sub>1</sub>R would be different in the human cortex than what was known from the rodent cortex. We furthermore investigated whether pyramidal neurons in the human cortex respond to caffeine indeed by acting on synaptic transmission and membrane properties through A<sub>1</sub>R, and by which synaptic fraction these possible effects would be mediated.

Research question 2:  
*Do A<sub>2A</sub>R influence synaptic plasticity in the rodent prefrontal cortex?*

Second, synaptic plasticity, thought to be the main neurochemical foundation for learning and memory, is potently altered by adenosine acting through A<sub>2A</sub>R as was previously shown in several brain areas, such as hippocampus, striatum, and nucleus accumbens. However, we know little about the effect of adenosine on synaptic transmission and plasticity in cortical regions. We therefore investigated in **chapter 3** with field recordings and whole-cell patch clamp recordings how A<sub>2A</sub>R are involved in synaptic transmission and plasticity in the medial prefrontal cortex. We induced plasticity in this network through an extracellular pipette, and could thereby evaluate the role of endogenous adenosine binding to A<sub>2A</sub>R at different synaptic fractions. We evaluated not only glutamatergic plasticity onto pyramidal neurons, but also glutamatergic plasticity onto interneurons, as these play an important role in network functioning in the mPFC.

Research question 3:  
*Does ACh facilitate disynaptic lateral inhibition between pyramidal neurons in the cortex?*

Third, acetylcholine released in the mPFC has large effects on low-threshold spiking Martinotti cells in which upon release of the neuromodulator acetylcholine action potential firing can be induced. In local cortical networks, these somatostatin-positive Martinotti cells are the most prominent interneuron type that participate in lateral inhibition. However, it is not known whether cholinergic inputs affect lateral inhibition between pyramidal neurons through Martinotti interneurons. Therefore, in **chapter 4** we made paired recordings from these interneurons and the connected pyramidal neurons that they receive projections from or project towards. We hypothesize that these interneurons become even stronger involved in disynaptic lateral inhibition when acetylcholine is released. To show whether this is true, we used mice expressing channelrhodopsin specifically in cholinergic terminals. Upon light activation, we were able to release endogenous acetylcholine and by recording from Martinotti cells and pyramidal
neurons we could evaluate changes of disynaptic inhibition mediated by the Martinotti cells.

In summary, the medial prefrontal cortex is a remarkable structure that serves as a hub for projections to and from many cortical and subcortical structures in the brain, making it ideally suited for the regulation of attentional processes. The function of the medial prefrontal cortex as well as other cortices is critically dependent on the neuromodulators acetylcholine and adenosine. Manipulation of these cortical neuromodulatory systems, such as by the most widely used psychoactive substances in the world nicotine and caffeine, can enhance or abrogate cognitive abilities. Understanding the influence of adenosine- or acetylcholine on cortical processing is not only relevant for understanding the underlying mechanism of the recreational use of caffeine and nicotine on behavior, but can also ultimately help to explain mechanisms underlying adenosine- or acetylcholine-dependent behavior. With this research, we hope to contribute to a deeper understanding of the cortical effect of caffeine, adenosine and acetylcholine on specific cellular subtypes in the cortex.
Caffeine controls glutamatergic synaptic transmission and pyramidal neuron excitability in human neocortex

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2.1 Abstract
Caffeine is the most widely used psychoactive drug, bolstering attention and normalizing mood and cognition, all functions involving cerebral cortical circuits. Whereas studies in rodents showed that caffeine acts through the antagonism of inhibitory A₁ adenosine receptors (A₁R), neither the role of A₁R nor the impact of caffeine on human cortical neurons is known. We here provide the first characterization of the impact of realistic concentrations of caffeine experienced by moderate coffee drinkers (50 μM) on excitability of pyramidal neurons and excitatory synaptic transmission in the human temporal cortex. Moderate concentrations of caffeine disinhibited several of the inhibitory A₁R-mediated effects of adenosine, similar to previous observations in the rodent brain. Thus, caffeine restored the adenosine-induced decrease of both intrinsic membrane excitability and excitatory synaptic transmission in the human pyramidal neurons through antagonism of postsynaptic A₁R. Indeed, the A₁R-mediated effects of endogenous adenosine were more efficient to inhibit synaptic transmission than neuronal excitability. This was associated with a distinct affinity of caffeine for synaptic versus extra-synaptic human cortical A₁R, probably resulting from a different molecular organization of A₁R in human cortical synapses. These findings constitute the first neurophysiological description of the impact of caffeine on pyramidal neuron excitability and excitatory synaptic transmission in the human temporal cortex, providing adequate ground for the effects of caffeine on cognition in humans.

2.2 Introduction
Coffee is the second most consumed beverage after water and its main constituent, caffeine, is the most widely consumed drug, improving attention and alertness, and normalizing mood and cognition (Fredholm et al., 1999; Smith, 2002). These central effects of caffeine result from the antagonism of adenosine receptors (Fredholm et al., 1999), in particular A₁ receptors (A₁R) and A₂A receptors (A₂A R), which are the main adenosine receptors in the brain (Fredholm et al., 2005). Because of its hydrophobic properties, brain concentrations of caffeine are similar to plasma concentrations (McCall et al., 1982, Fredholm et al., 1999), in the range of 20-70 μM upon moderate intake (Costenla et al., 2010; Kaplan et al., 1990; Thithapandha et al., 1972). These concentrations trigger the maximal psychostimulant effects of caffeine (Bruce et al., 1986). Careful consideration of caffeine concentration is important since most effects of caffeine are bell-shaped,
Caffeine controls human cortical function

being a psychostimulant with neuroprotective actions at moderate doses and a depressant with deleterious effects at higher doses (Doepker et al., 2016; Rogers and Dernoncourt, 1998; Smith, 2002).

Moderate doses of caffeine antagonize adenosine receptors (El Yacoubi et al., 2000; Daly et al., 1981; Huang et al., 2005; Karcz-Kubicha et al., 2003; Simões et al., 2016; Yang et al., 2009), whereas higher caffeine concentrations act on other targets such as the inhibition of phosphodiesterases, the modification of GABA<sub>A</sub> receptor function or the release of calcium from intracellular calcium stores (Boswell-Smith et al., 2006; Fredholm et al., 1999; Lopez et al., 1989; McPherson et al., 1991). These are likely associated with the toxic effects of caffeine (Yu et al., 2009). Since A<sub>1</sub>R are inhibitory, reducing excitatory synaptic transmission and neuronal excitability (Dunwiddie and Masino, 2001), whereas A<sub>2A</sub>R are mostly excitatory, facilitating synaptic plasticity processes (Cunha, 2016), it is assumed that the neurostimulant effect of caffeine mostly results from the partial antagonism of A<sub>1</sub>R whereas the neuroprotective effects of caffeine may results from limiting excessive A<sub>2A</sub>R activation (Cunha, 2016; Ferré, 2008). The role of A<sub>1</sub>R in the cerebral cortex has mostly been studied in rodents, where A<sub>1</sub>R are mostly located at synapses (Tetzlaff et al., 1987; Rebola et al., 2003), in particular at excitatory rather than at inhibitory synapses (e.g. Rombo et al., 2015). At excitatory synapses, A<sub>1</sub>R depress synaptic transmission and neuronal excitability through a combined presynaptic action decreasing glutamate release, postsynaptic actions decreasing the activation of glutamate receptors and voltage-sensitive calcium channels (de Mendonça et al., 1995; Klishin et al., 1995) as well as extrasynaptic actions through a decrease of neuronal excitability by controlling potassium rectifier channels (Greene et al., 1985), after-hyperpolarization potentials (Haas and Greene, 1984) and HCN (Li et al., 2011). Both in vivo and in vitro studies in rodents concluded that the inhibitory effect of A<sub>1</sub>R predominantly results from the presynaptic inhibition of glutamate release (Phillis et al., 1979; Thompson et al., 1992).

Despite the clear effects of caffeine in human subjects on cortical regions resulting in alterations of vigilance, mood and cognition (Doepker et al., 2016; Smith, 2002), the functional impact of caffeine on neuronal excitability and information flow in the human cerebral cortex is not yet characterized. On a molecular level, moderate doses of caffeine affect A<sub>1</sub>R binding (Boulenger et al., 1982) and occupancy in human cortical neurons (Elmenhorst et al., 2012; Paul et al., 2014).
While studies in rodents revealed an ability of caffeine to partially antagonize some $A_1$R-mediated effects (Phillis et al., 1979, Qi et al., 2016), responses in the human cortical network might differ as $A_1$R have clear inter-species differences, typified by a lower density (Boulenger et al., 1982; Dodd et al., 1986; Fastbom et al., 1987; Svenningsson et al., 1997), a higher affinity for agonists and a lower affinity for antagonists in human versus rodent cerebral cortex (Maemoto et al., 1997; Murphy and Snyder, 1982; Ferkany et al., 1986). Therefore, we here delineate the synaptic and subsynaptic localization of $A_1$R in the human cerebral cortex, and test how $A_1$R affect neuronal excitability and excitatory synaptic transmission. We report how caffeine at realistic concentrations reached in the brain parenchyma and experienced by coffee consumers after 1-2 cups of coffee, affects these neuronal and synaptic $A_1$R actions in human neocortex.

2.3 Methods

Human samples

All procedures on human brain resection material that had to be removed for the surgical treatment of deeper brain structures were performed with the approval of the Medical Ethical Committee of the VU University Medical Centre, written informed consent by patients involved, and in accordance with Dutch license procedures and the declaration of Helsinki, as previously described (Verhoog et al., 2016). Human brain samples were also collected at autopsies, performed at the Instituto Nacional de Medicina Legal e Ciências Forenses, which approved all procedures according to the rules of the European Consortium of Nervous Tissues: BrainNet Europe II, to protect the identity of individual donors, as previously described (Pliássova et al., 2016).

Membrane preparation and binding assays

Total membranes and synaptic membranes (from a synaptosomal preparation) were obtained by isopicnic and gradient centrifugations of homogenized brain tissue, as previously described (Pliássova et al., 2016; Rebola et al., 2005). To determine the enrichment and basic binding characteristics of $A_1$R in cortical synapses, we compared saturation binding isotherms of the selective $A_1$R antagonist $^3$H-DPCPX (0.1-10 nM; specific activity of 102.1 Ci/mmol; from DuPont NEN) in total and synaptosomal membranes (72-164 µg) incubated for 2 h incubation at room temperature in a buffer containing 50 mM Tris, 1 mM EDTA, 2 mM EGTA, pH 7.4, with adenosine deaminase (4 U/ml, Roche) before filtration.
Caffeine controls human cortical function through Whatman GF/C filters (Millipore), as previously described (Coelho et al., 2006; Rebola et al., 2003). To estimate the binding affinity of caffeine, we carried out displacement curves of $^3$H-DPCPX binding with caffeine (0.1-300 μM; from Sigma), as previously described (Coelho et al., 2006). Results are expressed as specific binding, determined by subtraction of the non-specific binding, which was measured in the presence of 2 μM 8-{(2-aminoethyl)amino}carbonylmethoxyphenyl)xanthine (XAC, a mixed $A_1R/A_2A_R$ antagonist; from Tocris) and normalized per amount of protein (bicinchoninic acid assay). To derive the binding parameters from saturation curves ($K_D$ and $B_{max}$ values) the data were fitted by a rectangular hyperbola using the GraphPad Prism software. For displacement binding curves, $IC_{50}$ values were converted to $K_i$ values by non-linear fitting of the semi-logarithmic curves derived from the competitions curves.

Subsynaptic fractionation and Western blot analysis

To separate the extrasynaptic (non-active) zone, presynaptic active zone and postsynaptic fractions from synaptosomes, we used a fractionation method previously described in detail (Canas and Cunha, 2016; Rebola et al., 2005). The efficiency of separation is based on the segregation of different markers in the several fractions: SNAP-25 in the presynaptic active zone, PSD-95 in the postsynaptic density and synaptophysin outside the active zone (extrasynaptic fraction). Western blot analysis was performed with a rabbit anti-$A_1R$ antibody (1:500, Thermo Scientific), as previously described (Rebola et al., 2003).

Human brain slice preparation and electrophysiological recordings

Human brain slices were derived from resected tissue obtained from patients suffering from mild-to severe forms of epilepsy (n=6, of which 5 were diagnosed with meso-temporal epilepsy, 1 with dysplasia; average age 44.8±10.0). All obtained tissue is derived from the temporal lobe area (n=6), away from the focal area of the epilepsy. Slices were prepared as described previously (Verhoog et al., 2016). Briefly, blocks of resected cortical tissue were transported to the laboratory in carbogen-saturated (95% O$_2$, 5% CO$_2$ at pH 7.4) ice-cold choline-based slicing solution containing (in mM): 110 choline chloride, 11.6 sodium ascorbate, 2.5 KCl, 1.3 NaH$_2$PO$_4$, 7 MgCl$_2$, 0.5 CaCl$_2$, 26 NaHCO$_3$, 10 glucose. Cortical slices (350–400 μm) were prepared in the same ice-cold solution as used for transport, and then transferred to holding chambers with artificial cerebrospinal fluid (aCSF) containing (in mM) 125 NaCl, 3 KCl, 1.25 NaH$_2$PO$_4$, 1 MgSO$_4$, 2 CaCl$_2$, 26 NaHCO$_3$, 10 glucose. Here they were stored for 30 min at 34 ºC and subsequently at room temperature for at least one hour before recording.
Following recovery, recordings from cells in deeper layers of the human temporal cortex were made in oxygenated aCSF (flow rate of 2-3 ml/min, 32°C). Whole-cell patch-clamp recordings were made with borosilicate glass pipettes (3-6 MΩ) filled with an intracellular solution containing (in mM): 111 K-gluconate, 8 KCl, 10 HEPES, 4 Mg-ATP, 10 K₂HPO₄, 0.4 GTP, 0.2 EGTA). Biocytin (0.5%) was added to all solutions for post-hoc cell identification, and osmolarity was adjusted to 290-295 mOsm. Pyramidal neurons were visualised with differential interference contrast microscopy, selected based on their large and pyramidal shape and further identified by their spike profile. During recordings, cells were kept at a holding potential close to -70 mV. Recordings were made using MultiClamp 700 A/B amplifiers (Axon Instruments, CA, USA), sampling at 10 kHz and low-pass filtering at 3–4 kHz. Recordings were digitized with an Axon Digidata 1440A and acquired using pClamp software (Axon). Acquired data were stored for off-line analysis.

To characterize the electrophysiological properties of pyramidal neurons, we used a step protocol to calculate the input resistance (Rin) as the slope of the linear fit through the current–voltage relationship in 10 pA steps. The membrane time constant was obtained by fitting a single exponential function to the membrane potential deflection in response to a −50 pA current injection. The sag was calculated as the percentage of the difference between transient and stable membrane potentials to a hyperpolarization amplitude of −10 mV after injecting a negative current. The rheobase was defined as the minimal current amplitude of 1 s duration that resulted in the first action potential (AP). Analysis of spike waveforms was performed on single APs elicited by depolarizing threshold current pulses. The AP half-width was defined as the spike width at its half amplitude. To study synaptic transmission, we evaluated spontaneous events that were detected using MiniAnalysis software. The average amplitude and frequency of excitatory post-synaptic potentials (EPSP) was determined over a time span of 5 minutes.

The tested drugs, adenosine (20 μM, Sigma), caffeine (50 μM, Sigma) or DPCPX (100 nM, Tocris), were used in concentrations previously tested in rodent preparations (Costenla et al., 2010; Cunha et al., 1998; Sebastião et al., 2000) and were dissolved in aCSF at the desired concentration and bath applied during the experiments.

Morphological analysis
After recording, slices were fixed at 4°C for at least 24 h in 100 mM phosphate
buffer saline containing 4% paraformaldehyde (pH 7.4), for subsequent neuronal visualization and reconstruction as previously described (Mohan et al., 2015). Slices containing biocytin-filled neuronal pairs were processed using a protocol described previously (Mohan et al., 2015). Slices were incubated in 0.1% Triton X-100 solution containing avidin biotinylated horseradish peroxidase (ABC-Elite; Camon); subsequently, they were reacted using 3,3-diaminobenzidine as a chromogen under visual control until the dendritic and axonal arborization was clearly visible (usually after 2–4 min). Slices were dehydrated and then mounted on slides, embedded in Mowiol and enclosed with a thin coverslip.

Biocytin-labeled neurons were examined under the light microscope. Representative pairs were photographed at low magnification to document the dendritic and axonal arborization. Subsequently, neurons were reconstructed with the aid of Neurolucida software (MicroBrightField).

**Statistical Analysis**

Data are means ± SEM. Paired Student’s t-test or Wilcoxon signed-rank test (n < 10) was used for statistical comparisons between two groups and one-way ANOVA followed by post hoc Tukey’s test for statistical comparisons among multiple groups. The normality of the distribution of values was determined by the Kolmogorov–Smirnov test. Statistical significance was set at P < 0.05.

### 2.4 Results

**Synaptic localization of A1R in the human cerebral cortex**

To define if A1R were enriched in synapses of the human cerebral cortex, as occurs in rodents (Tetzlaff et al., 1987; Rebola et al., 2003), we compared the binding density of the previously validated selective A1R antagonist ^3^H-DPCPX (Svenningsson et al., 1997) in membranes from synaptosomes (purified synapses) and in total membranes (mostly representing non-synaptic neuronal and astrocytic membranes) from human neocortical tissue. **Figure 2.1A** shows that the binding density of A1R was larger in synaptosomal membranes ($B_{max}$ = 755±22 fmol/mg protein, n=5) than in total membranes ($B_{max}$ = 421±17 fmol/mg protein, n=5; P<0.001 versus synaptosomal membranes; unpaired Student’s t test).

Interestingly, ^3^H-DPCPX displayed a higher affinity to bind A1R in synaptosomal membranes ($K_D$ = 2.68 nM, 95% confidence interval: 2.12-3.24 nM, n=5) than in total membranes ($K_D$ = 4.58 nM, 95% confidence interval: 3.69-5.46 nM, n=5;
P=0.0079 versus synaptosomal membranes; unpaired Student’s t test). These different binding properties of synaptic versus extra-synaptic A₁R in the human cerebral cortex were confirmed by the different apparent affinity of caffeine for these two populations of A₁R. In fact, competition curves of ³H-DPCPX binding by caffeine (a non-selective adenosine receptor antagonist; Daly et al., 1981) showed that caffeine displaced ³H-DPCPX binding with greater efficiency in synaptosomal membranes (Kᵢ= 11.53 μM, 95% confidence interval: 7.89-15.17 μM, n=5) than in total membranes (Kᵢ= 33.02 μM, 95% confidence interval: 25.18-40.86 μM, n=5; P=0.0079 versus synaptosomal membranes; unpaired Student’s t test) (Fig 2.1B). This is suggestive of a possible difference in human cerebral cortical synaptic

![Graphs showing specific binding and binding curves](image)

**Figure 2.1** A₁R are enriched and evenly distributed in human cortical synapses, and have greater affinity for caffeine and selective A₁R antagonist compared to extra-synaptic A₁R. **A**: The selective A₁R antagonist ³H-DPCPX binds with greater affinity and to a larger number of A₁R in synaptosomal compared to total membranes of the human temporal cortex. Data are mean±SEM of 5 subjects. **B**: Caffeine has a greater affinity to displace the binding of the selective A₁R antagonist ³H-DPCPX (2 nM) from synaptosomal compared to total membranes of the human temporal cortex. Data are mean±SEM of 5 subjects. **C**: Western blots of subsynaptic fractions prepared from synaptosomes (SYN) showing the subsynaptic distribution of A₁R in the human temporal cortex (n=2). A₁R are present in all the subsynaptic fractions, outside (EXTRA) and inside the presynaptic active zone (PRE) and postsynaptic density (POST).
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and extra-synaptic membranes of the homo- or heterodimerization of A$_1$R, which has been shown to affect the binding of caffeine and A$_1$R antagonists (Ciruela et al., 2006; Gracia et al., 2013).

We further detailed the localization of A$_1$R within human cerebral cortical synapses. Using different subsynaptic fractions prepared from synaptosomes of the human temporal cortex, we concluded that A$_1$R are present in all the subsynaptic fractions on somatodendritic (postsynaptic) and axon terminal (presynaptic) compartments, inside and outside the presynaptic active zone and postsynaptic density. Such a widespread distribution predicts a complex role for the A$_1$R in the control of synaptic communication in the human cerebral cortex.

**Adenosine effects on intrinsic membrane properties of pyramidal neurons are mediated by A$_1$R**

Adenosine, at non-toxic concentrations (i.e. in low μM range), is known to affect intrinsic membrane properties of layer 2-3 and layer 5 pyramidal neurons in the rodent cerebral cortex through the activation of A$_1$R (Phillis et al., 1979; van Aerde et al., 2015). Since A$_1$R activation also controls excitability in the human brain (Klaft et al., 2016) by still undefined mechanisms, we first characterized the effect of exogenous adenosine application on membrane properties of pyramidal neurons in the human temporal cortex. On average, adenosine (20-100 μM) did not decrease the resting membrane potential (RMP; control: -67.2±2.3 mV; adenosine: -67.2±3.4 mV; difference: 0.01±3.7 mV, n=12, paired t-test: p=0.99), while it did lower the input resistance (R$_{input}$; control: 45.9±16.7 mΩ; adenosine: 40.4±14.5 mΩ; difference: 5.5±6.5 mΩ, n=12, paired t-test: p=0.01; Fig 2.2C,F) and increased the current injection needed before firing of the first action potential, expressed as the rheobase value [Rheobase; control: 251.7±130.5 pA; adenosine: 344.5±149.9 pA; difference: 92.8±45.0 pA, n=12, paired t-test: p<0.0001; Fig 2.2B,E). Altogether these changes lead to a decreased neuronal excitability and a need for a larger input to fire an action potential. Furthermore, adenosine decreased the voltage sag (Sag; control: 2.1±1.2 mV; adenosine: 1.6±1.1 mV; difference: 0.45±0.44 mV, n=12, paired t-test: p=0.003; Fig 2.2D,G), reflecting an adenosine-induced partial block of HCN channels, as described in rodents (Li et al., 2011). These effects of adenosine were not seen at 5 μM and were concentration-dependent in the range of 20-100 μM.
The effects of adenosine on membrane intrinsic properties were due to A<sub>1</sub>R activation, since the selective A<sub>1</sub>R antagonist DPCPX (100 nM) blocked the effects of adenosine on the rheobase: (DPCPX: 275.0±125.5 pA; DPCPX+adenosine: 293.6±120.4 pA; difference: 16.4±25.0 pA, n=7, paired t-test: p=0.13; Fig 2.2B,E), input resistance (DPCPX: 42.2±20.5 mΩ; DPCPX+adenosine: 33.0±12.9 mΩ; difference: 2.2±5.5 mΩ, n=7, paired t-test: p=0.33; Fig 2.2C,F) and sag (DPCPX: 2.3±1.2 mV; DPCPX+adenosine: 2.5±0.6 mV; difference: 0.1±0.6 mV, n=7, paired t-test: p=0.72; Fig 2.2D,G). By itself, DPCPX (100 nM) did not significantly alter these membrane properties: Rheobase (control: 350.0±151.2 pA; DPCPX: 275.0±125.5 pA; difference: 75.0±125.5 pA, n=7, paired t-test: p=0.13; Fig 2.2B,E).
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pA; difference 75.0±133.8 pA, n=8, paired t-test: p=0.16; (Fig 2.2B,E). Input resistance (control: 44.4±22.7 mΩ; DPCPX: 42.2±20.5 mΩ; difference: 0.2±5.8 mΩ, n=8, paired t-test: p=0.92; (Fig 2.2C,F) or sag (control: 2.1±0.7 mV; DPCPX: 2.3±1.2 mV; difference: 0.3±0.6 mV, n=8, paired t-test: p=0.17; (Fig 2.2D,G). This indicates the lack of a constitutive effect of endogenous adenosine on the A1R controlling membrane intrinsic properties of pyramidal neurons in human cortical slices.

Caffeine does not modify intrinsic membrane properties of pyramidal neurons

As with DPCPX, there were no effects of caffeine (50 μM) by itself on intrinsic membrane properties of pyramidal neurons in human temporal-cortical slices. The rheobase (control: 192.9±73.4 pA; caffeine: 196.4±69.8 pA; difference 18.2±72.9 pA, n=11, paired t-test: p=0.42; (Fig 2.2B,E), input resistance (control: 67.5±21.0 mΩ; caffeine: 68.2±16.3 mΩ; difference: 4.1±10.3 mΩ, n=11, paired t-test: p=0.21; (Fig 2.2C,F) and sag were unaltered (control: 1.4±0.7 mV; caffeine: 1.6±0.8 mV; difference: 0.3±0.6 mV, n=11, paired t-test: p=0.12; (Fig 2.2D,G). Since the selective A1R antagonist DPCPX had no effect by itself and thereby ruled out constitutive activation of A1R in human cortical slices, the lack of a direct effect of caffeine on intrinsic membrane properties suggests that A2A R are also not constitutively altering intrinsic membrane properties in human pyramidal neurons in vitro (reviewed in Cunha, 2016).

We next tested if concentrations of caffeine reached in the brain parenchyma by moderate coffee drinkers (50 μM) could effectively antagonize the A1R-mediated control of the intrinsic membrane properties of pyramidal neurons. The effect of exogenously applied adenosine on the rheobase was still observed in the presence of caffeine (50 μM). Thus, when adenosine (20 μM) was added in the presence of caffeine (50 μM), the rheobase increased significantly (control: 192.9±73.4 pA; caffeine+adenosine: 318.2±110.8 pA; difference: 116.4±108.7 pA, n=11, paired t-test: p=0.005; (Fig 2.2B,E). In contrast, caffeine partially blocked the effects of adenosine on the input resistance (control: 67.5±21.0 mΩ; caffeine+adenosine: 55.8±13.6 mΩ; difference: 4.1±10.3 mΩ, n=11, paired t-test: p=0.21; (Fig 2.2C,F) and voltage sag (control: 1.4±0.7 mV; caffeine+adenosine: 1.3±0.5 mV; difference: 0.3±0.7 mV, n=11, paired t-test: p=0.12; (Fig 2.2D,G). This indicates that caffeine concentrations experienced by coffee drinkers can interfere with effects of adenosine on the intrinsic membrane properties of pyramidal neurons in human neocortex.
Adenosine controls synaptic transmission through A1R

The impact of caffeine on cerebral cortical-mediated behavioral responses may also result from its ability to control glutamatergic synaptic transmission, as observed in rodents (Costenla et al., 2010; Phillis et al., 1979; Qi et al., 2016; Thompson et al., 1992). To evaluate the effect of caffeine on glutamatergic synaptic transmission in the human neocortex, we made whole-cell recordings of large deeper-layer pyramidal neurons of the human temporal cortex and recorded spontaneous excitatory transmission. Adenosine (20 μM) significantly decreased both the amplitude of excitatory events (control: 41.7±10.6 pA; adenosine: 38.1±6.6 pA; difference: 3.9±4.9 pA, n=10, paired t-test: p=0.03, Fig 2.3D,E) as well as the frequency of excitatory events (control: 1.8±1.6 Hz; adenosine: 0.6±0.5 Hz; difference: 1.2±1.4 Hz; n=10, paired t-test: p=0.02; Fig 2.3A). Both effects were reversible upon washout of adenosine (Fig 2.3E; EPSC amplitude: wash-out: 43.6±12.2 pA; difference vs. adenosine: 5.8±6.6 pA; n=10, paired t-test: p=0.02; EPSC frequency: wash-out: 2.8±2.2 Hz; difference vs. adenosine: 2.1±1.8 Hz; n=9, paired t-test: p=0.009).

The effects of adenosine were mediated by A1R, since the selective A1R antagonist DPCPX (100 nM) blunted the effect of exogenously added adenosine (20 μM) on frequency (control: 2.0±1.8 Hz; DPCPX+adenosine: 1.9±1.2 Hz; difference: 0.1±0.9 Hz; n=6, paired t-test: p=0.77; Fig 2.3B) and amplitude of excitatory postsynaptic events (control: 41.8±12.9 pA; DPCPX+adenosine: 51.7±30.0 pA; difference: 9.7±17.2 pA, n=7, paired t-test: p=0.18; Fig 2.3F). DPCPX (100 nM) by itself increased the frequency (control: 2.0±1.8 Hz; DPCPX: 3.0±2.0 Hz; difference: 1.1±0.6 Hz; n=6, paired t-test: p=0.01; Fig 2.3B) but not the amplitude of excitatory synaptic events (control: 41.8±12.9 pA; DPCPX: 49.8±24.9 pA; difference: 8.0±13.0 pA, n=8, paired t-test: p=0.13; Fig 2.3F), which may suggest a constitutive activity of presynaptic A1R inhibiting synaptic release of glutamate.

We next investigated the effect of caffeine concentrations experienced by most coffee drinkers (50 μM) on synaptic transmission in the presence of adenosine. Caffeine (50 μM) blocked the effect of adenosine (20 μM) on the amplitude of spontaneous excitatory events which indicates a postsynaptic effect of caffeine (control: 38.9±10.3 pA; caffeine+adenosine: 36.7±11.5 pA; difference: 2.1±8.9 pA, n=9, paired t-test: p=0.49; Fig 2.3G), but did not prevent the effect of adenosine on the frequency of spontaneous events (control: 1.1±0.7 Hz; caffeine+adenosine:
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0.2±0.1 Hz; difference: 0.9±0.7 Hz; n=9, paired t-test: p=0.007; **Fig 2.3C**. Similar to the adenosine-only group, effects of adenosine in the case of pre-incubated caffeine could be washed out (wash-out: 0.5±0.4 pA; difference vs. adenosine: 0.3±0.3 pA; n=8, paired t-test: p=0.04; **Fig 2.3C**). In contrast to DPCPX, caffeine (50 μM) by itself had no effect on spontaneous glutamatergic synaptic transmission.

**Figure 2.3** A1R-mediated effects of adenosine on synaptic transmission are postsynaptically blocked by caffeine. **A**: Adenosine (20 μM, green) significantly decreased the frequency of excitatory events onto pyramidal neurons in a reversible manner. Paired t-test comparing adenosine (20 μM) to ACSF condition: * p<0.05; ** p<0.01. Boxes depict average ± SD. **B**: Direct application of DPCPX (100 nM, red) significantly increased the frequency of excitatory events and blocked adenosine-induced inhibition (green). Paired t-test comparing DPCPX (100 nM) to ACSF condition: * p<0.05. Boxes depict average ± SD. **C**: Caffeine pre-incubation (50 μM, blue) did not affect the potency of adenosine (20 μM, green) to decrease the frequency of excitatory events onto pyramidal neurons. Wash-out of adenosine and caffeine restored the frequency of events. Paired t-test comparing adenosine (20 μM) to ACSF control/wash-out condition: * p<0.05; ** p<0.01. Boxes depict average ± SD. **D**: Example trace of the average effect of adenosine application onto the amplitude of excitatory events onto a pyramidal neuron. **E,F,G**: Adenosine (20 μM, green) significantly decreased the amplitude of excitatory events onto pyramidal neurons in a reversible manner (**E**). Pre-incubation with either DPCPX (100 nM, red, **F**) or caffeine (50 μM, blue, **G**) completely abolished this effect. Paired t-test comparing adenosine (20 μM) to ACSF control/wash-out condition: * p<0.05.
It did not modify the amplitude (control: 38.9±10.3 pA; caffeine: 40.1±12.9 pA; difference: 1.1±0.6 pA, n=9, paired t-test: p=0.59; Fig 2.3G) or frequency of spontaneous excitatory postsynaptic events (control: 1.1±0.7 Hz; caffeine: 0.6±0.2 Hz; difference: 0.5±0.7 Hz; n=9, paired t-test: p=0.06; Fig 2.3C). This may suggest that, in contrast to DPCPX, moderate concentrations of caffeine are not sufficient to interfere with constitutive activity of presynaptic A\textsubscript{1}R. Together, our findings show that caffeine concentrations experienced by coffee drinkers interfere with adenosine signalling when there is an additional adenosine load, allowing to restore excitatory synaptic transmission and pyramidal neuron excitability in the human neocortex.

2.5 Discussion
In this study, we provide the first characterization of the impact of caffeine concentrations experienced by moderate coffee drinkers (50 μM) on neuronal excitability in pyramidal neurons and excitatory synapses of the human temporal cortex. Caffeine had a larger affinity for synaptic A\textsubscript{1}R compared to extra-synaptic A\textsubscript{1}R in the human temporal cortex, which contrasts with the affinity profile of caffeine in the rodent cortex; this difference probably results from a different molecular organization of A\textsubscript{1}R within human cortical synapses compared to rodent cortical synapses. Caffeine interfered with several of the inhibitory A\textsubscript{1}R-mediated effects of adenosine in the human cortex, similar to previous observations in the rodent brain. Caffeine blunted the effects of adenosine on intrinsic membrane properties and prevented the amplitude reduction of excitatory synaptic transmission through antagonistic actions at postsynaptic A\textsubscript{1}R.

A distinct interaction of caffeine with A\textsubscript{1}R in the human cerebral cortex compared with rodent cortex is in agreement with several previous studies describing differences of binding properties of brain A\textsubscript{1}R between humans and different animal models (Boulenger et al., 1982; Dodd et al., 1986; Fastbom et al., 1987; Ferkany et al., 1986; Maemoto et al., 1997; Murphy and Snyder, 1982; Svenningsson et al., 1997). Our results confirmed previous evidence that the binding density of A\textsubscript{1}R in the temporal cortex is lower in humans compared to mice or rats (Boulenger et al., 1982; Dodd et al., 1986; Fastbom et al., 1987; Svenningsson et al., 1997). We also confirmed previous evidence that human A\textsubscript{1}R display a different affinity for xanthine-based selective antagonists, which tend to
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have lower affinity in humans compared to rodents (Ferkany et al., 1986; Maemoto et al., 1997; Murphy and Snyder, 1982). Despite these different pharmacological properties, human \( \mathrm{A}_1 \)R seem to exert an overall inhibitory effect in the cerebral cortex that is similar to that observed in rodents: in fact, the exogenous administration of adenosine decreased the excitability of pyramidal neurons in the human temporal cortex in a manner similar to the inhibition previously reported to occur through \( \mathrm{A}_1 \)R activation in the cerebral cortex of rodents (Li et al., 2011; Phillis et al., 1979). In rodents (Thompson et al., 1992), tonic adenosine inhibition is dominated by \( \mathrm{A}_1 \)R-mediated inhibition of excitatory transmission. In human neocortex, both intrinsic membrane properties of pyramidal neurons as well as excitatory synaptic transmission received by these neurons, were inhibited by somatodendritic postsynaptic \( \mathrm{A}_1 \)R. Whether one of these mechanisms dominates effects of adenosine on cortical overall excitability in human neocortex cannot be concluded based on our present results. We did find a high density of \( \mathrm{A}_1 \)R in human cortical synapses, both at somatodendritic (postsynaptic) and axon terminals (presynaptic) compartments, as well as a greater affinity for DPCPX of synaptic \( \mathrm{A}_1 \)R in comparison with extra-synaptic \( \mathrm{A}_1 \)R, which may point to a stronger control of synaptic transmission by \( \mathrm{A}_1 \)R. Since one of the few factors that has been documented to regulate the affinity of \( \mathrm{A}_1 \)R for antagonists is their relative homomerization (Gracia et al., 2013) or heteromerization (Ciruela et al., 2006), the present findings are suggestive of a different molecular arrangement of \( \mathrm{A}_1 \)R in synapses of the human cerebral cortex.

To characterize the impact of caffeine on human cortical neurons, we used a concentration of caffeine within the range of concentrations reached by caffeine in the brain parenchyma upon moderate consumption of caffeine (20-70 \( \mu \)M) (Costenla et al., 2010; Duarte et al., 2012; Kaplan et al., 1990; Silva et al., 2013; Thithapandha et al., 1972), which are similar to plasma concentrations of caffeine that cause maximal psycho-activating responses in humans (Bruce et al., 1986). Many studies have described neuronal effects of caffeine using high millimolar or submillimolar concentrations of caffeine (Grigoryan et al., 2012; Lee et al., 1987; Margineanu & Klitgaard, 2004; Martin & Buno, 2003; Vyleta and Smith, 2008), which represent toxic effects of caffeine found in extreme cases of caffeinism (Gilliland and Andress, 1981). Using concentrations of caffeine realistic for normal human consumption, we found that caffeine only targets inhibitory \( \mathrm{A}_1 \)R, since caffeine mimics the effects of DPCPX. This is in agreement with PET imaging studies that documented a decreased occupancy of cortical \( \mathrm{A}_1 \)R in subjects
consuming caffeine (Elmenhorst et al., 2012; Paul et al., 2014). Importantly, we now report that caffeine displays a greater affinity for synaptic \( \text{A}_1 \text{R} \) compared to extra-synaptic \( \text{A}_1 \text{R} \). This may suggest that synaptic \( \text{A}_1 \text{R} \) are more strongly affected by caffeine and that interference with inhibitory effects of adenosine on transmission at excitatory synapses is a major effect in the human cerebral cortex. In contrast to effects in rodent neocortex, we find that effects of caffeine in human cortical neurons interfered both with adenosine effects on neuronal excitability and excitatory synaptic transmission through postsynaptic \( \text{A}_1 \text{R} \), while in rodents, caffeine mostly controls synaptic transmission through presynaptic \( \text{A}_1 \text{R} \) and affect the release of excitatory neurotransmitters (Greene et al., 1985; Phillis et al., 1979). Nevertheless, also in rodent neocortex, caffeine can exert a postsynaptic effect (Greene et al., 1985; Simons et al., 2011) in accordance with the ability of postsynaptic \( \text{A}_1 \text{R} \) to induce robust responses in rodent cortical neurons (Qi et al., 2016; van Aerde et al., 2015). Differences in effects of caffeine in human and rodent neocortex may result from the previously discussed different molecular organization of \( \text{A}_1 \text{R} \) or to a different subsynaptic distribution of \( \text{A}_1 \text{R} \) in rodent and human cortical synapses; thus, \( \text{A}_1 \text{R} \) seem to be more abundant presynaptically in rodent synapses (Rebola et al., 2003; Tetzlaff et al., 1987), whereas we find here in human neocortex an even distribution of \( \text{A}_1 \text{R} \) in all subsynaptic fractions.

For our experiments, we used neocortical tissue derived from epileptic and tumour patients that underwent surgery for treatment of deeper brain structures. Although the resected tissue was from regions outside the focal area of epilepsy or tumour, and was not part of the disease, our observations could be affected by disease and medication history. Earlier, adenosine \( \text{A}_1 \text{R} \) density was shown to be up- or downregulated in surrounding neocortical areas of patients suffering from temporal lobe epilepsy (Glass et al., 1996; Angelatou et al., 1993) and adenosine \( \text{A}_1 \text{R} \) agonists are explored as therapeutic targets to treat epilepsy (Boison, 2016). Therefore, the possibility that epileptic conditions affect the adenosine neuromodulation system in the cerebral cortex (Rebola et al., 2005) should be considered as a factor influencing the presently reported effects of caffeine. However, the impact of caffeine in epileptic conditions is still largely unclear (Dworetzky et al. 2010; Samsonsen et al., 2013) and seems to be more evident in the developing than in the mature brain (e.g. Tchekalarova et al., 2006), with reports of proconvulsant (e.g. Chu et al., 1981) and anticonvulsant effects of caffeine (e.g. Rigoulot et al., 2003). It is also important to keep in mind that the present study focused on the human temporal cortex and it remains to be
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confirmed if the effects of caffeine are similar in other cortical regions, although both the density of $A_1R$ and the occupancy of $A_1R$ by caffeine seem to be similar in different cortical areas (Svenningsson et al., 1997; Diukova et al., 2012; Elmenhorst et al., 2012; Park et al., 2014).

The present characterization of the effects of a physiological concentration of caffeine (50 $\mu$M) on the human temporal cortex provides an important insight in the subtle effects of cortical exposure to human caffeine consumption. By acting mainly postsynaptically, caffeine is able to counteract adenosine-induced inhibition of the received excitatory signal. This provides a mechanism to explain the enhancement by caffeine on human cognition (Borota et al., 2014), which is not readily observed in adult rodents (e.g. Dall’Ignna et al., 2007; Duarte et al., 2012; Espinosa et al., 2013; Kaster et al., 2015), where caffeine intake mostly normalizes rather than bolsters learning and memory performance. Thus, these findings provide the first neurophysiological description of the impact of caffeine on excitatory synaptic transmission in the human temporal cortex, providing adequate ground for the effects of caffeine in normal consumption amounts on cognition in humans.
Adenosine A2A receptors control glutamatergic synaptic plasticity onto fast spiking interneurons in the prefrontal cortex

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3.1 Abstract
Adenosine A$_{2A}$ receptors (A$_{2A}$R) are activated upon increased synaptic activity to assist in the implementation of long-term plastic changes at synapses. While it is reported that A$_{2A}$R are involved in the control of prefrontal cortex (PFC)-dependent behavior such as working memory, reversal learning and effort-based decision making, it is not known whether A$_{2A}$R control glutamatergic synapse plasticity within the medial PFC (mPFC). To elucidate that, we tested whether A$_{2A}$R blockade affects long-term plasticity (LTP) of excitatory postsynaptic potentials (EPSP) in pyramidal neurons and fast spiking interneurons in layer 5 of the mPFC and of population spikes. Our results show that A$_{2A}$R are enriched at mPFC synapses, where their blockade reversed the direction of plasticity at excitatory synapses onto layer 5 fast spiking (FS) interneurons from LTP to long-term depression (LTD), while their blockade had no effect on the induction of LTP at excitatory synapses onto layer 5 pyramidal neurons. At the network level, extracellularly induced LTP of population spikes was reduced by A$_{2A}$R blockade. The interneuron-specificity of A$_{2A}$R in controlling glutamatergic synapse LTP may ensure that during periods of high synaptic activity, a proper excitation/inhibition balance is maintained within the mPFC.

3.2 Introduction
The prefrontal cortex (PFC) is involved in the control of cognitive and executive functions, such as decision making, working memory, inhibitory control, attention, and behavioral flexibility (Dalley et al., 2004; Euston et al., 2013). The flexible regulation of these types of behavior makes it possible to properly respond to a changing environment (Arnsten, 2009). Such flexibility is thought to require plastic changes in the strength of synaptic connections (Kandel, 1976; Mayford et al., 2012), which is heavily dependent on the action of several neuromodulators (Pawlak et al., 2010; Bloem et al., 2014a; Dembrow and Johnston, 2014).

One of the neuromodulators that can impact synaptic plasticity is adenosine, which is released in an activity-dependent fashion at synapses (Cunha et al., 1996; Wall and Dale, 2013). Its actions are mediated by a balanced activation of the inhibitory A$_{1}$ receptors (A$_{1}$R) and the facilitatory A$_{2A}$ receptors (A$_{2A}$R) (Cunha, 2008b), which act predominantly on glutamatergic but also on GABAergic signaling (Shen et al., 2008; Rombo et al., 2015; Qi et al., 2017). While A$_{1}$R control basal synaptic transmission, A$_{2A}$R are selectively engaged in events where long-term
A2A R control PFC synaptic plasticity

potentiation (LTP) is induced (d’Alcantara et al., 2001; Rebola et al., 2008; Simões et al., 2016).

A2A R are present in the PFC (Van Dort et al., 2009; Pandolfo et al., 2013; van Aerde et al., 2013) and affect PFC-dependent behavior. Indeed, genetic elimination of A2A R decreases effort-based decision-making (Pardo et al., 2012), while enhancing working memory (Zhou et al., 2009; Wei et al., 2011) and reversal learning (Wei et al., 2011). Their possible pathophysiological relevance is highlighted by the ability of selective A2A R antagonists to attenuate working memory deficits (Horita et al., 2013; Li et al., 2015), and by the ability of caffeine, which antagonizes both adenosine receptors, to counteract cognitive behavioral deficits both in human subjects suffering from attention deficit hyperactivity disorder (ADHD; Leon 2000) as well as in a rat model of ADHD (Pandolfo et al., 2013).

Despite the effects of A2A R on PFC-dependent behavior, it is not known how A2A R control the information flow and whether A2A Rs affect glutamatergic synaptic plasticity of information within the local PFC circuit. Therefore, we studied the impact of A2A R on synaptic transmission and plasticity at excitatory synapses onto pyramidal neurons and interneurons and at the network level in the medial PFC (mPFC). We found that A2A R are enriched at mPFC synapses, where A2A R blockade shifts the direction of plasticity at excitatory synapses onto layer 5 fast spiking (FS) interneurons from LTP to LTD, while it is ineffective at excitatory synapses onto layer 5 pyramidal neurons and reduces plasticity at the network level.

3.3 Materials and Methods

Animals
All studies were conducted in accordance with the principles and procedures outlined as "3Rs" in the guidelines of EU (210/63), FELASA, and the National Centre for the 3Rs (the ARRIVE; Kilkenny et al., 2010), and were approved by the Animal Care Committee of the Center for Neuroscience and Cell Biology (ORBEA 78/2013) or by the VU University’s Animal Experimentation Ethics Committee (DEC) and were in accordance with institutional and Dutch license procedures. Rats were housed in a temperature and humidity-controlled environment with 12 h light on/off cycles and ad libitum access to food and water.

Membrane-binding assay
The density of A2A R in total membranes or synaptosomal membranes from the PFC was estimated by a radioligand-binding assay using a supramaximal concentration.
of the A2A R antagonist [3]H-SCH58261 (6 nM; provided by E. Ongini, Schering-Plough, Milan, Italy), as described previously (Kaster et al., 2015; Viana da Silva et al., 2016). Specific binding was determined by the subtraction of nonspecific binding measured in the presence of 3 μM XAC (Tocris).

**Subsynaptic fractionation of mPFC synaptosomes and Western blot analysis**

To separate the extra-synaptic (non-active) zone, presynaptic active zone and post-synaptic fractions from synaptosomes, we used a fractionation method previously described in detail (Rebola et al., 2005; Canas and Cunha, 2016). The efficiency of separation is based on the segregation of different markers in the several fractions: SNAP-25 in the presynaptic active zone, PSD-95 in the postsynaptic density and synaptophysin outside the active zone (extra-synaptic fraction). Western blot analysis was performed with an anti-A2A R antibody (1:500; sc-32261 from Santa Cruz Biotechnology; Santa Cruz, CA, USA), which selectivity was confirmed by the lack of signal in A2A R knockout mice (Rebola et al., 2005).

**Whole-cell-recordings**

Male Wistar rats (5-6.5 week-old) purchased from Charles River (Harlan) were decapitated, their brain was carefully removed and the mPFC was sliced in carbogen buffered (pH 7.4) ice-cold choline-based slicing solution containing (in mM): choline chloride 110, sodium ascorbate 11.6, KCl 2.5, NaH2PO4 1.3, MgCl2 7, CaCl2 0.5, NaHCO3 26, and glucose 10. Slices (350 μm) were kept at room temperature in aCSF oxygenated with carbogen in a holding chamber. Following recovery for at least 1h, recordings from cells in L5 of the mPFC were made in oxygenated aCSF (flow rate of 2-3 ml/min, 32°C). Whole-cell patch-clamp recordings were made with borosilicate glass pipettes (3-6 MΩ) filled with an intracellular solution containing (in mM): K-gluconate 111, KCl 8, HEPES 10, Mg-ATP 4, K2phosphocreatine 10, GTP 0.4, EGTA 0.2. Biocytin (0.2-0.5%) was added to all solutions for post-hoc cell identification, and osmolarity was adjusted to 290-295 mOsm. Pyramidal L5 cells were visualized with differential interference contrast microscopy, selected on their large and pyramidal shape and further identified by their spike profile. FS interneurons were selected based on their small, round shape and further identified by their spike profile. During recordings, neurons were kept at a holding potential close to -70 mV. Recordings were made using MultiClamp 700 A/B amplifiers (Axon Instruments, CA, USA), with sampling at 10 kHz and low-pass filtering at 3–4 kHz. Recordings were digitized with an Axon Digidata 1440A and acquired using pClamp software (Axon). After experiments were completed, slices were stored in 4% paraformaldehyde for subsequent
neuronal visualization and reconstruction as previously described (Mohan et al., 2015).

**Spontaneous EPSCs:** Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded 5-10 min before and 25-30 min after drug incubation. Acquired data were stored for off-line analysis and events were detected using MiniAnalysis software. EPSC amplitude and frequency were determined and averaged over a 5-min time-course in each condition.

**Evoked EPSCs:** Excitatory postsynaptic currents (EPSCs) were evoked (eEPSCs) every 3.5 s using bipolar stimulating electrodes in glass pipettes filled with aCSF positioned 100–150 μm along the cell’s apical dendrite. Duration (0.5 ms) and amplitude (100-350 mA) of extracellular stimulation were controlled by Isoflex stimulators (A.M.P.I., Jerusalem, Israel) to generate a monosynaptic response. After recording a baseline for 5-10 min, drugs were added and the eEPSC response was recorded for another 20 min. In pyramidal neurons, 15 datapoints were determined and averaged in each condition (baseline, 5 min after incubation and 15 min after incubation). In FS cells, EPSC amplitude was averaged over a 5-min time-course in all conditions (baseline, 5 min after incubation and 15 min after incubation).

**Long-term potentiation:** Excitatory postsynaptic potentials (EPSPs) were evoked every 7 s (0.14 Hz) using bipolar stimulating electrodes in glass pipettes filled with aCSF positioned 100–150 μm along the cell’s apical dendrite. The duration (0.5 ms) and amplitude (100-350 μA) of extracellular stimulation were controlled by Isoflex stimulators (A.M.P.I., Jerusalem, Israel) to generate a monosynaptic response. Baseline EPSP was defined with an input/output curve, stimulating at below half maximum response. After obtaining a stable baseline of 3-5 min (30–43 EPSPs), long-term potentiation was induced within 15 min of whole-cell configuration with an unpaired theta burst stimulation (TBS) protocol (10 bursts of 5 pulses each at 100 Hz, repeated 3 times). This protocol triggered an optimal potentiated response in the cells, which was more reliable than other tested protocols such as spike timing dependent potentiation (STDP), although it was still highly variable especially in pyramidal neurons. Timing of EPSPs and the induction protocol was controlled by a Master-8 stimulator (A.M.P.I.). The slope of the initial 2 ms of the EPSP was taken as a measure of EPSP strength. The change in synaptic strength was defined as the percent change in EPSP slope 20–30 min after the TBS relative to baseline. Cell input resistance was monitored by applying a hyperpolarizing
pulse at the end of each sweep (-30 pA). After LTP induction, membrane potential was returned to approximate baseline value by modest current injection. Criteria for inclusion of recordings were: (1) baseline resting membrane potential <-60 mV, (2) smooth rise of EPSP and clear separation from stimulation artifact, (3) stable baseline EPSP slope, (4) less than 30% change in input resistance, (5) no AP-firing evoked by extracellular stimulation in post-pairing period. In total, 5 cases of extreme EPSP rundown (slope <20% of baseline) were excluded from analysis.

**Extracellular recordings**

Male Wistar rats (6-8 week-old) were purchased from Charles River Laboratories (Barcelona, Spain). Rats were anesthetized under halothane atmosphere, decapitated and the brain rapidly removed from the skull and submerged in ice-cold artificial cerebrospinal fluid (aCSF) solution of the following composition, in mM: NaCl 125, KCl 3, MgSO₄ 1, CaCl₂ 2, Na₂HPO₄ 1.25 NaHCO₃ 25-26 and glucose 11, pH 7.4 (osmolality, ~300 mOsmol.kg⁻¹), oxygenated with carbogen (95% O₂ + 5% CO₂). Coronal slices (300 µm-thick) containing the medial prefrontal cortex (mPFC) were cut with a Vibratome 1500 sectioning system (Vibratome, Germany). The slices were then transferred to a pre-chamber containing aCSF under continuous oxygenation to recover at 32ºC for at least 1 h. Slices were then transferred to a submerged recording chamber where they were continuously superfused at a rate of 2-3 ml/min with oxygenated aCSF at 30-32 ºC. A bipolar concentric stimulation electrode SNE-100 (Kopf, Germany) was placed on the layer II/III of the mPFC delivering rectangular pulses (80-160 µA) of 0.1 ms duration applied with a Digitimer DS3 stimulator (Digitimer LTD, U.K.) once every 20 s. The evoked population spikes were recorded through an extracellular borosilicate microelectrode (filled with 4 M NaCl, 2–4 MΩ resistance) placed in the layer V of the mPFC, using an Axopatch 200B amplifier (Axon Instruments, Inc. USA), coupled to an analogue/digital acquisition board (Digidata 1322A; Axon Instruments, Inc., USA). Responses were digitized at 10 kHz and continuously monitored on a personal computer via WinLTP 1.1 software (Anderson and Collingridge, 2007). Responses were quantified as the amplitude of the population spike recordings. After stabilizing the response, the input/output curve was obtained. Then the intensity of the stimulus was regulated to obtain 40-50% of the maximum response before induction of long-term potentiation (LTP). LTP was induced by delivering a train of 100 Hz (50 pulses, 0.5 s duration) for a priming effect, which was 15 min later followed by 4 trains of 100 Hz (50 pulses, 0.5 s duration, 1 every 10s). Due to difficulties in inducing LTP in rat PFC slices, LTP protocols were
extensively tested and this protocol, which has been used by Gemperle et al (2003), was the most reliable one in our hands.

**Experimental design and statistics**

For membrane binding assays, PFC from 5 adult male Wistar rats were used, and the density of A2A-R in synaptosomal membranes was compared to that in total membranes using unpaired t-test. For Western blotting of sub-synaptic fractions, we pooled together mPFC tissue from 22 rats (30-45 days-old). This was due to the requirement of 1 g of tissue for the sub-synaptic fractionation step. For pharmacology in electrophysiology experiments, all the drugs used were dissolved in aCSF at the desired concentration and bath applied during the experiments. The drugs were diluted from stock solutions made in dimethylsulfoxide (DMSO) to their final concentrations: SCH58261 (50-100 nM from 5 mM stock solution, Tocris). All experiments were performed without application of synaptic blockers. In extracellular recordings, due to high variability in LTP magnitude, whenever SCH58261 was tested, a control slice was also done in parallel. In the end, data from 25 slices per group (from 25 different rats) were pooled together for statistical comparison using an unpaired t-test. For plasticity in whole-cell patch-clamp experiments, due to cellular variability, strict exclusion criteria (see above), long duration of the experiment and high quality of slices needed, more animals were needed than are presented in our figures. When relevant and possible, we recorded one cell in control and one cell in drug condition from every animal. For the pyramidal-TBS experiment, 51 animals were used. For the FS interneuron-TBS experiment, 27 animals were used. Raw data was analyzed using Clampfit 10.4 and custom Matlab scripts. For all LTP experiments, we used the percentage of increase in EPSP slope (whole-cell recordings) or population spike amplitude (extracellular recordings) induced by the LTP protocol per cell (whole-cell recordings) or per slice (extracellular recordings) as input for statistical tests. An unpaired t-test was used to compare two groups consisting of multiple such experiments, comparing the percentage of LTP induction in control experiments versus the percentage of LTP induction in A2A-R antagonist-treated experiments. This method for comparing differences in LTP induction in control versus drug treated slices is adopted from our previous research on LTP induction (Mansvelder and McGehee, 2000; Couey et al., 2007; Meredith et al., 2007; Rebola et al., 2008; Verhoog et al., 2013, 2016; Simões et al., 2016). For whole-cell spontaneous recordings, 12 slices from 10 different animals were used in sEPSC on pyramidal neurons; 2 recordings were excluded for rundown reasons (>20% change in resistance). For sEPSC on FS interneurons, 32 recordings of interneurons were
made from slices of 21 different animals. Of these, 13 cells were actual FS interneurons; 1 was excluded for rundown. For eEPCS experiments on pyramidal neurons, 14 slices were used from 4 different animals. For eEPSC experiments on FS interneurons, 8 slices from 3 animals were used, 4 animals were used in total for these experiments. All recorded sEPSCs were analyzed with MiniAnalysis software (Synaptosoft, version 6.0.7). All the statistical analysis was performed using Prism 6 (GraphPad software). Data was analyzed by using the appropriate parametric statistical test as mentioned in the text and p<0.05 was taken as statistically significant.

3.4 Results

A$_2$AR are enriched in synaptosomal membranes and present in all sub-synaptic fractions

To investigate the density and synaptic distribution of A$_2$AR, we compared the binding of $^3$H-SCH58261 in total and synaptosomal membranes from the PFC. The binding density of $^3$H-SCH58261 was higher (n=5; t$_8$=4.56; p=0.0018; unpaired t-test) in the synaptosomal membrane fraction (39.0±3.6 fmol/mg protein) compared to the total membrane fraction (19.4±2.4 fmol/mg protein; n=5) from the PFC (Fig 3.1A).

**Figure 3.1 A$_2$AR are enriched in synaptosomal membranes and present in all subsynaptic fractions.** A: the binding density of a supra-maximal concentration of a selective A$_2$AR antagonist $^3$H-SCH58261 (6 nM) was higher in synaptosomal when compared to total membranes from Wistar rat PFC. Data are mean±SEM of 5 rats; *p<0.05, unpaired Student’s t test. B: Western blots of subsynaptic fractions showing the subsynaptic distribution of A$_2$AR in the mPFC (pooled from 22 rats due to the requirement of large sample size – 1 g of tissue - at the start of the subsynaptic fractionation procedure). The efficiency of separation is based on the segregation of different markers in the several fractions: SNAP-25 in the presynaptic active zone, PSD-95 in the postsynaptic density and synaptophysin outside the active zone (extrasynaptic fraction). A$_2$AR are present in all the subsynaptic fractions, inside and outside the presynaptic active zone and postsynaptic density. However, there is an enrichment outside the presynaptic active zone and postsynaptic density.
A$_{2A}$R control PFC synaptic plasticity

Given this enrichment of A$_{2A}$R in synaptosomal membranes, we used mPFC synaptosomes (pooled from 22 rats) to separate the different subsynaptic fractions, and probed for the subsynaptic distribution of A$_{2A}$R. A$_{2A}$R were present in all the subsynaptic fractions, inside and outside the presynaptic active zone and postsynaptic density, with a higher A$_{2A}$R density observed outside the presynaptic active zone and postsynaptic density (Fig 3.1B). The presence of A$_{2A}$R in all PFC sub-synaptic fractions suggests a role for A$_{2A}$R in the control of synaptic communication in the mPFC.

A$_{2A}$R do not control spontaneous and evoked excitatory synaptic transmission

To determine whether spontaneous excitatory postsynaptic currents (sEPSCs) or evoked excitatory postsynaptic currents (eEPSCs) are affected by A$_{2A}$R, we recorded sEPSCs and eEPSCs onto both pyramidal neurons and fast spiking (FS) interneurons, the two largest groups of neurons in the PFC (Markram et al., 2004) and tested the effect of the selective A$_{2A}$R antagonist SCH58261. After recording a baseline in ACSF, SCH58261 (100 nM) was incubated into the bath and cells were...
recorded for another twenty to thirty minutes. Spontaneous events onto pyramidal neurons were unaffected by incubation of SCH58261 (100 nM; Fig 3.2A-E) in both frequency (Fig 3.2B,C; Frequency mean control: 1.22±1.2 Hz, n=10; 5 min after SCH58261: 1.12±0.83 Hz, n=10; 25 min after SCH58261: 0.78±0.46 Hz, n=10; difference: F_{2,9}=6.05, p=0.21, ANOVA) and amplitude (Fig 3.2D,E; Amplitude mean control: 36.38±6.2 pA, n=10; 5 min after SCH58261: 38.18±8.9 pA, n=10; 25 min after SCH58261: 37.50±6.5 pA, n=10; difference: F_{2,9}=0.95, p=0.39, ANOVA). Similarly, spontaneous events onto FS interneurons were unaffected by incubation of SCH58261 (100 nM; Fig 3.2I-L) in both frequency (Fig 3.2I,J; Frequency mean control: 3.25±2.7 Hz, n=12; 5 min after SCH58261: 3.50±2.64 Hz, n=12; 25 min after SCH58261: 3.72±2.7 Hz, n=12; difference: F_{2,11}=1.52, p=0.24, ANOVA) and amplitude (Fig 3.2K,L; Amplitude mean control: 

Figure 3.2 A²AR do not affect spontaneous or evoked excitatory synaptic transmission in the mPFC. A,H (top): Biocytin staining of layer 5 pyramidal neuron (A) and fast spiking (FS) interneuron (H) from coronal slice of rat prelimbic mPFC. Scale bars: 100 μm. A,H (bottom): Voltage responses to hyperpolarizing (-50 pA) and depolarizing (+225 pA) somatic current injections to the soma of a L5 pyramidal neuron (A) and to hyperpolarizing (-60 pA) and depolarizing (+400 pA) somatic current injections to the soma of a FS interneuron (H). Scale bars: 20 mV, 200 ms. B,C: Bath application of the selective A²AR antagonist, SCH58261 (100 nM, blue) did not affect the frequency of sEPSCs onto pyramidal neurons. A representative trace is depicted in control (black) and prolonged SCH58261 (100 nM, blue) wash-in condition (B). The frequency over 5 min does not change after SCH58261 (100 nM, blue) wash-in; data are mean±SD of n=10, individual lines show the average of one cell over the course of 5 min per condition (C). Scale bars: 30 pA, 50 ms. D,E: Bath application of SCH58261 (100 nM, blue) did not affect the amplitude of sEPSCs onto pyramidal neurons. The average sEPSC amplitude of a representative cell is depicted in control (black) and prolonged SCH58261 (100 nM, blue) wash-in condition (D). The average amplitude over 5 min does not change after SCH58261 (100 nM, blue) wash-in; data are mean±SD of n=10 (E). Scale bars: 10 pA, 1 ms. F,G: Bath application of SCH58261 (100 nM, blue) did not affect the amplitude of eEPSCs onto pyramidal neurons. The eEPSC amplitude of a representative cell is depicted during 15 sweeps in all three conditions; control (black), short- (light blue) and prolonged SCH58261 (100 nM, blue) wash-in conditions are shown (F). The average amplitude does not change after SCH58261 (100 nM, blue) wash-in; data are mean±SD of n=14 (G). I,J: Bath application of SCH58261 (100 nM, blue) did not affect the frequency of sEPSCs onto interneurons. A representative trace is depicted in control (black) and prolonged SCH58261 (100 nM, blue) wash-in condition (I). The frequency over 5 min does not change after SCH58261 (100 nM, blue) wash-in; data are mean±SD of n=12, individual lines show the average of one cell over the course of 5 min per condition (J). Scale bars: 30 pA, 50 ms. K,L: Bath application of SCH58261 (100 nM, blue) did not affect the amplitude of sEPSCs onto interneurons. The average sEPSC amplitude of a representative cell is depicted in control (black) and prolonged SCH58261 (100 nM, blue) wash-in condition (K). The average amplitude over 5 min does not change after SCH58261 (100 nM, blue) wash-in; data are mean±SD of n=12 (L). Scale bars: 10 pA, 1 ms. M,N: Bath application of SCH58261 (100 nM, blue) did not affect the amplitude of eEPSCs onto pyramidal neurons. The average eEPSC amplitude of a representative cell is depicted over a time-course of 30 min, in which control (black), short- and prolonged SCH58261 (100 nM, blue) wash-in conditions are shown (M). The average amplitude over 5 min does not change after SCH58261 (100 nM, blue) wash-in; data are mean±SD of n=8 (N). Paired one-way ANOVA; all data are non-significant (P>0.05).
A$_2A$R control PFC synaptic plasticity

40.34±9.3 pA, n=12; 5 min after SCH58261: 41.83±11.5 pA, n=12; 25 min after SCH58261: 41.34±10.5 pA, n=12; difference: F$_{2,11}$=0.32, p=0.65, ANOVA). Also, eEPSCs onto both pyramidal neurons (Fig 3.2F,G) and interneurons (Fig 3.2M,N) were unaffected by incubation of SCH58261. Specifically, the amplitude of eEPSCs onto pyramidal neurons did not differ between baseline and incubation conditions (Fig 3.2F,G; Amplitude mean control: 522.6±160.4 pA, n=14; 5 min after SCH58261: 574.7±218.9 pA, n=14; 15 min after SCH58261: 554.1±239.1 pA, n=14; difference: F$_{2,13}$=0.67, p=0.46, ANOVA) and likewise, the amplitude of eEPSCs onto FS interneurons did not differ between baseline and incubation conditions (Fig 3.2M,N; Amplitude mean control: 242.5±107.3 pA, n=8; 5 min after SCH58261: 246.5±115.4 pA, n=8; 15 min after SCH58261: 237.9±116.1 pA, n=8; difference: F$_{2,7}$=0.14, p=0.79, ANOVA). Thus, A$_2A$R do not seem necessary for excitatory synaptic transmission in the mPFC as its blockade does not affect either sEPSCs or eEPSCs in pyramidal neurons and FS interneurons.

A$_2A$R blockade does not affect glutamatergic synapse LTP in layer 5 pyramidal neurons

Under endogenous levels of adenosine, A$_2A$R mainly act as a modulator of processes in which plasticity is engaged (D’Alcantara et al., 2001; Rebola et al., 2008; Costenla et al., 2011). Therefore, we tested whether A$_2A$R blockade affected the induction of glutamatergic synaptic plasticity in mPFC pyramidal neurons. We made whole-cell recordings from L5 pyramidal neurons (Fig 3.3A,B) and glutamatergic EPSPs were evoked by extracellular stimulation. To induce LTP, a theta-burst stimulation protocol was applied (Larson and Munkácsy, 2015). After recording a stable baseline of EPSPs, 10 bursts of 5 pulses each were given at 100 Hz (Fig 3.3C), and this was repeated 3 times within 30 seconds. Following this induction protocol, the slope of EPSPs was increased in a sustained manner, 20-30 minutes after the induction protocol (128.2±46.6%, n=32; Fig 3.3D-F,H). When slices were pre-incubated with SCH58261 (100 nM), the increase in EPSP slope (122.8±59.8%, n=17; Fig 3.3D-F,H) was not significantly different from control experiments without SCH58261 (t$_{47}$=0.35, p=0.731, unpaired t-test). Indeed, in the absence of SCH58261, 53% of cells showed TBS-induced LTP (17 out of 32), 28% did not show a change in EPSP slope (9 out of 32) and 19% showed a reduction in EPSP slope (6 out of 32), while in the presence of SCH58261 (100 nM), 47% of the pyramidal cells showed TBS-induced LTP (8 out of 17), 29% showed no change (5 out of 17) and 24% showed a reduction in EPSP slope (4 out of 17). These distributions were not significantly different between control and SCH58261.
In both conditions - control and presence of SCH58261 – cells had on average similar resting membrane potential and input resistance (mean RMP of control: -67.6±0.4 mV; mean RMP with SCH58261: -69.1±0.7 mV, t_{49}=1.82, p=0.07, unpaired t-test; mean R input of control: 52.3±4.7 mΩ; mean R input with SCH58261: 54.4±7.6 mΩ, t_{49}=0.26, p=0.80, unpaired t-test). Thus, blockade of A_{2A}R has no significant effect on the induction of glutamatergic synaptic plasticity in L5 pyramidal neurons in mPFC slices.
A2AR blockade shifts the direction of plasticity from LTP into LTD at excitatory synapses onto layer 5 fast spiking (FS) interneurons

Next, we tested the effects of A2AR blockade on glutamatergic synaptic plasticity in FS interneurons. Glutamatergic synapses on FS interneurons can undergo LTP, albeit through different mechanisms than pyramidal neurons (Lamsa et al., 2007; Lu et al., 2007; Sarihi et al., 2008; Nissen et al., 2010; Sambandan et al., 2010; Huang et al., 2013). To test whether A2AR are involved in this type of plasticity, we made whole-cell recordings from mPFC L5 FS interneurons (Fig 3.4A). These neurons had fast spiking patterns, short action potential half widths, showed no inter-spike interval adaptation, and displayed fast hyperpolarization time constants and minimal hyperpolarization amplitude (Fig 3.4B). To induce LTP, we applied the same TBS protocol as in the pyramidal neuron recordings (Fig 3.4C). This induced a robust potentiation of EPSP slope in FS interneurons (159.4±44.9%, n=10; Fig 3.4D-G,H). When slices were pre-incubated with SCH58261 (100 nM), stimulation with the TBS protocol induced long-term depression, rather than potentiation (64.4±25.2%, n=10; Fig 3.4D-G,H), which was significantly different from control (t18=5.84, p<0.0001, unpaired t-test). In the two conditions - absence or presence of SCH58261 - the resting membrane potential and input resistance were similar (mean RMP of control: -71.2±0.9 mV; mean RMP with SCH58261: -69.6±1.9 mV, t18=0.75, p=0.46, unpaired t-test; mean R input of control: 164.3±17.8 mΩ; mean R input with SCH58261: 160.2±19.2 mΩ, t18=0.15, p=0.88, unpaired t-test). In the absence of SCH58261, 70% of all cells displayed LTP, compared to 0% in the SCH58261 group. Conversely, 70% of all cells displayed LTD in the SCH58261 group, whereas none of the cells in the control condition displayed LTD (Fig 3.4G). This shows that A2AR control the direction of plasticity at glutamatergic synapses onto FS interneurons in the mPFC.

A2AR control LTP of population spikes in the layer V mPFC (mPFC)

Since population spikes represent the integrated responses of all local cells, i.e. responses from both pyramidal cells and interneurons, we next recorded population spikes to determine whether A2AR affect plasticity on the neuronal network level. To that end, we recorded extracellularly evoked AMPA receptor-mediated population spikes in mPFC layer 5 (L5) in acute brain slices, upon stimulation of L2/3 (Figure 3.5A,B). Bath application of SCH58261 (50 nM) affected the stimulus-response relationship of the network by increasing the maximum amplitude of population spikes (1.86±0.03 mV in SCH58261, n=23; 1.58±0.04 mV in control, n=26; t47=5.34, p<0.0001, unpaired t-test; Fig 3.5C). LTP of the population
Figure 3.4 A₂AR blockade shifts reverses LTP to LTD at excitatory synapses in layer 5 FS interneurons.

A: Biocytin reconstruction of a FS interneuron from coronal slice of rat mPFC showing relative positions of recording and stimulating electrodes. B: Voltage responses to hyperpolarizing (-80 pA) and depolarizing (+360 pA) somatic current injections to the soma of a FS interneuron. Scale bars: 20 mV, 200 ms.

C: Plasticity induction protocol. Theta burst stimulation (TBS) was induced by stimulation of 10 bursts of 5 pulses each at 100 Hz, repeated 3 times. Scale bars: 2 mV, 20 ms.

D: After obtaining a baseline measure of EPSPs, TBS-LTP was induced. EPSPs were then recorded for up to 30 min to observe changes in EPSP slope. Slices were pre-incubated in either control ACSF (black traces) or in ACSF with added SCH58261 (100 nM; blue traces). Scale bars: 2 mV, 20 ms.

E,F: Representative TBS-LTP experiments in control (E) and 100 nM SCH58261 (F) conditions showing slope and input resistance (top and bottom panels, respectively) versus time. Grey shading indicates time of TBS induction.

G: Summary plot of control (black symbols) and SCH58261 (100 nM; blue symbols) experiments, showing a robust LTP in control condition, and a strong LTD in SCH58261 pre-incubated cells.

H, left panel: The fraction of cells that obtain plasticity is reversed in control versus SCH58261 conditions. In control, 70% of cells display LTP, whereas in SCH58261, 70% of cells display LTD.

H, right panel: Summary bar chart of control and SCH58261 (100 nM) TBS-LTP experiments, showing percentage change in EPSP slope for both conditions (mean±SEM; control: n=10; SCH58261: n=10). ****p<0.0001 compared to the respective control (black dots), unpaired Student’s t-test. **p<0.01 compared to the hypothetical value of 100, one-sample t-test. ***p<0.001 compared to the hypothetical value of 100, one-sample t-test.
spike was induced by applying a single train of high-frequency stimulation (HFS), followed 15 mins later by four HFS trains (50 pulses at 100 Hz, 0.5 second duration, delivered every 10 seconds). This induction protocol was run in the absence or presence of SCH58261, and for each experiment a naïve mPFC slice was used. Blockade of A2A R by SCH58261 decreased the magnitude of population spike LTP (120.7±2.9% in SCH58261, n=25; 130.9±3.4% in control slices, n=25; \( t_{48}=2.28, p=0.027 \), unpaired \( t \)-test; **Fig 3.5D-F**). These findings show that A2AR control plasticity at a neuronal network level in mPFC.

### 3.5 Discussion

In the present study, we show that in the mPFC, A2A R control LTP at excitatory synapses onto fast-spiking interneurons rather than onto pyramidal neurons. A2ARs did not affect spontaneous or evoked synaptic transmission in either cell type. A similar predominant role of A2A R on plasticity has been observed in other brain areas, including hippocampus (Rebola et al., 2008), amygdala (Simões et al., 2016) and striatum (d’Alcantara et al., 2001; Shen et al., 2008; Li et al., 2015). As in the hippocampus (Rebola et al., 2005), A2A R in the mPFC are enriched at synapses. However, mPFC A2AR are enriched outside the presynaptic active zone and postsynaptic density (PSD), whereas most of the hippocampal A2AR are located inside the presynaptic active zone and PSD (Rebola et al., 2005). It is conceivable that this different sub-synaptic distribution could translate into A2AR playing by different rules to control information flow within the mPFC. Indeed, our results show an effect of A2AR antagonism on the induction of LTP at excitatory synapses specifically in FS interneurons, while the antagonist was ineffective at excitatory synapses onto pyramidal neurons. At excitatory connections to FS interneurons, the blockade of A2AR led to a long-term depression of their excitatory synapses, meaning that without active A2AR, LTD would occur at these glutamatergic synapses onto FS interneurons. Thus, A2AR activation would be particularly important for the induction of synaptic potentiation of glutamatergic synapses in FS interneurons, while not affecting glutamatergic synapses in mPFC pyramidal neurons. At the mPFC neuronal network level, blockade of A2AR reduced LTP induction, suggesting a role for A2AR at the network level.

Target cell specificity of A2AR modulation has also been found in the hippocampus, although with the difference that activation of hippocampal A2AR increased excitatory transmission to CA1 pyramidal cells but not to inhibitory interneurons.
The PFC is unique in the magnitude and variety of interneurons, where FS interneurons represent the largest group (Markram et al., 2004). FS interneurons are activated by feedback and feedforward excitation, and they target perisomatic regions of pyramidal neurons (Tremblay et al., 2016) to control the output of pyramidal neurons by exerting fast, powerful and uniform inhibition of their firing (Kvitsiani et al., 2013; Sparta et al., 2014). Both LTP and LTD can be generated in FS interneurons, although LTP seems to be the dominant form of plasticity expressed in this neuron subtype (Lamsa et al., 2007; Lu et al., 2007; Sarihi et al., 2008; Nissen et al., 2010; Sambandan et al., 2010). In contrast to long-term plasticity of excitatory synapses onto pyramidal neurons, long-term plasticity of excitatory synapses onto pyramidal neurons (Rombo et al., 2015).

Figure 3.5 A<sub>2A</sub>R facilitate LTP of population spikes in mPFC layer 5. A: positioning of the stimulating (layer 2/3 mPFC) and recording electrodes (layer 5 mPFC). B: the recorded population spikes were abolished by the AMPA/kainate receptor antagonist, CNQX 10 µM. C: bath application of the selective A<sub>2A</sub>R antagonist, SCH58261 (50 nM; blue dots) increased the number and synchrony of cells discharging action potentials as indicated by the increase in the maximum response when the input / output response was assessed 20 min after SCH58261 superfusion. D: representative averaged traces at baseline and 30 min after the induction of LTP, in the absence and presence of SCH58261 (50 nM); SCH58261 decreased LTP magnitude. E: Time course showing that SCH58261 decreased the magnitude of LTP of the population spike responses triggered by a priming train of high-frequency stimulation (HFS), followed 15 min later by four HFS trains (50 pulses at 100 Hz, 0.5 s duration, delivered every 10 s). F: Summary plot displaying the variability of LTP magnitude, estimated 30 min after the induction of LTP, in the absence and presence of SCH58261 (50 nM). Data are mean±SEM of 25 slices (from 25 rats) per group. ####p<0.0001 comparing the estimated maximum response in the presence of SCH58261 to control (black bar/dots), unpaired Student's t-test. *p<0.05 comparing SCH58261 to control (black bar/dots), unpaired Student's t-test. ****p<0.0001 compared to the hypothetical value of 100%, one-sample t-test.
A$_2$A$_R$ control PFC synaptic plasticity

plasticity at glutamatergic synapses in FS neurons is predominantly independent of NMDA receptors (Lamsa et al., 2007; Sarihi et al., 2008; Nissen et al., 2010; Sambandan et al., 2010; Huang et al., 2013). In most cases, an essential role for group I metabotropic glutamate receptors (mGluRs) has been demonstrated in LTP and LTD induction in these FS interneurons (Perez et al., 2001; Lu et al., 2007; Sarihi et al., 2008; Huang et al., 2013). Whether LTP or LTD can be induced in these synapses is dependent on postsynaptic calcium fluctuations during LTP induction (Alle et al., 2001; Sambandan et al., 2010; Huang et al., 2013). A$_2$A$_R$ control both NMDA receptors and voltage-sensitive calcium channels, thus potentially contributing to modulate the pattern of plasticity (Mogul et al., 1993; Gonçalves et al., 1997; Rebola et al., 2008; Azdad et al., 2009; Higley and Sabatini, 2010). Furthermore, A$_2$A$_R$ heteromerize with mGluR5 (Ferré et al., 2002) and tightly interact with mGluR5 receptor function in the hippocampus, changing the efficiency of NMDA receptors (Tebano et al., 2005; Sarantis et al., 2015; Viana da Silva et al., 2016). Whether either of these mechanisms is responsible for the observed effects, should be subject to further investigation.

We here show the role of A$_2$A$_R$ in normal, non-pathological, conditions by targeting the endogenous pool of adenosine acting at A$_2$A$_R$ with the A$_2$A$_R$ antagonist SCH58261. In these conditions, A$_2$A$_R$ act mainly as modulators of synaptic plasticity (d'Alcantara et al., 2001; Rebola et al., 2008; Simões et al., 2016). A$_2$A$_R$ have an additional role in pathological conditions, where they can control microglia and astrocytes (Rebola et al., 2011; Matos et al., 2015; Orr et al., 2015; Cunha, 2016). Targeting A$_2$A$_R$ with an A$_2$A$_R$ agonist would mimic the situation of an additional load onto these microglia-and astrocytic located A$_2$A$_R$, thereby recruiting A$_2$A$_R$ that are only active in pathological conditions (Matos et al., 2013; Orr et al., 2015). As we are specifically interested in the role of A$_2$A$_R$ in non-pathological conditions, we only evaluated plastic changes under influence of the A$_2$A$_R$ antagonist.

The alteration in glutamatergic synapse strength in FS interneurons by A$_2$A$_R$ can have a major impact on cortical function. A decreased synaptic strength at FS interneurons has been linked to a loss of temporal fidelity of pyramidal-to-pyramidal signaling (Lamsa et al., 2005), leading to a loss of information processing (Pouille and Scanziani, 2001). Also, the selective control by A$_2$A$_R$ of plasticity at glutamatergic synapses onto FS interneurons might have important implications for the excitation-inhibition balance. Indeed, if the activity of interneurons is experimentally reduced in mPFC, LTP of excitatory to pyramidal neurons is impaired (Konstantoudaki et al., 2016). Adenosine, by acting at A$_2$A$_R$ at synapses of
FS interneurons, could therefore provide a homeostatic mechanism by which inhibition is ensured, thereby maintaining a proper excitation-inhibition balance (Zhang et al., 2015). FS interneurons, in particular the parvalbumin-positive FS cells, have been shown to support working memory and cognitive flexibility (Murray et al., 2015), and to be central for the control of attention (Kim et al., 2016). Therefore, abnormal A<sub>2A</sub>R function might lead to impaired behavioral functioning through changes in plasticity at FS interneuron synapses. An overexpression of A<sub>2A</sub>R specifically in the PFC is indeed related to cognitive and attentional deficits in a rat model of attention deficit and hyperactivity disorder (Pandolfo et al., 2013). Furthermore, genetic elimination of A<sub>2A</sub>R also interferes with behaviors that involve information processing in the PFC, including working memory (Zhou et al., 2009; Wei et al., 2011) and reversal learning (Wei et al., 2011). Future research targeting selectively A<sub>2A</sub>R in PFC FS interneurons will be needed to elucidate whether specifically A<sub>2A</sub>R located on glutamatergic synapses in FS interneurons control PFC-related behavior.

In short, we present here a first characterization of the role of endogenous adenosine acting at A<sub>2A</sub>R to affect synaptic plasticity in the mPFC, showing that A<sub>2A</sub>R specifically affect plasticity of glutamatergic synapses in cortical FS interneurons. An effect of A<sub>2A</sub>R manipulation on plasticity at these synapses was never shown before, therefore, further explorations into synaptic plasticity of FS interneurons in the PFC should be considered to reveal the underlying mechanism of A<sub>2A</sub>R manipulation.
A2AR control PFC synaptic plasticity
Lateral Inhibition by Martinotti Interneurons is Facilitated by Basal Forebrain Cholinergic Inputs in Human and Mouse Neocortex

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In submission
4.1 Abstract
A variety of inhibitory pathways encompassing different interneuron types shape activity of neocortical pyramidal neurons. While Basket cells (BCs) mediate fast lateral inhibition between pyramidal neurons, Somatostatin-positive Martinotti cells (MCs) mediate a delayed form of lateral inhibition. Neocortical circuits are under control of acetylcholine released from basal forebrain (BF) inputs, which is crucial for cortical function and cognition. How BF cholinergic inputs affect cortical lateral inhibition is not known. Here, we find that cholinergic inputs selectively augment and speed up lateral inhibition between pyramidal neurons mediated by MCs, but not by BCs. Optogenetically-activated cholinergic inputs depolarize MCs through activation of β2 subunit-containing nicotinic AChRs, not muscarinic AChRs, without affecting glutamatergic inputs to MCs. We find that these mechanisms are conserved in human neocortex. BF cholinergic inputs thus enable cortical pyramidal neurons to recruit more MCs, and can thereby dynamically highlight specific circuit motifs, favoring MC-mediated pathways over BC-mediated pathways.

4.2 Introduction
Inhibition of pyramidal neurons by GABAergic interneurons is essential for cortical computation. Several circuit motifs have been identified by which interneurons shape cortical signal propagation, among which are feed forward inhibition, feedback inhibition and disinhibition (Fino et al., 2013a; Tremblay et al., 2016). In each of these motifs, several distinct types of interneurons can be involved. For instance, lateral inhibition, a form of feedback inhibition generated by activity in local circuits of pyramidal neurons and interneurons, can be mediated by parvalbumin (PV)-positive fast spiking Basket cells as well as somatostatin (SOM)-positive interneurons (Pouille and Scanziani, 2004; Kapfer et al., 2007; Silberberg and Markram, 2007). Because of the profound difference in projection targets on pyramidal neuron dendrites between PV and SOM axons, whereby PV neurons innervate perisomatic regions and SOM neurons generally target distal dendrites, lateral inhibition by PV neurons may be more involved in rapidly silencing action potential firing in neighboring pyramidal neurons, while lateral inhibition through SOM neurons will control synaptic integration, burst firing and dendritic regenerative phenomena (Pouille and Scanziani, 2004; Gentet et al., 2012a; Gidon and Segev, 2012; Tremblay et al., 2016). What the precise impact will be of lateral
inhibition by a given interneuron type at any point in time will depend among other things on neuromodulatory conditions, but this is poorly understood. Both PV and SOM interneurons are modulated by various neurotransmitters (Tremblay et al., 2016) and in particular SOM interneurons are strongly modulated by acetylcholine (Beierlein et al., 2000; Couey et al., 2007; Fanselow et al., 2008; Poorthuis et al., 2013a; Xu et al., 2013; Chen et al., 2015) The cortex receives cholinergic inputs from the basal forebrain (Bloem et al., 2014b; Do et al., 2016). How cholinergic inputs from the basal forebrain affect lateral inhibition is not known. It is also not known whether lateral inhibition between pyramidal neurons exists in human neocortical circuits. Here, we address the question of how acetylcholine from basal forebrain inputs affects lateral inhibition between pyramidal neurons in mouse and human neocortex.

Both PV-positive Basket cells (BCs) and SOM-positive Martinotti cells (MCs) form disynaptic inhibitory microcircuits with pyramidal neurons that enable them to alter activity of surrounding pyramidal cells (Silberberg and Markram, 2007; Berger et al., 2009). It was shown that a single pyramidal cell can activate BCs and MCs when spiking at high frequencies, which in turn leads to lateral inhibition of neighboring pyramidal cells (Silberberg and Markram, 2007). Whereas only a subgroup of BCs show a response to acetylcholine, both muscarinic and nicotinic acetylcholine receptors strongly depolarize MCs and induce action potential firing in these neurons (Beierlein et al., 2000; Couey et al., 2007; Fanselow et al., 2008; Poorthuis et al., 2013a; Xu et al., 2013), which has been implied to be involved in cholinergic modulation of cortical function (Kawaguchi and Kubota, 1997; Fanselow et al., 2008; Hasselmo and Sarter, 2011; Demars and Morishita, 2014). Here, we investigate the mechanisms by which cholinergic inputs from the basal forebrain affect fast and delayed disynaptic inhibition between pyramidal cells (PCs). In simultaneous recordings from synaptically connected neocortical neurons we find that only delayed lateral inhibition via MCs is modulated by BF cholinergic inputs, while fast lateral inhibition via BCs is not. We demonstrate that somatic depolarization of MCs, rather than changes in synaptic strength, induced by endogenously released ACh from basal forebrain projections augments lateral inhibition in both supragranular and infragranular layers in both the medial PFC and the somatosensory cortex. In addition, we show that lateral inhibition is evolutionary conserved in the human neocortex and is facilitated by ACh through similar mechanisms.
4.3 Results

Delayed lateral inhibition is selectively enhanced by basal forebrain cholinergic inputs

Pyramidal neurons in the neocortex can inhibit neighboring pyramidal cells (PCs) by feedforward activation of inhibitory interneurons (Silberberg and Markram, 2007; Berger et al., 2009). This process of disynaptic inhibition has been observed in several cortical area’s and in different cortical layers (Kapfer et al., 2007; Tremblay et al., 2016). As the majority of interneurons in the cortex express acetylcholine receptors (AChRs) (Porter et al., 1999; Christophe et al., 2002; Couey et al., 2007; Gulledge et al., 2007; Poorthuis et al., 2013a; Rudy et al., 2013), we tested whether acetylcholinergic (ACh) inputs from the basal forebrain (BF) modulate disynaptic lateral inhibition between pyramidal neurons. We recorded from up to 4 pyramidal cells simultaneously in L2/3 or L5 in acute brain slices of the mPFC or the somatosensory cortex (Fig 4.1A). To recruit disynaptic inhibitory loops, presynaptic pyramidal cells (Pre-PC) were triggered to fire 15 action potentials (APs) at 100Hz, which induced stereotypic fast or delayed inhibitory postsynaptic responses (IPSPs) in pyramidal cells (Post-PC), as reported by Silberberg and Markram (2007) and Kapfer et al., (2007) (Fig 4.1B-D, Pre-PC and Post-PC traces). Cholinergic BF projections were stimulated optogenetically by blue light activation of channelrhodopsin (ChR2) expressed by BF ChAT-positive neurons (see Methods). Since cholinergic neurons of the basal forebrain fire in bursts during wakefulness (Lee et al., 2005; Hay et al., 2016), and to approximate physiologically relevant rates of ACh release, we used five blue light pulses with a frequency of 25 Hz to release endogenous ACh. One hundred milliseconds after the first blue light stimulus, the Pre-PC cells were triggered to fire 15 APs with a frequency of 100 Hz (Fig 4.1B). Optogenetic activation of cholinergic basal forebrain inputs resulted in a shorter onset latency of delayed, but not fast disynaptic IPSPs in postsynaptic L5 pyramidal cells of the mPFC (Fig 4.1C,D). Furthermore, optogenetic stimulation of BF cholinergic inputs altered the kinetics of delayed disynaptic inhibition, increasing both the time course (Fig 4.1C) and the amplitude (Fig 4.1D) of delayed disynaptic IPSPs. In contrast, BF cholinergic inputs did not affect the duration (Fig 4.1D,F) or amplitude (Fig 4.1D,F) of fast disynaptic inhibition. In L2/3 of the primary sensory cortex (S1) we observed similar effects of optogenetic activation of BF cholinergic projections on delayed disynaptic IPSPs (see Fig 4.3). This shows that cholinergic BF inputs to the neocortex modulate delayed but not fast lateral inhibition between pyramidal neurons.
Cholinergic facilitation of lateral inhibition via Martinotti interneurons

Previous work has shown that induction and kinetics of lateral inhibitory postsynaptic potentials correlate with firing frequencies of presynaptic pyramidal neurons (Silberberg and Markram, 2007). We asked whether the modulation of lateral inhibition by BF cholinergic projections is less pronounced when the presynaptic pyramidal cell (Pre-PC) is firing trains of APs at lower frequencies. To address this question, we stimulated presynaptic pyramidal neurons to fire trains of 15 APs at different frequencies (40, 60, 80, 100 Hz) and simultaneously recorded inhibitory responses in postsynaptic pyramidal cells (Fig 4.2B). Independent from the firing frequency of the presynaptic pyramidal neuron, optogenetic activation of BF cholinergic projections led to a shorter onset delay (Fig 4.2A,D), an increased

Figure 4.1. Delayed, but not fast lateral inhibition between pyramidal neurons is enhanced by BF cholinergic projections. A. Digital reconstruction of two Biocytin-filled layer 5 (L5) pyramidal neurons in coronal slices of the mouse medial prefrontal cortex (mPFC). B. Example trace of APs induced in a presynaptic L5 pyramidal neuron (Pre-PC). Grey trace: the Pre-PC cell is stimulated to fire 15 APs at 100Hz. The presynaptic AP train coincides with optogenetic activation of ChR2-expressing BF cholinergic fibers with 5 short blue light flashes at 25 Hz, 100 ms preceding the first AP (blue bars). C. Example trace of a delayed disynaptic inhibitory response in the postsynaptic pyramidal cell (Post-PC) in absence (OFF, black trace) or presence (ON, blue trace) of endogenous ACh release. D. As (C), but in contrast, a typical fast disynaptic inhibitory response in the Post-PC. E. Summary charts showing that activation of BF cholinergic projections shortens the onset delay, increases the time course and amplitude of delayed disynaptic inhibition in mPFC L5 (Delay: light OFF 104±9 ms, light ON 87±8 ms, paired t-test, two-tailed, p<0.01, t=4.8, df=9; Duration: light OFF 141±16 ms, light ON 274±41 ms, Wilcoxon signed-rank test, p<0.01; Amplitude: light OFF 1.01±0.16 mV, light ON 1.40±0.19 mV; paired t-test, two-tailed, p<0.001, t=5.9, df=9; n=10, mean±s.e.m.). F. As (E), showing that in mPFC L5 fast disynaptic lateral inhibition is not affected by optogenetic activation of BF cholinergic projections (Delay: light OFF 5.58±0.356 ms, light ON 5.88±0.3 ms, p=0.1249, paired t-test, two-tailed, t=1.783, df=6; Duration: light OFF 140±29.12 ms, light ON 136.9±28.3 ms, p=0.7733, paired t-test, two tailed, t=0.3014, df=6; Amplitude: light OFF 1.316±0.26 mV, light ON 1.36±0.24 mV, p=0.6425, paired t-test, two-tailed, t=0.4885, df=6; n=7; mean±s.e.m.).
duration (Fig 4.2A,D) and a larger amplitude (Fig 4.2A,D) of disynaptic IPSPs. Facilitation by BF cholinergic projections of onset (Fig 4.2D), duration (Fig 4.2D) and amplitude (Fig 4.2D) of disynaptic inhibition was not different between applied frequencies. These results indicate that BF cholinergic projections facilitate lateral inhibition independent of pyramidal neuron firing frequencies.

The neuromodulator ACh can shape neuronal circuits by activation of muscarinic and nicotinic acetylcholine receptors (mAChRs, nAChRs respectively), and cortical interneurons have been shown to express both types of receptors (Beierlein et al., 2000; Couey et al., 2007; Fanselow et al., 2008; Poorthuis et al., 2013a; Xu et al., 2013). To determine whether the modulation of delayed disynaptic inhibitory
Figure 4.3. BF cholinergic projections facilitate lateral inhibition by activating heteromeric nAChRs. A. Recording from two layer 5 pyramidal neurons in the mPFC showing disynaptic inhibition. Top trace: Example trace of AP firing by the Pre-PC (15 AP at 100Hz) combined with or without blue light for optogenetic activation of BF cholinergic projections (blue bars), starting 100ms before the electrical stimulation. Middle trace: Average trace of a inhibitory response in the Post-PC neuron in absence (OFF, black trace) or presence (ON, blue trace) of BF cholinergic projection activation. Bottom trace: As middle trace, in presence of DHßE (10 µM). B. Summary chart showing that ACh shortens the onset delay of lateral inhibition. Modulation of lateral inhibition by BF cholinergic projection activation is unaffected by mAChR antagonist atropine (light OFF 101±11 ms, light ON 79±8 ms, paired t-test, two-tailed, p<0.05, t=3.5, df=7, n=8). Modulation of lateral inhibition by BF cholinergic projection activation is blocked by DHßE, an antagonist of heteromeric nAChRs (light OFF 111±11 ms, light ON 112±12 ms, paired t-test, two-tailed, p=0.8, t=0.3, df=6, n=7). Bath application of ACh leads to decreased onset latency (Ctrl. 96±11 ms, ACh 77±6 ms, wash 90±8 ms, paired t-test, two-tailed, p<0.05, t=2.895, df=5, n=6) similar to BF cholinergic projection activation. In somatosensory cortex(S1) the modulation of lateral inhibition by cholinergic projection activation is comparable to modulation of the mPFC (light OFF 81±7 ms, light ON 56±8 ms; paired t-test, two-tailed, p<0.05, t=4.5, df=5, n=6). C. As in (B) showing that the increase in duration of lateral is both present in presence ofmAChRs blockers (atropine: light OFF 163±11 ms, light ON 185±13 ms, Wilcoxon signed-rank test, p<0.05, n=8) as well as heteromeric nAChRs blockers (DHßE: light OFF 171±23 ms, light ON 284±18 ms, Wilcoxon signed-rank test, p=0.01, n=6). D. As in (B) and (C) showing that the cholinergic modulation of the amplitude of lateral inhibition depends on activation of heteromeric nAChRs (atropine: light OFF 0.88±0.16 mV, light ON1.42±0.25 mV, paired t-test, two-tailed, p<0.01, t=4.7, df=8, n=9; DHßE: light OFF 1.40±0.19 mV, light ON 1.36±0.18 mV, paired t-test, two-tailed, p=0.27, t=1.2, df=6, n=7; bath application: paired t-test, two tailed, p=0.3648, t=0.9963, df=5, n=6; S1: light OFF 1.27±0.42 mV, light ON 1.56±0.46 mV; paired t-test, two-tailed, p<0.05, t=2.8, df=5, n=6, mean±s.e.m.).
loops by BF cholinergic projections is mediated by mAChRs, nAChRs, or both, we applied either the mAChR antagonist atropine or the heteromeric nAChR antagonist DHβE (Fig 4.3A). Atropine (400nM) did not affect the modulation of disynaptic inhibition by optogenetic activation of BF cholinergic projections (Fig 4.3B-D). In the presence of atropine, optogenetic activation of BF cholinergic projections sped up the onset latency and the amplitude of the IPSPs in the postsynaptic pyramidal cells similar to control conditions (Fig 4.3B, 4.3D). In contrast to atropine, the nAChR antagonist DHβE (10µM) abolished the modulatory effects of endogenous ACh on onset delay, duration and amplitude (Fig 4.3A-D). These pharmacological manipulations show that modulation of disynaptic inhibitory loops by BF cholinergic inputs is mediated almost exclusively by heteromeric nAChRs and not by mAChRs.

Cholinergic projections from the basal forebrain have been shown to co-release other neurotransmitters, such as GABA (Saunders et al., 2015). To exclude potential involvement of other neurotransmitters, we tested whether bath application of ACh in the presence of atropine would modulate disynaptic inhibition in a similar fashion as optogenetic activation of BF cholinergic projections. Upon application of ACh (1 mM, atropine 400nM) we observed a decrease in the onset delay of the disynaptic IPSPs, similar to optogenetic activation of BF cholinergic projections (Fig 4.3B) as well as an increase in the time course of disynaptic IPSPs (Fig 4.3C). After 15 minutes washout of ACh, the onset delay returned to baseline before ACh application (Fig 4.3B). IPSP amplitude was not modulated by bath application of ACh (Fig 4.3C). Taken together, these results show that ACh inputs from the basal forebrain speed up the action and increase the amplitude of lateral inhibition between neocortical pyramidal neurons via activation of heteromeric nAChRs.

**BF cholinergic projections directly depolarize Martinotti Cells**

Previous studies have shown that Martinotti Cells (MCs) in the neocortex mediate lateral inhibition through disynaptic loops between pyramidal neurons (Pouille and Scanziani, 2004; Kapfer et al., 2007; Silberberg and Markram, 2007). In addition, MCs express a mixed population of somatic α7 and non-α7 nAChRs, but nicotinic receptor currents are dominated by heteromeric nAChRs containing β2 subunits (Couey et al., 2007; Poorthuis et al., 2013). To address the question whether a change in MC activity caused by ACh release is responsible for the modulation of disynaptic inhibition between pyramidal neurons, we recorded from GFP-expressing MCs in the ‘GIN’ mouse line (Ma et al., 2006) and either optogenetically
Figure 4.4. BF cholinergic projections depolarize Martinotti cells. A. Left: schematic illustration of the experiment. Right: Example trace (Blue trace) of an nAChR-mediated response in a L5 Martinotti cell in the mPFC recorded in a Gin/Chat-ChR2-EYFP mouse. Optogenetic activation of BF cholinergic projections was induced by applying five short blue light flashes at 25 Hz. The blue light evoked response was blocked by DHßE (10 µM, grey trace). B. Left: Summary chart showing the maximum amplitude of nAChR-EPSPs in L5 Martinotti cells recorded in the mPFC evoked by optogenetic activation of BF cholinergic projections (light OFF -60.4 ±1.08 mV, light ON -58.73±1.91 mV, DHßE -60.08±1.08 mV, F(6, 12)= 6.115, one-way ANOVA, p<0.05, n=7; mean±s.e.m.). Right: Summary chart indicating the depolarization of the membrane potential of L2/3 Martinotti cells by application of ACh (1 mM) (Ctrl. -61.8±0.75 mV, ACh -57.2±0.94 mV, wash -61.0±1.48 mV, paired t-test, two-tailed, p=0.001, t=8.288, df=11, n=12). C. Right: schematic representation of the experiment. Middle: Example traces of simultaneously recorded synaptically connected pre-PC and postsynaptic Martinotti cell (Post-MC). Middle top: Pre-PC cell fired a train of 15 AP at 100 Hz. Optogenetic activation of BF cholinergic projections was induced by 5 blue light pulses at 25 Hz starting 100ms preceding the first AP. Middle bottom: Postsynaptic responses recorded in a mPFC L5 Post-MC in absence (Black trace) or presence of optogenetic activation of BF cholinergic projections (Blue trace). The potentiation that is induced by BF cholinergic projections is blocked by DHßE (Grey trace). Right: Summary plot. The combination of glutamatergic EPSPs from the PC and cholinergic excitatory input leads to a significant stronger depolarization of the membrane potential (light OFF 1.54±SEM mV, light ON 3.30±SEM mV). This increase is blocked by bath application of DHßE (1.07 mV, One-way ANOVA F(2,13)=16.81, p<0.001, n=6). D. Left: Example trace of a glutamatergic EPSP (Black trace) and nAChR-mediated (Blue trace) EPSPs. The co-occurring of glutamatergic and cholinergic EPSPs (Green trace) leads to larger depolarization. Right: The summation of the singular recorded glutamatergic and nAChR-mediated EPSPs (Expected value, Purple trace, 2.54 mV±SEM) did not differ from the recorded combined EPSP (2.88 mV±SEM, p=0.2766, paired t-test, two-tailed, t=1.221, df=5, n=6; mean±s.e.m.), showing that both signals summate linearly.
activated BF cholinergic projections or bath applied ACh (1mM) (see Methods; Fig 4.4A). For optogenetic activation of BF cholinergic projections, acute mPFC slices of CHAT-ChR2 mice crossed with ‘GIN’ mice were used and 5 short (10 or 20 ms duration) blue light pulses (25 Hz) were applied. In the experiments using ACh bath application, ACh was washed in for 15 minutes in the presence of atropine (400nM). Optogenetic activation of BF cholinergic projections resulted in a depolarization of the membrane potential in the MCs (Fig 4.4B). This effect was abolished by bath application of DHßE (10µM) (Fig 4.4B). Bath application of ACh (1mM) depolarized MCs to a similar degree (Fig 4.4B), which was reversed following washout (Fig 4.4B, p<0.001). These results show that both in S1 L2/3 and mPFC L5, ACh from BF projections depolarizes MCs by activation of postsynaptic heteromeric nAChRs.

Next, we simultaneously recorded from synaptically-connected pyramidal-Martinotti cell pairs, and tested whether MC depolarization by BF cholinergic projections linearly summates with the depolarizations induced by synaptic inputs received from presynaptic pyramidal cells (Pre-PC, Fig 4.4C). Excitatory postsynaptic potentials (EPSPs) received by MCs in response to AP firing of presynaptic pyramidal cells were compared with EPSPs that co-occurred with optogenetic activation of BF cholinergic projections. Optogenetic activation of BF cholinergic projections occurred 100 ms before the onset of AP firing of presynaptic pyramidal cells. MC depolarizations were strongly increased by combined optogenetic activation of BF cholinergic projections and EPSPs received from Pre-PCs (Fig 4.4C). The nAChR antagonist DHßE blocked the ACh induced increase of the depolarization of the MC (Fig 4.4C). To determine whether these depolarizations summate linearly, we calculated the linear sum of the depolarization induced by the EPSPs and cholinergic inputs separately (Fig 4.4D), and compared this with the recorded depolarization when these events occurred simultaneously. We observed no significant difference between the recorded (green trace) and expected amplitude (purple trace) of depolarizations (Fig 4.4D). These findings indicate that depolarizations by BF cholinergic inputs and synaptic EPSPs summate linearly to depolarize MCs.

**ACh does not affect synaptic strength between pyramidal and Martinotti cells**

Somatic depolarization of MCs may be sufficient to explain cholinergic modulation of lateral inhibition between pyramidal neurons. However, nAChRs can also be expressed on presynaptic terminals directly affecting neurotransmitter release and synaptic strength (McGehee et al., 1995; Gray et al., 1996; Mansvelder et al.,
In recordings from synaptically connected pyramidal neurons and MCs in L2/3 of the somatosensory cortex, we tested whether ACh affected synaptic strength between pyramidal neurons and MCs (Fig 4.5A,C). Presynaptic pyramidal neurons were driven with current pulses to trigger 8 APs at a frequency of 30 Hz, a frequency at which postsynaptic MCs are unlikely to fire APs, and recorded EPSPs in post-MCs in the presence or absence of ACh. We observed no difference in EPSP amplitudes, kinetics or facilitation of EPSPs in postsynaptic MCs between control, ACh wash in and washout conditions (Fig 4.5B). Similarly, the IPSP amplitudes recorded in postsynaptic pyramidal cells that were induced by presynaptic MC stimulation were not significantly affected by ACh application (Fig 4.5D). These data indicate that augmentation of disynaptic inhibitory loops by ACh is not due

Figure 4.5. The synaptic strength between pyramidal neurons and Martinotti cells is not affected by ACh. A. Left: schematic representation of the simultaneous recording of a presynaptic pyramidal cell (Pre-PC) and a postsynaptic Martinotti cell (Post-MC). Example trace recorded from a PC-Pre cell injected with current to evoke 8 APs at a frequency of 30 Hz (Black trace) and the EPSPs in the Post-MC cell (Grey trace). B. Summary plot of the normalized amplitude of EPSPs recorded in post-MCs. The amplitude was normalized to the last EPSP. Bath application and washout of ACh (1mM) did not alter synaptic strength between the Pre-PC and Post-MC ($F_{(2, 21)}=0.2511$, $p=0.7802$, One-Way ANOVA; $n=6$). C. Simultaneous recording of a presynaptic Martinotti cell (Pre-MC) and a postsynaptic pyramidal cell (Post-PC). A 30 Hz AP train was induced in the Pre-MC cell (Black trace) that induced a series of IPSPs in the Post-PC cell (Grey trace). D. Summary plot showing the normalized amplitude of the IPSP in the Post-PC cell. The amplitude is normalized to the first IPSP. The amplitude of the IPSP is not changed in the presence of ACh (1mM) or during ACh washout ($F_{(2,23)}=0.2705$, $p=0.7656$; One-way ANOVA; $n=7$, mean±s.e.m.)
to altered synaptic strength (or efficacy) between pyramidal and MCs, nor due to changes in the release machinery that would affect the time course of IPSP depression and EPSP facilitation.

**BF cholinergic inputs advance and prolong Martinotti cell AP firing**

Since depolarization of MCs is the most likely mechanism by which BF cholinergic projections modulate lateral inhibition between pyramidal cells, we tested whether BF cholinergic projections alter action potential firing of MCs in response to activation of presynaptic pyramidal cells. In simultaneous recordings from synaptically-connected presynaptic pyramidal cells (Pre-PC) and MCs (Post-MC) in the mPFC, we tested whether ACh modulates the delay, number and frequency of APs in MCs. Similar to previous experiments, presynaptic pyramidal cells were driven to fire 15 APs at 100 Hz and APs were recorded in the postsynaptic MCs (Fig 4.6A,B example traces). Optogenetic activation of BF cholinergic projections was induced by five blue light pulses at 25 Hz starting 100 ms before the presynaptic pyramidal neuron stimulation. Only recordings were included for analysis in which the postsynaptic MC reliably fired APs in all experimental conditions (Fig 4.6A). Combining presynaptic stimulation with light-evoked activation of BF cholinergic projections resulted in shortening of the latency to post-MC AP firing (Fig 4.6A,C) and an increased number of APs in Post-MCs compared to Pre-PC stimulation alone without BF cholinergic projection activation (Fig 4.6A,C). Similarly, bath application of ACh in L2/3 of the somatosensory cortex led to a significant decrease of the delay time from the start of the Pre-PC AP firing until the first AP in the postsynaptic MC (Fig 4.6B,C). In addition, the number of APs fired by the postsynaptic MC following ACh application increased significantly (Fig 4.6B,C). However, we observed no change of the AP threshold potential or of the frequency of AP firing. These results show that ACh reduces the delay time for the first AP in MCs, which can explain the advanced disynaptic inhibition upon ACh release, as well as an increase in the number of APs fired, which can account for the increase in time course and amplitude of the lateral inhibition.

**Lateral inhibition in human temporal cortex**

The concept of lateral inhibition between pyramidal neurons is reported in rat and mouse cortices (Silberberg and Markram, 2007; Roux and Buzsáki, 2015). However, it is not known whether this mechanism also exists in the human brain. To test this, we simultaneously recorded from up to 4 neighboring L2/3 pyramidal neurons in acute human neocortical slices (Fig 4.7A) from temporal cortex tissue resected during surgical treatment of epilepsy or tumor patients to gain access to
deeper structures (Testa-Silva et al., 2010, 2014, Verhoog et al., 2013, 2016). Electrical stimulation of pyramidal neurons (Pre-PC) to induce 15 APs at different frequencies (50-150Hz) induced characteristic lateral inhibition in postsynaptic pyramidal neurons (Post-PC) (Fig 4.7B, blue traces). With increasing AP frequency fired by Pre-PCs, the onset delay decreased, the duration of the inhibition increased as well as the amplitude (Fig 4.7A,C). Presynaptic electrical stimulation with a frequency below 50 Hz rarely resulted in lateral disynaptic IPSPs in postsynaptic pyramidal neurons. Our results indicate that disynaptic lateral inhibition exists between pyramidal neurons in layer 2/3 of the human neocortex.

Figure 4.6. BF cholinergic projections facilitate AP firing by Martinotti cells. A. Simultaneous recording from a presynaptic pyramidal (Pre-PC) and a postsynaptic Martinotti cell (Post-MC) in LS of the mPFC. A 100 Hz AP train by the Pre-PC cell induces AP firing in the Post-MC cell (Black trace, OFF condition). BF cholinergic inputs are optogenetically activated by five short blue light pulses at 25 Hz preceding the first induced AP by 100 ms (Blue trace). Simultaneous stimulation of the pre-PC and cholinergic inputs leads to a shorter onset delay and increased number of spikes in the MC-Post cell (Blue trace). B. Simultaneous recordings of a PC and MC recorded in L2/3 of the somatosensory cortex (S1). Representative traces of Post-MC APs in presence or absence of ACh (1mM) (orange and black traces). C. Left: Summary plot of onset delay of the first AP. Right: the number of APs in the Post-MC cell (paired t-test, p<0.05, optogenetic stimulation n=7; ACh bath application n=8). Optogenetic activation as well as bath application of ACh leads to a significant shortening of the onset delay time of Post-MC APs (light OFF 102.1 ±10.79 ms, light ON 85.73 ±10.18 ms, paired t-test, two-tailed, p<0.01; Ctrl. 99±1 ms, ACh 69±1 ms, paired t-test, two-tailed, p<0.01, t=3.4, df=7, n=8) and increase in number of APs during lateral inhibition (light OFF 3.429±0.9724 APs, light ON 7.571±1,744 APs, paired t-test, p<0.05; Ctrl. 2.1±0.4 APs, ACh 3.8±0.6 APs, paired t-test, two-tailed, p<0.01, t=4.1, df=7, n=8). However, we observed no change of the AP threshold potential or of the frequency of AP firing (p=0.681, p= 0.165, paired t-test, two-tailed, mean±s.e.m.).
Since lateral inhibition in the neocortex of rodents is mediated by somatostatin-positive MCs (Kapfer et al., 2007; Silberberg and Markram, 2007; Berger et al., 2009), we asked whether this specific inhibitory cell type is also mediating disynaptic inhibitory loops in the human neocortex. In rodents, MCs have unique morphological and cellular properties that distinguish them from other interneuron types, such as axonal projections to L1 and marked rebound APs (Kozloski, 2001; Goldberg et al., 2004; Karube, 2004; Wang et al., 2004; Silberberg and Markram, 2007). In our recordings, we found interneurons that share similar morphological and cellular characteristics as MCs in rodent neocortex (Fig 4.8) (Karube, 2004; Wang et al., 2004; Kawaguchi et al., 2006; Silberberg and Markram, 2007). These human interneurons had axons projecting to L1, a bipolar dendritic morphology where the dendritic tree is significantly smaller than the axonal tree and an oval soma (Fig 4.8A). Furthermore, these interneurons also had a low spiking threshold with a prominent rebound spike (Fig 4.8C), responded with a sag to hyperpolarizing current steps and showed AP accommodation to depolarizing current steps (Fig 4.8C). Excitatory EPSPs from Pre-PCs showed facilitation and

Figure 4.7. Lateral inhibition between pyramidal neurons in human temporal cortex. A. Digital reconstruction of two biocytin-filled L2/3 pyramidal neurons in human temporal cortex. B. Lateral inhibition between two pyramidal neurons in layer 2/3 of the human temporal cortex. Top trace: The Pre-PC fires 15 APs at 100 Hz. Middle traces: Example traces of disynaptic inhibitory responses in the Post-PC neuron following Pre-PC APs at 50, 100 and 150 Hz (n=6). (Bottom) Summary plot showing that the amplitude of lateral inhibition increased depending on the Pre-PC AP frequency (50 Hz $0.14\pm0.05$ mV, 100 Hz $0.29\pm0.06$ mV, 150 Hz $0.52\pm0.09$ mV). C. Summary plots showing that lateral inhibition decreased in latency (Top panel, 50 Hz 277.2±66.98 ms, 100 Hz 131.5±17.83 ms, 150 Hz 102.1±15.08 ms) and increased in duration (Bottom panel, 50 Hz 76.04±9.36 ms, 100 Hz 189.2±26.21 ms, 150 Hz 254.1±18.63 ms) depending on the firing frequency of the Pre-PC (n=6, mean±s.e.m.).
summated to supra-threshold AP firing (Fig 4.8B, blue arrows). These findings indicate that in layer 2/3 of the human cortex, low threshold spiking cells exist that share numerous morphological and physiological characteristics with MCs in rodents. In addition, when recording from all three components of a disynaptic lateral inhibition loop in human L2/3 we found the same features of disynaptic lateral inhibition as described in rodent cortex (Silberberg and Markram, 2007): as in rodent cortex, high frequency AP firing (100Hz, 15 APs) by presynaptic pyramidal neurons (Pre-PC), (Fig 4.8E black trace) led to facilitating EPSPs in low threshold spiking interneurons, which in its turn, resulted in AP firing (Fig 4.8E blue trace) that caused time-locked IPSPs in the postsynaptic pyramidal neuron (Fig 4.8E grey trace). Our findings show that disynaptic lateral inhibition exists in the human neocortex and is mediated by low threshold spiking interneurons that share several features with rodent MCs.

Figure 4.8. Lateral inhibition in the human cortex is mediated by putative Martinotti interneurons. A. Digital reconstruction of a complete disynaptic loop in human neocortex between two biocytin-filled pyramidal neurons mediated by a putative Martinotti interneuron (PC-MC-PC). B. Left: Schematic representation of the experiment: a presynaptic pyramidal (Pre-PC) and a postsynaptic low threshold spiking interneuron (Post-MC) recorded in L2/3 of the human temporal cortex. Right: Example trace from a PC-Pre cell firing 15 APs at 100 Hz (Grey trace) and facilitating EPSPs (blue arrows) in the synaptically connected Post-MC cell (blue trace). C. Left: Schematic representation of the experiment: simultaneous recording from a presynaptic MC and postsynaptic Post-PC. Right: Example trace showing a firing pattern characteristic of MCs, with rebound spiking, the sag in response to hyperpolarization and spike frequency accommodation in a response to depolarizing current injection. D. Simultaneous recording of a Pre-MC and a Post-PC in L2/3 of the human temporal cortex. The Pre-MC was stimulated to fire 100 Hz AP trains (15 APs) (Blue trace) resulting in the postsynaptic inhibition in the Post-PC cell (Grey trace, average shown in black). E. A complete disynaptic lateral inhibitory loop in layer 2/3 of the human temporal cortex. Triggering a train of APs (100 Hz, 15 APs) in the presynaptic pyramidal neuron (Pre-PC, black trace) led to the activation of a facilitating connection in the postsynaptic MC (Mid-MC, blue trace). The excitatory inputs evoked an AP in the mid-MC that resulted in IPSPs in the synaptically connected pyramidal neuron (Post-PC, grey trace, average indicated in black).
Acetylcholine enhances lateral inhibition by activating nAChRs in human temporal cortex

Since ACh is facilitating disynaptic lateral inhibition in rodent neocortex, we asked whether cholinergic modulation of lateral inhibition is conserved in human cortex. To test this, we induced lateral inhibition by electrically stimulating presynaptic pyramidal neurons (Pre-PC) to fire 15 APs with 100Hz while recording IPSPs in the postsynaptic pyramidal cell (Post-PC, Fig 4.9A). Following wash-in of ACh (1mM), the onset delay time of disynaptic IPSPs in Post-PCs was reduced and the duration of inhibition increased, while the amplitude was not affected (Fig 4.9A,B). Bath application of DHßE (10µM) blocked these effects by ACh (Fig 4.9A,B, light green trace). Blocking muscarinic acetylcholine receptors by bath application of atropine (400nM) did not affect the onset, duration or amplitude of lateral IPSPs (Fig 4.9A,B, dark green trace). These findings suggest that lateral inhibition in L2/3 of the human temporal neocortex is facilitated by activation of heteromeric nAChRs and not muscarinic acetylcholine receptors.

ACh depolarizes human putative Martinotti cells and alters AP firing properties

As we showed above, cholinergic facilitation of lateral inhibition in rodent neocortex is mediated by heteromeric nAChRs that depolarize MCs. Since interneurons in the human neocortex express also various types of nAChRs (Alkondon et al., 2000; Obermayer et al., 2017), we asked whether depolarization of putative MCs by ACh mediates cholinergic facilitation of lateral inhibition in human neocortex. To test this, we recorded from human putative MCs in L2/3 (Fig 4.10A), identified by the morphological and electrophysiological criteria described above (Fig 4.10B). Bath application of ACh (1mM) for 15 minutes depolarized these neurons (Fig 4.10C,E). In 2 out of 8 recordings, ACh application induced spontaneous AP firing in putative MC (Fig 4.10C). This suggests that somatic depolarization of putative MC interneurons may mediate facilitation of lateral inhibition in the human cortex.

To test whether ACh modulates AP firing in putative MCs during lateral inhibition, we simultaneously recorded from synaptically-connected pyramidal neurons and putative MCs in L2/3 of the human temporal cortex. We electrically stimulated the presynaptic pyramidal cell (Pre-PC) to fire 15 APs at a frequency of 100Hz and recorded AP firing by the postsynaptic putative MC. Recordings were only included for analyses in which the postsynaptic interneuron reliably fired APs following presynaptic stimulation in all experimental conditions. Presynaptic stimulation
combined with ACh application (1 mM) led to a shorter latency of the first AP (Fig 4.10D,E) and more APs in the postsynaptic interneuron compared to presynaptic stimulation alone (Fig 4.10D,E). The AP threshold potential and frequency of APs in the postsynaptic interneuron was not changed. Our findings show that ACh depolarizes human putative MCs mediated by heteromeric nAChRs, advancing both AP firing in MCs and lateral inhibition between human pyramidal neurons.

Figure 4.9. ACh facilitates lateral inhibition in human neocortex. A. Left: schematic illustration of the experiment: simultaneous recording of pyramidal neurons in L2/3 of the human temporal cortex showing lateral inhibition. Right: top trace: 15 APs at 100 Hz (black trace) in the Pre-PC. Middle trace: Example trace of lateral inhibitory response in the Post-PC neuron in ACSF (grey trace) or in presence of ACh (1 mM) (green trace). Bottom trace: as middle traces, ACh (1 mM) was bath applied in presence of DHβE (10 µM) (light green trace) or DHβE (10 µM) and Atropine (400 nM) (dark green trace). B. Summary plots showing that the onset delay time of lateral inhibition in the Post-PC is decreased ($F_{(5, 15)} = 24.37$, p<0.01; Two-way ANOVA, multiple comparison, n=6) and the duration increased following ACh application $F_{(5, 15)} = 4.669$, p<0.01; Two-way ANOVA, multiple comparison, n=6). The heteromeric nAChR blocker DHβE blocks these effects. Atropine had no effect on lateral inhibition. The amplitude was not affected by ACh ($F_{(5, 15)} = 7.153$, p<0.01; Two-way ANOVA, multiple comparison, n=6, mean±s.e.m.).
In this study, we addressed the question whether cholinergic projections from the basal forebrain modulate cortical lateral inhibition between pyramidal neurons. We find that (1) in L2/3 and L5, optogenetic activation of BF projections shortens the delay time and increases the duration of delayed lateral inhibition via Martinotti cells of neighboring pyramidal neurons, while fast lateral inhibition is not affected by cholinergic inputs. (2) Cholinergic facilitation of lateral inhibition is independent of firing frequencies of presynaptic pyramidal neurons. (3) We show

**Figure 4.10. ACh facilitates AP firing by human putative Martinotti cells. A.** Schematic illustration of the experiment: recording of synaptically connected human pyramidal (Pre-PC) and putative Martinotti cell (Post-MC). **B.** Spiking profile of the Post-MC. **C.** Example trace showing that Post-MCs can start to firing APs upon ACh (1mM) application (n=2 of 8). **D.** Recording of synaptically connected human pyramidal cell (Pre-PC) and putative MC (Post-MC). Top-trace: The pyramidal neuron is electrically stimulated to fire a train of 15 APs at 100 Hz. Bottom trace: Typical traces showing the excitatory input in the Post-MC without (grey trace) and with ACh (green trace) application. **E.** Summary plots showing that ACh depolarized putative MCs (from -63.54 ±0.85 mV to -60.15 ±0.88 mV, p<0.05; paired t-test; two-tailed; t=3.699, df=7; n=8), decreased the spike delay (from 76.12 ±17.51 ms to 51.43 ±12.21, p<0.05; paired t-test; two-tailed; t=2.89, df=7; n=8) to the first spike and increased the number of APs (from 1.713±0.48 APs to 2.531±0.41 APs, p<0.05; paired t-test; two-tailed, t=2.427, df=7; n=8). The spiking threshold and firing frequency was not affected by ACh (p=0.439, paired t-test; two-tailed, t=0.8199, df=7, n=8; p=0.094, paired t-test; two-tailed, t=2.185, df=4, n=5, mean±s.e.m.)

4.4 Discussion

In this study, we addressed the question whether cholinergic projections from the basal forebrain modulate cortical lateral inhibition between pyramidal neurons. We find that (1) in L2/3 and L5, optogenetic activation of BF projections shortens the delay time and increases the duration of delayed lateral inhibition via Martinotti cells of neighboring pyramidal neurons, while fast lateral inhibition is not affected by cholinergic inputs. (2) Cholinergic facilitation of lateral inhibition is independent of firing frequencies of presynaptic pyramidal neurons. (3) We show
that heteromeric nAChRs containing β2 subunits, rather than muscarinic AChRs, mediate this cholinergic modulation. (4) The mechanism of cholinergic facilitation of lateral inhibition between pyramidal neurons relies on direct depolarization of MCs mediated by postsynaptic heteromeric nAChRs. Co-occurrence of glutamatergic and cholinergic excitatory inputs summate linearly. Strength of synapses between pyramidal cells and MCs is not affected by ACh. (5) ACh leads to a significant decrease of the onset delay of AP firing and increases the number of AP fired in MCs, which can account for the earlier onset and prolonged duration of disynaptic inhibition. (6) In addition, we show that delayed disynaptic lateral inhibition between pyramidal neurons is conserved in the human cortex and is modulated by putative MCs. (7) In the human cortex, mechanisms of cholinergic modulation of lateral inhibition are similar to rodent cortex. In short, BF cholinergic inputs selectively augment disynaptic lateral inhibition via MCs in rodent and human cortex by increasing excitability of MCs.

Pyramidal neurons in the cortex can suppress activity of surrounding pyramidal neurons through lateral inhibition mediated by MCs and PV-positive interneurons (Pouille and Scanziani, 2004; Kapfer et al., 2007; Silberberg and Markram, 2007). Excitatory synapses between pyramidal neurons and most types of interneurons are depressing, but excitatory synapses between pyramidal neurons and SOM-positive MCs are facilitating (Beierlein et al., 2000; Thomson and Bannister, 2003; Pouille and Scanziani, 2004; Kapfer et al., 2007; Silberberg and Markram, 2007; Xu et al., 2013). With increasing firing frequencies of pyramidal neurons, stronger synaptic facilitation occurs in MCs and the probability of generating action potentials increases. As a consequence, higher firing frequencies of the presynaptic pyramidal neurons speed up the discharge of the MC, which leads to an earlier onset of AP firing and more APs (Silberberg and Markram, 2007; Berger et al., 2010). Glutamatergic synapses can be facilitated by nAChRs located on presynaptic glutamatergic terminals (McGehee et al., 1995; Gray et al., 1996; Mansvelder et al., 2000), and BF cholinergic inputs can alter the strength of glutamatergic synapses in a layer-dependent fashion (Verhoog et al., 2016). Furthermore, depolarization of presynaptic pyramidal cells can increase glutamatergic facilitation in the pyramidal cell to MC synaptic pathway, which augments lateral inhibition between pyramidal neurons (Zhu et al, 2011). In our experiments, ACh did not alter presynaptic facilitation of EPSPs between pyramidal and MCs. We did find that basal forebrain ACh inputs depolarize MCs, giving rise to an earlier onset and a higher number of APs in MCs. Furthermore, we showed that basal forebrain ACh inputs speed up the onset and increase the duration of
disynaptic inhibition in neighboring pyramidal neurons. The increased amplitude of disynaptic IPSPs following ACh release from basal forebrain inputs in the mPFC might therefore be the result of a larger number of MCs that reach firing threshold and take part in lateral inhibition. Disynaptic inhibition produced by MCs can affect a substantial fraction of neighboring pyramidal neurons as a result of high connection probability between MCs and pyramidal neurons, reported in both juvenile and adult rodent neocortex (Berger et al., 2009; Fino et al., 2013a; Jiang et al., 2015). By depolarizing MCs, BF cholinergic projections can dynamically facilitate recruitment of MCs by pyramidal neurons to take part in lateral inhibition.

Fast disynaptic lateral inhibition is modulated by PV-positive interneurons (Silberberg and Markram, 2007). A subgroup of these interneurons express fast nicotinic AChR currents mediated by α7-containing nAChRs in layer 5 of the mPFC (Poorthuis et al., 2013a). These nicotinic currents act on PV interneurons on a time scale similar to currents acting at glutamatergic synapses, and they can be activated by optogenetic activation of basal forebrain ACh inputs (Arroyo et al., 2014). We showed that fast lateral inhibition is not affected by BF cholinergic projections. Possibly in lateral inhibition, PV-positive interneurons are recruited that do not express nAChRs (Poorthuis et al., 2013a). PV-positive interneurons target perisomatic regions of pyramidal neurons, and are well-suited to control timing of action potentials, whereas SOM-positive MCs target distal areas of pyramidal neuron dendrites, affecting dendritic integration, calcium spikes, and action potential burst generation (Pouille and Scanziani, 2004; Murayama et al., 2009; Gentet, 2012; Gidon and Segev, 2012). Since lateral inhibition via MCs is ACh sensitive, BF cholinergic inputs can selectively alter inhibitory pathways between pyramidal neurons, shifting the balance between somatic and dendritic processing.

Cholinergic receptors are widely distributed among different cell types in the cortex (Gulledge et al., 2007; Poorthuis et al., 2013a; Ballinger et al., 2016; Obermayer et al., 2017). In the neocortex, optogenetic ACh release from BF projections predominantly activate nicotinic AChR currents (Arroyo et al., 2012; Bennett et al., 2012; Hedrick and Waters, 2015; Hay et al., 2015; Verhoog et al., 2016). A prominent feature of SOM-positive interneurons is the strong membrane depolarization caused by agonists of both muscarinic and nicotinic AChRs (Beierlein et al., 2000; Couey et al., 2007; Fanselow et al., 2008; Poorthuis et al., 2013a; Xu et al., 2013; Chen et al., 2015). ACh released from projections from the basal forebrain could in principle activate both types of receptors expressed by
MCs. In the thalamus, endogenous release of ACh by optogenetic stimulation results in a biphasic response caused by activation of both nicotinic and muscarinic ACh receptors (Pita-Almenar et al., 2014). However, in our experiments blocking muscarinic AChRs, cholinergic facilitation of disynaptic inhibition was not affected. Furthermore, we found that BF cholinergic projections and ACh induced a prominent depolarization in MCs, which was completely abolished by nicotinic AChR blockers. This suggests that although MCs express muscarinic AChRs, BF cholinergic inputs mainly activated nAChRs and not muscarinic AChRs. The activation of nAChRs by BF inputs does not have to reach firing threshold by itself, unlike in the visual cortex, where supra-threshold cholinergic recruitment of SOM-positive interneurons alters local network activity to a more desynchronized state (Chen et al., 2015). Cholinergic modulation of lateral inhibition by BF inputs can occur without supra-threshold cholinergic activation of the SOM-positive interneurons. Sub-threshold depolarization by cholinergic inputs is sufficient to facilitate lateral inhibition between pyramidal neurons, and advance action potential firing of SOM-positive interneurons induced by pyramidal neuron inputs.

Recently, several studies highlighted similarities and differences in cellular and synaptic function between rodent and human neocortical circuitry (Molnár et al., 2008, 2016; Testa-Silva et al., 2010; Verhoog et al., 2013, 2016; Mohan et al., 2015; Eyal et al., 2016; Szegedi et al., 2017). Although inhibition mediated by fast spiking interneurons is described (Szegedi et al., 2017), we found here that layer 2/3 pyramidal neurons in the human cortex modulate activity of surrounding pyramidal neurons through delayed lateral inhibition mediated by putative MCs. Although it was reported that single AP firing in the presynaptic pyramidal neuron can trigger complex events in the human cortex (Szegedi et al., 2017), we did not observe fast lateral inhibition between pyramidal neurons in our recordings.

Various reports show that fast cholinergic signaling plays an important role in modulating cellular activity and microcircuits in the rodent brain (Poorthuis et al., 2013a, 2014). However, little is known about whether cholinergic modulation of information processing in the human neocortex follows similar mechanisms. EM studies show that 67% of all varicosities on cholinergic axons in the human temporal cortex can be identified as point-to-point synapses, in contrast to only 15% in rodent cortex, which suggests that in human neocortex fast cholinergic signaling may be more abundant (Smiley et al., 1997). Pyramidal neurons and interneurons in the human cortex express α7-containing and β2-containing nAChRs acting on a fast time scale (Alkondon et al., 2000; Verhoog et al., 2016;
Obermayer et al., 2017). Our results show that ACh facilitates the onset and duration of delayed disynaptic lateral inhibition by activating heteromeric nAChRs. Cholinergic depolarization, advancement of spiking onset and higher spiking rate of putative MCs appear to be conserved in human neocortex.

Cholinergic modulation of interneurons plays an important role in cortical functions depending on cortical processing (Kawaguchi and Kubota, 1997; Fanselow et al., 2008; Hasselmo and Sarter, 2011; Demars and Morishita, 2014; Chen et al., 2015; Hangya et al., 2015). For example, disinhibitory pathways activated by cholinergic inputs to interneurons in superficial layers of the auditory cortex control auditory fear conditioning (Letzkus et al., 2011). Whether similar mechanisms are in place in the mPFC is not known. However, cholinergic control of mPFC circuits is behaviorally relevant. In mice lacking nAChR β2 subunit attentional performance is reduced (Guillem et al., 2011), and during attention behavior the amount of ACh in the mPFC rapidly increases to make a shift from monitoring cues towards a cue evoked goal directed response (Howe et al., 2013; Parikh et al., 2017). Modulation of lateral inhibition between pyramidal neurons by basal forebrain ACh inputs via MCs in the mPFC may play a role in these cognitive processes and serve to enhance, on demand, signal-to-noise ratio in pyramidal neuron activity.

4.5 Experimental Procedures

**Mouse brain slice preparation**

Coronal medial prefrontal cortex (mPFC) or parasagittal somatosensory (S1) slices were prepared from P14-25 male or female C57Bl/6 mice, Gin mice [FVBTg(GadGFP)45704Swn/J from the Jackson Laboratory] or the F1 of matings between Gin mice with Chat-Chr2-EYFP mice [B6.Cg-Tg(Chat-COP4*H134R/EYFP)6Gfng/J from the Jackson Laboratory (Jax, USA). Following decapitation, the brain was carefully removed from the scull and maintained and sliced in carbogen buffered (95 % O2, 5 % CO2 at pH 7.4) ice cold slicing solution containing (in mM): 125 NaCl, 3 KCl, 1.25 NaH2PO4, 7 MgSO4, 0.5 CaCl2, 26 NaHCO3, and 10 glucose. Acute brain slices (350 µm) were incubated for one minute at 34°C in N-Methyl-D-glucamin solution (NMDG solution; in mM: NMDG 93, KCl 2.5, NaH2PO4 1.2, NaHCO3 30, HEPES 20, Glucose 25, NAC 12, Sodium ascorbate 5, Sodium pyruvate 3, MgSO4 10, CaCl2 0.5, at pH 7.4 adjusted with 10M HCl). For recovery, slices were maintained at room temperature in artificial cerebrospinal fluid (aCSF) containing (in mM) 125 NaCl, 3 KCl, 1.25 NaH2PO4, 1
MgSO₄, 2 CaCl₂, 26 NaHCO₃, and 10 glucose in a holding chamber for at least one hour prior to recordings.

**Human brain slice preparation**
All performed procedures on human tissue were in line with the Dutch license procedures and the declaration of Helsinki and approved by the Medical Ethical Committee of the VU University Medical Centre. To reach deeper brain regions for surgical treatment, human anterior and medial temporal cortex had to be removed. For this study, we obtained tissue from the temporal lobe from five patients (3 females, 2 males, aged 32-52 years) with written informed consent. The dissected temporal tissue showed no abnormalities on preoperative MRI and was classified by neuropathologists as non-pathological. All patients were diagnosed with meso-temporal epilepsy and had mild to severe forms of epilepsy.

Slice preparation from human brain tissue followed the same procedures as described previously (Testa-Silva et al., 2010, 2014, Verhoog et al., 2013, 2016; Mohan et al., 2015; Eyal et al., 2016). Resected cortical tissue blocks from the temporal cortex were transported and sliced in ice-cold choline slicing solution containing (in mM): 110 choline chloride, 26 NaHCO₃, 10 D-glucose, 11.6 sodium ascorbate, 7 MgCl₂, 3.1 sodium pyruvate, 2.5 KCl, 1.25 NaH₂PO₄ and 0.5 CaCl₂. The slice preparation started maximal 10 minutes after the tissue resection. Human cortical slices with a thickness of 350 µm were prepared and transferred to a holding chamber with aCSF for 30 min at 34 °C. Subsequently the slices were incubated for recovery at room temperature for at least one hour before starting recordings. The recordings were performed in aCSF at 32°C and a flow rate of 2-3 ml per minute.

**Electrophysiology**
Simultaneous whole cell recordings from up to four connected pyramidal (PC) and Martinotti cells (MCs) in L5 of the mPFC or L2/3 of the somatosensory cortex (S1) or slices from the human temporal cortex were made in oxygenated aCSF (flow rate of 3-4 ml/min, 32°C). For recordings, borsiclicate glass pipettes (3-6 MΩ) filled with a potassium based internal solution (in mM): K-gluconate 135, NaCl 4, Hepes 10, Mg-ATP 2,K2Phos 10, GTP 0.3, EGTA 0.2 were used. MCs were identified in the GIN mice by expression of GFP (Ma et al., 2006), spike profile and bipolar morphology. PCs were identified by spike profile and DIC image. We minimized the exposure to blue light to avoid long lasting activation of ChR2 and let the tissue recover for at least 5 minutes before recording. In recordings without MCs, there was no exposure to blue light preceding the recordings. During recordings, PC and
MCs were kept at a holding membrane potentials close to -60 mV. To quantify disynaptic or monosynaptic connections, presynaptic neurons were injected with 2nA pulses of 2 ms to evoke a train of 15 APs at a frequency of 100Hz with an inter-train interval of 7s. Fifteen APs per train were also used for experiments were presynaptic cells were stimulated to fire AP trains at different frequencies. In multiple cell recordings, each cell was stimulated with an interval of 60s in an alternating manner. The postsynaptic excitatory or inhibitory response were analysed and quantified by averaging 5-20 traces. Amplitudes were calculated as the difference between the peak value and the average baseline of 100 ms before the stimulation onset. The onset latency was calculated from the start of the stimulation in the pre-synaptic cell to the threshold of the response at 20% of the maximum amplitude, response duration was calculated as the time difference between the onset threshold and the offset threshold at 20% of the maximal amplitude.

**Optogenetically evoked endogenous acetylcholine release**

In Gin/Chat-ChR2-EYFP-crossed mice, cholinergic fibers were stimulated by blue light activation of channelrhodopsin (ChR2) (five light pulses, 470 nm, @ 25 Hz) using a DC4100 4- channel LED-driver (Thorlabs, Newton, NJ) or a Fluorescence lamp (X-Cite Series 120q, Lumen Dynamics). In experiments where light stimulation was combined with presynaptic electrical stimulation the first light pulse started 100 ms before the first AP in the presynaptic neuron. The presynaptic stimulation was either with light off or with light on, alternating with 60 s interval. In some experiments we observed feedforward inhibitory responses by blue light, as was reported previously (Arroyo et al., 2012). These recordings were excluded from analysis. In layer 5, we sometimes observed feedforward excitatory responses by blue light, which was prevented by reducing the field of illumination.

**Pharmacology**

All drugs used were dissolved in aCSF at the final concentration and bath applied during the experiments. Drug concentrations used were: acetylcholine (1 mM, Sigma-Aldrich), atropine (400nM; Sigma-Aldrich), DHβE (10µM; Tocris Bioscience). All experiments were performed without application of synaptic blockers.

**Analysis and statistics**

Raw data was analysed using Clampfit 10.4. or custom-written Igor Pro scripts (Igor Pro 7 waveMetrics). Statistical analysis was performed using Prism 6 (GraphPad software). Data was analyzed by using the appropriate parametric statistical test.
as mentioned in the Figure legends, data shown are mean±sem, and p<0.05 was taken as level of significance.

4.6 Author contributions

4.7 Acknowledgements
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5

General discussion
5. General discussion

The medial prefrontal cortex is a remarkable structure that serves as a hub for projections to and from many cortical and subcortical structures in the brain, making it ideally suited for the regulation of attentional processes. The function of the medial prefrontal cortex as well as other cortices is critically dependent on the presence of neuromodulators. Our understanding of the role of the neuromodulators adenosine and acetylcholine on network signaling in the rodent prefrontal cortex and in human cortical areas is however yet limited. The aim of this thesis was to understand the actions of these two neuromodulators and their short- and long-term effect on network signaling in cortical areas of the rodent and human brain.

Part of this interest was drawn by the wide use of psycho-active substances such as caffeine, which is known to enhance cognitive behavior, but of which there is a lack of knowledge on how it modulates signaling in the cortex. We show in chapter 2 that caffeine in normal consumption amounts is capable of altering the cellular response to adenosine, an ATP metabolite that is present in the extracellular space around active cells. To reveal short-and long-term effects of endogenous adenosine in the prefrontal cortex, the area mainly responsible for attentive behavior we functionally evaluated neuronal responses in the rodent cortex using electrophysiology. We showed in chapter 3 that endogenous adenosine acts through A2AR to affect long-term plasticity especially at synapses that project to local fast-spiking (FS) interneurons. The neuromodulator acetylcholine has especially strong effects on Martinotti cells, an interneuron subtype crucially involved in lateral inhibition. In chapter 4 we show that through release of the neuromodulator acetylcholine, a disynaptic loop is enhanced that can thereby provide faster, longer and stronger inhibition to a network of surrounding pyramidal neurons.

In this final chapter, I discuss the relevance and the implications of our findings.

5.1 Caffeine affects cellular signaling in the human brain

Caffeine is the most widely used psychoactive drug in the world. Coffee, the main source of caffeine, is part of our cultural values: in the western world, more than 50% of people above 18 years old report to drink coffee on a daily basis (International Coffee Organization EU/Harvard). This is not surprising, as we know caffeine to be a cognitive enhancer that can elevate our mood, increase our
alertness, postpone sleep and elevate attention (Barry et al., 2007; Hewlett and Smith, 2007; Borota et al., 2014; Wilhelmus et al., 2017). Compared to other stimulants, its effects are rather modest, but yet clearly detectable on larger cohorts, even when doses are used that equal one single cup of coffee (Wilhelmus et al., 2017). As cognitive behavior relies on neuronal activity in cortical areas, it is generally assumed that caffeine in normal consumption amounts can affect neuronal activity in the cortex. Indeed, studies using fMRI, PET and other imaging techniques show that network activity of populations of neurons is altered when caffeine is present in the brain (Nehlig and Boyet, 2000; Park et al., 2014a; Xu et al., 2015). However, little was yet known on how caffeine affects specific populations of neurons in the human cortex. To this end, we recorded from single pyramidal neurons in dissected human brain tissue from the temporal lobe region, reported in chapter 2. Using this technique, we could show that caffeine indeed affects signaling of individual pyramidal neurons in response to adenosine, and that this happens specifically at the postsynaptic side, together with changes in intrinsic membrane properties of these cells (Kerkhofs et al., 2018b).

More specifically, caffeine was able to disinhibit some, but not all, of the A1R-mediated adenosine-induced effects onto excitatory transmission and intrinsic membrane properties of pyramidal neurons in the human cortex. First, caffeine could inhibit adenosine-induced effects on the hyperpolarization-activated cationic depolarizing current (Ih) and on the input resistance of pyramidal neurons, both important actors in cellular excitability and signal propagation (Kandel, 1976; Williams and Stuart, 2000). Thus, in the presence of caffeine, the input resistance and Ih of pyramidal neurons would stay constant even when there was an additional load of adenosine. As adenosine alone decreased the input resistance and Ih, these results indicate that caffeine can prevent adenosine-induced inhibition of excitability in pyramidal neurons. However, caffeine was unable to counteract the effect of adenosine on another intrinsic membrane property, the rheobase or threshold value for action potential firing, which was greatly increased upon an increased adenosine load. Caffeine thus only has partial effects on the net excitability of neurons in the human temporal lobe.

Caffeine could also fulfill only a partial blockade of adenosine-induced inhibition of synaptic transmission. Caffeine only controlled synaptic transmission through postsynaptic A1R, while being ineffective on presynaptic A1R, measured as the frequency and amplitude of spontaneous excitatory events, respectively. Our results show that caffeine is only capable of controlling the received electrical
signal, and not the released neurotransmitter, while those are both under clear control of adenosine. Thus, whereas adenosine had an effect on all recorded measures of excitability and excitatory transmission onto pyramidal neurons of the human temporal cortex, caffeine only affected postsynaptic transmission and some measures of excitability, likely explaining why caffeine only moderately affects signaling on a network scale when this is measured using EEG or fMRI (Nehlig and Boyet, 2000; Park et al., 2014a; Xu et al., 2015).

These results show that human neurons respond differently to caffeine than rodent neurons; although pyramidal neurons of the human brain responded to adenosine with a similar reduction in membrane excitability and synaptic transmission as neurons of the rodent brain (van Aerde et al., 2013, Li et al., 2011, Phillis et al., 1979, Thompson et al., 1992), there are some striking differences in their response to caffeine. First, caffeine onto rodent neurons affected membrane properties even without exogenously applied adenosine (Greene et al., 1985; Phillis et al., 1979; Simons et al., 2011). More specifically, modest caffeine incubation (100 μM) in the rodent hippocampus decreased the resting membrane potential, decreased the fast afterhyperpolarization (fAHP) and increased the input resistance of hippocampal neurons (Greene et al., 1985). Also, in the rodent hippocampus and cortex caffeine was shown to control synaptic transmission through both an A₁R-mediated presynaptic control of the release of excitatory neurotransmitters (Greene et al., 1985; Phillis et al., 1979), as well as through a facilitation of postsynaptic responses (Greene et al., 1985; Simons et al., 2011). These postsynaptic- rather than membrane effects of caffeine in the human cortex were associated with a greater affinity of caffeine for synaptic compared to extrasynaptic A₁R in the human cortex, different from the affinity profile of caffeine in the rodent cortex. This difference probably results from a different molecular organization of A₁R within human cortical synapses compared to rodent cortical synapses: in rodents, the total binding density of A₁R is higher than in the human temporal cortex (Boulenger et al., 1982; Dodd et al., 1986; Fastbom et al., 1987; Svenningsson et al., 1997), while there is a higher number of A₁R in human cortical synapses, as well as a greater affinity for DPCPX of synaptic A₁R in comparison with extra-synaptic A₁R, more than in rodents (Ferkany et al., 1986; Maemoto et al., 1997; Murphy and Snyder, 1982). Since one of the few factors that has been documented to regulate the affinity of A₁R for antagonists is their relative homomerization (Gracia et al., 2013) or heteromerization (Ciruela et al., 2006), the present findings are suggestive of a different molecular arrangement of A₁R in synapses of the human cerebral cortex.
To characterize the impact of caffeine on human cortical neurons, we were particularly careful to select a concentration of caffeine within the range of concentrations reached by caffeine in the brain parenchyma upon moderate consumption of caffeine; these were estimated by our group (Costenla et al., 2010; Duarte et al., 2012; Silva et al., 2013) and by others (Kaplan et al., 1990; Thithapandha et al., 1972) to be in the range of 20-70 μM in rodents, similar to the plasma concentrations of caffeine that cause maximal psycho-activating responses in humans (Bruce et al., 1986). This aspect is of particular importance in view of the numerous studies that have described neuronal effects of caffeine using high millimolar or submillimolar concentrations of caffeine (Grigoryan et al., 2012; Lee et al., 1987; Margineanu & Klitgaard, 2004; Martin & Buno, 2003; Vyleta and Smith, 2008), which can only represent toxic effects of caffeine found in extreme cases of caffeinism (Gilliland and Andress, 1981). We find here that in this concentration range, effects are partial, subtle and only observed in relation of an increased adenosine load.

We looked in our study at human neurons of the temporal lobe area. Unfortunately, we were unable to study fresh tissue of regions outside of the temporal lobe, such as the prefrontal cortex, which is identified by fMRI and PET studies as a main target area for caffeine (Nehlig and Boyet, 2000; Park et al., 2014a; Xu et al., 2015), or brain areas associated with aging-, mood- and attention disorders, in which caffeine is a known restorative and preventive agent (Cunha and Agostinho, 2010). However, it is noteworthy that the temporal lobe area has been identified by imaging studies as a target area for caffeine (Svenningsson et al., 1997; Elmenhorst et al., 2012; Xu et al., 2015) and A₁R are expressed in the temporal lobe area in a similar density as in other cortical areas (Svenningsson et al., 1997; Elmenhorst et al., 2012; Xu et al., 2015). When tissue of other areas would become available, it would be interesting to see whether caffeine in this concentration range would impact neurons of those areas similarly, or whether results are region-specific.

In our study, we looked specifically at the effect of caffeine on glutamatergic synapses onto pyramidal neurons. Adenosine has been shown to have mainly effects on glutamatergic synapses in the rodent cortex, regarding both intrinsic membrane properties (van Aerde et al., 2013) and synaptic transmission (Qi et al., 2016). In the rodent cortex, effects of adenosine or caffeine on GABAergic transmission have rarely been reported (Cunha et al., 2008). However, there are indications that in other areas of the brain such as in hippocampus, GABAergic
transmission is affected by caffeine (Greene et al., 1985; Isokawa, 2016). Also in the human cortex, there are indications for the involvement of caffeine on GABAergic signaling (Cerqueira et al., 2006; Cunha et al., 2008). Future studies on the effects of caffeine on inhibitory signaling on individual neurons in the temporal lobe region could further advance our understanding of caffeine as an agent in control of the excitation-inhibition balance in the cortex.

In short, our study shows for the first time the effect of caffeine on specifically excitatory neurons in the human cortex, revealing new insights of caffeine as only a partial antagonist that works more subtle and more specific than what had previously been shown in the cortex.

5.2 Endogenous release of the neuromodulators adenosine and acetylcholine affects mPFC

The mPFC is a highly interconnected area with many different subtypes connecting to each other or to other brain areas. The largest part of the mPFC consists of pyramidal neurons, while about 20-30% of all neurons are local GABAergic interneurons. The two main types of GABAergic interneurons in the mPFC are those that have a fast-spiking profile and express parvalbumin (FS-PV), and those that have a low-threshold-spiking electrophysiological profile and express somatostatin (LTS-SOM) (Markram et al., 2004; Fino et al., 2013b; Tremblay et al., 2016). Both FS-PV and LTS-SOM control the output of network signaling, but in a different manner: lateral inhibition by PV neurons may be more involved in rapidly silencing action potential firing in neighboring pyramidal neurons, while lateral inhibition through SOM neurons will control synaptic integration, burst firing and dendritic regenerative phenomena (Pouille and Scanziani, 2004; Gentet et al., 2012; Gidon and Segev, 2012; Tremblay et al., 2016). These interneuron subtypes are under tight control of neuromodulators such as acetylcholine. What the precise impact will be of FS-PV and LTS-SOM inhibition will largely depend on this neuromodulatory environment.

We show in this thesis two ways that neuromodulation can impact network functioning within the mPFC via effects on FS and LTS-SOM interneurons and their synapses.

5.2.1 Adenosine affects plasticity at FS synapses

The neuromodulator adenosine, a metabolite of the cellular source of energy ATP, becomes available upon activity of neurons (Fredholm et al., 2005; Pajski and Venton, 2010) and as such, adenosine levels are highest when there is high-
frequency firing of neurons in the network (Cunha et al., 1996). High-frequency firing of neurons induces plasticity of synapses that will last minutes to hours (Bliss and Lømo, 1973; Larson and Munkácsy, 2015), an effect that occurs in connections between excitatory presynaptic terminals and glutamatergic pyramidal neurons as well as between excitatory presynaptic terminals and interneurons (Kullmann and Lamsa, 2007; Sarihi et al., 2008). As our group and others have shown that in the cortex, adenosine receptors are located mainly on synapses (Kerkhofs et al., 2018; Tetzlaff et al., 1987; Rebola et al., 2005), the activation of these receptors was hypothesized to impact the formation of plasticity at synapses.

In this thesis, we show that in the rodent prefrontal cortex, adenosine indeed acts on $A_{2A}R$ to reverse plasticity; however, this is only the case on synapses between glutamatergic presynaptic terminals and FS interneurons and not at the glutamate-pyramidal synapse.

These results are in contrast to findings in other extrastriatal brain areas such as the hippocampus and amygdala. There, the manipulation of $A_{2A}R$ largely affected glutamatergic plasticity of either the network under a field electrode, or of glutamatergic neurons specifically (d'Alcantara et al., 2001; Rebola et al., 2008; Simões et al., 2016). For example, in synapses between mossy fibers and CA3 of the hippocampus, $A_{2A}R$ affected a postsynaptic form of plasticity dependent on CA3 NMDA receptors (Rebola et al., 2008), while in nucleus accumbens and amygdala, antagonism of $A_{2A}R$ affected plasticity at glutamatergic synapses (d'Alcantara et al., 2001; Simões et al., 2016). The different role of $A_{2A}R$ in the mPFC could be due to its primarily peri- or extrasynaptic location, compared to a primarily synaptic location in hippocampus and striatum (Kerkhofs et al., 2018; Rebola et al., 2005, 2008).

Although the impact of adenosine had not been shown before in relation to glutamatergic plasticity of interneurons, other neuromodulators can indeed influence the state of interneurons and thereby affect plasticity. For example, in the mPFC of young animals, the non-selective acetylcholine agonist nicotine enhances the glutamatergic load on interneurons such as FS-PV and LTS-SOM, preventing the induction of LTP at glutamatergic synapses onto pyramidal neurons (Couey et al., 2007). In visual cortex, the activation of adrenergic receptors enabled the induction of Hebbian bidirectional STDP in FS-PV interneurons and in LTS-SOM interneurons, that would otherwise not obtain plasticity in control conditions (Huang et al., 2013).
The finding that A$_{2A}$R blockade specifically reverses plasticity onto FS interneurons without affecting pyramidal neuron plasticity has important implications. For the maintenance of temporally precise signal integration in local pyramidal neurons, coordinated activity between interneurons and pyramidal neurons is necessary; this is termed the excitation-inhibition (E/I) balance (Pouille and Scanziani, 2001; Lu et al., 2007; Kullmann and Lamsa, 2011). Disruptions in the E/I balance can disrupt the formation of both long- and short-term plasticity (Konstantoudaki et al., 2016). Neuromodulators that specifically target either interneuron- or pyramidal neuron plasticity can therefore largely influence the temporal fidelity of the network through changes in the E/I balance (Meunier et al., 2017). We showed that inhibition of A$_{2A}$R specifically targets plasticity at FS interneurons in the mPFC. Fast spiking interneurons in the mPFC, in particular the parvalbumin-positive FS interneurons, have been shown to support working memory and cognitive flexibility (Murray et al., 2015) and to be central for the control of attention (Kim et al., 2016) and social behavior (Yizhar et al., 2011). Disruptions in FS interneuron activity or expression leads to cognitive deficits often associated with suppressed gamma oscillations (Sohal et al., 2009; Murray et al., 2015; Kim et al., 2016). A disrupted expression of A$_{2A}$R affecting FS interneuron plasticity could therefore lead to changes in the cognitive domain. Indeed, in fronto-cortical A$_{2A}$R knockout and overexpression models, disruptions of A$_{2A}$R expression lead to impairments in cognitive behavior, such as spatial learning, decision-making and working memory (Giminez-Llort et al., 2007; Shen et al., 2008a; Zhou et al., 2009; Wei et al., 2011; Pardo et al., 2012; Horita et al., 2013; Coelho et al., 2014). Also, in rodent models for cortex-associated disorders such as ADHD, A$_{2A}$R are overexpressed and associated with aberrant behavior (Pandolfo et al., 2013). In these rodent models, it is unknown whether A$_{2A}$R on pyramidal neurons or interneurons are responsible for this effect. Future research targeting selectively A$_{2A}$R in mPFC FS interneurons will be needed to elucidate whether specifically A$_{2A}$R located on glutamatergic to FS synapses are in control of mPFC-related behavior.

In short, blockade of adenosine acting through A$_{2A}$R reverses LTP to LTD on excitatory synapses onto FS interneurons, while plasticity at the excitatory-pyramidal neuron synapse remains unaffected. Alterations in A$_{2A}$R expression in mPFC, such as observed in ADHD, could therefore majorly impact the E/I balance and consequently cortical functioning. Further research targeting selectively A$_{2A}$R in FS interneurons needs to elucidate whether these targeted effects are indeed relevant to cognitive functioning.
5.2.2 Acetylcholine-induced depolarization of Martinotti cells enhances disynaptic inhibition

The neocortex receives cholinergic input from the basal forebrain nuclei (Rye et al., 1984), which become active during waking states and conditions that involve attention and memory (Sarter and Bruno, 1999; Hasselmo, 2006). The released acetylcholine can strongly depolarize low-threshold spiking interneurons (SOM-LTS) and induce action potential firing in these neurons (Gulledge et al., 2007; Fanselow et al., 2008; Poorthuis et al., 2013). We hypothesized that these interneurons would become even stronger involved in disynaptic lateral inhibition when cholinergic inputs are active. In chapter 4 of this thesis, we could show that indeed, acetylcholine acting on Martinotti cells, a specific subtype of these SOM-LTS interneurons, can ensure that pyramidal neurons inhibit surrounding pyramidal neurons faster, longer and stronger.

The mechanism that we describe by which cholinergic signaling facilitates lateral inhibition between pyramidal neurons, is by direct depolarization of the membrane potential of the MCs through activation of postsynaptic nAChRs. As MCs and pyramidal neurons have a high connection probability (Berger et al., 2009; Fino et al., 2013; Jiang et al., 2015), basal forebrain ACh inputs may this way increase the fraction of pyramidal neurons that are affected by lateral inhibition. Interestingly, we find here that modulation of lateral inhibition by cholinergic inputs can occur without direct cholinergic activation of the SOM-positive interneurons. A sub-threshold membrane depolarization by cholinergic inputs is sufficient to facilitate lateral inhibition between pyramidal neurons, and advance action potential firing of SOM-positive interneurons induced by pyramidal neuron input. This contrasts findings in the visual cortex, where supra-threshold cholinergic recruitment of SOM-positive interneurons alters local network activity to a more desynchronized state (Chen et al., 2015). Whether the subthreshold depolarization that we observe similarly leads to desynchronization, is yet unknown.

Our results show that this effect is specific to MCs, and is not mediated by FS-PV interneurons, as fast lateral inhibition mediated by FS-PV interneurons was clearly unaffected. This could implicate that for fast lateral inhibition, a subtype of PV-FS interneuron is recruited that does not express nAChRs. Such a subtype-specific targeting of ACh in the mPFC has important implications for cortical functioning. As axons from PV-positive interneurons target the perisomatic regions and SOM-
positive MCs axons target the distal dendritic areas of pyramidal neurons (Gentet, 2012; Gidon and Segev, 2012; Murayama et al., 2009; Pouille and Scanziani, 2004), the function of these interneuron subtypes is substantially different: activity of PV-interneurons is suitable for the timing of AP firing and synchronization of network activity, whereas SOM-positive MCs affect dendritic integration of inputs, dendritic calcium spikes and action potential burst generation (Gentet, 2012; Gidon and Segev, 2012; Kim et al., 2016; Murayama et al., 2009; Pouille and Scanziani, 2004). Therefore, cholinergic signaling might shift inhibition from somatic to dendritic areas of the pyramidal neurons, thereby highlighting specific inhibitory motifs.

Whether these findings translate to the human cortex is of interest, as cholinergic signaling in the human cortex is under study of several different groups that show the relation of cholinergic signaling and several disease types, such as in Alzheimer's disease (Pabst et al., 2016). Little is yet known about whether and how cholinergic modulation of information processing in the human neocortex can modulate cellular activity and microcircuits. However, pyramidal neurons and interneurons in the human cortex do express nAChRs (Alkondon et al., 2000; Obermayer et al., 2017; Verhoog et al., 2016) and EM studies show that 67% of all varicosities on cholinergic axons in the human temporal cortex can be identified as point-to-point synapses, in contrast to only 15% in rodent cortex, suggesting that in human neocortex cholinergic signaling may be even more abundant (Smiley et al., 1997). We were therefore curious to see whether our findings could translate to the human cortex. Our results indeed showed that ACh facilitates delayed disynaptic lateral inhibition by activating heteromeric nAChRs in human neocortex, likely mediated by MCs as these indeed show ACh-dependent depolarization. Unlike others that reported that single AP firing in the presynaptic pyramidal neuron can trigger complex events in the human cortex (Molnár et al., 2008; Szegedi et al., 2017), we did not observe fast lateral inhibition between pyramidal neurons in our recordings. These exciting findings show that mechanisms of delayed lateral inhibition are evolutionary conserved in the human neocortex and are facilitated by ACh through similar mechanisms as in rodent neocortex.

Our results, showing the readiness of Martinotti cells to depolarize upon acetylcholine release to participate in lateral inhibition, could advance our knowledge on how acetylcholine release can affect cortical functioning. During attention behavior and successful responding to cues, acetylcholine levels rise and fall on a time scale of milliseconds to seconds in the prefrontal cortex (Parikh et al., 2017; Teles-Grilo Ruivo et al., 2017). Since Martinotti cells are readily depolarized
by cholinergic basal forebrain inputs, our findings show that the basal forebrain can rapidly highlight inhibitory circuit motifs in the cortical network and steer cortical computation. Indeed, acetylcholine release can control cortical function and information flow (Kawaguchi and Kubota, 1997; Fanselow et al., 2008; Hasselmo and Sarter, 2011; Demars and Morishita, 2014). Several of these effects were traced back to acetylcholine acting on inhibitory interneurons (Beierlein et al., 2000; Fanselow et al., 2008; Arroyo et al., 2012; Lovett-Barron et al., 2014; Chen et al., 2015). In specific, acetylcholine acting on LTS-SOM interneurons is crucially involved in coordinating firing patterns of many cortical neurons over a distance of several hundred microns (Beierlein et al., 2000; Fanselow et al., 2008). The contribution of this pathway on behavior remains to be elucidated in the mPFC, however, a recent evaluation of a similar pathway in the hippocampus shows that acetylcholine can specifically activate LTS-SOM interneurons in CA1 area of the hippocampus to selectively inhibit integration of the excitatory input carrying information to CA1, thereby preventing fear learning (Lovett-Barron et al., 2014). In neocortex, acetylcholine activates a disinhibitory neocortical microcircuit required for fear learning, although here the inhibitory subtype through which these effects are mediated was not uncovered (Letzkus et al., 2011).

In short, our results show that acetylcholine release facilitates lateral inhibition through Martinotti cells, possibly affecting cognitive processes. We show that this effect is mediated by nAChRs on Martinotti cells, ensuring longer, stronger and faster lateral inhibition upon an increased acetylcholine load.

5.2.3 Interaction acetylcholine and adenosine
Adenosine and acetylcholine also interact with each other in the prefrontal cortex. Acetylcholine release in the prefrontal cortex is affected by both adenosine receptors; agonists of A2aR facilitate acetylcholine release, while activating A1R decreased acetylcholine release in vivo in the prefrontal cortex (van Dort et al., 2011). These neuromodulatory systems also interact in conditions where plasticity is induced, such as shown in thalamocortical synapses, where adenosine receptors are crucially involved in acetylcholine-gated forms of plasticity (Chun et al., 2013, Blundon et al., 2011). These interactions might affect how the commonly consumed psychoactive drugs caffeine and nicotine can lead to behavioral effects, such as on attention and arousal. Indeed, caffeine and nicotine are often shown to interact on behavioral outcomes such as arousal (Tanda Goldberg, 2000, Rose and Behm, 1991) and are commonly assumed to interact on the basis of their mutual effect on the dopaminergic system (Tanda and Goldberg, 2000). In our
experiments, we did not address interaction effects of these two neuromodulators. However, for translation of these results to the in vivo situation, one should keep in mind that the influence of adenosine and acetylcholine on the cortical network in the rodent prefrontal cortex and human temporal cortex is likely dependent on each other’s presence or absence.

5.3 Conclusion
Neuromodulatory systems define for a large part the information flow of cortical networks. This thesis is an attempt to shed more light on the role of two prominent neuromodulators on cortical processing in the prefrontal cortex and human cortical areas. To this end, we revealed in chapter 2 the effects of caffeine as a partial adenosine antagonist on excitatory signaling in human pyramidal neurons, and showed that caffeine can mainly target post- and not presynaptic signaling when used in amounts that mimic normal physiological amounts of caffeine intake. In chapter 3, we showed the important role of the facilitatory adenosine receptors A2aR on the induction of excitatory plasticity. Only excitatory plasticity onto FS interneurons was dependent on the activation of A2aR by endogenous adenosine, implicating that disruptions in A2aR expression would affect the balance between excitation and inhibition in the prefrontal cortex. In chapter 4, we showed that acetylcholine enhances disynaptic inhibition through the Martinotti cell in both rodent and human cortex, which has important implications for cortical signaling. Together, these results offer substantial insights into the actions of the neuromodulators adenosine and acetylcholine on cortical signaling, revealing the crucial involvement of neuromodulators on inhibitory cortical signaling.
References
6. References


Chapter 6


References


Cunha RA (2008b) Different cellular sources and different roles of adenosine: A1 receptor-mediated


de Mendonça A, Sebastião AM, Ribeiro JA (1995) Inhibition of NMDA receptor-mediated currents in isolated rat hippocampal neurones by adenosine A1 receptor activation. Neureport 6: 1097-100


Demars MP, Morishita H (2014) Cortical parvalbumin and somatostatin GABA neurons express distinct endogenous modulators of nicotinic acetylcholine receptors. Mol Brain 7:75..


Gentet L (2012) Functional diversity of supragranular GABAergic neurons in the barrel cortex. Front


Haas HL, Greene RW (1984) Adenosine enhances afterhyperpolarization and accommodation in hippocampal pyramidal cells. Pflugers Arch 402: 244-7


Kirk IP, Richardson PJ (1995) Inhibition of striatal GABA release by the adenosine A2A receptor is not mediated by increases in cyclic AMP. J Neurochem 64:2801–2809.


Meunier CNJ, Chameau P, Fossier PM (2017) Modulation of synaptic plasticity in the cortex needs to
understand all the players. Front Synaptic Neurosci 9.


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Poorthuis RB, Bloem B, Verhoog MB, Mansvelder HD (2013b) Layer-Specific Interference with Cholinergic Signaling in the Prefrontal Cortex by Smoking Concentrations of Nicotine. 33:4843–


References


Svenningsson P, Hall H, Sedvall G, Fredholm BB (1997) Distribution of adenosine receptors in the


Chapter 6


Nederlandse samenvatting
7. Nederlandse samenvatting
Het schrijven van een thesis. Wie zichzelf ooit voor een dergelijke taak heeft gesteld, weet dat geduld, aandacht en concentratie belangrijke ingrediënten zijn om dit tot een goed einde te brengen. Vele jaren onderzoek hebben ons geleerd dat deze gedragingen voor een belangrijk gedeelte gebaseerd zijn op de signalen die plaatsvinden in de prefrontale cortex.

De prefrontale cortex is te beschouwen als een knooppunt op de snelweg van verschillende neurotransmitter- en neuromodulatorroutes. Die neurotransmitters en neuromodulatoren zorgen voor een juiste overdracht van signalen. Belangrijk, want zonder de juiste mate aan signalen die op het juiste moment verstuurd worden, zouden gedragingen zoals concentratie en geduld niet mogelijk zijn. Neurotransmitters zorgen in dat geval voor de signaaloverdracht, en de neuromodulatoren verfijnen deze signalen, zodat deze inderdaad op de juiste tijd in de juiste mate aankomen bij de ontvangende hersencel. Omdat de prefrontale cortex zo’n verzamelpunt is voor neurotransmitter- en neuromodulatorroutes, is deze bij uitstek geschikt voor het integreren van informatie om complex cognitief gedrag te reguleren. De twee neuromodulatoren die centraal staan in deze thesis, adenosine en acetylcholine, worden afgegeven in dit hersengebied en spelen hierin een belangrijke rol.

Hoewel de rol van zowel adenosine als acetylcholine op cognitief gedrag al enige tijd bewezen is, zijn de onderliggende mechanismen nog steeds slecht begrepen. Het doel van dit proefschrift is daarom om de werking van deze twee neuromodulatoren en hun korte- en langetermijneffect op hersencelcommunicatie in de cortex beter te begrijpen.

Een deel van deze interesse werd gewekt doordat velen van ons cafeïne gebruiken als dagelijks oppepmiddel. Cafeïne is een antagonist: het blokkeert de werking van adenosine en is op die manier in staat om hersencellen te activeren waar adenosine deze zou deactiveren. We hadden echter nog geen idee hoe cafeïne de communicatie tussen menselijke hersencellen zou beïnvloeden. Om dit proces beter te begrijpen, hebben we de signalen tussen hersencellen in de menselijke cortex gemeten onder invloed van een hoeveelheid cafeïne die normaal in de hersenen zouden voorkomen na het drinken van een sterke kop koffie. We zagen dat cafeïne erg goed in staat is om adenosine te blokkeren, vooral aan de kant van de ontvangende hersencel. Dat is bijzonder, want eerder werd, aan de hand van dieronderzoek, gedacht dat cafeïne vooral effect had op de kant van de
verzendende hersencel. Cafeïne had op zichzelf geen effect, het blokkerende effect werd pas zichtbaar als ook adenosine werd toegediend. Mogelijk verklaren degelijke mechanismen de gematigde werking van cafeïne als oppepmiddel en biedt dit aanwijzingen dat cafeïne enkel zou werken als er al een vermoeidheidssignaal in de hersenen aanwezig is.

Afgezien van het beïnvloeden van hersensignalen op de korte termijn, hebben cafeïne en adenosine ook lange termijn effecten op de hersenen, namelijk bij het opbouwen van contactpunten tussen hersencellen. Hoewel we uit eerder onderzoek wisten dat een bepaalde ontvanger van het adenosinesignaal, de A<sub>2A</sub> receptor, betrokken is bij deze processen en daarmee bijvoorbeeld het verloop van de ziekte van Alzheimer kan beïnvloeden, wisten we nog weinig over de rol van deze receptor in de cortex. Met metingen aan zowel individuele hersencellen als aan groepen van hersencellen konden we aantonen dat de A<sub>2A</sub> receptor heel specifiek invloed heeft op de opbouw van contactpunten naar een bepaald type hersencel, de snel-vurende interneuronen. Dit zijn neuronen die veel en snel actiepotentialen vuren en dus een intensieve communicatie hebben met andere hersencellen. Deze interneuronen zijn betrokken bij werkgeheugen, cognitieve taken en aandacht. De invloed van de A<sub>2A</sub> receptor op de opbouw van contactpunten naar deze snel-vurende interneuronen wijst er dus op dat adenosine aandacht zou kunnen beïnvloeden via deze weg.

De neuromodulator acetylcholine wordt vrijgegeven in de prefrontale cortex bij cognitieve taken, zoals geheugen en aandacht. De vrijgekomen acetylcholine kan een bepaald type hersencel, de laagdrempelig vurende interneuronen, activeren en actiepotentialen in deze neuronen veroorzaken. Deze interneuronen zijn nauw verweven in een netwerk van andere hersencellen. In dit netwerk zorgen zij er voor dat activiteit die één hersencel veroorzaakt in een netwerk van andere hersencellen, geremd wordt, een noodzakelijk mechanisme om er voor te zorgen dat individuele signalen van hersencellen betekenisvol blijven. We veronderstelden dat deze interneuronen nog sterker betrokken zullen raken bij dit proces wanneer ze onder invloed van acetylcholine staan. Inderdaad zagen we dat acetylcholine, door in te werken op deze interneuronen, ervoor kan zorgen dat een hersencel naburige hersencellen sneller, langer en sterker kan afremmen. Dit mechanisme komt zowel in de menselijke hersenen als in muizenhersenen voor en lijkt dus evolutionair bewaard te zijn gebleven.

Samen bieden deze resultaten inzichten in de werking van de neuromodulatoren adenosine en acetylcholine op hersencelcommunicatie.
8. English summary

Writing a thesis. Anyone who has ever set himself up for such a task knows that patience, attention and concentration are important ingredients to bring this to a good end. Many years of research have taught us that these behaviors are for a large part based on the signals that take place in the prefrontal cortex.

The prefrontal cortex can be regarded as a junction on the highway of various neurotransmitter and neuromodulator routes. These neurotransmitters and neuromodulators ensure the correct transmission of signals. Important, because without the right amount of signals that are sent at the right time, behaviors such as concentration and patience would not be possible. Neurotransmitters provide the signal transfer, whereas neuromodulators refine these signals, so that these indeed arrive at the right time and in the right amount at the receiving brain cell. Because the prefrontal cortex is a junction for neurotransmitter and neuromodulator routes, it is ideally suited for integrating information to regulate complex cognitive behavior. The two neuromodulators that are central to this thesis, adenosine and acetylcholine, are released in this brain region and play an important role in these processes.

Although the role of both adenosine and acetylcholine on cognitive behavior has been known for some time, underlying mechanisms are still poorly understood. The aim of this thesis is therefore to better understand the functioning of these two neuromodulators and their short and long-term effect on brain cell communication in the cortex.

Part of this interest was raised because many of us use caffeine to give a boost to the day. Caffeine is an antagonist: it blocks the action of adenosine and is thus able to activate brain cells where adenosine would de-activate them. However, we had no idea how caffeine would affect communication between human brain cells. To better understand this process, we measured the signals between brain cells in the human cortex under the influence of an amount of caffeine that would normally occur in the brain after drinking a strong cup of coffee. We saw that caffeine is very well able to block adenosine, especially on the side of the receiving brain cell (postsynaptically). This is special, because previously, on the basis of animal research, caffeine was thought to have an effect on the side of the sending brain cell (presynaptically). Caffeine in itself had no effect, the effect only became visible when adenosine was also administered. Possibly these mechanisms may explain
the moderate effect of caffeine as a stimulant and provide indications that caffeine will only work if there is already a fatigue signal in the brain.

Apart from influencing brain signals in the short term, caffeine and adenosine also have long-term effects on the brain, namely in building contact points between brain cells. Although we knew from previous research that a certain recipient of the adenosine signal, the $A_{2A}$ receptor, is involved in these processes and can, for example, influence the course of Alzheimer's disease, we knew little about the role of this receptor in the cortex. Through recordings on both individual brain cells and groups of brain cells we were able to demonstrate that the $A_{2A}$ receptor has a very specific influence on the build-up of contact points to a specific type of brain cell, the fast-spiking interneurons. These are neurons that fire many fast action potentials and thus have intensive communication with other brain cells. These interneurons are involved in working memory, cognitive tasks and attention. The influence of the $A_{2A}$ receptor on the build-up of contact points to these fast-spiking interneurons thus indicates that adenosine could influence attention through this mechanism, although follow-up research is needed to elucidate that.

The other neuromodulator acetylcholine is released in the prefrontal cortex in cognitive tasks, such as memory and attention. The released acetylcholine can activate a certain type of brain cells, the low-threshold spiking interneurons, and trigger action potentials in these neurons. These interneurons are closely intertwined in a network of other brain cells. In this network they ensure that the communication of one brain cell that causes activity in a network of brain cells is inhibited, a necessary mechanism to ensure that individual signals from brain cells remain sparse and meaningful. We assumed that these interneurons would become even more involved in this process if they were under the influence of acetylcholine. Indeed, we saw that acetylcholine, by acting on these interneurons, can ensure that a brain cell can slow down neighboring brain cells faster, longer and stronger. This mechanism is evolutionary conserved, as it existed both in rodent as well as in human brain.

Together, these results offer substantial insights into the actions of the neuromodulators adenosine and acetylcholine on cortical signaling.
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