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Effects of Opioids on Neuronal Nicotinic Acetylcholine Receptors

Reeta Talka

ACADEMIC DISSERTATION

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ABSTRACT

Tobacco use is the leading cause of preventable death worldwide. Nicotine is the primary addictive component of tobacco, and repeated nicotine exposure often leads to dependence in humans. Nicotine is one of the most commonly co-used substances among polysubstance abuse patients and combined use of nicotine and other drugs of abuse, such as opioids, increases the use of one or both substances. The health consequences associated with polysubstance abuse exceed those of either drug alone. The current pharmacotherapeutic options are ineffective among opioid-substituted patients and the levels of successful smoking cessation are low. At the cellular level, nicotine and opioids have their own molecular mechanisms of action, yet both drugs increase the activity of the reward pathway by increasing dopamine (DA) transmission.

The purpose of these studies was to investigate the possible effects of different opioid ligands on human neuronal nicotinic acetylcholine receptors (nAChRs) expressed in cell cultures. In the first part, the effects of morphine on nAChRs was investigated with ligand-binding and functional studies, and in the second part the effects of methadone were studied with similar methods. Since these opioids showed effects on nAChRs, the next step was to study the effect of prolonged drug treatments on nAChR numbers and function because the nAChRs are known to be upregulated by chronic nicotine exposure. Additionally, the effects of other opioid ligands, buprenorphine, codeine, oxycodone, tramadol, naloxone and naltrexone were also studied.

Our results showed that morphine has a partial agonist effect at α4β2 nAChRs, a very weak antagonist effect at α3* nAChRs (where * denotes other nAChR subunits that may not have been identified) and a positive synergistic effect with nicotine on α7 nAChR function. We found that methadone acts as a non-competitive antagonist (NCA) at α4β2 and α3* nAChRs. We also confirmed that methadone is a human α7 nAChR agonist. In the prolonged studies with methadone and morphine, we found that human α3*, α4β2 and α7 nAChRs are differentially regulated by prolonged exposure to methadone and morphine. Methadone and morphine up-regulate α3* and α7 nAChRs, whereas α4β2 nAChRs are down-regulated. Methadone-induced up-regulation of α3* nAChRs has no effect on the function of cell surface receptors, while methadone and morphine-induced down-regulation of α4β2 nAChRs changes the function of receptors on the cell surface. Buprenorphine was shown to be a weak antagonist at α4β2, α3*, and α7 nAChRs, and codeine had a positive modulatory effect on α4β2 nAChRs and a weak NCA effect on α3* nAChRs. Oxycodone seemed to have a mixed competitive/non-competitive effect on α4β2 nAChRs and a weak NCA effect on α3* nAChRs. Tramadol was shown to be a NCA of α3* nAChRs and a weak NCA of α4β2 nAChRs. Naltrexone and naloxone were mixed competitive/non-competitive antagonists of α4β2 nAChRs, weak NCAs of α3* nAChRs and weak antagonists of α7 nAChRs.

Taken together, these studies showed that many opioid ligands have effects on nAChRs that are independent of their agonist or antagonist properties at opioid receptors. These findings suggest that some effects of the nicotine–opioid interaction seen in humans can be partially mediated through the receptor-level interplay of these substances. These results, together with earlier findings, highlight the
complexity of different nAChRs and the multiplicity of responses to opioid ligands. This variability should be taken into account when designing treatments for polysubstance dependence.
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:


II Talka R, Salminen O, Tuominen RK. Methadone is a non-competitive antagonist at the α4β2 and α3* nicotinic acetylcholine receptors and an agonist at the α7 nicotinic acetylcholine receptor. Basic Clin Pharmacol Toxicol. 116:321-8, 2015

III Talka R, Tuominen RK, Salminen O. Methadone's effect on nAChRs -a link between methadone use and smoking? Biochem Pharmacol. 97:542-9, 2015

IV Talka R, Tuominen RK, Salminen O. Effects of prolonged methadone and morphine treatment on nAChR function and receptor number in cell lines expressing α7, α4β2 and α3* nAChRs. Manuscript

The publications are referred to in the text by their roman numerals. Reprints were made with permission from the copyright holders.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>α-Bgtx</td>
<td>α-bungarotoxin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>$B_{\text{max}}$</td>
<td>maximum specific binding</td>
</tr>
<tr>
<td>$[\text{Ca}^{2+}]_i$</td>
<td>concentration of free intracellular calcium</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CPP</td>
<td>conditioned place preference</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>concentration activating 50% of maximum</td>
</tr>
<tr>
<td>$[^3\text{H}]$EPI</td>
<td>$[^3\text{H}]$epibatidine</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein–coupled receptor</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>concentration inhibiting 50% of maximum</td>
</tr>
<tr>
<td>$K_d$</td>
<td>equilibrium binding constant</td>
</tr>
<tr>
<td>$K_i$</td>
<td>equilibrium dissociation constant</td>
</tr>
<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
</tr>
<tr>
<td>Meca</td>
<td>mecamylamine</td>
</tr>
<tr>
<td>MHb</td>
<td>medial habenula</td>
</tr>
<tr>
<td>MLA</td>
<td>methyllycaconitine</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NAc</td>
<td>nucleus accumbens</td>
</tr>
<tr>
<td>nAChR</td>
<td>neuronal nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>NAM</td>
<td>negative allosteric modulator</td>
</tr>
<tr>
<td>NCA</td>
<td>non-competitive antagonist</td>
</tr>
<tr>
<td>PAM</td>
<td>positive allosteric modulator</td>
</tr>
<tr>
<td>$^{86}\text{Rb}^+$</td>
<td>rubidium-86 isotope</td>
</tr>
<tr>
<td>SAM</td>
<td>silent allosteric modulator</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>TSS</td>
<td>Tyrode’s salt solution</td>
</tr>
<tr>
<td>VOCCs</td>
<td>voltage-operated Ca$^{2+}$ channels</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
</tr>
</tbody>
</table>
Introduction

1. INTRODUCTION

Tobacco use is a worldwide public health problem and is the leading cause of preventable death. The vast majority of lung cancer deaths are caused by tobacco smoking (Islami et al. 2015). In Finland, smoking caused approximately 4 300–4 500 deaths in 2012 (Jääskeläinen and Virtanen 2015). Nicotine is the primary addictive component in tobacco, and repeated nicotine exposure often leads to dependence in humans. In addition to the problem of nicotine dependence in the general population, tobacco smoking among individuals with substance-use disorders presents another public health concern. Nicotine is one of the most commonly co-used substances among polysubstance abuse patients (McClure et al. 2014; Goodwin et al. 2014). Clinical and epidemiological studies suggest that nicotine, when combined with other drugs of abuse, increases consumption of one or both substances (Mello et al. 1980; Henningfield and Griffiths 1981; Chait and Griffiths 1984; Higgins et al. 1994). The health consequences associated with polysubstance abuse exceed those of either drug alone and the mortality rate of polysubstance users is significantly increased due to tobacco-related causes (Hurt et al. 1996).

Almost all methadone-maintained patients are tobacco smokers (Nahvi et al. 2006; Richter et al. 2007; Elkader et al. 2009; Pajusco et al. 2012; Chisolm et al. 2013). In addition to methadone, the use of other opioid agonists, such as buprenorphine and heroin, also increase smoking prevalence (Mello et al. 1980; Mello et al. 1985; Mutschler et al. 2002; Pajusco et al. 2012). Furthermore, smokers are more likely to be abusers of prescription opioids (Zale et al. 2015). Nicotine use increases methadone self-administration and reinforcing properties, and smoking combined with methadone use also increases subjective ratings of smoking satisfaction (Chait and Griffiths 1984; Spiga et al. 1998). Although smoking cessation is more difficult for maintenance patients than for the general population, quitting smoking also improves opioid abstinence, thus, effective treatment for tobacco dependence is particularly beneficial among these patients (Lemon et al. 2003).

At the cellular level, nicotine and opioids both have their own molecular mechanisms of action. Nicotine interacts directly with neuronal nicotinic acetylcholine receptors (nAChRs), which are pentameric ligand-gated ion channels. Opioid agonists act through G protein-coupled opioid receptors. A commonality among all addictive drugs is their ability to increase extracellular DA levels in the nucleus accumbens (NAc) (Nestler 2005; Ross and Peselow 2009). Both nicotine and opioids increase DA by indirect mechanisms that affect DA cell firing. The activation of μ-opioid receptors increases DA transmission by inhibiting γ-aminobutyric acid-ergic (GABAergic) interneurons that normally provide tonic inhibition to dopaminergic neurons in the ventral tegmental area (VTA) (Kosten and George 2002). Nicotine binds to the nAChRs located on the dopaminergic cell bodies and on GABAergic and glutamatergic neurons in the VTA (Pidoplichko et al. 1997; Mansvelder and McGehee 2002), which causes a shift from tonic firing of dopaminergic neurons to burst firing, resulting in an increase in DA levels in the NAc and the prefrontal cortex (Rao et al. 2003; Rice and Cragg 2004). Furthermore, the endogenous opioid system influences nicotine reward and antinociception. The levels of endogenous opioid peptides, such as enkephalins and β-endorphin, are increased following nicotine administration (Dhatt et al. 1995). These endogenous peptides bind to μ-opioid receptors located on the GABA interneurons in the VTA, which further increases DA release in the NAc by disinhibiting the GABAergic interneurons (Davenport et al. 1990; Bergevin et al. 2002). Nicotine may thus potentiate opioid-induced antinociception via nAChR activation.
Introduction

The acute rewarding aspects of drug use and conditioned learning associated with craving and relapse seem to be mediated by the mesolimbic dopaminergic pathway, whereas adaptations in the mesocortical and corticofugal glutamatergic pathways play a role in the loss of inhibitory control and continued drug seeking behaviors (Feltenstein and See 2008). Other brain regions and systems, such as the insula, thalamus and cerebellum and stress-related brain systems, also have modulatory functions in the development and maintenance of drug dependence (Baler and Volkow 2006; Zorrilla et al. 2014; Korpi et al. 2015). Since the mechanisms, brain regions and systems involved in polysubstance drug dependence are greatly diverse and complex, it is no wonder that effective pharmacological treatments are challenging to develop. The ideal pharmacotherapeutic treatment should be able to treat withdrawal symptoms, induce abstinence, reduce substance use and prevent relapse.
2. REVIEW OF THE LITERATURE

2.1 Neuronal nicotinic acetylcholine receptors

2.1.1 Structure and classification

The nAChRs belong to the cys-loop receptor superfamily of ligand-gated ion channel receptors, which includes GABA\textsubscript{A}, glycine, and 5-HT\textsubscript{3} (serotonin) receptors and the muscle-type nicotinic receptors (Miller and Smart 2010). They are often referred as the cys-loop receptors since they share a similar topology with a disulphide-bridged cys-loop in the extracellular domain. Mammalian nAChRs are composed of five subunits arranged around a pore filled with water (Fig. 1) (Dani 2015; Fasoli and Gotti 2015). Each subunit is composed of a long extracellular N-terminal domain, where the ligand binding site is located, followed by four hydrophobic transmembrane regions (M1–M4). Between transmembrane regions M3 and M4 there is a large intracellular loop, where the phosphorylation sites for proto-oncogene tyrosine-protein kinase Src family kinases are (Charpantier et al. 2005). The M4 region ends with a short extracellular C-terminus.

Figure 1. The structure of α7, α3β4 and α4β2 nAChRs. The nAChRs are pentameric ion channel receptors with long extracellular N-terminal domains, followed by four transmembrane regions (M1–M4). The M4 region ends with a short extracellular C-terminus. The ionic pore of nAChRs is mainly lined by the M2 transmembrane segment. The heteromeric receptors have two orthosteric ligand binding sites at the extracellular N-terminal domain, whereas the homomeric receptors have five binding sites.

The ionic pore of nAChRs is mainly lined by the M2 transmembrane segment with some contribution from the M1 segment (Hucho et al. 1986; Karlin 2002). The ionic pore contains amino acid residues important for the ion selectivity, permeability, and channel gating of nAChRs. The other transmembrane segments (M1, M3 and M4) separate the M2 segment from the hydrophobic cell membrane (Papke 2014). The intracellular loop between M3 and M4 transmembrane segments, which holds the phosphorylation sites for Src family kinases, is the most variable element in nAChRs and has a profound
Review of the Literature

influence upon receptor assembly, targeting and ion channel properties (Charpantier et al. 2005; Kracun et al. 2008; Pollock et al. 2009).

The extracellular N-terminal domain contains the orthosteric agonist-binding site in nAChRs (Brejc et al. 2001; Bartos et al. 2009). The orthosteric binding site is composed of many amino acid residues grouped into loops A, B, and C (the principal component) and D, E, and F (the complementary component) (Fig. 2) (Changeux and Taly 2008). The agonists bind to a hydrophobic pocket formed by loops A, B, D, and F, after which loop C closes this binding pocket. When antagonists bind, loop C stays in the open conformation.

![Figure 2. Structure of the acetylcholine (ACh)-binding site. Schematic representation of the ACh-binding site illustrating the loops A-C (the principal component) and loops D-F (the complementary component). Upon agonist binding, loop C closes the binding pocket formed by loops A, B, D and F.](image)

The mammalian nAChR subunits are divided into alpha (α2–α7, α9, and α10) (the α8 nAChRs are only found in avian species) and beta (β2–β4) subunits based on the presence of adjacent cysteine groups in the extracellular domain of only the α subunits (Gotti et al. 2009). These subunits form either homo- or heteromeric pentameric receptors; the great diversity of different combinations also makes the functionality diverse. Two main classes of nAChR subtypes have been identified: α-bungarotoxin (α-Bgtx)-sensitive and -insensitive receptors. The α-Bgtx-sensitive receptors are homomeric or heteromeric receptors made up of α7, α9 and/or α10 subunits, and they bind α-Bgtx with high affinity. The α-Bgtx-insensitive receptors are heteromeric receptors formed by combinations of α (α2–α6) and β (β2–β4) subunits, which do not bind α-Bgtx. The α-Bgtx-insensitive receptors have a higher affinity for nicotine and other nicotinic agonists than the α-Bgtx-sensitive receptors (Gotti and Clementi 2004).

The homomeric α7 and α9 nAChRs have five identical binding sites to which the same subunit contributes both the principal and complementary component (Fig. 1) (Zoli et al. 2015). The heteromeric nAChRs have two agonist binding sites at the interface between two adjacent, asymmetric subunits. The principal component of the binding site in heteromeric nAChRs is formed by the α2, α3, α4, α6, α7, or α9 subunits and the complementary site is formed by the β2, β4, α7, α9, or α10 subunits (Figs. 1 and 2). The α5 subunit is not a true α subunit since it only forms functional receptors when co-expressed with a principal and complementary subunit. The α10 subunit cannot act as a principal subunit at the agonist binding
site, and only functions when it is associated with the \(\alpha 9\) subunit (Sgard et al. 2002). The \(\beta 3\) subunit is also an accessory subunit since it needs to be co-expressed with a principal and complementary subunit. The presence of accessory subunits has an effect on the pharmacological and functional properties of nAChRs.

In addition to different subunit combinations, subunit stoichiometries also have an effect on nAChR function. The \(\alpha 4\beta 2\) and \(\alpha 3\beta 4\) nAChR subtypes are example of receptors that can exist in two different stoichiometric arrangements: \((\alpha 4)_2(\beta 2)_3\) or \((\alpha 4)_3(\beta 2)_2\), and \((\alpha 3)_2(\beta 4)_3\) or \((\alpha 3)_3(\beta 4)_2\) (Moroni et al. 2006; Krashia et al. 2010). The different stoichiometric combinations can have variable agonist sensitivities and \(\text{Ca}^{2+}\) permeabilities (Tapia et al. 2007). The \(\alpha 4\beta 2\) subtype exhibits biphasic agonist concentration–response curves since the \((\alpha 4)_2(\beta 2)_3\) combination has a higher sensitivity for agonists, such as nicotine and ACh, than the \((\alpha 4)_3(\beta 2)_2\) stoichiometry (Zwart and Vijverberg 1998; Nelson et al. 2003; Marks et al. 2010). Chronic nicotine exposure favors increased assembly of the high-sensitivity \((\alpha 4)_2(\beta 2)_3\) stoichiometry (Moroni et al. 2006; Fasoli et al. 2016; Fasoli et al. 2016). The two stoichiometries of the \(\alpha 3\beta 4\) subtype exhibit similar agonist sensitivities, but only the subtype with two \(\alpha\) subunits is susceptible to enhancement by low \(\text{Zn}^{2+}\) concentrations (Krashia et al. 2010). The two stoichiometries also exhibit substantially different channel conductance and kinetics.

2.1.2 Distribution and localization

The nAChRs are widely and unevenly distributed in the brain and, in most cases, they have presynaptic or preterminal localization, but some are also located post-synthetically in somatodendritic synapses (Fig. 3) (Jones et al. 1999). The nAChRs operate in the brain by multiple mechanisms, yet the most well-studied process is the modulation of neurotransmitter release by presynaptic nAChRs (Gray et al. 1996; Role and Berg 1996; Albuquerque et al. 1997; Wonnacott 1997; Dani and Bertrand 2007). When presynaptic nAChRs are activated, the level of intracellular calcium rises, which enhances neurotransmitter release (Vijayaraghavan et al. 1992; Vernino et al. 1992; Rathouz and Berg 1994; Engelman and MacDermott 2004). Axonal and preterminal nAChRs modulate neuron excitability and neurotransmitter release indirectly by activating voltage-operated \(\text{Ca}^{2+}\) channels (VOCCs) and initiating action potentials (Lena et al. 1993; Albuquerque et al. 2000). Somatodendritic nAChRs modulate plasticity and information flow by initiating or modulating synaptic inputs to the cell body (Pidoplichko et al. 2013).
Review of the Literature

**Figure 3. Different localization of nAChR subtypes at synaptic sites.** A) Postsynaptic nAChRs bind ACh released from the presynaptic terminal. This type of nicotinic cholinergic synaptic transmission is fast and direct. B) The presynaptic nAChRs can influence the release of synaptic vesicles. Presynaptic nAChRs initiate a direct and indirect increase of calcium in the presynaptic terminal, which enhances the release of the neurotransmitter. C) The preterminal or axonal nAChRs are located in a position along the axon where they can influence the excitability of the axon.

The α4* nAChRs bind radiolabeled nicotine with the highest affinity and the α4 subunit is predominantly expressed with the β2 subunit in the vertebrate brain (Flores et al. 1992; Clementi et al. 2000). The α4β2 nAChRs are implicated in nicotine self-administration, reward and dependence, and in Alzheimer’s disease and epilepsy (Picciotto et al. 1998; Tapper et al. 2004; Steinlein and Bertrand 2010; Jurgensen and Ferreira 2010). This subtype is widely expressed in the mammalian brain and in specific subregions such as the cerebral cortex, striatum, superior colliculus, nucleus geniculatus lateralis and cerebellum (Zoli et al. 2002; Turner and Kellar 2005; Gotti et al. 2005). In addition to the β2 subunit, the α4 subunit can also assemble with other subunits, such as β4 or α5 subunits (Kuryatov et al. 2008; Hamouda et al. 2009). The α4β2 subtype is expressed in both dopaminergic and non-dopaminergic cells in the striatum, whereas the α4α5β2 subtype is localized in dopaminergic terminals (Zoli et al. 2002).

The α7 nAChRs are highly expressed in the brain (e.g., in the cortex, hippocampus and subcortical limbic regions) and have presynaptic, postsynaptic or somatic localization (Picciotto et al. 2001; Jones and Wonnacott 2004). The localization of α7 nAChRs can be readily studied with the α7-specific ligand, α-Bgtx. The channel kinetics of α7 nAChRs are rapid and they are highly permeable to calcium. In addition to the homomeric α7 nAChRs, emerging evidence demonstrates the existence of heteromeric α7 nAChRs, in which α7 subunits are co-assembled with β2 subunits to form a novel type of α7β2 nAChR (Wu et al. 2016). The α7β2 nAChRs have been found in rodents as well as in human basal forebrain neurons and cerebral cortical neurons (Moretti et al. 2014; Thomsen et al. 2015). Compared to the homomeric α7 nAChRs, α7β2 nAChRs have slower whole-cell current amplitudes and decay kinetics (Liu et al. 2009; Liu et al. 2012; Zwart et al. 2014).

The α3* nAChRs are expressed in multiple brain areas such as the pineal gland, medial habenula (MHB), dorsal nucleus of the vagus nerve, anterior thalamus, dopaminergic ventral midbrain, locus coeruleus, and retinal ganglionic neurons (Zoli et al. 1998; Lena et al. 1999; Whiteaker et al. 2002). α3β2* nAChRs are found in the visual pathway of the retina, superior colliculus and nucleus geniculatus lateralis (Gotti
et al. 2005), whereas α3β4* nAChRs are localized in the pineal gland, cerebellum, retina, hippocampus and the habenulo-interpeduncular pathway (Luo et al. 1998; Grady et al. 2001; Hernandez et al. 2004; Turner and Kellar 2005).

Distribution of the α6 subunit in the central nervous system (CNS) is limited. α6* nAChRs are highly expressed in regions such as the substantia nigra, VTA, locus coeruleus, retina, interpeduncular nucleus and MHB, where it often co-localizes with the β3 subunit (Le Novere et al. 1996; Champtiaux et al. 2003). The α6 subunit co-assembles with the β2 subunit in the striatum and retina (Champtiaux et al. 2002; Gotti et al. 2007). The two major α6* subtypes in rodent striatum and retina are the α6α4β2β3 and α6β2β3 nAChRs, which have different binding affinities and sensitivities to α-conotoxin MII and methyllycaconitine (MLA) (Zoli et al. 2002; Champtiaux et al. 2003).

α2* nAChRs are expressed in small amounts in different brain regions (e.g., in the interpeduncular nucleus, putamen, globus pallidus, motor and somatosensory cortex and thalamus) (Wada et al. 1989). Expression of the α5 subunit is also restricted, with the highest expression in the substantia nigra, VTA and MHB (Picciotto et al. 2001).

2.1.3 Functionality of nAChRs

The subunit composition of each nAChR subtype determines the functional characteristics of the channel. Depending on the subunit composition, each subtype has unique channel kinetics, ion conductance and ion selectivity (Albuquerque et al. 2009). Channel gating is a reversible process that leads to channel opening or closure after a ligand has been bound to the receptor. The gate is located at the ion channel pore where the M2 segment provides the amino acid residues necessary for channel opening and closure. In the closed conformation, the hydrophobic residues located in the middle of the channel approach each other to narrow the channel. Ion flow in a closed state is prevented by the hydrophobic environment, which is energetically unfavorable for ion permeation (Unwin and Fujiyoshi 2012). The channel opens upon agonist binding by concerted tilting of the M2 helices, the M2–M3 loop, and the M3 segment, which increases the diameter of the pore near the middle of the membrane (Taly et al. 2009). The agonist binding region is linked to the channel through a series of interacting residues which transmit the conformational change of the receptor upon ligand binding (Lee and Sine 2005; Lee et al. 2009).

nAChRs are permeable to small mono- and divalent cations that are small enough to fit through the channel in the open conformation (Dani and Eisenman 1987; Albuquerque et al. 2009). Sodium and potassium ions contribute to the majority of the ion current, but some nAChR subtypes, such as the homomeric α7 nAChRs, are especially permeable to calcium (Fucile et al. 2003; Fucile 2004). Binding of an agonist stabilizes the open conformation of the nAChR, so that small cations flow through the channel for several milliseconds before the channel closes by going back to a resting state or a desensitized state that is unresponsive to agonists. The cation flow causes depolarization of the cell.

The Ca2+ influx through nAChRs modulates several Ca2+-dependent cellular processes, such as neurotransmitter release, synaptic plasticity and cell motility. The subunit composition of nAChRs influences their Ca2+ permeability. Heteromeric nAChRs have a fractional Ca2+ current of 2–5%, whereas with homomeric α7 nAChRs the fractional Ca2+ current ranges from 6% to 12% in vitro (Fucile et al. 2003;
The incorporation of accessory subunits may change the Ca\textsuperscript{2+} permeability of nAChRs. For instance, the Ca\textsuperscript{2+} permeability is increased if the α5 subunit is coassembled with α3 subunit (Gerzanich et al. 1998). The flow of Ca\textsuperscript{2+} through nAChRs causes an increase in intracellular calcium levels. In addition to this direct flow of ions, VOCCs are also activated by depolarization, which augments the primary Ca\textsuperscript{2+} signals (Fig. 4) (Dajas-Bailador et al. 2002). Furthermore, Ca\textsuperscript{2+} is released from the intracellular stores which creates long-lasting Ca\textsuperscript{2+} signals (Brain et al. 2001). The Ca\textsuperscript{2+} release from ER is regulated by inositol-1,4,5-trisphosphate receptors (IP\textsubscript{3}R), ryanodine receptors (RyR) and sarco-/endoplasmic reticulum calcium ATPase (SERCA) (Dajas-Bailador et al. 2002; Stutzmann and Mattson 2011).

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**Figure 4. Calcium homeostasis in a neuronal cell.** Sources of calcium influx include the glutamate-type receptors (N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors), neuronal nicotinic acetylcholine receptors (nAChRs), voltage-operated calcium channels (VOCCs) and conventional transient receptor potential (TRPC) channels. Calcium efflux is mediated by plasma membrane calcium ATPase (PMCA) and sodium-calcium exchanger (NCX). Intracellular calcium levels are also regulated by mitochondria, calcium binding proteins, and the endoplasmic reticulum (ER), where the calcium homeostasis is regulated by inositol-1,4,5-trisphosphate receptors (IP\textsubscript{3}R), ryanodine receptors (RyR) and sarco-/endoplasmic reticulum calcium ATPase (SERCA).

The nAChRs have three basic conformational states: rest, open, and desensitized (Fig. 5) (Katz and Thesleff 1957; Changeux et al. 1984). In the resting state, the channel is closed until the conformation – upon agonist binding – changes to the open state, allowing ions to flow through the channel. Upon prolonged exposure to an agonist, the receptor conformation changes to a desensitized state, where the channel is closed. The desensitized receptor has a higher affinity for agonists than the resting or open conformations, and thus slow application of low agonist concentrations can cause desensitization.
without activation. The kinetics of transitions between different conformational states depends, among other things, on receptor subtype. Therefore, the same agonist may cause different conformational responses depending on the nAChR subtype. For instance, agonist concentrations needed for α4α5β2 nAChR desensitization are, on average, 8-fold higher than what is needed for desensitization of the α4β2 subtype (Wageman et al. 2014). The kinetics of conformational changes are also ligand-dependent. The onset and recovery from desensitization, for example, depend on both duration of ligand exposure and ligand concentration. Long exposures to low concentrations of agonists cause “deeper”, longer-lasting levels of desensitization (Lester and Dani 1994; Dani and Heinemann 1996).

**Figure 5. The basic conformational states of nAChRs: rest, open, intermediate and desensitized states.** In the resting state, the channel is closed and affinity for antagonists is high. In the open state, the channel is open and affinity is low for agonists and there is little if any affinity for antagonists. In the desensitized state, the channel is closed and affinity for agonists and antagonists is high. Agonists, such as ACh or nicotine, stabilize the open conformation which transiently lets through small cations for several milliseconds before closing upon switch to the resting state or the desensitized state that is unresponsive to agonists. Prolonged exposure to agonists, such as nicotine, produces significant desensitization of nAChRs to the unresponsive closed state. The intermediate state is a conformation between ligand binding and channel opening and between open state and full desensitization. Modified from Changeux et al., (1984).

### 2.1.4 Regulation of nAChR numbers

Chronic nicotine exposure paradoxically increases the number of nAChRs, whereas long-lasting repeated agonist stimulation usually leads to a reduction in the number of receptors (Creese and Sibley 1981; Wonnacott 1990). nAChRs are upregulated in the brain of tobacco smokers, which has been detected by [3H]nicotine binding in postmortem brains of smokers and by single-photon emission computed tomography (SPECT) and positron emission tomography (PET) imaging (Benwell et al. 1988; Breese et al. 1997; Perry et al. 1999; Mamede et al. 2007; Mukhin et al. 2008; Wullner et al. 2008; Brody et al. 2013; Jasinska et al. 2014). Furthermore, chronic nicotine has been shown to cause upregulation of nAChRs in numerous *in vitro* and *in vivo* experiments (Marks et al. 1983; Schwartz and Kellar 1983; Marks et al.
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Nicotine-induced upregulation is brain region-specific; upregulation is found in the brain stem, cerebellum, prefrontal cortex, and corpus callosum, but not in the thalamus (Pauly et al. 1991; Marks et al. 1992; Nguyen et al. 2003; Mukhin et al. 2008; Brody et al. 2013).

In addition to region specificity, upregulation is also nAChR subtype-specific. α4β2 nAChRs have been shown to be upregulated both by low (≤100 nM) and high (>1 μM) nicotine concentrations (Peng et al. 1994; Srinivasan et al. 2011); the high-sensitivity (α4)(β2)3 stoichiometry is preferentially upregulated by nicotine (Fasoli et al. 2016). The concentration activating 50% of maximum (EC50) for the nicotine-induced upregulation of α4β2 nAChRs varies between tens to hundreds of nM, depending on the assay type (Peng et al. 1994). The steady state plasma concentration of nicotine in the blood of smokers is approximately 150–200 nM (Benowitz 1990; Schneider et al. 2001), and the calculated typical concentrations of free brain nicotine after one cigarette are 265 nM after overnight abstinence and 465 nM during afternoon smoking (Rose et al. 2010). Therefore, it is likely, that the α4β2 nAChRs are upregulated by smoking, which is also supported by postmortem human studies (Benwell et al. 1988; Breese et al. 1997; Perry et al. 1999). α3β4* nAChRs on the other hand are not believed to be upregulated at nicotine concentrations achieved by smoking, as they require much higher (≥10 μM) nicotine concentrations for upregulation (Peng et al. 1997; Mazzo et al. 2013). α3β2 nAChRs are more sensitive to nicotine-induced upregulation than α3β4 nAChRs (Walsh et al. 2008). Similar to α4β2 nAChRs, α3β4 nAChRs also exist in two stoichiometries and the (α3)(β4)3 stoichiometry is preferentially upregulated by chronic nicotine exposure (Mazzo et al. 2013). α7 nAChRs are upregulated only by high concentrations of nicotine, not achieved by moderate smoking, and the magnitude of upregulation is smaller than for α6* and α4* nAChRs (Peng et al. 1997). The regulation of α6* nAChRs depends on the subunit composition: α4α6β2* nAChRs are not upregulated by nicotine, whereas α6(non-α4)β2* are upregulated by high nicotine concentrations (Tumkosit et al. 2006; Perez et al. 2008; Walsh et al. 2008). α6(non-β3)* nAChRs in the striatum are downregulated by nicotine, whereas those containing β3 are unaffected (Perry et al. 2007).

The nicotine-induced upregulation of nAChRs is concentration-dependent in humans (Breese et al. 1997; Perry et al. 1999), in vivo (Rowell and Li 1997) and in vitro (Gopalakrishnan et al. 1997; Govind et al. 2012). Heavy smokers (more than 14 cigarettes a day) have 25-330% higher nAChR levels compared to non-smokers. The upregulation of nAChRs is reversible: smokers, who have been abstinent more than 3 weeks, have nAChR levels similar to non-smokers (Breese et al. 1997; Mamede et al. 2007). In in vivo animal models nAChR levels return to normal after 8–14 days of withdrawal, depending on assay type (Marks et al. 1983; Collins et al. 1990; Fasoli et al. 2016). Maximal upregulation levels are reached in 1-14 days in vitro (Peng et al. 1994; Walsh et al. 2008; Srinivasan et al. 2011) and in 10-14 days in vivo (Marks et al. 1983; Nashmi et al. 2007).

Ion flow through nAChRs or receptor activation are not necessary for nAChR upregulation since both NCAs, such as mecamylamine (Meca), and competitive antagonists, such as dihydro-β-erythroidine, upregulate nAChRs (Peng et al. 1994; Kishi and Steinbach 2006). Meca also has an additive effect on upregulation, when administered with nicotine (Peng et al. 1994). Furthermore, upregulation without activation of nAChRs has been shown by using loss-of-function-mutated receptors which allow nicotinic ligands to bind without opening the channel (Kuryatov et al. 2005). Nevertheless, nicotinic agonists are more potent pharmacological chaperones than antagonists, thus inducing upregulation more readily.
since the assembly of activated or desensitized conformations is more efficient than the closed conformation. Although nAChR activation is not a requirement for upregulation (Peng et al. 1994), the binding of a nicotinic ligand is necessary. If ligand binding to nAChRs is impaired using mutated nAChRs, the upregulation of α4β2 nAChRs is diminished or completely abolished (Kishi and Steinbach 2006).

The nicotine-induced upregulation of nAChRs is independent of transcriptional events since the messenger ribonucleic acid (mRNA) levels are unchanged in response to nicotine (Marks et al. 1983; Marks et al. 1992; Peng et al. 1994; Bencherif et al. 1995). Several posttranslational mechanisms have been proposed to contribute to nicotine-induced upregulation, such as nAChR trafficking to the cell surface, reduced receptor turnover, nAChR subunit maturation and assembly in the ER, inhibition of subunit degradation in the ER, changes in subunit stoichiometry and nAChR conformational changes (Fig. 6) (Peng et al. 1994; Harkness and Millar 2002; Nashmi et al. 2003; Nelson et al. 2003; Darsow et al. 2005; Sallette et al. 2005; Kuryatov et al. 2005; Ficklin et al. 2005; Vallejo et al. 2005; Moroni et al. 2006; Rezvani et al. 2007).

### Figure 6. The suggested mechanisms for nAChR upregulation

The major models of nicotine-induced nAChR upregulation are: 1) increased nAChR trafficking to the cell surface, 2) reduced receptor turnover, 3) increased nAChR subunit maturation and assembly in the ER/Golgi, 4) inhibition of subunit degradation in the ER, 5) changes in subunit stoichiometry and 6) nAChR conformational changes.

One of the mechanisms suggested to contribute to the nicotine-induced upregulation is increased receptor trafficking to the cell surface (Harkness and Millar 2002). The nAChRs are transported from the ER/Golgi to the plasma membrane through the secretory pathway, which is tightly controlled so that only fully assembled receptors eventually reach the cell surface (Wang et al. 2002). In order to be transported from the ER, the nAChRs must be correctly folded into pentamers and recruited at the ER-exit sites. The correct assembly of the five subunits buries a motif responsible for the retention of unassembled subunits, thus, only assembled pentamers are allowed to be delivered to the membrane. When the
secretory pathway from the Golgi apparatus is blocked with brefeldin A, nicotine-induced upregulation of surface α4β2 nAChRs is blocked, whereas upregulation of the total binding sites is not inhibited (Darsow et al. 2005). This indicates that the upregulation of intracellular nAChRs is independent of the transport mechanism through the secretory pathway, but this pathway is necessary for the upregulation of surface nAChRs.

The reduced turnover of cell-surface receptors has been suggested to contribute to the nicotine-induced upregulation of nAChRs since nicotine was shown to slow down the turnover of surface α4β2 nAChRs (Peng et al. 1994). The nAChRs treated with high concentrations of nicotine (5 μM) remained on the plasma membrane longer than untreated nAChRs. Subsequent studies produced mixed results: some groups were unable to replicate these results (Vallejo et al. 2005; Darsow et al. 2005; Sallette et al. 2005), whereas others succeeded (Kuryatov et al. 2005). More studies are still needed to provide evidence regarding whether nAChR stability and turnover is altered by chronic nicotine exposure.

Increased receptor assembly and subunit maturation in the ER is one suggested mechanism for nAChR upregulation, and many studies have shown that receptor assembly and maturation are increased during α4β2 nAChR upregulation (Harkness and Millar 2002; Nashmi et al. 2003; Sallette et al. 2005; Kuryatov et al. 2005). The increase in the assembly process leads to growing receptor numbers on the cell surface. Nicotine may promote the assembly of subunits by acting as a molecular chaperone in the ER and may also increase the half-life of surface receptors (Kuryatov et al. 2005).

Degradation and trafficking of nAChRs is regulated partly by the ubiquitin–proteosome system (Yi and Ehlers 2007). Blocking the ER-associated proteosome-mediated degradation of nAChRs can increase the receptor numbers expressed on the cell surface. In the case of α7 nAChRs, nicotine has been shown to inhibit the activity of the proteasome directly, without activating nAChRs, which leads to increased receptor numbers (Rezvani et al. 2007). Overexpression of ubiquilin-1, a ubiquitin-like protein with the capacity to interact with both the proteosome and ubiquitin ligases, abolishes the nicotine-induced upregulation of surface α3* nAChRs by promoting ER-associated proteosome-mediated degradation (Ficklin et al. 2005).

α4β2 nAChRs exist in two different stoichiometries and nicotine selectively upregulates the high-sensitivity stoichiometry (α4)(β2)3 (Nelson et al. 2003; Kuryatov et al. 2005; Tapia et al. 2007; Son et al. 2009; Srinivasan et al. 2011). The affinity of nicotine for the high-sensitivity α4β2 nAChRs is about 100-fold higher than for the low-sensitivity α4β2 nAChRs; thus, nicotine acts as a chaperone mainly at the high-sensitivity subtype to which it binds best (Nelson et al. 2003; Kuryatov et al. 2005). When oocytes are injected with different subunit ratios of mRNAs coding for α4 and β2 subunits, α4β2 nAChRs with high- and low-sensitivity are formed, yet only the high-sensitivity type is notably upregulated by nicotine (Lopez-Hernandez et al. 2004). Nicotine-induced stabilization of the high-sensitivity α4β2 nAChRs happens at the level of the ER and its exit sites, where the ER export of the high-sensitivity subtype is enhanced (Srinivasan et al. 2011) and the number of high-sensitivity α4β2 nAChRs on the cell surface is increased (Moroni et al. 2006).

The change in the nAChR conformation is one of the suggested mechanisms of nicotine-induced upregulation. According to this theory, there is a nicotine-induced increase in the number of high-affinity binding sites, with no increase in the number of receptors or change in the assembly, trafficking, or cell-
surface turnover of α4β2 nAChRs (Vallejo et al. 2005). Nicotine regulates the transition between two conformational states: the resting and the upregulated state. In the resting state, the affinity for agonists is low/normal and nicotine binding activates and desensitizes the receptors as usual. The suggested mechanism is that chronic nicotine exposure induces a transition to the upregulated state, where the affinity for the agonists is high and an increase in the agonist binding sites is observed. The conformational change also converts the functional state of the receptor. Nicotine is able to induce functional upregulation of α4β2 nAChRs (Buisson et al. 2000; Buisson and Bertrand 2001), caused by a conformational change in which the desensitization rate, single-channel conductance and ligand concentration-dependence of activation are changed. On the other hand, it has been demonstrated that the increase in α4β2* nAChR binding sites in mouse brain results from increases in assembled nAChR subunit proteins (Marks et al. 2011).

Since there is a lot of data about other upregulation mechanisms, the conformational change of nAChRs induced by chronic nicotine is unlikely to be the sole regulatory mechanism of upregulation. Instead, upregulation seems to be a multiple-step process occurring at different rates and through different mechanisms. The kinetic studies of upregulation have shown that there are two components of upregulation: an initial fast component that saturates after approximately 4h and a second slower phase which results in higher receptor numbers (Govind et al. 2012). It is hypothesized that the initial component is due to nicotine-induced conformational changes in nAChRs, and the second component results from increased receptor assembly and decreased subunit degradation (Darsow et al. 2005; Ficklin et al. 2005; Vallejo et al. 2005; Govind et al. 2012).

2.2 Nicotinic acetylcholine receptor ligands

2.2.1 Nicotinic agonists and antagonists

nAChRs exhibit multiple ligand-binding sites: the orthosteric site, the allosteric sites and the ion channel (Cecchini and Changeux 2015). Nicotinic agonists are ligands that bind to the orthosteric binding site of the receptor located in the extracellular domain at the interface between subunits leading to the activation of the receptor. Partial agonists also bind to the orthosteric binding site, but they only have partial efficacy at the receptor in relation to full agonists. For instance, cytisine and varenicline are partial α4β2 nAChR agonists that produce a more moderate and sustained increase in DA levels in the reward pathway compared to nicotine, a full agonist (Peng et al. 2013). At the same time, partial agonists prevent the binding of other ligands to the orthosteric site, thus blocking their effect. Competitive antagonists, on the other hand, bind to the orthosteric site, have no efficacy, prevent the binding of other orthosteric ligands and prevent the allosteric transition to the open channel state (Dwoskin and Crooks 2001; Wyllie and Chen 2007). Classical competitive nAChRs antagonist are, for example, dihydro-β-erythroidine, MLA, lobeline and α-Bgtx. NCAs either physically block the ion channel or act via allosteric mechanisms (Arias et al. 2006). Channel blockers are NCAs, that bind to the transmembrane domain of nAChRs and prevent ion flux by sterically occluding the channel pore. Many anesthetic agents act as nAChR channel blockers (Tassonyi et al. 2002).
2.2.2 Allosteric modulation

Allosteric modulators are compounds that are able to modulate nAChR function by binding to sites distinct from the orthosteric binding site (Chatzidaki and Millar 2015). Positive allosteric modulators (PAMs) potentiate the effects of agonist activation with no intrinsic activity. There are several proposed mechanisms of action: PAMs may reduce the energy barrier between closed and open conformations or increase the energy barrier between open and desensitized states, leading to increased agonist efficacy (Bertrand and Gopalakrishnan 2007; Williams et al. 2011; Cecchini and Changeux 2015). Some PAMs may potentiate activation of nAChRs at low agonist concentrations, whereas at high agonist concentrations their effect is weak. PAMs can be divided into type I and type II modulators based on their functional properties (Bertrand and Gopalakrishnan 2007). Type I modulators enhance agonist-induced nAChR activation without affecting desensitization kinetics, while type II modulators enhance agonist-induced nAChR activation by stabilizing the open-channel conformation and slowing down desensitization. Allosteric agonists are ligands that can induce nAChR activation in the absence of an orthosteric agonist (Chatzidaki and Millar 2015). Allosteric agonists may structurally resemble type II PAMs, with only a minor change in the ligand structure (Gill et al. 2012; Gill-Thind et al. 2015).

Negative allosteric modulators (NAMs) inhibit agonist-induced activation without binding to the orthosteric binding site and are thus NCAs of nAChRs (Arias 2010; Chatzidaki and Millar 2015). NAMs inhibit agonist-induced activation either by stabilizing a non-conducting conformational state of the nAChR or by increasing the nAChR desensitization rate. The binding sites of NAMs differ from those of NCAs, although these binding sites are similarly located within the ion channel or at the extracellular-transmembrane interface. For instance, the ethidium binding site is localized at the extracellular portion of the receptor and the quinacrine binding site is localized within the transmembrane domain (Arias 1998; Pratt et al. 2000).

Silent allosteric modulators (SAMs) are compounds that do not have a positive or negative modulatory effect on responses evoked by an orthosteric ligand, but they can block the effect of other allosteric modulators (Chatzidaki and Millar 2015; Gill-Thind et al. 2015). SAMs bind to the same allosteric site as other allosteric modulators or to an overlapping allosteric site. The chemical structure of SAMs is similar to PAMs and NAMs, with only a small change in the structure (e.g., a methyl substitution of a single aromatic ring).

2.3 The connection between opioid ligands, nAChRs and nicotine

2.3.1 Mechanism of nicotine dependence

Nicotine is considered to be the main addictive chemical in tobacco smoke (Benowitz 2009; Le Foll and Goldberg 2009). Following a cigarette puff, nicotine enters the bloodstream from the lungs and crosses the blood–brain barrier, reaching the brain within 10–20 seconds (Le Houezec 2003; Tutka et al. 2005). Tobacco addiction is described as a chronic disorder with compulsive drug-seeking and drug-taking behavior (McLellan et al. 2000). Although most smokers want to quit smoking, only a small percentage succeeds. Chronic tobacco use induces adaptive changes in the CNS, such as desensitization and
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upregulation of nAChRs, which could contribute to the development of drug dependence. In addition to nicotine, tobacco smoke contains several hundred other chemical substances, some of which may enhance the addictive and reinforcing effects of nicotine (Fowler et al. 1996a; Fowler et al. 1996b).

The basis of nicotine addiction is a combination of positive reinforcement and avoidance of the negative consequences (i.e., withdrawal symptoms), in addition to conditioning. Tobacco smoking and nicotine produce feelings of pleasure and reward and reduce stress and anxiety (De Biasi and Dani 2011). Some of the effects of smoking can be positive, such as improved concentration, better reaction time, and better performance at certain tasks (Levin et al. 2006). The withdrawal symptoms, on the other hand, are negative: irritability, depression, restlessness, anxiety, difficulty concentrating, insomnia and craving (McLaughlin et al. 2015). Nicotine withdrawal often causes anhedonia, inability to experience pleasure from activities usually found enjoyable (Cook et al. 2015). The withdrawal symptoms, mood disorders, anhedonia and tobacco craving are thought to result from a relative deficiency in DA release.

Activation of nAChRs by nicotine releases several neurotransmitters in the brain, most importantly DA, in a similar manner as other drugs of abuse (Imperato et al. 1986; Di Chiara and Imperato 1988). Nicotine induces DA release in multiple brain areas (e.g., the mesolimbic area, corpus striatum, and frontal cortex). The mesolimbic pathway, connecting the VTA to the NAc, is particularly important for the rewarding effects of nicotine (Dani and De Biasi 2001; Nestler 2005). Acute nicotine administration increases brain reward function by releasing DA, and if this release is blocked by, for instance, lesioning DA neurons in vivo, nicotine self-administration is reduced (Corrigall et al. 1992). In addition to DA release, other neurotransmitters, such as ACh, serotonin, GABA, norepinephrine, glutamate and endorphins, also mediate the effects of nicotine. Nicotine-induced release of neurotransmitters is both direct and indirect, the latter being the primary mode of action (Wonnacott 1997). Nicotine enhances glutamatergic inputs and inhibits the GABAergic inputs to the DA neurons in the VTA, which leads to a net increase in excitation of the DA neurons and augmented DA release (Mansvelder and McGhee 2002). Tobacco smoke contains components that inhibit brain monoamine oxidase A (MAO-A) and B (MAO-B) activity, which can potentiate nicotine’s addictive effects by increasing the levels of monoamine neurotransmitters, such as DA and norepinephrine (Yu and Boulton 1987; Berlin and Anthenelli 2001; Lewis et al. 2007). Nicotine self-administration studies in rats indicate that MAO inhibition (particularly MAO-A inhibition) increases the rewarding effect of low doses of nicotine, possibly via dopaminergic and serotonergic mechanisms (Villegier et al. 2007; Villegier et al. 2011; Smith et al. 2016).

Chronic nicotine administration leads to adaptive mechanisms, such as desensitization and upregulation of nAChRs (Wonnacott 1990; Wang and Sun 2005). Desensitization has been suggested to be one of the mechanisms contributing to nicotine dependence and tolerance (Picciotto et al. 2008). Typical daily cigarette smoking leads to nearly complete occupancy of the α4β2 nAChRs, indicating that nAChR saturation and desensitization is maintained throughout the day (Brody 2006). During longer periods of nicotine abstinence (e.g., during sleep or smoking cessation), nicotine levels drop and some of the desensitized nAChRs recover to a responsive state, which is suggested to be the cause of nicotine withdrawal and craving symptoms (Dani and Heinemann 1996). It is speculated that smoking maintains α4β2 nAChRs in a desensitized state so that these negative symptoms are avoided.

In addition to maintaining plasma nicotine levels high enough to prevent the withdrawal symptoms, smokers may also continue smoking because of the conditioned reinforcers associated with smoking.
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(Chiamulera 2005; Le Foll and Goldberg 2005a; Conklin 2006; Stoker and Markou 2015). Conditioning is the association between smoking cues and anticipated drug effects which results in the urge to smoke. The cues can be, for example, environmental situations, social interactions or the taste or feel of smoke, which becomes repeatedly associated with the pleasurable effects of smoking. Conditioned cues can also be unpleasant experiences associated with tobacco abstinence, such as irritability provoked by not smoking (Baker et al. 2004). Smokers may even come to perceive irritability from any source, such as stress, as a cue for smoking after repeated experiences (Perkins and Grobe 1992; Childs and de Wit 2010).

The high rate of relapse observed in smokers wanting to quit is most likely related to conditioning factors, since smoking cues are long-lasting and resistant to interventions. Exposure to smoking-related cues in nicotine-deprived smokers activate both brain reward and attention circuits (Due et al. 2002), whereas cues associated with nicotine withdrawal decrease brain reward function (Kenny and Markou 2005). The effects of environmental stimuli on the reinforcing effects of nicotine can be studied in vivo, for instance, intravenous nicotine self-administration and conditioned place preference (CPP) procedures (Goldberg and Henningfield 1988; Tzschentke 1998; Caggiula et al. 2002; Le Foll and Goldberg 2005b).

2.3.2 Opioid receptors and their signaling

The classical opioid receptors expressed in the CNS are the μ-, δ- and κ-receptors (Waldhoer et al. 2004; Trescot et al. 2008). Although the nociceptin opioid receptor is genetically related to classical opioid receptors, it is not classed as one since it does not bind the same ligands and has rather a modulatory role in μ-opioid receptor-mediated actions (Toll et al. 2016). Opioid receptors are expressed primarily in the cortex, brain stem and limbic system in the brain (Le Merrer et al. 2009). The classical opioid receptors belong to the class A γ-subgroup of the G protein-coupled receptor (GPCR) superfamily (Fredriksson et al. 2003; Katritch et al. 2013). The GPCRs all have a similar structure with seven transmembrane domains. GPCRs associate with heterotrimeric G-proteins composed of three different subunits: α, β, and γ. Upon opioid receptor activation, guanosine-5'-triphosphate (GTP) is converted to guanosine diphosphate (GDP) and the G protein dissociates into active Ga and Gβγ subunits (Stein 2016). The Ga subunit inhibits adenyl cyclase and reduces the levels of adenosine 3', 5'-cyclic monophosphate (cAMP), and the Gβγ subunit interacts with different ion channels expressed on the cell membrane. The opioid receptors modulate presynaptic Ca\(^{2+}\) channels by suppressing the influx of Ca\(^{2+}\), lowering the excitability of neurons (Tedford and Zamponi 2006). Opioid receptor activation also prevents neuronal excitation and the propagation of action potentials by opening G protein-coupled inwardly-rectifying K\(^+\) channels (Luscher and Siesinger 2010; Nockemann et al. 2013). Furthermore, opioid agonists inhibit Na\(^+\) channels, VOCCs, transient receptor potential vanilloid-1 and acid-sensing ion channels (Gold and Levine 1996; Endres-Becker et al. 2007; Cai et al. 2014).

Opioid receptor activation commonly prevents the elevation of free intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) by inhibiting VOCCs, yet opioid receptor activation can also increase [Ca\(^{2+}\)]\(_i\) (Samways and Henderson 2006). The opioid receptor-mediated increase in [Ca\(^{2+}\)]\(_i\), usually requires simultaneous activation of G\(_q\)-coupled receptors, which leads to Ca\(^{2+}\) release from intracellular stores via the inositol phosphate pathway (Connor and Henderson 1996; Werry et al. 2003). Nevertheless, opioid agonists have also been reported to be able to increase [Ca\(^{2+}\)]\(_i\) without concomitant G\(_q\)-coupled receptor activation (Okajima et al. 1993; Allouche et al. 1996; Spencer et al. 1997; Thorlin et al. 1998). Opioids have also been shown to increase [Ca\(^{2+}\)]\(_i\), by stimulating Ca\(^{2+}\) entry across the plasma membrane in some cell types,
possibly via L-type \(Ca^{2+}\) channels (Jin et al. 1992; Bao et al. 2003). Furthermore, opioids may increase \(Ca^{2+}\) influx into cells by increasing \(Ca^{2+}\) flux through ligand-gated cation channels, such as the P2X receptors (Chizhmakov et al. 2005).

### 2.3.3 Opioid dependence and pharmacological treatment options

Opioid addiction results from repeated, long-lasting exposure to opioids which leads to changes in the mesolimbic dopaminergic system, increased tolerance and to receptor desensitization and downregulation. In particular, DA is implicated in the establishment of reward in opioid addiction and also in the rewarding effects of other drugs of abuse (Nutt et al. 2015). Opioids increase the firing rate of DA neurons and thereby increase the DA levels in the NAc (Di Chiara and Imperato 1988; Volkow and Morales 2015). Another neurotransmitter particularly involved in opioid addiction is norepinephrine, which is implicated in motivating drug-seeking behaviors and establishing drug–environment pairings necessary for CPP (Weinshenker and Schroeder 2007). Other neurotransmitters, such as GABA, serotonin, opioid peptides and glutamate, have been implicated indirectly in the acute reinforcing properties and may cooperate with the DA system or work via independent pathways of reinforcement (Le Merrer et al. 2009; Kranz et al. 2010).

The pharmacological treatment options for opioid addiction can generally be divided into abstinence-oriented treatments and opioid maintenance (Lobmaier et al. 2010). The abstinence-oriented treatments involve a preliminary phase where the withdrawal symptoms are eliminated or reduced by pharmacological treatments, after which, abstinence is further supported (Gowing and Ali 2006). The most common treatments used in opioid detoxification are tapered methadone, other opioid agonists, clonidine, lofexidine, and buprenorphine (Gish et al. 2010; Meader 2010; Ducharme et al. 2012; Amato et al. 2013). Opioid maintenance is an option for treatment if the abstinence-oriented approaches have failed. The purpose is not to achieve a drug-free state, but to replace the illicit opioid with a safer option. Methadone is the most widely used opioid in maintenance therapy, but buprenorphine is also increasingly used (Bell 2014). The opioid antagonist naloxone is used in the treatment of opioid overdose and for opioid dependence combined with buprenorphine, and naltrexone is in use as an extended-release injection for opioid dependence (Gerra et al. 2006; van Dorp et al. 2007; Syed and Keating 2013).

### 2.3.4 Methadone

Methadone is a \(\mu\)-opioid receptor agonist with low affinity for \(\delta\)- and \(\kappa\)-opioid receptors (Kristensen et al. 1995). Similar to other opioid agonists, methadone is in clinical use for treating moderate to severe pain (Bieter and Hirsch 1948; Fredheim et al. 2008), but it has also been used since the 1960s for the maintenance replacement treatment of opioid addiction (Kramer 1970; Sim 1973; Bell 2014). Smoking rates are unusually high among methadone maintenance patients with approximately 77–97% of them being smokers (Nahvi et al. 2006; Richter et al. 2007; Elkader et al. 2009; Pajusco et al. 2012; Chisolm et al. 2013). As the methadone dose increases, the satisfaction experienced from smoking rises, which leads to increased smoking (Chait and Griffiths 1984). Heavy smokers also use higher doses of methadone (Frosch et al. 2000). Methadone users have more severe nicotine dependence than regular smokers (Clarke et al. 2001), and they are less successful at smoking cessation (Stein et al. 2006; Okoli et al. 2010).
Methadone is commonly used in maintenance treatment for heroin addiction, and heroin use is also linked to increased smoking rates (Mello et al. 1980). Heroin addicts smoke more cigarettes when heroin self-administration is allowed than during drug-free periods. Quitting smoking appears to be more difficult than quitting opioid use for addicts (Story and Stark 1991).

At the receptor level, methadone has been shown to be an agonist of human α7 nAChRs (Pakkanen et al. 2005). Methadone inhibits \([^3H]\)MLA and \([^3H]\)epibatidine (\([^3H]\)EPI) from binding to nAChRs. Both optical isomers of methadone increase \([\text{Ca}^{2+}]\) in SH-SY5Y and SH-EP1-hα7 cell lines and evoke nAChR-mediated inward currents in patch-clamp studies in SH-SY5Y cells. Furthermore, nAChR antagonists are able to block these methadone-induced responses. Methadone exposure for 3 days increases \([^3H]\)EPI binding in the SH-SY5Y cell line. In addition to interaction with the α7 nAChRs, both enantiomers of methadone also have non-competitive antagonistic activity at α3β4 nAChRs in rats (Xiao et al. 2001).

2.3.5 Morphine

Morphine is a classical μ-receptor agonist used as an analgesic (Wolff et al. 1940; Pasternak and Pan 2013). Morphine’s effect on nAChRs has not been studied on the receptor level, yet in vivo studies have revealed the interplay between morphine and nicotine dependence. Both nicotinic and opioid antagonists reduce CPP to both morphine and nicotine in mice (Zarrindast et al. 2003). Furthermore, the reinstatement of morphine-induced CPP is diminished by pretreatment with specific α4β2 and α7 nAChR-subtype antagonists, dihydroxy-β-erythroidine and MLA, which suggests that these nAChR subtypes may contribute to the reinstatement of morphine-induced CPP (Feng et al. 2011). Nicotine is able to reduce naloxone-induced withdrawal symptoms in morphine-tolerant mice (Zarrindast and Farzin 1996). The effect of morphine on locomotor activity and reinforcement is further enhanced by chronic nicotine administration (Vihavainen et al. 2006; Vihavainen et al. 2008). The α3β4* nAChRs are involved in the mediation of physical dependence on morphine, since mice with increased expression of α5, α3 and β4 nAChR subunits exhibit enhanced somatic signs of morphine withdrawal, and the blockade of these nAChRs attenuates morphine withdrawal symptoms (Muldoon et al. 2014).

2.3.6 Other opioids

Buprenorphine is a partial agonist of the μ-opioid receptor, antagonist of the κ-opioid receptor and also a partial agonist of the nociceptin receptor (Cowan et al. 1977; Leander 1987; Kamei et al. 1995; Bloms-Funke et al. 2000). Buprenorphine does not have the same intrinsic activity as full μ-opioid agonists, such as heroin or methadone, which makes it safer, compared to full agonists, with respect to respiratory depression and overdose (Kimber et al. 2015). Buprenorphine has a long duration of action and a relatively low addiction potential (Jasinski et al. 1978). Nevertheless, buprenorphine is increasingly misused (Lofwall and Walsh 2014) and it is the most common intravenously abused opioid in Finland (Simojoki and Alho 2013). Clinically, buprenorphine is used as an analgesic and for the treatment of opioid addiction (Johnson et al. 2005; Vadivelu and Anwar 2010; Bell 2014). Smoking rates are increased during opioid substitution therapy with buprenorphine in a similar manner as in methadone maintenance therapy (Mello et al. 1985; Mutschler et al. 2002; Pajusco et al. 2012), and buprenorphine detoxification
treatment reduces smoking (Patrick et al. 2014). Smoking cessation treatment with bupropion has shown to be ineffective among buprenorphine-maintained patients (Mooney et al. 2008).

Opioid antagonists naloxone and naltrexone are clinically used for the treatment of opioid overdose, for opioid addiction combined with buprenorphine and for alcohol dependence (Fudala et al. 1998; Bart 2012; Zindel and Kranzler 2014; Noble et al. 2015). The effect of naloxone and naltrexone in smoking cessation is controversial (David et al. 2013). In vitro, naloxone and naltrexone inhibit nAChRs (Madsen and Albuquerque 1985; Almeida et al. 2000; Almeida et al. 2004) and naltrexone blocks nicotine-induced upregulation of α4β2 nAChRs (Almeida et al. 2000).

Dextromethorphan is structurally related to opioid agonists and commonly used as an antitussive (Brown et al. 2004; Taylor et al. 2016). Dextromethorphan is a NCA at α3β4, α4β2 and α7 nAChRs (Hernandez et al. 2000; Damaj et al. 2005) and has been shown to reduce nicotine self-administration in rats (Glick et al. 2001; Briggs et al. 2016). Another antitussive agent with an opioid structure, codeine, is a PAM of α4β2 and α7 nAChRs (Storch et al. 1995; Iorga et al. 2006). Tramadol, a weak μ-receptor agonist, used as an analgesic, has been shown to inhibit the function of α7 nAChRs (Lewis and Han 1997; Shiraishi et al. 2002). Furthermore, tramadol use increases the severity of nicotine dependence in tramadol addicts dose-dependently, and the increase in nicotine dependence seems to have the same effect on tramadol intake (Shalaby et al. 2015).
3. AIMS OF THE STUDY

The purpose of this study was to find out whether opioid ligands have any effect on nAChRs at the receptor level. This question was approached by studying the receptor binding and functional effect of opioid ligands, both after acute drug treatment and after prolonged incubation. More specifically, the aims were:

- To determine, whether opioid agonists, such as morphine and methadone, bind to nAChRs expressed in SH-SY5Y, SH-EP1-hα4β2 and SH-EP1-hα7 cells (I, II).
- To investigate the acute functional effects of methadone and morphine on nAChRs expressed in SH-SY5Y, SH-EP1-hα4β2 and SH-EP1-hα7 cells (I, II).
- To investigate whether buprenorphine, codeine, oxycodone, tramadol, naloxone and naltrexone bind to nAChRs expressed in SH-SY5Y, SH-EP1-hα4β2 and SH-EP1-hα7 cells and whether they have any effect on nAChR function (III, unpublished results).
- To characterize the effects of prolonged methadone and morphine treatments on nAChR numbers and function (IV).
4. MATERIALS AND METHODS

4.1 Drugs and reagents

The (–)-nicotine hydrogen tartrate, bovine serum albumin, Meca hydrochloride, MLA citrate salt hydrate, naltrexone hydrochloride, Bradford reagent, poly-d-lysine, poly(ethyleneimine) solution, cytisine and carbamylcholine chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). The bovine serum albumin standards were from Thermo Fisher Scientific Inc. (Rockford, IL, USA). The rubidium-86 isotope (\(^{86}\text{Rb}^+\)) and \(^{[3]}\text{H}\)EPI radioligand were purchased from PerkinElmer (Waltham, MA, USA). The nicotine ditartrate dehydrate was from Acros Organics (Geel, Belgium). Morphine hydrochloride was purchased from University Pharmacy (Helsinki, Finland). The (±)-methadone hydrochloride was purchased from Star (Tampere, Finland) and from University Pharmacy (Helsinki, Finland). Cell culture plasticware was purchased from Nunc (Roskilde, Denmark) and cell culture media and media supplements and Fluo-3AM were from Invitrogen (Carlsbad, CA, USA). Potassium phosphate buffer was composed of 50 mM K\(_2\)HPO\(_4\), 50 mM KH\(_2\)PO\(_4\), 1 mM ethylenediaminetetra-acetic acid (EDTA) and the pH was adjusted to 7.4. Phosphate-buffered saline (PBS) was composed of 150 mM NaCl, 8 mM K\(_2\)HPO\(_4\), 2 mM KH\(_2\)PO\(_4\), and the pH was adjusted to 7.4. \(^{86}\text{Rb}^+\) efflux buffer was composed of 130 mM NaCl, 5.4 mM KCl, 2 mM CaCl\(_2\), 5 mM glucose and 50 mM HEPES and the pH was adjusted to 7.4. Tyrode’s salt solution (TSS) was composed of 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 0.2 mM NaH\(_2\)CO\(_3\) and 5.5 mM glucose, pH adjusted to 7.4.

4.2 Cell culture

The cell lines used were SH-SY5Y, SH-EP1-hα4β2 and SH-EP1-hα7 cells. The SH-SY5Y cell line is a neuroblastoma cell line of human origin that is thrice cloned from the SK-N-SH line (Ross et al. 1983; Gould et al. 1992; Lukas et al. 1993; Peng et al. 1997; Groot Kormelink and Luyten 1997). SH-SY5Y cells natively express α3, α5, α7 and β2, β3, β4 subunit cDNAs, and these subunits form functional pentameric receptors of different combinations, such as α3β4 and α7 nAChRs. In addition to nAChRs, the SH-SY5Y cells also express functional muscarinic and μ- and δ-opioid receptors (Adem et al. 1987; Kazmi and Mishra 1987; Kukkonen and Mishra 1987; Kukkonen et al. 1992).

The SH-EP1 cell line has no native expression of nAChRs, but expresses the μ- and δ-opioid receptors (Baumhaker et al. 1993). SH-EP1-hα4β2 and SH-EP1-hα7 cells were transfected with human cDNAs coding for α4 and β2 subunits or α7 subunit, respectively, and thus express these human nAChR subtypes exclusively (Peng et al. 1999; Zhao et al. 2003; Eaton et al. 2003). Both of these cell lines were kindly provided by Dr. Ronald J. Lukas (Barrow Neurological Institute, St Joseph’s Hospital and Medical Center, Phoenix, AZ, USA). The SH-EP1-hα4β2 cell line was constructed as described by Eaton et al. (2003). In brief, cDNAs encoding human α4 and β2 subunits we used to generate the pcDNA3.1/zeo-hα4 and pcDNA3.1/hygro-hβ2 constructs. Native nAChR-null SH-EP1 cells were simultaneously transfected with both α4 and β2 constructs using electroporation. The culture medium was supplemented with selection antibiotics zeocin and hygromycin B in order to sort out the cells expressing dual drug resistance. Ring cloning was used to isolate single, resistant cell colonies, which were then expanded. Functional screening was done with the \(^{86}\text{Rb}^+\) efflux assay, and a clone exhibiting high expression of α4β2 nAChRs
was further subcloned by dilution and the ring-cloning method. The SH-EP1-hα7 cells were constructed as described in Zhao et al. (2003). Briefly, the SH-EP1 cells were transfected using electroporation with cDNA encoding the human α7 subunit. Hygromycin was used as the selection antibiotic, and positive transfectants were isolated by ring cloning. The expression of α7 nAChRs was confirmed by radiolabeled α-Bgtx binding.

All cell cultures were maintained in 5% CO₂/humidified air at 37 °C, the cells were split twice weekly and passage numbers 10–30 were used in experiments. SH-SY5Y cells were grown in a mixed (1:1) Dulbecco’s modified Eagle:Ham’s F12 (DMEM/Ham F12) medium supplemented with fetal bovine serum (12.7%), penicillin and streptomycin (1.7%) and non-essential amino acids (0.8%). SH-EP1-hα7 cells were grown in DMEM (with high glucose, l-glutamine and sodium pyruvate) supplemented with horse serum (8%), fetal bovine serum (4.3%), penicillin and streptomycin (0.9%), amphotericin B (0.7%) and hygromycin (0.7%), and the same mixture supplemented with zeocin (0.3%) was used for the SH-EP1-hα4β2 cells.

4.3 [³H]Epibatidine binding

Epibatidine is an alkaloid that was extracted from the skin of *Epipedobates tricolor*, an endemic Ecuadorian frog (Daly et al. 1978). Epibatidine is a potent analgesic and its analgesic action is not blocked by the opioid antagonist naloxone, whereas the nACHR antagonist Meca is able to antagonize its analgesic effect (Qian et al. 1993; Li et al. 1993; Badio and Daly 1994). Both isomers of epibatidine have an equal affinity for nACHRs, whereas their affinities for muscarinic, opioid, serotonin, adrenergic or GABA receptors are weak or nonexistent (Badio and Daly 1994). Although epibatidine is known to be 200 times more potent than morphine in terms of its analgesic effect, its toxicity makes it unsuitable for clinical use. Doses not much higher than what are needed for antinociception cause hypertension, respiratory paralysis, seizures and death *in vivo* (Sullivan et al. 1994; Bonhaus et al. 1995).

Epibatidine’s high affinity for nACHRs makes it an excellent ligand for studying nACHRs. [³H]EPI binds with high affinity to α2, α3, α4, β2 and β4 subunit-containing nACHRs expressed in *X. laevis* oocytes or HEK 293 cells (Parker et al. 1998; Xiao and Kellar 2004) and to α6β2* and α6β4* nACHRs expressed in *X. laevis* oocytes (Kuryatov et al. 2000). In mouse brain, [³H]EPI binding is biphasic by nature meaning that there are binding sites with both high affinity (equilibrium binding constant (Kᵦ) = 0.02 nM) and low affinity (Kᵦ ≈ 5 nm) (Gerzanich et al. 1995; Marks et al. 2006). Deletion of α7, β2, and β4 nACHR subunits eliminates virtually all [³H]EPI binding sites in mouse brain (Marks et al. 2006). The lower affinity binding sites are α-Bgtx-sensitive, α7-containing receptors, and α-Bgtx-resistant receptors, which contain either β2 or β4 subunits. The high affinity binding sites can be divided into cytisine-sensitive sites, which require the β2 subunit, and to cytisine-resistant sites, which require the presence of either a β2 or β4 subunit.

[³H]EPI binding can be used to assess the site of action for ligands interacting with nACHRs (Lukas et al., 2002). Additionally, radio-labeled agonist binding can be used to measure the change in receptor numbers (e.g., upregulation). Ligands that act as competitive antagonists or agonists of nACHRs inhibit the binding of [³H]EPI to these receptors. NCAs have either no effect on [³H]EPI binding or inhibit it only at very high concentrations. In order to measure the affinities of different nACHR ligands by [³H]EPI binding, the number of binding sites must be assessed by saturation binding. The amount of specific
binding is determined by measuring both total and nonspecific binding (the binding of $[^3H]$EPI to something other than its designated receptors) and subtracting the nonspecific binding from the total.

$[^3H]$EPI binding was used for saturation binding studies and for competition binding. For the acute nicotine and opioid studies, the cells were grown in 175 cm$^2$ flasks. The medium was aspirated at confluency, followed by three washes with ice-cold PBS. Cells were mechanically harvested into potassium phosphate buffer and homogenized using an ultrasonic homogenizer (Ringo Ultrasonics Bio 70, Romanshorn, Switzerland) (75% amplitude, 2×15 s) on ice. Homogenates were next centrifuged at 13,000 g for 20 min at 4 °C. The supernatant was discarded and the pellet was resuspended in potassium phosphate buffer and frozen at −70 °C. The frozen cell homogenates were thawed and re-homogenized by sonication, and protein concentrations were measured by the Bradford method. $[^3H]$EPI binding was done in 96-well filter plates (Millipore MultiScreen HTS FC) in a final volume of 250 μl with samples containing 5–50 μg of protein. For saturation binding studies, the cell homogenates were incubated with 25–5000 pM $[^3H]$EPI for 2 h at room temperature. In competition binding assays, the cell membranes were incubated with 150 pM $[^3H]$EPI and serial dilutions of nicotinic and/or opioid ligands. Nicotine (1 mM) was used to determine the amount of nonspecific binding. Optiphase HiSafe 3 scintillant (Perkin Elmer, Turku, Finland) was added to the wells and radioactivity was quantified by scintillation counting (1450 MicroBeta TriLux Liquid Scintillation Counter & Luminometer, PerkinElmer, Waltham, MA, USA).

For prolonged nicotine and opioid studies, cells grown in 175 cm$^2$ flasks were treated (final concentration) with either nicotine (1 and 10 μM), methadone (1 and 10 μM) or morphine (0.1 and 1 μM). The studied drugs were added to cell culture medium and flasks were maintained at 37 °C in 5% CO$_2$/humidified air for 3 days. For the control group, cells were incubated with normal cell culture medium for 3 days. On the day of assay, cultures were rinsed for 3×10 min with warm medium; then incubated for 3 h at 37°C before a final wash with PBS to remove all traces of drugs from the cultures. After that, the cell homogenates were prepared as described above. Cell membranes were incubated with 25–6400 pM $[^3H]$EPI; otherwise the protocol was the same as described above.

4.4 Calcium fluorometry

Cells were grown in 75 cm$^2$ flasks, from which the cells were seeded into 96-well BD Falcon microplates (in a 100 μl volume) coated with poly-d-lysine the day before the experiment. Culture medium was removed from 96-well plates and the cells were washed twice with TSS. The loading medium was composed of Fluo-3 AM (10 μM) and probenecid (2.5 mM) in TSS. Probenecid was prepared fresh every day as a stock concentration of 250 mM solubilized 1:1 in 1.0 M NaOH and TSS. Cells were incubated with the loading medium (50 μl/well) at room temperature for 1 h in the dark. After incubation, cells were washed twice with TSS (200 μl/well). After washings, 100 μl of TSS with or without antagonists was added, and the plate was transferred to a FlexStation microplate reader (Molecular Devices, Sunnyvale, CA, USA). If pretreatments were used, the cells were preincubated with drugs for 10 min in the dark. The test drug was then injected (50 μl/well), and the fluorescence was monitored for 80 s at 485/525 nM wavelength. The magnitudes of the drug-induced signals were calculated by subtracting the background signal (measured for 10 s before injection) from the drug-induced signal. The cells were also stimulated by injecting vehicle (TSS) as a control in each experiment. The changes in [Ca$^{2+}$], were expressed as a percentage of nicotine-evoked increase in fluorescence.
4.5 Rb⁺ efflux

nAChR function can be studied with the Rb⁺ efflux assay, which is a specific and highly sensitive ion flux assay, where the Rb⁺ acts as a tracer for potassium movement across the cell membrane (Lukas and Cullen 1988; Lukas et al., 2002). When nAChRs are stimulated, the Rb⁺ ions flow out of the cell down a concentration gradient and the amount of outflow ions can be measured using Cerenkov counting. The Rb⁺ ions are loaded by the Na⁺/K⁺-ATPase into cells by incubating them with cell medium containing a fixed amount of Rb⁺. The Rb⁺ has a short half-life (18.66 days), thus, the amount of Rb⁺ in the loading medium must be adjusted to an adequate level before every experiment. The “flip-plate” method, where cells are seeded to 24-well plates and liquid changes are handled by flipping the plates, offers gentle sample handling, high throughput, good temporal accuracy and reproducibility. In this method, the rinse buffer and ligands being studied are also pipetted into 24-well plates and the liquid changes are handled by aligning the plates on top of each other so that liquid from all 24 original wells flows to the other 24 wells almost simultaneously. Liquid aspiration is handled by a multichannel aspiration manifold, so that several wells can be handled at the same time.

For the Rb⁺ efflux assays, the cells were grown in 75 cm² flasks, which were maintained at 37 °C in 5% CO₂/humidified air. For the prolonged drug treatments, the cell culture medium was supplemented with drugs being studied (nicotine, methadone or morphine) and flasks were maintained in the incubator for 3 days. Cells were detached from flasks with trypsin-EDTA and seeded in a 500 μl volume to 24-well plates. Plates were placed in a 37 °C incubator for at least four hours to let the cells adhere. Next, the culture medium was replaced with medium (250 μl per well) supplemented with 250,000–300,000 cpm of Rb⁺ and the drugs being studied for the prolonged drug treatments. A day after seeding the cells, the Rb⁺ efflux was measured with the “flip-plate” method (Lukas et al., 2002). Each well was rinsed once with 1.2 ml of Rb⁺ efflux buffer for 1 min to remove extracellular Rb⁺, except for prolonged studies where each well was rinsed twice with buffer for altogether 10 min, in order to remove any traces of drugs. Cells were exposed to efflux buffer containing the ligands studied for 5 min. Six of the wells on each plate were reserved for controls: three for maximum Rb⁺ efflux (1 mM carbamylcholine) and three for non-specific Rb⁺ efflux (buffer only). Radioactivity was counted with Cerenkov counting after inserting the cross-talk-minimizing inserts into wells. Specific efflux in each well was obtained by subtracting the nonspecific efflux from the total efflux. Typical values for specific maximum Rb⁺ efflux (depending on cell type and density and the concentration of Rb⁺ in the loading medium) were 25,000–50,000 cpm over the non-specific backgrounds of 6,000–15,000 cpm. The amount of remaining intracellular Rb⁺ was counted to ensure that the sum of Rb⁺ released into the efflux plate and Rb⁺ remaining in the cell plate were the same for each well. To determine the amount of remaining intracellular Rb⁺, 0.1% sodium dodecyl sulfate/0.1 M NaOH (1.5 ml per well) was added to lyse the cells and the plates were counted with Cerenkov counting. The Rb⁺ efflux assays could not be conducted with the SH-EP1-hα7 cell line because α7 nAChRs are rapidly inactivating channels that are not open long enough to give significant ion flux signal (Lukas et al., 2002).

4.6 Statistical analysis

All data were analyzed using GraphPad Prism (version 5.02 in study I, version 6.02 in studies II and IV); GraphPad Software, Inc., La Jolla, CA, USA). Briefly, the Kd and maximum specific binding (Bmax) values...
Materials and Methods

from saturation binding experiments in studies I and II were determined by non-linear regression by fitting the data points to a one-site or two-site ligand binding model. The equilibrium dissociation constant (K_i) values in the competition binding assay were calculated using one-site and two-site curve-fitting models, and the K_i values were obtained from best fit (p-value < 0.05, extra sum-of-squares F test). The calcium responses were calculated as the percentage of the increase in fluorescence produced by a maximally effective concentration of nicotine (10 μM for SH-EP1-hα4β2 and SH-EP1-hα7 cells and 30 μM for SH-SY5Y cells). The data were analyzed statistically using one-way analysis of variance (ANOVA) and Dunnett’s multiple-comparison post hoc test. Specific 86Rb⁺ efflux signal was defined as total 86Rb⁺ efflux (1 mM carbamylcholine) minus non-specific 86Rb⁺ efflux (buffer) and drug-induced 86Rb⁺ efflux was normalized to specific efflux after subtraction of nonspecific efflux. The specific 86Rb⁺ efflux was fit to the variable slope model or the biphasic concentration–response model after comparing these two different fits (p-value < 0.05, extra sum-of-squares F test). EC50 values were determined using the biphasic concentration–response model, and concentration inhibiting 50% of maximum (IC50) values were determined using the variable slope model.

In study IV, the B_max and K_d values for the saturation binding assays were calculated using one-site specific binding with Hill slope model. Changes in the B_max values were analyzed using one-way ANOVA and Dunnett’s multiple comparisons test. Values of p < 0.05 were taken to be statistically significant. In the 86Rb⁺ efflux assay, the EC50 and logEC50 values were determined using the biphasic concentration–response model after comparing it to the variable slope model. The biphasic concentration-response model was a better fit (p-value < 0.05, extra sum-of-squares F test).
5. RESULTS

5.1 Methadone

5.1.1 Effects of acute methadone treatment (II)

Methadone displaced \(^{3}H\)EPI in SH-SY5Y and SH-EP1-hα7 cells, but not in SH-EP1-hα4β2 cells. \(K_i\) and log\(K_i\) values are presented in Table 1. In SH-SY5Y cells, methadone displaced \(^{3}H\)EPI with high and low affinity, and the fraction of high-affinity binding sites was 49 ± 20%. Methadone significantly increased \([Ca^{2+}]\), in SH-SY5Y and SH-EP1-hα7-cells, but not in SH-EP1-hα4β2 cells. In SH-EP1-hα4β2 cells, pre-incubation with methadone (10 and 50 μM) inhibited the nicotine (10 μM) -induced increase in \([Ca^{2+}]\), by 72 ± 6% and 85 ± 14%, respectively. In SH-SY5Y cells, pre-incubation with methadone (10 and 50 μM) inhibited the nicotine (30 μM) -induced increase in \([Ca^{2+}]\), by 65 ± 12% and 74 ± 17%, respectively. In SH-EP1-hα4β2 cells, pre-incubation with Meca (50 μM) inhibited the methadone (100 μM) -induced increase in \([Ca^{2+}]\), by 43 ± 9%. MLA, naloxone and naltrexone had no effect on the methadone-induced response. In SH-SY5Y cells, pre-incubation with Meca (100 μM), MLA (50 μM), naloxone (100 μM) and naltrexone (100 μM) inhibited the methadone (100 μM) -induced response in \([Ca^{2+}]\), by 36 ± 4%, 53 ± 10%, 40 ± 10% and 37 ± 8%, respectively. In SH-EP1-hα7 cells, pre-incubation with Meca (50 μM), MLA (100 μM), naloxone (50 μM) and naltrexone (100 μM) inhibited the methadone (100 μM) -induced increase in \([Ca^{2+}]\), by 30 ± 7%, 30 ± 7%, 34 ± 9% and 32 ± 8%, respectively.

Methadone had no effect on the \(^{86}Rb^+\) efflux in SH-EP1-hα4β2 and SH-SY5Y cells. However, methadone did inhibit nicotine-induced \(^{86}Rb^+\) efflux in SH-EP1-hα4β2 and SH-SY5Y cells in a concentration-dependent manner. To determine whether this antagonism is competitive or non-competitive, nicotine concentration–response assays were also performed in the presence of several different methadone concentrations, all of which inhibited the nicotine-induced \(^{86}Rb^+\) efflux non-competitively in both cell lines studied. \(IC_{50}\) and log\(IC_{50}\) values are presented in Table 2.
Table 1. Equilibrium dissociation constant (K_i and logK_i) values for opioid ligands in [^3H]EPI competition binding in the SH-EP1-hα4β2, SH-SY5Y and SH-EP1-hα7 cell lines.

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<tbody>
<tr>
<td></td>
<td>K_i (μM)</td>
<td>logK_i ± SD</td>
<td>K_iHi/K_iLow (μM)</td>
</tr>
<tr>
<td>Methadone</td>
<td>&gt;3000 NA</td>
<td>19.4, 1008</td>
<td>-4.71±0.28, -3.00±0.50</td>
</tr>
<tr>
<td>Morphine</td>
<td>13.2 -4.88±0.03</td>
<td>0.2, 126</td>
<td>-6.80±0.42, -3.90±0.12</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>&gt;3000 NA</td>
<td>1234</td>
<td>-2.09±0.45</td>
</tr>
<tr>
<td>Codeine</td>
<td>366.9 -3.44±0.05</td>
<td>165.5</td>
<td>-3.78±0.08</td>
</tr>
<tr>
<td>Oxycodone</td>
<td>296.3 -3.53±0.03</td>
<td>105.5</td>
<td>-3.98±0.06</td>
</tr>
<tr>
<td>Tramadol</td>
<td>&gt;3000 NA</td>
<td>&gt;3000 NA</td>
<td>NA</td>
</tr>
<tr>
<td>Naloxone</td>
<td>&gt;3000 NA</td>
<td>0.3, 44.5</td>
<td>-6.47±0.59, -4.35±0.13</td>
</tr>
<tr>
<td>Naltrexone</td>
<td>&gt;3000 NA</td>
<td>3.9, 131.8</td>
<td>-5.41±0.36, -3.88±0.18</td>
</tr>
</tbody>
</table>

K_iHi/K_iLow, the equilibrium dissociation constant value for the high-/low-affinity binding sites; logK_iHi/logK_iLow, the log equilibrium dissociation constant values for the high-/low-affinity binding sites; SD, standard deviation; NA, not applicable; -, not measured.

Table 2. IC_{50} and logIC_{50} values for opioid ligands in ^86Rb^+ efflux assay in the SH-EP1-hα4β2 and SH-SY5Y cell lines.

<table>
<thead>
<tr>
<th>Compound</th>
<th>SH-EP1-hα4β2</th>
<th>SH-SY5Y</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nicotine 10 μM</td>
<td>Nicotine 1 μM</td>
</tr>
<tr>
<td></td>
<td>IC_{50} (μM)</td>
<td>logIC_{50} ± SD</td>
</tr>
<tr>
<td>Methadone</td>
<td>7.4</td>
<td>-5.13±0.03</td>
</tr>
<tr>
<td>Morphine</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>68.3</td>
<td>-4.17±0.10</td>
</tr>
<tr>
<td>Codeine</td>
<td>1721</td>
<td>-2.76±0.42</td>
</tr>
<tr>
<td>Oxycodone</td>
<td>247</td>
<td>-3.61±0.05</td>
</tr>
<tr>
<td>Tramadol</td>
<td>64.7</td>
<td>-4.19±0.03</td>
</tr>
<tr>
<td>Naloxone</td>
<td>104.2</td>
<td>-3.98±0.03</td>
</tr>
<tr>
<td>Naltrexone</td>
<td>1866</td>
<td>-2.73±0.09</td>
</tr>
</tbody>
</table>

IC_{50}, the half-maximal inhibitory concentration; logIC_{50}, the log half-maximal inhibitory concentration; SD, standard deviation.

5.1.2 Effects of prolonged methadone treatment (IV)

Prolonged exposure to methadone (1 and 10 μM) increased the [^3H]EPI binding sites in SH-SY5Y (Fig. 7B) and SH-EP1-hα7 (Fig. 7C) cells, but decreased [^3H]EPI binding sites in SH-EP1-hα4β2 cells (Fig. 7A).
Results

Figure 7. [$^3$H]EPI saturation binding profiles in SH-EP1-hα4β2 (A), SH-SY5Y (B) and SH-EP1-hα7 (C) cell membranes. The cells were exposed for 72 h to either normal cell culture medium or to cell culture medium containing 1 or 10 μM methadone. The [$^3$H]EPI saturation binding curve for no pre-treatment is presented in each picture as a control. The assay mixtures contained 25-6400 pM [$^3$H]EPI and 5-50 μg cell protein. The data were fit to a one-site competition binding model, after comparing it with the two-sites model (the better fit was determined using the extra sum-of-squares F test, p-value < 0.05). The values are the mean ± standard deviation (SD) from five to six assays, with each point assayed in triplicate at least.

In the $^{86}$Rb$^+$ efflux assay, nicotine showed biphasic full agonist activity in the SH-EP1-hα4β2 cell line with EC$_{50}$ values of 0.1 μM and 1.3 μM (Fig. 8A). The EC$_{50}$ values for nicotine were 0.08 μM and 5.7 μM when pre-treated with 1 μM methadone for 4 days, and 0.07 μM and 1.6 μM with 10 μM methadone pre-treatment. Nicotine also showed biphasic full agonist activity in the SH-SY5Y cell line, with EC$_{50}$ values of 2.5 μM and 11.9 μM (Fig. 8B). When SH-SY5Y cells were pre-treated with 1 μM methadone for 4 days, the EC$_{50}$ values for nicotine were 3.2 μM and 10.5 μM, and with 10 μM methadone the EC$_{50}$ values were 1.4 and 7.5 μM.

Figure 8. Dose–response profiles of nAChR function for nicotine using an $^{86}$Rb$^+$ efflux assay in SH-EP1-hα4β2 (A) and SH-SY5Y (B) cells. The specific $^{86}$Rb$^+$ efflux was determined in the presence of nicotine and the cells were pretreated with 1 or 10 μM methadone for 96 h. The specific efflux for each drug concentration was assessed using 1 mM carbamylcholine (total efflux) and efflux buffer (non-specific efflux) controls. The cells were exposed to nicotine for 5 min. The data were fit to the biphasic concentration–response equation. The values are the mean ± SD from six separate assays.
Results

5.2 Morphine

5.2.1 Effects of acute morphine treatment (I)

Morphine displaced $[^{3}H]$EPI from all cell lines studied. The $K_i$ and log$K_i$ values are presented in Table 1. Morphine was also able to increase $[\text{Ca}^{2+}]_i$ in all cell lines, and in SH-EP1-hα7 cells the effect was comparable to that of nicotine. The effect of morphine on the nicotine-induced response in SH-EP1-hα4β2 cells was statistically significant only at 100 μM, inhibiting 74% of the nicotine-induced response. In SH-SY5Y cells, morphine (10 μM and 50 μM) preincubation increased the nicotine-induced response by 70% and 49%, respectively. In SH-EP1-hα7 cells, morphine (50 μM) preincubation increased the nicotine-induced response by 61%. In the $^{86}$Rb$^+$ efflux assay, concentration–response profiles showed partial agonist efficacy for morphine in SH-EP1-hα4β2 cells with an $EC_{50}$ value of 53.3 μM. Meca concentration-dependently attenuated the morphine-induced $^{86}$Rb$^+$ efflux response. Opioid antagonists naloxone and naltrexone modified the morphine-induced $^{86}$Rb$^+$ efflux response by shifting the curve to the right. Morphine enhanced the $^{86}$Rb$^+$ ion efflux induced by low nicotine concentrations. In SH-SY5Y cells, morphine showed no efficacy in the $^{86}$Rb$^+$ efflux assay. Morphine had inhibitory efficacy on nicotine-induced $^{86}$Rb$^+$ ion flux. In order to clarify the mechanism of nAChR antagonism or agonism, the $^{86}$Rb$^+$ efflux nicotine concentration–response studies were also performed in the presence of multiple morphine concentrations. In SH-EP1-hα4β2 cells, morphine increased the nicotine-stimulated response at low nicotine concentrations and decreased the nicotine-stimulated response at high nicotine concentrations. In SH-SY5Y cells, morphine had virtually no effect on the nicotine-stimulated response.

5.2.2 Effects of prolonged morphine treatment (IV)

Prolonged exposure to morphine (0.1 and 1 μM) increased $[^{3}H]$EPI binding sites in SH-SY5Y (Fig 9B) and SH-EP1-hα7 (Fig 9C) cells, but decreased $[^{3}H]$EPI binding sites in SH-EP1-hα4β2 cells (Fig 9A).

![Figure 9](image_url)

*Figure 9. $[^{3}H]$EPI saturation binding profiles in SH-EP1-hα4β2 (A), SH-SY5Y (B) and SH-EP1-hα7 (C) cell membranes.*

The cells were exposed for 72 h to either normal cell culture medium or to cell culture medium containing 0.1 or 1 μM morphine. The $[^{3}H]$EPI saturation binding curve without any pre-treatment is presented in each picture as a control. The assay mixtures contained 25-6400 pM $[^{3}H]$EPI and 5-50 μg cell protein. The data were fit to a one-site competition binding model, after comparing it with the two-sites model (the better fit was determined using the extra sum-of-squares $F$ test, $p$-value < 0.05). The values are the mean ± SD from five to six assays, with each point assayed in triplicate at least.
Results

In the $^{86}$Rb⁺ efflux assay, nicotine had biphasic full agonist activity with EC$_{50}$ values of 0.1 μM and 1.3 μM in SH-EP1-hα4β2 cells (Fig. 10A). The EC$_{50}$ values for nicotine were 1.3 μM and 21.4 μM when pre-treated with 0.1 μM morphine for 4 days, and 0.2 μM and 7.8 μM with 1 μM morphine pre-treatment. Nicotine also showed biphasic full agonist activity in the SH-SY5Y cell line, with EC$_{50}$ values of 2.5 μM and 11.9 μM (Fig 10B). After pre-treatment with 0.1 μM morphine for 4 days the EC$_{50}$ values for nicotine were 2.2 μM and 10.5 μM, and with 1 μM morphine the EC$_{50}$ values were 2.8 and 11.8 μM.

Figure 10. Dose–response profiles of nAChR function for nicotine using an $^{86}$Rb⁺ efflux assay in SH-EP1-hα4β2 (A) and SH-SY5Y (B) cells. The specific $^{86}$Rb⁺ efflux was determined in the presence of nicotine and the cells were pretreated with 0.1 or 1 μM morphine for 96 h. The specific efflux for each drug concentration was assessed using 1 mM carbamylcholine (total efflux) and efflux buffer (non-specific efflux) controls. The cells were exposed to nicotine for 5 min. The data were fit to the biphasic dose–response equation. The values are the mean ± SD from six separate assays.

5.3 Buprenorphine (unpublished)

5.3.1 Effects of acute buprenorphine treatment

Buprenorphine displaced [$^{3}$H]EPI in SH-EP1-hα7 cells, but not in SH-EP1-hα4β2 or SH-SY5Y cells (Fig. 11). The Kᵢ and logKᵢ values are presented in Table 1. Preincubation with buprenorphine inhibited the nicotine-induced increase in [Ca$^{2+}$] in SH-SY5Y, SH-EP1-hα4β2 and SH-EP1-hα7 cells (Fig. 12). In SH-SY5Y cells, buprenorphine (10 μM, 50 μM and 100 μM) preincubation decreased the nicotine-induced response by 68%, 89% and 99%, respectively. The effect of buprenorphine on the nicotine-induced response in SH-EP1-hα4β2 and SH-EP1-hα7 cells was statistically significant only at 50 μM and 100 μM, inhibiting 75% and 82% of the nicotine-induced response in SH-EP1-hα4β2 cells and 59% and 70% in SH-EP1-hα7 cells.
Results

Figure 11. [³H]EPI competition binding profiles in SH-EP1-hα4β2 (A), SH-SY5Y (B) and SH-EP1-hα7 (C) cell membranes for nicotine and buprenorphine. The assay mixtures contained 150 pM [³H]EPI and 5–50 μg cell protein and nicotine or buprenorphine at increasing concentrations. The data were fit to a one-site competition binding model. The values are the mean ± SD from three to five assays, with each point assayed at least in triplicate. Kᵢ and logKᵢ values are provided in Table 1.

Figure 12. Effects of buprenorphine on nicotine-induced increases in [Ca²⁺] in SH-SY5Y, SH-EP1-hα4β2 and SH-EP1-hα7 cells. The Fluo-3 AM-loaded cells were incubated for 10 min with vehicle (TSS) or 10 μM, 50 μM or 100 μM buprenorphine and then stimulated with 10 μM or 30 μM nicotine. The responses are expressed as the percentage of the nicotine response. The values are the mean ± standard error of mean (SEM) from three to six separate assays. In each experiment, there were at least six replicates for each condition. Significantly different from nicotine response **p < 0.01, and ***p < 0.001 using one-way ANOVA.

Buprenorphine (100 μM) induced increase in [Ca²⁺], in all cell lines studied (data not shown). Preincubation with nAChR partial agonist cytisine or with opioid antagonist naltrexone inhibited the buprenorphine-induced increase in [Ca²⁺], in SH-EP1-hα4β2, SH-SY5Y, and SH-EP1-hα7 cells (Fig. 13). Opioid antagonist naloxone was able to inhibit the buprenorphine-induced increase in [Ca²⁺], in SH-EP1-hα4β2 and SH-SY5Y cells.
Results

Figure 13. Effects of cytisine and opioid antagonists on buprenorphine-evoked increases in [Ca$^{2+}$] in SH-EP1-h$\alpha$4β2 (A), SH-SY5Y (B) and SH-EP1-h$\alpha$7 (C) cells. The Fluo-3 AM-loaded cells were incubated for 10 min with vehicle (TSS), cytisine (Cyt), naloxone (Nalo) or naltrexone (Nalt) and then stimulated with 100 μM buprenophine. The responses are expressed as the percentage of the buprenorphine response. The values are the mean ± SEM from three to six separate assays. In each experiment, there were at least six replicates for each condition. Significantly different from buprenorphine response *p < 0.05, **p < 0.01, using one-way ANOVA.

In the $^{86}$Rb$^+$ efflux assay, buprenorphine showed no efficacy in either SH-EP1-h$\alpha$4β2 or SH-SY5Y cells (data not shown). Buprenorphine had inhibitory efficacy on nicotine-induced $^{86}$Rb$^+$ ion flux in both cell lines (Fig. 14A, C). To determine the mechanism of nAChR antagonism, the $^{86}$Rb$^+$ efflux nicotine dose–response studies were also performed in the presence of multiple buprenorphine concentrations (Fig. 14B, D). In both cell lines, buprenorphine decreased the nicotine-stimulated response non-competitively. The IC$_{50}$ and logIC$_{50}$ values are presented in Table 2.
Results

Figure 14. \(^{86}\text{Rb}^+\) efflux dose–response profiles of nAChR function for buprenorphine in SH-EP1-h\(\alpha 4\beta 2\) (A) and SH-SY5Y (C) cells and the mechanisms of antagonism of nAChR function in SH-EP1-h\(\alpha 4\beta 2\) (B) and SH-SY5Y (D) cells. The specific \(^{86}\text{Rb}^+\) efflux was determined in the presence of various concentrations of nicotine and increasing concentrations of buprenorphine in SH-EP1-h\(\alpha 4\beta 2\) (A) and SH-SY5Y (C) cells. To determine the mechanism of antagonism the nicotine-stimulated specific \(^{86}\text{Rb}^+\) efflux was determined in the absence or presence of various concentrations of buprenorphine in SH-EP1-h\(\alpha 4\beta 2\) (B) and SH-SY5Y (D) cells. The cells were exposed to the ligands for 5 min. The data were fit to the variable slope model. The values are the mean ± SD from three to six separate assays. The IC\(_{50}\) and logIC\(_{50}\) values are provided in Table 2.

5.4 Codeine (unpublished)

5.4.1 Effects of acute codeine treatment

Codeine only displaced [\(^3\text{H}\)]EPI at high concentrations in SH-EP1-h\(\alpha 4\beta 2\) and SH-SY5Y cells (Fig. 15). The \(K_i\) and log\(K_i\) values are presented in Table 1. In the \(^{86}\text{Rb}^+\) efflux assay, codeine showed no efficacy in either SH-EP1-h\(\alpha 4\beta 2\) or SH-SY5Y cells (data not shown). Codeine had inhibitory efficacy on nicotine-induced \(^{86}\text{Rb}^+\) ion flux in SH-SY5Y cells (Fig. 16C) and this inhibition was non-competitive by nature (Fig. 16D). The IC\(_{50}\) and logIC\(_{50}\) values are presented in Table 2. In SH-EP1-h\(\alpha 4\beta 2\) cells, codeine had a potentiative effect on nicotine-induced \(^{86}\text{Rb}^+\) efflux at low nicotine concentrations (Fig. 16A, B).
Results

Figure 15. \([^3H]EPI\) competition binding profiles in SH-EP1-\(ха4\beta2\) (A) and SH-SY5Y (B) cell membranes for nicotine and codeine. The assay mixtures contained 150 pM \([^3H]EPI\), 5–50 \(\mu\)g cell protein and nicotine or codeine at increasing concentrations. The data were fit to a one-site competition binding model. The values are the mean ± SD from three to five assays, with each point assayed at least in triplicate. \(K_i\) and log\(K_i\) values are provided in Table 1.

Figure 16. \(^{86}\text{Rb}^+\) efflux dose–response profiles of nAChR function for codeine in SH-EP1-\(ха4\beta2\) (A) and SH-SY5Y (C) cells and the mechanisms of antagonism or agonism of nAChR function in SH-EP1-\(ха4\beta2\) (B) and SH-SY5Y (D) cells. The specific \(^{86}\text{Rb}^+\) efflux was determined in the presence of various concentrations of nicotine and increasing concentrations of codeine in SH-EP1-\(ха4\beta2\) (A) and SH-SY5Y (C) cells. To determine the mechanism of antagonism or agonism, the nicotine-stimulated specific \(^{86}\text{Rb}^+\) efflux was determined in the absence or presence of various concentrations of codeine in SH-EP1-\(ха4\beta2\) (B) and SH-SY5Y (D) cells. The cells were exposed to the ligands for 5 min. The data were fit to the variable slope model. The values are the mean ± SD from three to six separate assays. The \(IC_{50}\) and log\(IC_{50}\) values are provided in Table 2.
Results

5.5 Oxycodone (unpublished)

5.5.1 Effects of acute oxycodone treatment

Oxycodone only displaced [³H]EPI at high concentrations in SH-EP1-hα4β2 and SH-SY5Y cells (Fig. 17). The $K_i$ and log$K_i$ values are presented in Table 1. In the $^{86}$Rb$^+$ efflux assay, oxycodone showed no agonist efficacy in either SH-EP1-hα4β2 or SH-SY5Y cells (data not shown). Oxycodone had inhibitory efficacy on nicotine-induced $^{86}$Rb$^+$ ion flux in both cell lines (Fig. 18A, C) and this inhibition was non-competitive by nature in the SH-SY5Y cells and mixed competitive/noncompetitive antagonism in the SH-EP1-hα4β2 cells, since oxycodone increased the EC$_{50}$ value for nicotine, yet the surmountability of the blockage was not evident (Fig. 18B, D). The IC$_{50}$ and logIC$_{50}$ values are presented in Table 2.

![Figure 17. [³H]EPI competition binding profiles in SH-EP1-hα4β2 (A) and SH-SY5Y (B) cell membranes for nicotine and oxycodone. The assay mixtures contained 150 pM [³H]EPI, 5–50 μg cell protein and nicotine or oxycodone at increasing concentrations. The data were fit to a one-site competition binding model. The values are the mean ± SD from three to five assays, with each point assayed at least in triplicate. $K_i$ and log$K_i$ values are provided in Table 1.](image-url)
Results

Figure 18. $^{86}\text{Rb}^+$ efflux dose–response profiles of nAChR function for oxycodone in SH-EP1-hα4β2 (A) and SH-SY5Y (C) cells and the mechanisms of antagonism of nAChR function in SH-EP1-hα4β2 (B) and SH-SY5Y (D) cells. The specific $^{86}\text{Rb}^+$ efflux was determined in the presence of various concentrations of nicotine and increasing concentrations of oxycodone in SH-EP1-hα4β2 (A) and SH-SY5Y (C) cells. To determine the mechanism of antagonism, the nicotine-stimulated specific $^{86}\text{Rb}^+$ efflux was determined in the absence or presence of various concentrations of oxycodone in SH-EP1-hα4β2 (B) and SH-SY5Y (D) cells. The cells were exposed to the ligands for 5 min. The data were fit to the variable slope model. The values are the mean ± SD from three to six separate assays with each point assayed at least in triplicate. The IC$_{50}$ and logIC$_{50}$ values are provided in Table 2.

5.6 Tramadol (unpublished)

5.6.1 Effects of acute tramadol treatment

Tramadol did not displace [³H]EPI in either SH-EP1-hα4β2 or SH-SY5Y cells (Fig. 19). In the $^{86}\text{Rb}^+$ efflux assay, tramadol showed no efficacy in either SH-EP1-hα4β2 or SH-SY5Y cells (data not shown). Tramadol had inhibitory efficacy on nicotine-induced $^{86}\text{Rb}^+$ ion flux in both cell lines (Fig. 20A, C) and this inhibition was non-competitive by nature (Fig. 20B, D). The IC$_{50}$ and logIC$_{50}$ values are presented in Table 2.
Results

Figure 19. [3H]EPI competition binding profiles in SH-EP1-hα4β2 (A) and SH-SY5Y (B) cell membranes for nicotine and tramadol. The assay mixtures contained 150 pM [3H]EPI, 5–50 μg cell protein and nicotine or tramadol at increasing concentrations. The data were fit to a one-site competition binding model. The values are the mean ± SD from three to five assays, with each point assayed at least in triplicate.

Figure 20. 86Rb⁺ efflux dose–response profiles of nAChR function for tramadol in SH-EP1-hα4β2 (A) and SH-SY5Y (C) cells and the mechanisms of antagonism of nAChR function in SH-EP1-hα4β2 (B) and SH-SY5Y (D) cells. The specific 86Rb⁺ efflux was determined in the presence of various concentrations of nicotine and increasing concentrations of tramadol in SH-EP1-hα4β2 (A) and SH-SY5Y (C) cells. To determine the mechanism of antagonism, the nicotine-stimulated specific 86Rb⁺ efflux was determined in the absence or presence of various concentrations of tramadol in SH-EP1-hα4β2 (B) and SH-SY5Y (D) cells. The cells were exposed to the ligands for 5 min. The data were fit to the variable slope model. The values are the mean ± SD from three to six separate assays with each point assayed at least in triplicate. The IC_{50} and logIC_{50} values are provided in Table 2.
5.7 Naloxone and naltrexone (unpublished)

5.7.1 Effects of acute naloxone and naltrexone treatments

Naloxone and naltrexone displaced [³H]EPI in SH-SY5Y and SH-EP1-α7 cells, but not in SH-EP1-α4β2 cells (Fig. 21). The Kᵢ and logKᵢ values are presented in Table 1. Preincubation with naloxone and naltrexone inhibited the nicotine-induced increase in [Ca²⁺]ᵢ in SH-SY5Y and SH-EP1-α7 cells, but not in SH-EP1-α4β2 cells (Fig. 22).

**Figure 21.** [³H]EPI competition binding profiles in SH-EP1-α4β2 (A), SH-SY5Y (B) and SH-EP1-α7 (C) cell membranes for nicotine, naltrexone and naloxone. The assay mixtures contained 150 pM [³H]EPI, 5–50 μg cell protein and nicotine, naltrexone or naloxone at increasing concentrations. The data were fit to a one-site competition binding model. The values are the mean ± SD from three to five assays, with each point assayed at least in triplicate. Kᵢ and logKᵢ values are provided in Table 1.

**Figure 22.** Effects of naloxone and naltrexone on nicotine-induced increases in [Ca²⁺]ᵢ in SH-EP1-α4β2, SH-SY5Y and SH-EP1-α7 cells. The Fluo-3 AM-loaded cells were incubated for 10 min with vehicle (TSS) or 100 μM naloxone or naltrexone and then stimulated with 10 μM or 30 μM nicotine. The responses are expressed as the percentage of the nicotine response. The values are the mean ± SEM from three to six separate assays. In each experiment, there were at least six replicates for each condition. Significantly different from nicotine response: *p < 0.05, **p < 0.01, and ***p < 0.001 using one-way ANOVA.

In the ⁸⁶Rb⁺ efflux assay, naloxone and naltrexone showed no agonist efficacy in either SH-EP1-α4β2 or SH-SY5Y cells (data not shown). Naloxone and naltrexone had inhibitory efficacy on nicotine-induced ⁸⁶Rb⁺ ion flux in both cell lines, and this inhibition was non-competitive by nature in the SH-SY5Y cells and
Results

mixed competitive/noncompetitive antagonism in the SH-EP1-hα4β2 cells, since both naloxone and naltrexone increased the EC$_{50}$ value for nicotine, yet the surmountability of blockage was not evident (Fig. 23 and 24). The IC$_{50}$ and logIC$_{50}$ values are presented in Table 2.

Figure 23. $^{86}$Rb$^+$ efflux dose–response profiles of nAChR function for naloxone (A) and naltrexone (C) and the mechanisms of antagonism of nAChR function (B, D) in SH-EP1-hα4β2 cells. The specific $^{86}$Rb$^+$ efflux was determined in the presence of various concentrations of nicotine and increasing concentrations of naloxone or naltrexone in SH-EP1-hα4β2 cells. To determine the mechanism of antagonism, the nicotine-stimulated specific $^{86}$Rb$^+$ efflux was determined in the absence or presence of various concentrations of naloxone or naltrexone in SH-EP1-hα4β2 cells. The cells were exposed to the ligands for 5 min. The data were fit to the variable slope model. The values are the mean ± SD from three to six separate assays with each point assayed at least in triplicate. The IC$_{50}$ and logIC$_{50}$ values are provided in Table 2.
Figure 24. $^{86}$Rb efflux dose–response profiles of nAChR function for naloxone (A) and naltrexone (C) and the mechanisms of antagonism of nAChR function (B, D) in SH-SY5Y cells. The specific $^{86}$Rb efflux was determined in the presence of various concentrations of nicotine and increasing concentrations of naloxone or naltrexone in SH-SY5Y cells. To determine the mechanism of antagonism, the nicotine-stimulated specific $^{86}$Rb efflux was determined in the absence or presence of various concentrations of naloxone or naltrexone in SH-SY5Y cells. The cells were exposed to the ligands for 5 min. The data were fit to the variable slope model. The values are the mean ± SD from three to six separate assays with each point assayed at least in triplicate. The IC$_{50}$ and logIC$_{50}$ values are provided in Table 2.
6. DISCUSSION

6.1 Effects of opioids on α4β2 nAChRs

The most abundant nAChR subtype in the human brain, the α4β2 nAChRs, have an important role in cognitive functions such as memory, attention and learning and in mood and motor function (Grupe et al. 2015). The α4β2 nAChRs are implicated in nicotine dependence as well as various neurological diseases, such as Parkinson's disease, Alzheimer's disease, attention deficit hyperactivity disorder (ADHD), schizophrenia, depression and epilepsy (Picciotto et al. 1998; Tapper et al. 2004; Steinlein and Bertrand 2010; Jurgensen and Ferreira 2010; Quik and Wonnacott 2011; Sarter et al. 2012; Yu et al. 2014; Potter et al. 2014). In addition to substance use patients, smoking rates are especially high among people with psychiatric disorders (Besson and Forget 2016; Sharma et al. 2016). Smoking has been proposed to act as self-medication among psychiatric patients and both smoking and mental illness may share a common cause.

In our studies, only morphine was able to displace \(^{3}H\)EPI from α4β2 nAChRs expressed in SH-EP1-hα4β2 cells. Morphine was also the only opioid that showed partial agonist efficacy in the \(^{86}\text{Rb}^+\) efflux assay, and this response was attenuated completely and non-competitively by nicotinic antagonist Meca, confirming that the effect is nAChR mediated. The opioid antagonists naloxone and naltrexone inhibited the morphine-induced ion flux competitively. Morphine elevated the \(^{86}\text{Rb}^+\) ion flux induced by low nicotine concentrations and attenuated the ion flux with high nicotine doses. Furthermore, the nicotine-induced elevation of \([\text{Ca}^{2+}]_i\) was reduced by high morphine concentrations, whereas low morphine concentrations had a small additive effect with nicotine. These findings suggest that morphine acts as a partial agonist at α4β2 nAChRs.

All of the other opioid ligands studied, methadone, buprenorphine, codeine, oxycodone, tramadol, naloxone and naltrexone, either did not displace \(^{3}H\)EPI from α4β2 nAChRs or the concentrations needed for displacement were very high. In the \(^{86}\text{Rb}^+\) efflux assay, only methadone inhibited nicotine-induced ion flow with adequate concentration. None of these opioid ligands showed agonistic efficacy in the \(^{86}\text{Rb}^+\) efflux assay. Codeine had a potentiative effect on nicotine-induced \(^{86}\text{Rb}^+\) efflux at low nicotine concentrations. Interestingly, the EC\(_{50}\) values for nicotine in the presence of naloxone, naltrexone or oxycodone were higher than in the absence of these ligands, but surmountability of blockage was not clearly evident, suggesting a mixture of competitive and noncompetitive blockage. Methadone and buprenorphine inhibited the nicotine-induced rise in \([\text{Ca}^{2+}]_i\), whereas naloxone and naltrexone did not inhibit the nicotine-induced increase in \([\text{Ca}^{2+}]_i\). These results suggest that methadone is an NCA at α4β2 nAChRs, that codeine has a positive modulatory effect on α4β2 nAChRs, that naloxone, naltrexone and oxycodone are mixed competitive/noncompetitive antagonists of α4β2 nAChRs, and that tramadol and buprenorphine are weak NCAs at α4β2 nAChRs. Our results support the previous findings of codeine’s positive modulatory effect on α4β2 nAChRs (Storch et al. 1995; Iorga et al. 2006).

Both prolonged methadone and morphine treatment down-regulated α4β2 nAChRs and changed nicotine’s effect on α4β2 nAChRs, whereas sustained nicotine caused substantial upregulation. The α4β2 nAChRs are upregulated by chronic nicotine as well as by other nicotinic agonists and antagonists in vitro and in vivo (Peng et al. 1994; Gopalakrishnan et al. 1997; Whiteaker et al. 1998; Hussmann et al. 2011;
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Marks et al. 2015). Studies with non-competitive α4β2 nAChR antagonists and partial agonists have produced mixed results; some have been shown to cause upregulation, whereas some either have no effect on receptor numbers or have a down-regulating effect (Peng et al. 1994; Whiteaker et al. 1998; Xiao et al. 2006; Hussmann et al. 2012; Marks et al. 2015). Whereas smoking upregulates nAChRs, reduced α4β2 nAChR numbers are associated with many neuropsychiatric disorders, such as Parkinson’s disease and Alzheimer’s disease (Meyer et al. 2009; Kendziorra et al. 2011; Hurst et al. 2013). Furthermore, a low baseline level of α4β2* nAChRs has been suggested to be one of the predisposing factors for nicotine dependence since increased nicotine self-administration is associated with lower levels of midbrain α4β2 nAChRs (Le Foll et al. 2009). Our findings that both methadone and morphine downregulate α4β2 nAChRs and that prolonged exposure to these opioid agonists changes nicotine’s effect on α4β2 nAChRs may thus have clinical importance. The high smoking rates and difficulties in smoking cessation among patients on opioid maintenance therapy may partially be a consequence of this receptor-level interaction. If α4β2 nAChRs are down-regulated in the brains of opioid-maintained patients, they may smoke more in order to compensate the reduced effect of nicotine. The plasma concentration of methadone in maintenance patients can reach 1 μM (de Vos et al. 1995; Dyer et al. 1999) and the plasma concentration of morphine in cancer patients varies between 0.02–0.60 μM (Sakurada et al. 2010). The effects seen in our upregulation studies are likely to also be valid in the clinical setting, since the opioid concentrations used fall in the concentration ranges of 1–10 μM for methadone and 0.1–1 μM for morphine.

6.2 Effects of opioids on α3* nAChRs

The 15q25 gene cluster, which contains the CHRNA5/CHRNA3/CHRNB4 genes coding for the α5, α3 and β4 nAChRs, is linked to the risk of heavy smoking, nicotine dependence and smoking-related diseases (Bierut 2009). The role of α5, α3 and β4 nAChRs in nicotine withdrawal and aversion has also been confirmed in in vivo studies (Salas et al. 2004; Salas et al. 2009; Jackson et al. 2010; Frahm et al. 2011). The majority of α3β4* nAChRs reside in the MHb and interpeduncular nucleus, which are involved in nicotine withdrawal and intake (Quick et al. 1999; Whiteaker et al. 2002; Salas et al. 2009; Fowler and Kenny 2012).

The SH-SY5Y cells express the α3, α5, α7 and β2, β3, β4 subunits and these subunits form functional pentameric receptors of different combinations, such as the α3β4 or α7 nAChRs (Ross et al. 1983; Gould et al. 1992; Lukas et al. 1993; Peng et al. 1997; Groot Kormelink and Luyten 1997). [3H]EPI binds to all nAChR subtypes expressed in SH-SY5Y cells, however, the β2 subunit-containing receptors have a higher affinity for [3H]EPI than the β4 or α7 subunit-containing receptors (Gerzanich et al. 1995; Wang et al. 1996). In the [3H]EPI binding assay, methadone, morphine, naloxone and naltrexone displaced [3H]EPI, whereas buprenorphine, codeine, oxycodone and tramadol either did not inhibit [3H]EPI binding or the concentrations needed for inhibition were very high. Methadone, morphine, naloxone and naltrexone also displaced [3H]EPI from α7 nAChRs in the SH-EP1-hα7 cell line, so the [3H]EPI displacement identified in the SH-SY5Y cells could result from either α7 or α3* nAChRs.

In the 86Rb+ efflux assay, methadone, tramadol and buprenorphine inhibited the nicotine-induced ion flux with reasonably low IC50 values in the SH-SY5Y cells, whereas other opioids were less effective. The rank order for ion flux inhibition potency by IC50 value was methadone < tramadol < buprenorphine <
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naloxone < naltrexone < oxycodone < codeine < morphine. Buprenorphine and methadone also inhibited the nicotine-induced increase in [Ca^{2+}], however the effect of tramadol, oxycodone or codeine on [Ca^{2+}] was not studied. Methadone itself increased [Ca^{2+}] in a concentration-dependent manner, as has been shown before (Pakkanen et al. 2005). In the same study, preincubation with an α3* nAChR antagonist, dihydro-β-erythroidine, had no effect on methadone-evoked increases in [Ca^{2+}] in SH-SYSY cells, whereas an α7 nAChR antagonist, MLA, partially antagonized the effects of methadone. These results suggest that the methadone-induced increase in [Ca^{2+}] is at least partly mediated through the α7 nAChRs and that the α3* nAChRs expressed in SH-SYSY cells do not participate in the Ca^{2+} influx evoked by methadone. The α7 nAChRs have especially high permeability for Ca^{2+} (Quik et al. 1997), and therefore the methadone-induced Ca^{2+} flow is also most likely mediated through α7 nAChRs. In SH-SYSY cells, opioids require ongoing muscarinic receptor activation in order to mobilize Ca^{2+} from intracellular stores (Connor and Henderson 1996), and the activation of α7 nAChRs may provide the initial increase in [Ca^{2+}], allowing the opioid-like action of methadone.

Tramadol has previously been shown to inhibit the function of α7 and the other subclasses of nAChRs expressed in adrenal chromaffin cells, such as the α3β4 nAChRs (Shiraishi et al. 2002), and our results are in line with this study regarding the α3* nAChR inhibition.

Both prolonged methadone and morphine treatment, as well as nicotine, upregulated nAChRs in SH-SYSY cells, as measured by [3H]EPI binding; however, this upregulation had almost no effect on nicotine-induced ion flow in the 86Rb⁺ efflux assay. This may be explained by the fact that the 86Rb⁺ efflux assay measures the function of receptors on the cell surface only, whereas the majority of the increase in [3H]EPI binding sites takes place in the intracellular compartment of SH-SYSY cells (Peng et al. 1997). The intracellularly accumulated α3* nAChRs in SH-SYSY cells have been shown to be previously exposed on the cell surface by an antigenic modulation technique, which suggests that chronic nicotine may induce internalization of α3* nAChRs without decomposition of these receptors. Chronic treatment with nicotine produces a 500–600% increase in α3* nAChRs in SH-SYSY cells, but only a 30% increase in α7 nAChRs (Peng et al. 1997); consequently, the receptors upregulated by methadone and morphine treatments are most likely α3* nAChRs. Furthermore, since only the function of the α3* nAChRs can be measured by 86Rb⁺ efflux, the effect of possible α7 nAChR upregulation cannot be measured using this method. The upregulated nAChRs on the cell surface may also be nonfunctional and therefore no effect is seen although receptor numbers measured by [3H]EPI binding are elevated. Nevertheless, these results are in line with an earlier study where chronic nicotine treatment did not increase the affinity for nicotine despite upregulation (Peng et al. 1997). Prolonged methadone treatment has previously been shown to upregulate the nAChRs in SH-SYSY cells (Pakkanen et al. 2005), whereas there are no studies on morphine’s effect on nAChR numbers.

6.3 Effects of opioids on α7 nAChRs

α7 nAChRs are particularly expressed in brain regions linked with cognitive function, such as the hippocampus, cortex and subcortical limbic regions as well as in the thalamus and basal ganglia (Marks

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and Collins 1982; Clarke et al. 1985; Rubboli et al. 1994; Gotti et al. 2006). α7 nAChRs are implicated in the pathophysiology and pathogenesis of many diseases and disorders such as schizophrenia, autism, Alzheimer’s disease and Down syndrome (Young and Geyer 2013; Deutsch et al. 2015; Shen and Wu 2015). The expression of α7 nAChRs is reduced in postmortem brains of schizophrenic patients (Freedman et al. 1995; Court et al. 1999). Most schizophrenics are heavy smokers, and smoking increases the expression of CHRNA7 mRNA and protein levels, raising them up to the levels of healthy controls (Dalack et al. 1998; de Leon and Diaz 2005; Mexal et al. 2010). α7 nAChR protein expression and binding are elevated in the cerebellum of autistic patients (Lee et al. 2002; Martin-Ruiz et al. 2004). Losses in α7 nAChR binding sites have also been reported in several brain regions of Alzheimer’s disease patients (Burghaus et al. 2000).

Methadone, morphine, buprenorphine, naloxone and naltrexone displaced [3H]EPI from the α7 nAChRs expressed in SH-EP1-hα7 cells, with methadone and naltrexone having the lowest Kᵢ values. Also, all of these opioid ligands studied, except for morphine, inhibited the nicotine-induced increase in [Ca²⁺]. These results suggest that buprenorphine, naloxone and naltrexone are weak α7 antagonists. Naltrexone has previously been shown to be a non-competitive α7 nAChR antagonist and to block the nicotine-induced upregulation of α7 nAChRs (Almeida et al. 2000; Almeida et al. 2004). Morphine (50 μM) preincubation had an additive effect on the nicotine-induced increase in [Ca²⁺], and morphine itself also increased [Ca²⁺], in a similar manner as nicotine. This could result from a synergistic effect of morphine and nicotine acting through their own specific receptors or due to interaction at nAChRs via a positive allosteric mechanism. Morphine’s effect on nAChRs has not been studied before at the receptor level; however, in vivo pretreatment with specific α7 nAChR antagonist, MLA, diminished the reinstatement of morphine-induced CPP, which suggests that α7 nAChRs may contribute to the reinstatement of morphine-induced CPP (Feng et al. 2011).

An earlier in vitro study suggested that methadone is an agonist of human α7 nAChRs (Pakkanen et al. 2005), and our results are in line with this study. Methadone inhibits [3H]EPI binding in SH-EP1-hα7 cells as well as in the SH-SY5Y cell line, which expresses the α3, α5, α7, β2 and β4 nAChR subunits. Unfortunately, the ⁸⁶Rb⁺ efflux assay is not suitable for studying α7 nAChR function due to the very rapid desensitization kinetics (Lukas et al., 2002). Functional studies, however, can be conducted with calcium fluorometry since the α7 nAChRs have a high permeability to Ca²⁺ (Castro and Albuquerque 1995). Methadone increased the [Ca²⁺] levels in SH-EP1-hα7 cells in a similar manner as nicotine, which supports the theory of methadone as an α7 nAChR agonist. When SH-EP1-hα7 cells were pre-incubated with methadone, the nicotine-induced increase in [Ca²⁺] was reduced, presumably as a result of the desensitization of α7 nAChRs. Nicotinic ligands MLA and Meca, as well as opioid antagonists naloxone and naltrexone, inhibited the methadone-induced increase in [Ca²⁺], levels in SH-EP1-hα7 cells. This inhibition could result from inhibition of both the α7 nAChRs and opioid receptors expressed in SH-EP1-hα7 cells, since naloxone and naltrexone appear to be weak antagonists at the α7 nAChRs.

Both prolonged methadone and morphine treatment upregulated α7 nAChRs measured by [3H]EPI binding. α7 nAChRs are upregulated by chronic nicotine exposure to a lesser extent than α4β2 nAChRs, and the nicotine concentration required for the upregulation might be higher than what is attained in smokers’ blood (Fenster et al. 1997; Ke et al. 1998; Alkondon et al. 2000). Both chronic nicotinic agonist and antagonist treatments upregulate α7 nAChRs and may enhance receptor function (Molinari et al. 1998; Ridley et al. 2001; Nuutinen et al. 2006).
6.4 General discussion

Nicotine appears to interact with several opioid ligands, and this interplay seems to be at least in part mediated through their pharmacological activity at nAChRs. The main findings of this receptor level interaction are summarized in Table 3. The high prevalence of smoking with opioid use may also be a consequence of interactions at the level of DA, since nicotine has the ability to increase extracellular DA levels in mesolimbic brain regions, likewise for opioids and other drugs of abuse. Nicotine exposure alters the reinforcing and rewarding effects of opioids and vice versa; the use of other drugs may enhance the pleasurable effect of smoking (Henningfield and Griffiths 1981; Chait and Griffiths 1984; Amos et al. 2004). In addition to opioids, nicotine is also commonly used with other substances of abuse, such as stimulants, alcohol and cannabis (Burling and Ziff 1988; Roll et al. 1997; Viveros et al. 2006). Nicotine has, for instance, been shown to alleviate opioid and alcohol withdrawal (Elkader et al. 2009; Perez et al. 2015), to enhance the effects of stimulants (Wiseman and McMillan 1998), and to enhance opioid tolerance and antinociception (Zarrindast et al. 1997; Schmidt et al. 2001; Zarrindast et al. 2003).

Table 3. The main findings of the receptor-level effects of opioids studied in SH-EP1-hα4β2, SH-SY5Y and SH-EP1-hα7 cell lines.

<table>
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<tr>
<td>Methadone</td>
<td>NCA</td>
<td>NCA</td>
<td>agonist</td>
</tr>
<tr>
<td>Morphine</td>
<td>partial agonist</td>
<td>weak NCA</td>
<td>PAM?</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>weak NCA</td>
<td>weak NCA</td>
<td>weak antagonist</td>
</tr>
<tr>
<td>Codeine</td>
<td>PAM</td>
<td>weak NCA</td>
<td>-</td>
</tr>
<tr>
<td>Oxycodone</td>
<td>mixed CA/NCA</td>
<td>weak NCA</td>
<td>-</td>
</tr>
<tr>
<td>Tramadol</td>
<td>weak NCA</td>
<td>NCA</td>
<td>-</td>
</tr>
<tr>
<td>Naloxone</td>
<td>mixed CA/NCA</td>
<td>weak NCA</td>
<td>weak antagonist</td>
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<tr>
<td>Naltrexone</td>
<td>mixed CA/NCA</td>
<td>weak NCA</td>
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</tr>
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NCA, non-competitive antagonist; CA, competitive antagonist; PAM, positive allosteric modulator; -, not studied;

Smoking increases health risks not only in the general population, but to an even greater magnitude among patients on opioid maintenance therapy. Opioid users are at a greater risk of death due to respiratory diseases, cancer, and cardiovascular diseases, and the relative risk of death due to tobacco-related diseases is 1.8 times higher among opioid abusers than in the general population (Hurt et al. 1996; Maxwell et al. 2005). Smoking cessation is challenging for opioid maintenance patients, yet the majority of them are motivated to quit (Shoptaw et al. 2002; Nahvi et al. 2006). Smoking cessation would be very beneficial for opioid-maintained patients, since success in smoking cessation is also associated with greater abstinence from opioid use (Lemon et al. 2003). Unfortunately, the current pharmacotherapeutic options for smoking cessation, bupropion, varenicline and nicotine replacement therapy, are ineffective among opioid-substituted patients (Okoli et al. 2010; Zirakzadeh et al. 2013; Nahvi et al. 2014). The nicotinic–opioid interaction is a built-in property of varenicline, since its chemical structure is derived from morphine and cytisine (Coe et al. 2005; Rollema et al. 2007). Varenicline is a α4β2 and α6β2* nAChR partial agonist and a full agonist at α3β4* and α7 nAChRs (Coe et al. 2005; Grady et al. 2010), upregulating α4β2, α3β4*, α7 nAChRs and down-regulating α6β2* nAChRs (Turner et al. 2011; Marks et al. 2015). Both methadone and varenicline seem to regulate nAChR numbers
differentially, which might explain why combined use of these substances results in low smoking abstinence rates among methadone users. If α4β2 nAChRs are down-regulated in the brains of methadone-maintained patients, the effect of varenicline might be submaximal, and lead to lower smoking cessation rates. Patients on high methadone doses are more severely addicted to nicotine, and their withdrawal symptoms and nicotine cravings are higher compared to the general population (Clarke et al. 2001; Elkader et al. 2009). In our studies, chronic methadone down-regulated the α4β2 nAChRs and reduced nicotine’s potency and efficacy on α4β2 nAChRs; thus, it can be speculated that methadone-maintained patients smoke more to overcome this receptor-level interaction of nicotine and methadone. Even though a direct comparison of in vitro and human data might not be relevant, our findings support the hypothesis of nicotine–opioid interactions at the level of nAChRs, which should be taken into account in the planning of smoking cessation treatments and pharmacotherapy for opioid addicts. Polysubstance abuse represents a unique challenge for treatment schemes, requiring a more precise understanding of the behavioral and biochemical background of the nicotine–opioid interplay.
7. SUMMARY AND CONCLUSIONS

Although nicotine and opioids have both their own molecular mechanisms of action and designated receptors, it seems that these substances interact at the level of nAChRs. The aims of these studies were to investigate in vitro the effect of various opioid agonists and antagonists on nAChR function and whether these opioid ligands bind to the orthosteric binding site of nAChRs. Additionally, the effect of prolonged opioid treatment on nAChR numbers and function was studied with methadone and morphine. The major findings are as follows:

1. Morphine acts as a partial agonist and codeine as a PAM at α4β2 nAChRs. Methadone is a NCA at α4β2 nAChRs, whereas tramadol and buprenorphine are weak NCAs. Naloxone, naltrexone and oxycodone are mixed competitive/noncompetitive antagonists of α4β2 nAChRs.

2. All of the opioid ligands studied are NCAs of α3* nAChRs: methadone and tramadol have the lowest IC50 values, and morphine and codeine are the weakest inhibitors.

3. Methadone is an agonist of human α7 nAChRs, whereas buprenorphine, naloxone and naltrexone are weak α7 antagonists. Morphine has a positive synergistic effect with nicotine on α7 nAChR function.

4. α3*, α4β2 and α7 nAChRs are differentially regulated by prolonged exposure to methadone and morphine. Methadone and morphine upregulate α3* and α7 nAChRs, whereas α4β2 nAChRs are down-regulated. The down-regulation of α4β2 nAChRs changes nicotine’s effect on these receptors.
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