Challenging the cholinergic system : ageing, cognition & inflammation

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GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

CHOLINERGIC SYSTEM DISCOVERY AND PHYSIOLOGY

In 1921 Otto Loewi and his co-workers discovered the chemical transmission of nerve impulses, demonstrating that the parasympathetic substance (Vagusstoff, translated from German as 'Vagus Substance'), today known as acetylcholine, played an important role at the sympathetic nerve endings (Loewi, 1921). Based on these observations he received the Nobel Prize of Medicine in 1936, jointly with Sir Henry Dale, who actually identified acetylcholine in the first place with his colleague Arthur Ewins (Ewins, 1914). It was Loewi, however, who showed its important role in the nervous system.

The cholinergic system comprises organized nerve cells that use the neurotransmitter acetylcholine to activate other neurons, mostly by containing and releasing acetylcholine, propagating a nerve impulse. Initial experiments to investigate acetylcholine's central origin were performed. Acetylcholine or diisopropylfluorophosphonate (an acetylcholinesterase inhibitor) induced effects when the compounds were administered in an experimental *cerveau isolé* animal model via carotid injection, but not via de isolated hemisphere preparation, suggesting that acetylcholine production occurred in the brainstem (Jasper, 1965; Karczmar, 1967). Later on, the concepts were supported by the observations that brain electric stimulation increased cortical acetylcholine concentrations (MacIntosh and Oborin, 1953) and, thereafter with immunohistochemistry techniques (Koelle and Friedenwald, 1949). These investigations gave rise to the discovery of the cholinergic alerting mesodiencephalic system or, as we know it today, the (ascending) reticular activating system (ARAS), a network containing the cholinergic and adrenergic system that regulates wakefulness and sleep-awake transitions (Figure 1.1).

Acetylcholine is produced in several brain structures including the basal forebrain (nucleus basalis, diagonal band, medial septum and substantia innominata), ventral tegmental area, raphe and locus ceruleus. All these conglomerates of cholinergic cells project their axons to different areas of the brain exerting mainly an excitatory effect, binding to different acetylcholine receptors (AChRs) in the thalamus and cortex (Mesulam, 2013). The cholinergic system has been associated with a number of cognitive functions, including memory, selective attention, language, reaction time to stimuli and emotional processing (Furey, 2011). In the periphery, acetylcholine also has important functions binding to AChRs in autonomic ganglia and in the neuro-muscular junction (Brunton *et al*, 2011). The functions of acetylcholine are sub served by two different types of receptor: muscarinic and nicotinic.

THE MUSCARINIC ACETYLCHOLINE RECEPTOR

RECEPTOR CONFORMATION AND PHYSIOLOGY

Five subtypes (M_1-M_5) have been characterized. All 5 types are expressed in the CNS, where they play a role in learning and memory, arousal, rapid eye movement sleep, control of movement, thermoregulation and reward behaviour (Pennartz *et al*, 1994; Picciotto *et al*, 2012; Vazquez and Baghdoyan, 2001). In the periphery, muscarinic activation is associated with parasympathic autonomic functions such as a reduction in heart rate (M_2) and vasodilatation (M_1, M_2, M_3), increases in exocrine secretions from sweat, salivary and lacrimal glands (M_1, M_3), and contraction of smooth muscle in the gastrointestinal tract (M_2, M_3) or airways (M_3, M_4) (Caulfield, 1993). Muscarinic acetylcholine receptors (mAChR) are members of the 7 transmembrane guanine nucleotide-binding protein (G protein)-coupled receptor (GPCR) superfamily. In general, M_1, M_3, M_5 receptors act via activation of G proteins while M_2, M_4 receptors perform inhibitory functions. Activation of the receptor coupled G proteins result in activation of an enzyme to generate a second messenger or producing a response by interaction directly with an effector, usually an ion channel (Birnbaumer *et al*, 1990). Knock-out mice of the muscarinic receptor have provided information on the executive functions of the receptor in the CNS. M_1 knock-out mice have demonstrated subtle detrimental effects in learning and memory, however none significant effects on behaviour or motor and coordination functions (Miyakawa *et al*, 2001). M_4 mice showed a significant increase in basal motor activity, interestingly these mice were also hyper-responsive to stimulation with a D_1 dopamine receptor agonist, suggesting that M_4 AChRs play a predominant role in dampening D_1 -mediated effects on locomotor activity (Gomeza *et al*, 1999). M_3 AChR has been related to centrally mediated regulation of feeding behavior and body weight (Yamada *et al*, 2001) and M_5 might have an important role in addiction and motivational behaviour (Basile *et al*, 2002).

RECEPTOR LOCALIZATION, RELATED COGNITIVE FUNCTIONS, LINK TO DISEASES AND THERAPEUTIC POSSIBILITIES

Table 1.1 shows the localization and muscarinic receptor distribution in the rat brain. The highest density of M_1 receptors is located mainly in cortex, hippocampus and striatum, which is consistent with effects on memory and cognition (Flynn *et al*, 1997). Interest in cholinergic agonists to treat Alzheimer's disease (AD) has predominantly been driven by the 'cholinergic hypothesis', relying on the fact that a degeneration of cholinergic neurons is associated with the memory and cognitive loss observed with the disease (Coyle *et al*, 1983; Whitehouse *et al*, 1982). Interest in specific M_1AChRs agonists grew with the discovery that M_1AChRs has a major role in hippocampal-based memory and learning regulation of cognition and short-term memory, all functions affected in AD. Also, post-mortem studies have demonstrated decreased levels of M_1 and M_4 AChRs in patients diagnosed with schizophrenia (Dean *et al*, 1996, 2002). The first M_1 and M_4 agonists to reach late clinical development, xanomeline, provided evidence of the cognitive improvement after muscarinic stimulation in patients with AD (Bodick *et al*, 1997) and schizophrenia (Shekhar *et al*, 2008), however due to adverse events secondary to peripheral muscarinic stimulation the compound development was abandoned. Aiming for a more selective central mechanism of action led to the development of allosteric agonists and positive allosteric modulators such as TBPB, 77-LH-28-1, AC260584 and thereafter VU0186470 and VU0357017, which are currently in clinical development with promising early development results (Jones *et al*, 2012). More recently, interest increased even more after chronic use of M_1AChR agonists, namely AF102B and talsaclidine, in AD patients compared to controls resulted in a significant decrease of CSF Aβ in AD patients, whereas the acetylcholinesterase inhibitor (AChEIs) physostigmine, galantamine and donepezil did not (Hock *et al*, 2003; Nitsch *et al*, 2000; Parnetti *et al*, 2002). Additionally, M_1 localization in the striatum is consistent with its role in control of movement and the noted therapeutic benefit of M_1 antagonists for movement disorders like Parkinson's disease (PD) (Xiang *et al*, 2012). As shown in Table 1.1, the M_4AChR is highly expressed in the striatum, hippocampus, and neocortex and can modulate dopaminergic signaling, being able to reduce striatal dopamine release (Threlfell *et al*, 2010). M_4 allosteric modulators (LY2033298, VU0152099 and VU0152100) are in early phase development for cognitive symptoms of neurodegenerative diseases. M_5 positive allosteric modulators are still in preclinical development as potential cognitive enhancers (Bridges *et al*, 2009).

THE NICOTINIC ACETYLCHOLINE RECEPTOR

RECEPTOR CONFORMATION

Nicotinic acetylcholine receptors (nAChR) are ligand-gated neurotransmitter coupled ion channel receptors composed of eleven different subunits. The subunits can be sub-classified into two groups, containing eight α and three β subunits. The nAChR situated in the muscle is α_1, which is the key calcium channel receptor in the neuromuscular junction. The neuronal subunits are α_2-α_9, all of them determine the agonist binding (functional) subunits. The significance of β subunits which are categorized in β_2-β_4 is primarily structural, rather than functional. However it has been demonstrated that both α and β subunits contribute to the pharmaceutical specificity of the receptors (Gotti *et al*, 2006). Each subtype differs in properties such as localization, up-regulation, channel kinetics and desensitization. Each receptor is formed by 5 homologous subunits, either combining α and β or only α subunits (α_7, α_8, and α_9 are the only subunits that can form homomeric receptors). α_7 is the predominant homomeric nAChR distributed in the mammalian brain (Dani and Bertrand, 2007). Table 1.2 shows the predominant localization of the main nAChRs in the brain.

RECEPTOR PHARMACOLOGY

Brain nAChRs are expressed in postsynaptic terminals at neurons where binding exerts a fast excitatory synaptic transmission. On the other hand, most of the receptors are located presynaptically, where binding mainly modulates neurotransmitter release into the synaptic cleft (Clarke, 1993). For example, both $\alpha_4\beta_2$ and α_7 AChRs modulate the release of GABA in hippocampal CA1 interneurons (Alkondon and Albuquerque, 2001). Instead

of terminating in synaptic targets, it has been postulated that the majority of cortical and hippocampal cholinergic projections release sites are non-synaptic and contribute to diffuse volume transmission. In other words, activation of the system causes an effect in large areas of the brain (Descarries *et al*, 1997). Functionally, central nAChRs have high calcium permeability and the receptor is able to rectify the inward currents when the membrane is depolarized (Mathie *et al*, 1990). All nAChRs in the presence of agonist first open the ion channel in several millisecond bursts followed by a desensitized conformation characterized by a closed channel and higher binding affinity conformational change (Quick and Lester, 2002). The receptors are also able to induce allosteric modulation, which are conformational changes, which lead to a response caused by binding of ligands to sites different to the agonist-binding sites (Changeux and Edelstein, 1998). Steroids, specifically 17β-estradiol, are examples of ligands that modulate the human $\alpha_4\beta_2$ nAChR (Paradiso *et al*, 2001). Adding complexity to understanding the system, nAChR up- or down-regulation occurs independently of the receptor in the presence of a ligand. For example, nicotine-induced up-regulation of α_3-associated (predominantly $\alpha_3\beta_2$) and α_7 nAChRs in the same hippocampal neurons differ under the same nicotine concentration (Ridley *et al*, 2001). Furthermore, during the development of new compounds with α_7 nAChR agonistic activity, namely EVP-6124 and PHA543613, evidence of a bell-shaped or inversed-U concentration-effect curve has been described in *in vitro* experiments (Prickaerts *et al*, 2012; Yang *et al*, 2013) and also in the $\alpha_4\beta_2$ nAChR for nicotine and partial nicotinic receptor agonist varenicline (Rollema *et al*, 2007). This unique nAChR property might result from the afore-mentioned mechanisms and represents a challenge to determine the effective dose of subtype selective nicotinic receptor agonists.

REGULATION OF COGNITIVE FUNCTIONS

For the above-mentioned reasons, determining the nicotinic function in cognition only by localization and by isolated receptor physiology experiments is not possible. Several experiments with agonists and antagonists *in vivo* have been needed to investigate the role the nicotinic system has in cognition. Memory is improved by nicotinic agonists, and impaired by antagonists and lesions in cholinergic nuclei or tracts impair memory (Levin *et al*, 2006). Local infusion of methyllycaconitine (an α_7 antagonist) or dihydro-β-erythroidine (an $\alpha_4\beta_2$ antagonist) into the basolateral amygdala, the ventral and dorsal hippocampus impaired the working memory of rats in a 16-arm radial maze (Nott and Levin, 2006). In monkeys, while nicotine improved performance in visuo-spatial memory and spatial working memory tests, mecamylamine (a known non-selective nAChR antagonist) impaired visuo-spatial memory and fine motor performance (Katner *et al*, 2004).

DYSFUNCTION AND THERAPEUTIC OPTIONS OF THE NICOTINIC SYSTEM

Plaques originating from Amyloid-β peptides and neurofibrillary tangles are hallmarks of Alzheimer's disease (AD). Even though Aβ plaque deposition is pathognomonic for AD, treatments directed to eliminate the plaques have not yet proven effective as disease modifying or delaying strategies regardless of the fact that the plaques are effectively cleared in the parenchymal brain tissue of treated patients (Paquet *et al*, 2015). AD is characterized by progressive and irreversible cognitive dysfunction, particularly in learning and memory. Anatomically, AD affects limbic structures, subcortical nuclei, and cortical regions. As mentioned in the previous section, the most important neuronal loss in AD is in the cholinergic system, particularly cholinergic neurons in the basal forebrain, the medial septal nucleus, the horizontal and vertical diagonal bands of Broca, and the nucleus basalis of Meynert (Auld *et al*, 2002). Several nAChR subtypes, including the $\alpha_4\beta_2$, α_7 and α_3-related nicotinic receptors, are reduced in AD (Perry *et al*, 1998). Even though the underlying etiologic mechanisms (link between the presence of Amyloid β and cholinergic dysfunction) are unknown, it has been shown that Amyloid β binds to the α_7 nAChR producing its inactivation and down-regulation (Wang *et al*, 2000). Compounds with nAChR agonist activity have been considered not only as cognitive enhancers, but also as mediators of the innate inflammatory response. They may therefore have a therapeutic possibility as disease modifying treatment for AD (Hurst *et al*,

2013; Vallés *et al*, 2014). Vagus nerve electrical stimulation decreased tumour necrosis factor (TNF-α) secretion in macrophages induced by bacterial lipopolysaccharide by acting on α_7 nAChRs (Wang *et al*, 2003). Alpha$_7$ nAChRs are also expressed in microglia and therefore might also play an important role in the inflammation response in AD (Conejero-Goldberg *et al*, 2008). Functional changes in the nAChR have been reported in several diseases including schizophrenia (Court *et al*, 2000). Nicotinic agonists have also been tested for the treatment of negative symptoms derived from schizophrenia (Beinat *et al*, 2015). Cholinergic-dopaminergic interactions might be related to improvement in the symptoms related to patients with Attention Deficit Hyperactivity Disorder and Parkinson's Disease (Potter *et al*, 2006; Quik and Kulak, 2002). Administration of nicotine to adolescents diagnosed with Attention Deficit Hyperactivity Disorder improved cognition (Potter and Newhouse, 2004). The nAChRs on dopaminergic neurons of the substantia nigra may be especially sensitive to loss in Parkinson's Disease (PD), suggesting that nAChRs may be especially important in the etiology of PD and might be a potential drug target (Perez-Lloret and Barrantes, 2016; Pérez and Quik, 2011). Recently, a phase III clinical trial with AQW051, an α_7 nicotinic agonist, reported no improvement in dyskinesia, however administration of AQW051 50 mg significantly improved cognitive memory tests when compared to placebo (Trenkwalder *et al*, 2016). On the other hand, pre-clinical results are promising for other two α_7 nicotinic agonists, ABT-107 and ABT-126, which reduced Levodopa-induced dyskinesia (LID) in an experimental model of PD (Zhang *et al*, 2014, 2015). To date, several novel nicotinic agonists compounds have shown promising early clinical results but are still in development as treatments for neurodegenerative diseases such as AD, PD and for schizophrenia (Jones *et al*, 2012; Toyohara and Hashimoto, 2010; Vallés *et al*, 2014), and the scientific community waits impatiently the results of the phase III studies.

CHOLINERGIC PHARMACOLOGIC CHALLENGE MODELS

A pharmacologic challenge influences, preferably selectively, a system by means of a pharmacological agent, with a resulting transitory physiological change that may resemble a pathologic state. During this period the researcher can measure the resulting effect, repetitively if needed, under controlled conditions and obtain important information about the underlying mediating process. Pharmacological challenge test are also used to study the roles of pharmacological systems in health and disease. Pharmacological challenge tests are often used for research purposes but they are also used, mostly for diagnostic purposes, in clinical practice.

Children with growth hormone deficiency may undergo a challenge with the α_2-adrenergic agonist clonidine to guide de pediatric endocrinologist to the right diagnosis (Lanes *et al*, 1985). A challenge model can give the additional benefit of quantifying the magnitude of the effect and relate the disturbing stimulus (e.g.: plasma concentrations of the pharmaceutical compound) to the effect (Klein *et al*, 2013).

A pharmacological challenge model may also be used to test new pharmaceutical compounds in humans and animals, as a disease model or to study the impact of a drug on a pharmacological system. Challenge tests can therefore be important translational tools in drug development and to test and quantify pharmacokinetic and pharmacodynamic interactions. Scopolamine is a selective competitive muscarinic antagonist and has been widely used in the field of neuropharmacology as a standard test to induce dementia- and age-related temporary cognitive impairments to healthy subjects, patients and animals (Klinkenberg and Blokland, 2010). Recently, the effects of scopolamine as a challenge model have been successfully quantified in order to better understand the effect of muscarinic blockade in the human brain (Liem-Moolenaar *et al*, 2011) and these advances have helped to test novel compounds acting on the cholinergic system (Buccafusco, 2009; Liem-Moolenaar *et al*, 2010a, 2010b; Lines *et al*, 1993; Preston *et al*, 1988). Several authors have reported reversal of scopolamine effects in cognition using two acetylcholinesterase inhibitors, galantamine and donepezil (Baraka and Harik, 1977; Thomas *et al*, 2008). Mecamylamine is a selective and competitive nicotinic antagonist widely used in animal experimental models but it has not been validated in humans as a model of cognitive impairment and therefore, to date, a specific nicotinic challenge test lacks.

The aim of the thesis was to study the functional effects of muscarinic and nicotinic pharmacological blockage using different cholinergic challenge models. The challenge models were also evaluated in order to test compounds with cholinergic activity. In order to differentiate nicotinic and muscarinic effects in humans a trial comparing a muscarinic pharmacologic challenge (using scopolamine) and a nicotinic challenge (using 10 and 20 mg of mecamylamine, a nicotinic antagonist) was performed (chapter 3). The trial was also an exploratory study to determine a tolerable and safe mecamylamine dose to be used in a later validation study (chapter 4). The mecamylamine validation study presents the results of the validation study using a higher mecamylamine dose, namely 30 mg, and in order to validate this pharmacological challenge model to be used in drug development, nicotine (a nAChR agonist) and galantamine (an acetylcholinesterase inhibitor) were separately co-administered with mecamylamine to attenuate or even reverse the disturbances induced by mecamylamine administration. The effects of mecamylamine administration in the previously two mentioned studies (chapter 3 and 4) were analyzed using a PK-PD model (chapter 5). The objective of the analysis was to quantify the relationships between the plasma mecamylamine concentration (pharmacokinetics) and the measured effects (pharmacodynamics). None of the measured effects reflected muscarinic activity closely enough to serve as a good biomarker. Therefore, a composite muscarinic cholinergic index using scopolamine effects on the EEG with biomarker algorithms in the different frequency bands and used machine-learning techniques was developed (chapter 7). The index integrates information from multiple EEG biomarkers, which on one side increased the sensitivity of drug-induced changes in the EEG and might be a useful biomarker in clinical trials. Finally, chapter 6 contains *in vitro* experiments performed with lipopolysaccharide plus an adjuvant (aluminium hydroxide, adenosine tri-phosphate) to explore the immune-modulating effect of choline (a nicotinic agonist) in human whole blood and monocytic THP-1 cells.

CHOLINERGIC HYPOTHESIS OF AGEING

Added to the previously mentioned role of the AChR in disease, a cholinergic dysfunction has also been suggested as aetiology for the age-related cognitive impairment in healthy elderly (Bartus *et al*, 1982). Acquisition, processing and recall of information and the speed this is performed seems to be most affected by ageing (Hedden and Gabrieli, 2004). Longitudinal morphological studies have also provided evidence that brain areas rich in acetylcholine (*e.g.*: hippocampus, entorhinal and temporal cortex) shrink significantly with age in healthy subjects (Raz *et al*, 2005). Attempts have been made to quantify the extent of cholinergic disturbances with an anticholinergic challenge test to indirectly measure cholinergic impairment. A significant difference in performance between young adults compared to elderly subjects during scopolamine administration was observed in several cognitive tests evaluating short-term verbal and numeric (working) memory (Molchan *et al*, 1992; Ray *et al*, 1992; Zemishlany and Thorne, 1991), attention (Zemishlany and Thorne, 1991), acquisition (Zemishlany and Thorne, 1991), visuo-spatial praxis (Flicker *et al*, 1992) and episodic memory (Molchan *et al*, 1992) and have finally led to the hypothesis that a cholinergic dysfunction takes place in healthy subjects with increasing age as a process of healthy or normal ageing (Dumas and Newhouse, 2011; Ellis *et al*, 2009; Terry and Buccafusco, 2003). Similarly but less extensively studied, mecamylamine also induced a significantly greater cognitive impairment measured as an increase in reaction time and number of incorrect answers in a recognition memory task (Newhouse *et al*, 1994). Concentration-effects relationships were not performed in any of the previously mentioned trials and therefore it is still unknown if the exposure or the sensitivity across different ages is responsible for this differences.

In order to study these differences in effects between healthy subjects, the effects of a previously validated and broadly used cholinergic pharmacologic model with scopolamine were compared between subjects in a wide age range (between 18 and 78 years). For this analysis, a PK-PD model relating plasma scopolamine concentration and effects was used to detect differences in exposure (pharmacokinetics) and differences in sensitivity (pharmacodynamics) to scopolamine using age as covariate (chapter 2).

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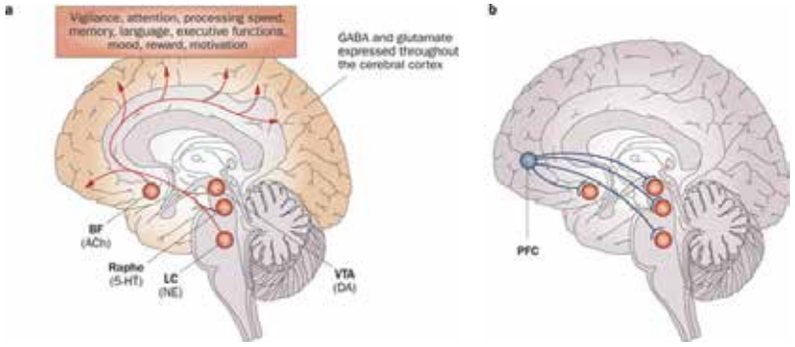
FIGURE 1.1 Cholinergic pathways in the human brain.

A. Cortical, cognitive and behavioral functions are regulated by ACh, NE, 5-HT and DA neurotransmitter systems. **B**. The prefrontal cortex in turn exerts top-down regulatory control over ascending modulatory systems, favouring the processing of incoming information and filtering of irrelevant stimuli. Damage, for example to the cholinergic system, may interfere with the processing of incoming verbal stimuli and favour the emergence of perseverations, omissions and semantic errors. Damage to the cholinergic system might be amenable to pharmacological treatment with cholinergic agonists. *Reprinted by permission from Macmillan Publishers Ltd: Nature reviews. Neurology (Berthier and Pulvermüller, 2011), copyright 2011.*



Abbreviations: 5-HT, 5-hydroxytryptamine; BF, basal forebrain; ACh, acetylcholine; DA, dopamine; GABA, γ-aminobutyric acid; LC, locus coeruleus; NE, norepinephrine; PFC, prefrontal cortex; VTA, ventral tegmental area.

TABLE 1.1 Distribution of muscarinic receptors in the brain.

Determined using **A**. high affinity radioligand [3 H] N-methylscopolamine (Flynn et al, 1997) or **B**. complementary nucleic acid sequences able to hybridize with parts of muscarinic receptor mRNA (Caulfield, 1993) in the rat brain.

Location	Receptor				
	M1	M2	M3	M4	M5
Cerebral cortex	+++++[a,b]	++[a] mainly occipital region	-	+[a] mainly occipital region	+[a] mainly outermost layer
Hippocampus	++++[a,b] mainly CA1 and CA2, followed by CA3	NA[b]	+[a,b]	+[a,b] CA1	+[a,b] CA1 and CA2
Striatum (caudate and putamen)	++++[a,b]	++ caudate (dorsal region)[a,b]; NA[b] putamen	+ [a,b] cortical laminae, CA1, CA2, and CA3	++++[a,b]	+[a,b]
Nucleus accumbens	+++[a]	++[a]	-	+[a]	+[a]
Dentate gyrus	+++[a] molecular layer	-	-	-	+[a] polymorphic layer
Olfactory bulb, olfactory tubercle	++a,b	++[a]	NA[b]	+[a,b] mainly anterior	-
Brainstem	+[a]	+++[a] parabrachial nuclei; ++[a,b] trigeminal motor and the facial nuclei	-	++[a] mainly pons, facial, and trigeminal motor nuclei; great overlap of M4 with M2 labelling	-
Amygdala	NA[b]	-	-	-	-
Superior and inferior colliculi	-	+++[a] superficial layers	-	-	+[a] only superior colliculus
Cerebellum	-	+[a,b]	-	-	-
Hypothalamus	-	NA[b]	-	-	-
Thalamus	-	-	NA[b]	-	-
Basal forebrain	-	-	-	-	++[a]
Substantia nigra	-	-	-	-	NA[b] pars compacta

NA: present but quantitative information not available.

TABLE 1.2 Distribution of nicotinic receptors in the brain.

Distribution of complementary nucleic acid sequences able to hybridize with parts of nicotinic receptor mRNA in human brain.

Location	Receptor						
	β2	β3	β4	α3	α4	α5	α7
Cerebral cortex	+ prefrontal, motor, entorhinal, cingular and temporal	+	+	++ prefrontal, motor and entorhina;+ cingular and temporal	+, ++ temporal	+	+ e ntorhinal, ++ prefrontal, +++ motor
Thalamus	+ dorsomedial and ventroposterolateral	+	+, ++ reticular, +++ lateroposterior	+++ dorsomedial and ventroposterolateral	-	+, +(+) reticular, ++ geniculate bodies	++ dorsomedial
Hippocampus	+(+)	-	-	+	-	++	++
Dentate gyrus	+(+)	-	-	+	-	-	++
Striatum (caudate and putamen)	+(+)	+	+	-	-	+	++
Cerebellum	+	+	+	+	+(+)	+	-

Adapted from Paterson and Nordberg, 2000.