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ACETYLCHOLINESTERASE INHIBITORS AND NICOTINE ADDICTION: RESEARCH STUDIES ON POTENTIAL EFFECTS OF ACETYLCHOLINESTERASE INHIBITORS IN ANIMAL MODELS OF NICOTINE DEPENDENCE

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Abstract

Tobacco use though cigarette smoking is the leading preventable cause of death in the developed world. The pharmacological effect of nicotine plays a crucial role in tobacco addiction. Nicotine dependence has a huge impact on global health and although several medications are available, including a wide range of nicotine-replacement therapies (NRTs), bupropion, and recently approved nicotinic receptor partial agonist varenicline, at best only about a fifth of smokers are able to maintain long-term (12 months) abstinence with any of these approaches. Thus, there is a need to identify more effective pharmacotherapy to aid smokers in maintaining long-term abstinence. Converging evidence from animal and human cognitive neurosciences studies indicate that cognitive functions, particularly inhibitory cognitive control, are linked closely to addictive behaviours. Drug addiction has been described as a disease of the brain reward system wherein drugs activate the neuronal circuitry involved in reward and memory. Because of the effect of cholinergic systems on reward and drug self-administration, the prevalence of acetylcholine (ACh) within the striatum, and the involvement of ACh in higher cognitive processes, ACh may play an important role in the addictive processes underlying nicotine dependence. These diverse functions are mediated by nicotinic (nAChRs) and muscarinic (mAChRs) receptors. Cholinergic neurons are either projecting neurons, terminating diffusely in the brain, or interneurons, which are located mainly in striatum and nucleus accumbens (NA). While cholinergic projection neurons are critical in cognitive function, cholinergic interneurons integrate cortical and subcortical information related to reward.

The cholinergic system interacts with the dopaminergic reward system at three levels: ventral tegmental area (VTA), NA and prefrontal cortex (PFC). In the VTA, both nAChRs and mAChRs stimulate the dopaminergic system. In the NA, cholinergic interneurons inhibit the dopaminergic system and integrate the cortical and subcortical information related to reward. In the PFC, the cholinergic system contributes to the cognitive control of addictive processes, although the neurobiological mechanism remains to be elucidate.

Acetylcholinesterase inhibitors (AChE-Is) have been developed and introduced into clinical practice for the treatment of cognitive deficits in neurological and psychiatric disorders. Their therapeutic action is mediated through increase extracellular acetylcholine (ACh) levels as a result of the inhibition of acetylcholinesterase (AChE), the enzyme that cleaves ACh into choline and an acetyl-moiety. The two AChE-Is galantamine and physostigmine exhibit allosteric potentiation ligand properties (APL) on nAChRs. Tacrine, the first AChE-I introduced into clinical practice, does not act as an APL at nAChRs.

The aim of this research is to investigate the role of ACh in nicotine dependence and how ACh modulates the mesocorticolimbic dopamine pathway. To address this question AChE-Is have been used as pharmacological tools to elevate the ACh level in the brain and the experimental paradigms applied in this research for investigating the addictive properties of nicotine were the drug discrimination (DD), self-administration (S/A) and reinstatement models. All these paradigms are operant conditioning models that mimic different phenomena of addictive behaviour. In particular, S/A directly measures the reinforcing and rewarding effects of drugs; drug discrimination provides information

regarding the interoceptive stimulus effect that a drug can exert and the reinstatement model corresponds to the human behaviour of relapse.

Galantamine, physostigmine and tacrine were initially tested in the drug discrimination model in rats trained to discriminate nicotine from saline. Galantamine and physostigmine partially generalized for nicotine discriminative stimulus; tacrine did not generalize to nicotine except for the highest tested dose. Physostigmine and tacrine were then selected to be tested in the nicotine self-administration model. Physostigmine and tacrine pre-treatment did not induce any significant changes on the number of nicotine infusions. Finally, tacrine was chosen as a test compound to be investigated in the extinction and relapse model. Tacrine administered chronically did not exert any effect either on extinction of nicotine self-administration behaviour, or on drug cues or nicotine priming reinstatement.

The present results from animal studies show a lack of effect of AChE-Is on different aspects of nicotine addiction behaviour, but a number of limitations need to be taken into account. These findings also need to be integrated with clinical data available on this pharmacological class of compounds in order to create a more comprehensive picture for the potential use of AChE-Is as treatment for nicotine addiction.

1.   Introduction

Cigarette smoking and other forms of tobacco use impose a large and growing global public health burden. Worldwide, tobacco use is estimated to kill about 5 million people annually, accounting for 1 in every 5 male deaths and 1 in 20 female deaths of those over age 30. On current smoking patterns, annual tobacco deaths will rise to 10 million by 2030 (Jha et al., 2006). Treatment for smoking cessation includes diverse methods from simple medical advice to pharmacotherapy. The powerful addictive properties of nicotine create a huge hurdle, even for those with strong desire to quit. Approximately 80% of smokers who attempt to quit on their own relapse within the first month of abstinence, and only 3-5% remain abstinent at 6 months (Hughes et al., 2004). The pharmacologic effect of nicotine plays a crucial role in tobacco addiction, and therefore pharmacotherapy is important to address this component of tobacco dependence in order to improve success rate (Benowitz, 2010). Present clinical practice guidelines categorise pharmacotherapy for the treatment of tobacco dependence into first-line [nicotine replacement therapy (NRT), bupropion and varenicline] and second-line medications (including nortriptyline and clonidine), although latter medications are used only in combination (Pelosa and Benowitz, 2011).

Dysfunction of cholinergic transmission and muscarinic and nicotinic acetylcholine (ACh) receptors have been associated with several psychiatric disorders characterized by gradual loss of cognitive functions (Pepeu and Giovannini, 2004) including Alzheimer's Disease (AD), schizophrenia (Breese et al., 2000; Freedman et al., 2000),

and drug addiction (Williams and Adinoff, 2008). An analogous, if not homologous, mechanism may be critical in modulating the acute and chronic effects of drugs of abuse. Dopaminergic fronto-cortical dysfunction has been shown to result from neuroadaptation at different modulatory systems, including the cholinergic system (Williams and Adinoff, 2008). As a result, long-lasting cognitive impairment may accompany detoxification and may also be a determinant factor for relapse to drug use (Block et al., 2002). Therefore, cognitive impairment (Rogers and Robbins, 2001), particularly the control of impulsivity and decision making has been recommended as a therapeutic target for drug addiction (Vocci et al., 2005).

The role of nicotinic ACh receptors (nAChRs) in cognitive processes has been widely demonstrated in laboratory animals, healthy volunteers and neuropsychiatric patients (Levin et al., 2006). nAChRs are expressed in brain areas, such as neocortex and ascending modulatory pathways, involved in cognitive, affective and motivational processes. nAChRs mediate the effects of ACh release at cholinergic nerve terminals and also modulate the effects of several other neurotransmitter systems (Mansvelder et al., 2006; Albuquerque et al., 2009). The nAChRs agonist nicotine has cognitive-enhancing and reinforcing properties in animal and human laboratory studies, as well in smokers and neuropsychiatric patients (Levin et al., 2006). Therefore, according to the widely distributed pattern of nAChRs in the brain and the addictive effects of nicotine, it is agreed that nAChRs may play an important role in mediating the cholinergic effects of not only tobacco, but also, more broadly, drug addiction. The ubiquitous brain projection of cholinergic pathways, the low temporal resolution of neurochemical assessments, the lack of selective (at receptor and/or anatomical level)

and feasible (devoid of side-effects) pharmacological tools are some of the critical factors that limited research advances in this field compared to others. Acetylcholinesterase Inhibitors (AChE-Is) have been developed and introduced in the clinical practice for the treatment of neurological diseases (Cummings, 2003), and psychiatric disorders characterized by cognitive deficits. Cholinomimetic drugs (Buccafusco 2004; Chiamulera and Fumagalli, 2007), including AChE-Is, significantly improved clinical endpoints such as sustained attention, working memory, visual detection, verbal fluency, and quality of life in psychiatric patients (Kirrane et al., 2001; MacEwan et al., 2001; Lenzi et al., 2003; Chouinard et al., 2007). Both, the proposed therapeutic effect (pro-cognitive), and the mechanism of action (cholinomimetic) strongly support studies with AChE-Is for the treatment of drug addiction. Reversible AChE-Is slow down ACh metabolism and increase extracellular ACh levels by inhibiting ACh-esterase (AChE), the enzyme which cleaves ACh into choline and an acetyl- moiety. AChE-Is currently approved for the treatment of dementia are; tacrine, donezepil, rivastigmine and galantamine (Ritchie et al., 2004). Galantamine is an AChE-I currently approved for the treatment of dementia, whereas physostigmine and tacrine are not widely used in clinic. However, physostigmine and tacrine are valuable tools to investigate the effects of increased synaptic ACh and its subsequent binding to nAChRs and muscarinic (mAChRs) receptors (Clarke and Pert 1985; Calabresi et al., 1989). Galantamine and physostigmine have been shown to exert a dual mechanism of action: selective competitive inhibition of AChE and positive allosteric modulator (APL) properties on nAChR response (Storch et al., 1995; Maelicke and Albuquerque, 2000; Samochocki

et al., 2003; Svobodova et al., 2005). APLs enhance the probability of agonist-mediated channel opening, stabilise the open-channel state and decrease the rate of desensitization of nAChRs (Schrattenholz et al., 1996). Although the mode of action by which galantamine and physostigmine enhance the sensitivity of nAChRs to agonists is not yet fully understood, it has been shown that both drugs directly interact with nAChRs, at sites close to, but distinct from, the ACh and nicotine binding sites. Potential binding sites have been located on the $\alpha4\beta2$ and $\alpha7$ nAChRs subunits (Texido' et al., 2005; Militante et al., 2008; Luttmann et al., 2009). Unlike galantamine and physostigmine, tacrine, the first AChE-I introduced in the clinical practice, does not act as an APL at nAChRs (Davis and Powchick, 1995).

1.1. Neuroanatomical and neurochemical interactions between cholinergic and dopaminergic systems

Cholinergic neurons are distributed through the central nervous system (CNS) and provide diffuse and sparse innervation to broad areas of the brain (Woolf, 1991). In particular, cholinergic neurons located in mesopontine nuclei (MN) and in striatum innervate the prefrontal cerebral cortex (PFC), amygdala, thalamus, ventral tegmental area (VTA), substantia nigra (SN) and hippocampus (Figure 1).

Primary Limbic Cholinergic Pathways
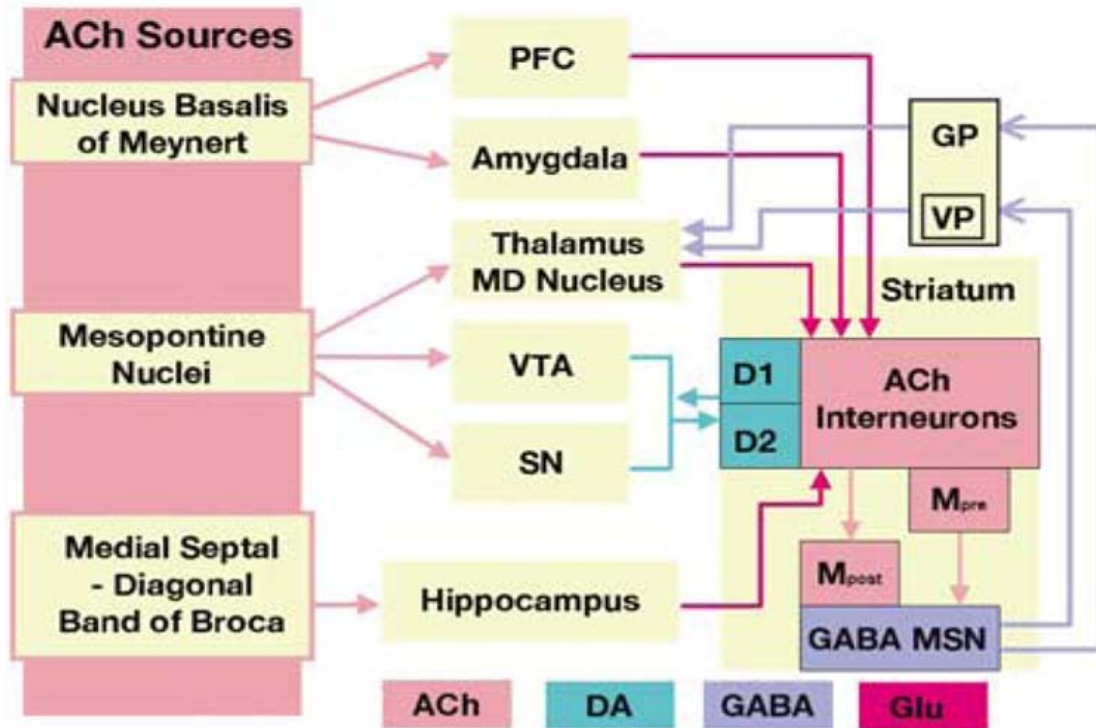
Figure 1. Picture taken from William & Adinoff (2008). The primary sources of cholinergic input are (1) the mesopontine nuclei, which provide ACh innervations to ventral tegmental area (VTA), substantia nigra (SN) and thalamus, (2) the nucleus basalis of Meynert (NBM) which provide ACh input to cerebral cortex and amygadala, and (3) the medial septal-diagonal band of Broca which provides ACh input to hippocampus. Striatal ACh interneurons are influenced by dopamine (DA) receptors D_1 and D_2. Stiatal muscarinic ACh interneurons primarily consist of M_1, M_2 and M_4; M_1 is post-synaptic (Mpost) and excitatory, whereas M_2 and M_4 are pre-synaptic (Mpre) and inhibitory. These interneurons synapse with γ-aminobutyric acid (GABA) medium spiny output neurons (MSNs). The ventral striatum projects output neurons to he ventral palladium (VP) of the globus pallidus (GP) and ,in turn, to the mediodorsal (MD) nucleus of the thalamus. The GP further projects, through GABAergic neurons, to the MD nucleus. Glutamatergic neurons from the MD project to prefrontal cortex (PFC).

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These brain areas are known to be the target of drugs of abuse and of addiction-related

processes such as drug self-administration (S/A) and drug-seeking behaviours (Box 1).

Box 1. Drug self-administration (S/A): definition, processes and procedures.

Drug self-administration is an operant conditioning assay in which a response (i.e. lever press or nose-poke) is followed by a reinforcer (i.e. food or drug). A drug is self-administered when the probability of a response increases overtime. Drugs can be self-administered by different routes (intravenously or orally are the most used). Self-administration is thought to measure the reinforcing effects of drugs, through the use of several schedule of reinforcement.

Reinforcer: It is a stimulus, most commonly a drug of abuse or food, which increases the probability of a response preceding it.

Reinforcement: An operation which increases the probability of a response. In operant behaviour, reinforcement refers to an operation which follows the emission of a response

Schedules of reinforcement: They are a set of instructions whereby a) reinforcers are presented to the animals; and b) response-reinforcement contingencies are regulated. Some elementary schedules of reinforcement include: Continuous schedule (a reinforcer is delivered after each response is emitted); Fixed Ratio (FR) schedule (a reinforcer is delivered after a fixed number of responses is emitted); Fixed Interval (FI) schedule (a reinforcer is delivered when a response is emitted after a fixed period of time).

Extinction: It is the learning process whereby a response is reduced overtime and approaches zero magnitude or frequency. A conditioned response is extinguished by presenting the conditioned stimulus without the unconditioned stimulus. An operant response is extinguished when it is not longer followed by a reinforcer.

Drug Seeking: It is a process, most commonly seen in self-administration assays, in which animals continue to respond when the reinforcer is removed or is substituted with a vehicle.

Reinstatement Behaviour: It is the resumption of a previously extinguished drug-reinforced behaviour in response to non-contingent drug delivery (i.e. priming), environmental stimuli previously associated to it (cue-induced reinstatement) or stressful stimuli.

Neuroanatomical studies on cholinergic receptor (AChRs) distribution strongly

implicate muscarinic and nicotinic receptors as mediators of the reinforcing effects of

drugs. mAChRs are G-protein-coupled receptors that mediate slow responses and

include five subtypes (M1–M5). mAChRs M1, M4 and M5 are mainly localised in

striatum, cortex and hippocampus. nAChRs are ligand-gated ion channels that open,

upon binding with ACh, to allow fast diffusion of cations (Albuquerque, 2009).

Neuronal nicotinic receptors are either heteromeric (e.g. $\alpha 4\beta 2$) or homomeric (e.g. $\alpha 7$) transmembrane proteins formed by different types of α ($\alpha 2$-$\alpha 10$) and β ($\beta 2$-$\beta 4$) subunit combinations ($\alpha 2$-$\alpha 6$, $\alpha 10$, and $\beta 2$-$\beta 4$). nAChRs receptors $\alpha 4\beta 2$ and $\alpha 7$ subtypes are expressed in striatal and mesolimbic dopaminergic neurons (Clarke and Pert, 1985). While mAChRs are expressed at high levels in both striatal and mesolimbic dopaminergic neurons, nAChRs are expressed at high level on both dopamine (DA) and GABA neurons in the VTA, and on both DA and GABA terminals in the striatum, but at low level on striatal cell bodies. Within the ventral striatum, the NA core is thought to process reinforced behaviours (Pontieri et al., 1995), whereas the NA shell processes and regulates reflexive autonomic and motor responses to drugs of abuse (Groenewegen et al., 1996). The role of the dorsal striatum in habit learning which then mediates drug-seeking behaviours after response acquisition is well established. Striatal cholinergic interneurons receive dopaminergic projections from the VTA and SN (Everitt and Robbins, 2005) and express both DA D_1 and D_2 receptors. Based on this, several studies have evaluated the effects of direct or indirect DA stimulation on ACh neurotransmission. For example, stimulation of D_1 receptors increased, whereas activation of D_2 receptors decreased, striatal ACh release (Consolo et al., 1999; Alcantara et al., 2003). Morphine, cocaine or food all altered cholinergic transmission in the NA (Hurd et al., 1990; Rada et al., 1996; Smith et al., 2004) through both D_1 and D_2 receptors, but also through mAChRs and nAChRs (Imperato et al., 1993[1]; Imperato et al., 1993[2]; Consolo et al., 1999; Pratt et al., 2004). It appears that, at least under acute conditions, the reinforcing effects of drugs are associated with an

increased striatal ACh release. Other studies have explored the effects of AChRs modulation on DA output. For example, intra-VTA administration of carbachol or oxotremorine (non-selective mAChRs agonists) increased DA concentrations in NA and VTA (Blaha and Winn, 1993; Blaha et al., 1996; Gronier et al., 2000). Furthermore, atropine (mAChRs antagonist) administered by reverse microdialysis into NA reduced remifentanil reinforcing effects (Crespo et al., 2006). Furthermore, when atropine was infused intravenously it inhibited nicotine-induced DA release in the NA (Sziráki et al., 1998). The same effect was blocked by the nAChRs antagonist mecamylamine, suggesting that nicotine-induced DA release involves by both mAChRs and nAChRs (Table 1).

	VTA[a]	NA[b]	BLA[c]	References
ACh	↑ ICSS			[Redgrave]
Atropine	↓ ICSS = Nicotine S/A	↓ Nicotine-induced DA ↓ Remifentanil reinforcement		[Yeomans ;Corrigall 1994, 2002 ; Sziraki; Crespo]
Carbachol	↑ CPP ↑ ICSS	↑ DA		[Ikemoto;Yeomans;Blaha]
Oxotremorine	↑ DA	↑ DA	↑ extinction amphetamine-induced CPP	[Schroeder; Gronier]
Neostigmine	↑ ICSS ↓ Nicotine S/A	↑ DA		[Yeomans;Corrigall 2002; Blaha]
Donepezil		↓ Cocaine- and morphine-induced CPP ↓methamphetamine-induced reinstatement		[Hikida; Hiranita]
Pysostigmine	↑ Cue-induced heroin reinstatement	↓ Cue-induced heroin reinstatement		[Zhou]
Scopolamine	= Nicotine S/A		↓ Cue-induced cocaine reinstatement	[Corrigall 2002; See]
Mecamylamine		↓ Nicotine-induced DA ↓ Remifentanil reinforcement		[Sziraki; Crespo]

Table 1. Summary of the neurochemical and behavioural effects of cholinergic agonists and antagonists microinjected into the ventral tegmental area (VTA), nucleus accumbens (NA) or basolateral amygdale (BLA). ↓ decrease of primary dependent variable; = no significant change; ↑ increase of primary dependent variable.

Overall, the literature indicates that mAChRs and nAChRs agonists enhance, whereas antagonists reduce brain DA release. Based on these studies it is predicted that cholinergic agonists would enhance, whereas cholinergic antagonists would decrease the reinforcing effects of drugs. However, selective brain lesions and direct administration of cholinergic agonists into the brain did not always produce consistent

results in behavioural assays. For example, carbachol and neostigmine (an AChE-I) have been shown to induce conditioned place preference (CPP) and intracranial self-stimulation (ICSS; Box 2) when they were injected into the posterior portion of the VTA (Yeomans et al., 1985; Ikemoto and Wise, 2002). Similarly, ACh potentiated ICSS (Redgrave and Horrell, 1976), whereas atropine shifted ICSS frequency curves to the right, i.e. increase the hedonic threshold, in rats (Yeomans and Baptista, 1997). The AChE-I physostigmine microinfused intra-VTA increased reinstatement of heroin-seeking induced by conditioned cues (Zhou et al., 2007), but decreased it when injected intra-NA. In a separate study, neostigmine microinjected into the VTA reduced nicotine S/A (Corrigall et al., 2002) (Table 1). Lesions of the peduncolopontine tegmental nucleus (PPTg) in the MN inhibited morphine- and amphetamine-induced CPP (Bechara and van der Kooy, 1989; Olmstead & Franklin, 1993). Intravenous nicotine S/A increased when the posterior portion of the PPTg was selectively lesioned with ibotenic acid (Alderson et al., 2006). In contrast, nicotine S/A was decreased by PPTg lesions (Lança et al., 2000), whereas it was unchanged after intra-VTA administration of atropine and scopolamine (Corrigall et al., 2002; Corrigall et al., 1994). It is important to recognize that several factors may have contributed to these inconsistent results, including the use of different schedules of reinforcement (Box 1), the type of procedure (CPP vs. self-administration), the assessment of the reinforcing effects in the acquisition phase in some studies and in the maintenance phase in others, as well as the lack of an accurate knowledge of the dose-effect relationship for intravenous S/A, and specificity in the lesion experiments. Collectively, the literature indicates that cholinergic agonists microinjected into the

VTA increased the reinforcing effects of drugs of abuse and enhanced DA release in the NA, whereas when they were microinjected into the NA they had opposite effects. These data pose some questions on the efficacy of cholinergic agonists on drug reinforcement after systemic administration and suggest that a systematic approach with AChE-Is could provide some answers.

Box 2. Other experimental paradigm for testing preclinical measures of drug addiction.

Intracranial self-stimulation (ICSS): It is an operant assay in which a response (i.e. lever press or nose-poke) is followed by intracranial electrical stimulation. The intensity and frequency of the electrical stimulation is increased and decreased stepwise in order to determine the minimal current needed to obtain a response (i.e. reward threshold). Drugs of abuse lower the reward threshold.

Conditioned Place Preference (CPP): It is an assay in which administration of a drug is paired with a specific context, whereas, administration of vehicle is paired with a different one. Drug pairing sessions are alternated with vehicle pairing sessions. After a sufficient number of sessions animal are tested in a drug free state in which both contexts are presented. If animals spend significantly more time in the context previously paired with the drug then the drug may have rewarding effects.

Drug-induced motor sensitization: It is an assay whereby a response is amplified by repeated drug administration. Drug-induced motor sensitization is a non-associative learning process that is observed with repeated administration of drugs of abuse such as amphetamine and cocaine.

1.2. Acetylcholinesterase inhibitors as experimental tools to increase ACh levels

Inhibition of AChE increases the synaptic concentration of ACh, thereby enhancing and prolonging the action of ACh on both mAChRs and nAChRs. The AChE-Is currently approved for the treatment of dementia are tacrine, donezepil, rivastigmine and galantamine (Ritchie et al., 2004). Although the primary mechanism of action of these drugs is inhibition of AChE, they differ in potency and selectivity, i.e. inhibition of AChE vs. butyrylcholinesterase (BChE). A significant increase in brain ACh can occur not only after AChE inhibition, but also after BChE inhibition as shown under some conditions (Cerbai et al., 2007). For example, tacrine, a reversible AChE-I, is slightly more potent for BChE than AChE (Davis and Powchick, 1995). On the other side of the specificity profile is donepezil highly selective for AChE (Villarroya et al., 2004). Physostigmine and galantamine, but not donepezil and rivastigmine, also act as nAChRs APL (Samochocki et al., 2003). Physostigmine and galantamine bind to a site distinct from the ACh-binding site on nAChRs subunits. This site is insensitive to blockade by competitive nAChR antagonists and has been detected even when the receptors were desensitized by large concentrations of agonists (Pereira et al., 1994). Others have shown that chronic treatment with AChE-Is increases nAChRs expression in rodents (Bhat et al., 1990). For example, chronic administration of donepezil, rivastigmine and galantamine increases non-α7 nAChR expression in rat hippocampus, and both non-α7 and α7 nAChR expression in cerebral cortex (Reid & Sabbagh, 2008; Takada-Takatori et al., 2008). In general, in vitro and in vivo data

have indicated that AChE-Is may increase nAChRs expression by increasing synaptic ACh levels, and not by direct agonist activity at the nAChRs (Kume et al., 2005). Collectively, these data indicate that inhibition of AChE (primary mechanism) may be slightly different across AChE-Is in terms of potency and selectivity. Moreover, further pharmacological differences may arise from secondary mechanisms such as nAChRs APL properties and receptor up-regulation complicating the pharmacological profile of the AChE-Is. Thus, the use of AChE-Is as experimental tools for understanding the mechanisms underlying the cholinergic component of drug reinforcement should be considered only after choice of route of administration (intracranial or systemic) and of their secondary pharmacological effects, in particular alternative mechanism on nAChRs, are taken into account.

1.3. Preclinical effects of acetylcholinesterase inhibitors on drug reinforcement

Several studies have shown that AChE-Is have biological effects in animal models of drug reinforcement and that these effects depend on route of administration. Direct brain administration of AChE-Is resulted in differential effects depending on the neuroanatomical site of injection (Table 1). Behavioural studies combined with cholinergic cell ablation, reported that elimination of cholinergic cell in the NA markedly enhances sensitivity to cocaine in both acute and long-lasting behavioural changes associated with cocaine addiction (Hikida et al., 2001). This study revealed that ACh is released from cholinergic interneurons within the NA and acts concertedly

but oppositely to dopamine on the NA neuronal circuit and that the elimination of cholinergic neurons in NA increases behavioural effects of cocaine rewarding. In a subsequent experiment, Hikida et al.(2003), showed that ablation of the NA cholinergic neurons enhanced not only the sensitivity to morphine in CPP but also negative reinforcement of morphine withdrawal in conditioned place aversion. Remarkably, the AChE-I donepezil, suppressed both cocaine- and morphine-induced CPP and blocked the induction and persistence of cocaine-evoked hyperlocomotion. Importantly, this inhibition was abolished by ablation of the NA cholinergic neurons. These results demonstrate that centrally active AChE-Is prevent long-lasting behavioural abnormalities associated with cocaine and morphine addictions by potentiating the action of ACh released from NA cholinergic neurons (Hikida et al., 2003). Hiranita et al., (2006) demonstrated that reinstatement of methamphetamine (MAP) -seeking behaviour is mediated by ACh and can be attenuated by donepezil. In fact systemic nicotine and donepezil administration attenuated MAP-associated cues and MAP-priming reinstatement. The AChE-Is physostigmine, produced dose-dependent inhibition of cue-induced reinstatement of heroin-seeking behaviour (Zhou et al., 2007). In the same study Zhou et al., demonstrated that microinjection of physostigmine in the NA prior to presenting conditioned cues inhibited the reinstatement of heroin-seeking. In contrast, microinjection of physostigmine in the VTA augmented the reinstatement induced by conditioned cues and extinction responding. Inactivation of either NA or VTA by tetradotoxine microinjection blocked both extinction and cue-induced reinstatement. These data demonstrate that cholinergic transmission influences heroin self-administration and reinstatement.

Moreover, cue-induced reinstatement was inhibit by physostigmine in the NA and potentiated by cholinergic stimulation in the VTA.

In addition, selective cholinergic lesions (i.e. induced by an immunotoxin) of NA neurons prevented the inhibitory effects of donepezil on morphine-induced CPP (Kaneko et al., 2000), suggesting that the NA was the site of action of donepezil. Unfortunately, these studies did not evaluate the effects of donepezil in the VTA. Such a study would have clarified the differential responses generated when an AChE-I is microinjected into the VTA compared to the NA. More recently, it has been shown that, in addition to VTA and NA, the BLA is also critical for conditioned memory formation. For example, when scopolamine was infused into the BLA it dose-dependently disrupted cocaine-induced reinstatement (See, 2005). Furthermore, oxotremorine facilitated extinction of amphetamine-induced CPP (Schroeder & Packard, 2004). Taken together, these results indicate that increase of ACh neurotransmission in the NA and BLA decreases the reinforcing effects of drugs.

When AChE-Is were administered systemically (Table 2) the effects were similar to those obtained after microinjection into the NA. For example, systemic administration of physostigmine produced dose-dependent inhibition of cue-induced heroin-seeking behaviour (Zhou et al., 2007). Furthermore, systemic administration of physostigmine reduced cocaine S/A in rhesus monkeys (De La Garza & Johanson, 1982). Tacrine inhibited cocaine S/A in rats (Grasing et al., 2008) and systemic administration of donepezil reduced morphine- and cocaine-induced CPP as well as hyperlocomotor activity in mice (Hikida et al., 2003). Systemic administration of galantamine and donepezil also blocked cocaine-induced motor sensitization, suggesting a potential

role of AChE-Is in neuroadaptation induced by psychostimulants. Furthermore, intraperitoneal administration of donepezil attenuated cue- and methamphetamine-induced reinstatement in rats (Hiranita et al., 2006). Interestingly, these effects were blocked by mecamylamine and not by scopolamine, suggesting that they were mediated nAChRs. In contrast, others have demonstrated that systemic administration of physostigmine reduced heroin S/A and seeking behaviour in rats and these effects were blocked by scopolamine suggesting that were mediated by mAChRs (Zhou et al., 2007).

	CPP [a]	LMA [b]	Drug taking [c]	Drug seeking [d]	Psychological symptoms [e]	Behavioural symptoms [f]	References
	preclinical	preclinical	preclinical	preclinical	clinical	clinical	
Physostigmine		.	↓Cocaine ↓Heroin ↓Nicotine	↓Heroin			[De La Garza 2008; Zhou]
Galantamine		↓Cocaine			=Alcohol =Smoking	↓Alcohol ↓Smoking	[Hikida; Mann; Diehl]
Donepezil	↓Morphine ↓Cocaine	↓Morphine ↓Cocaine					[Hikida; Jovanovski]
Rivastigmine					↓ Methamph	↓ Methamph	[Jovanovski]
Tacrine			↓Cocaine ↓Nicotine				[Grasing]

Table 2. Summary of systemically administered AChE-Is effects on preclinical (primary dependent variables in laboratory animal models) and clinical addiction (clinical endpoints) measures. [a] Conditioned Place Preference in mice; [b] Locomotor activity or motor sensitization in mice or rats; [c] Drug S/A; [d] Cue- or drug priming –induced drug seeking relapse; [e] Psychological subjective measures such as craving, anxiety, etc.; [f] Behavioural objective measures such as relapse or consumption. ↓ Decrease of primary dependent variable or clinical endpoint; = No significant change.

In general, systemic administration of AChE-Is decreased the reinforcing effects of drugs although the exact mechanism (i.e. nAChRs vs. mAChRs) by which this occurred has not been completely clarified. Taken together, the data published so far indicate that the ability of systemically administered AChE–Is to reduce the reinforcing effects of drugs is consistent with a potentiation of cholinergic neurotransmission in the NA. Although, additional preclinical studies should be conducted to evaluate differences related to the different routes of AChE-Is administration (i.e. intra-NA, -VTA and systemic administration), these data indicate that systemic administration of AChE-Is inhibits the reinforcing effects of drugs of abuse in laboratory animals. Thus, these preclinical data strongly suggest a potential clinical utility of AChE-Is for the treatment of drug addiction.

1.4. Aim

This research originated from the experimental evidence that ACh contributes to homeostatic regulation in the mesolimbic dopamingergic pathway, which has a fundamental role in behavioural adaptations that occurs with repeated administration of drug of abuse, including nicotine.

The aim of this research is to investigate the role of ACh in nicotine dependence, and how ACh modulates the mesocorticolimbic dopamine pathway. To address this question AChE-Is have been used as pharmacological tools to elevate the ACh level in the brain. The recent availability of neurochemical ACh measurements at a sub-second temporal scale (Giuliano et al., 2008) is not yet widely used, thus, behaviourally correlated ACh changes have at best, 10-15 minutes temporal resolution as assessed by microdialysis methods.

The experimental paradigms applied in this research for investigating the addictive properties of nicotine were the drug discrimination, self-administration and reinstatement models. All these paradigms are operant conditioning models. The term operant conditioning describes one type of associative learning in which there is a contingency between a behaviour and the presentation of a biologically significant event (e.g. reinforcer). A positive reinforcement (e.g. nicotine administration) occurs when a behaviour (lever press) is followed by a stimulus which is appetitive or rewarding, increasing the frequency of that behaviour. Some psychoactive drugs

including nicotine are abused because of their ability to act as reinforcers. As a consequence behavioural patterns (such as drug seeking/drug taking behaviour) are promoted that ensure further drug consumption. Addiction cannot be modelled in animals, at least a whole, however different procedures of operant behaviour can be applied as rodent analogues of addiction's major elements including discriminative effect, drug seeking and relapse (Ator and Griffiths, 2003; Sanchis-Segura & Spanagel, 2006). The most common apparatus used in conducting an operant task is the so-called "Skinner box" (Figure 2).
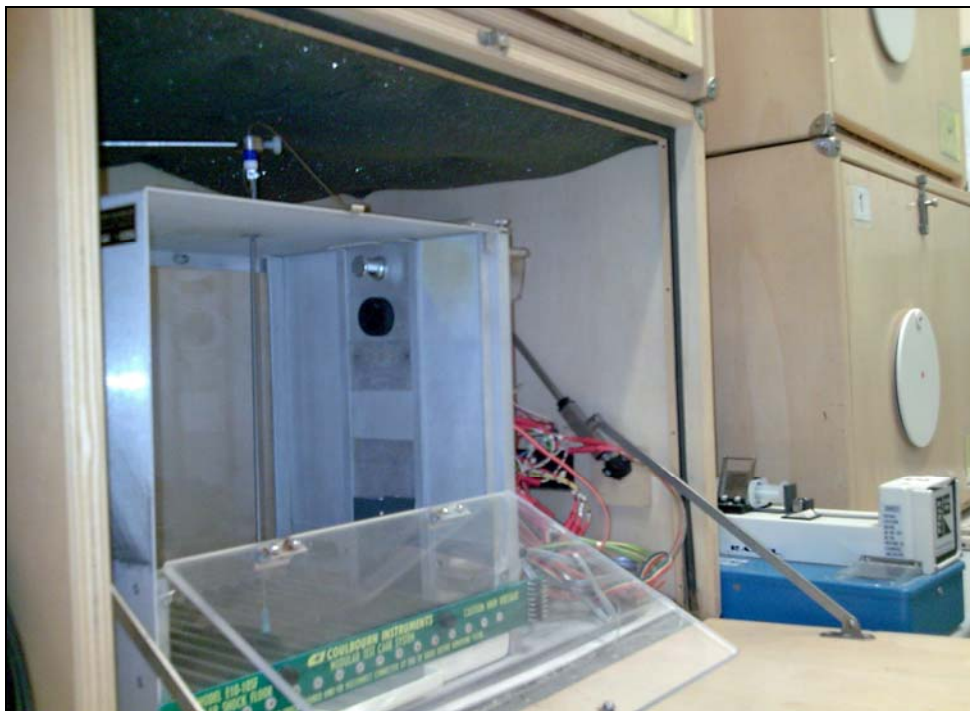

Figure 2. Skinner box

The potential interpretative outcome is to integrate circumstantial data coming from scientific evidence in animal models and clinical data into a comprehensive picture for the potential use of AChE-Is in nicotine addiction. In particular, last-cognitive impairment may act as a determinant factor for relapse to drug use (Block et al., 2002), and these findings suggest the importance of studying the potential therapeutic value of 'cognitive enhancers' for prevention of relapse

1.5. Behavioural paradigms

The models applied in this research, are based on the experimental paradigm of operant conditioning. The two major experimental paradigms are the drug discrimination and the drug self-administration, respectively for the assessment of discriminative and reinforcing stimulus properties. In laboratory animals, conditioning tests take place inside a Skinner box (Figure 2), within a sound-insulated cubicle, and provided with levers, cue lights and speakers in order to set different experimental contingencies. A module-based system interfaces and computers with properly compiled software independently control all boxes. Briefly, experimental subjects are trained to receive a reward if they exhibit a specific behavioural response. The reward increases the probability of responding occurrence, therefore acting as a 'reinforcer'. This mechanism is physiological and it sustains motivated behaviours for seeking and obtaining natural rewards such as food, water, sex, etc. Drugs of abuse may act as primary reinforcers, as it is observed in addicts and as it is characterised in laboratory studies in humans and animals.

Moreover, drugs of abuse may also act as a discriminative stimulus able to induce, maintain or enhance responding for a natural or drug reinforcer; this property may be evident in processes such as subjective psychoactive effects, incentive salience, conditioned reinforcement and drug priming.

1.5.1. Drug Discrimination

The discriminative stimulus properties of a drug are related to their subjective effects, and DD procedures in animals have often been used as animal models of the subjective effects of the compounds (Schuster and Johanson, 1988). In DD research, animal subjects are required to perceive the differences between the effects of drug and vehicle injections in order to solve a choice problem to receive food or other reinforcement. Although there are many variants of the procedure, the DD paradigm typically involves training animals to make an operant response to obtain a food pellet when treated with a specific drug (the so-called "training drug"), but to make an alternative response when treated with a placebo injection. Drugs successfully used as training drugs have ranged from antipsychotic to almost every class of abused drugs. Animals are trained under a discrete trial schedule for food pellet delivery to respond on one lever after an injection of nicotine training dose and on the other lever after an injection of vehicle. Two types of training conditions are involved. During one type of training condition experimental sessions are preceded by injection of nicotine; during the other type of training conditions sessions are preceded by injection of vehicle. The two different training conditions alternate daily. During nicotine-training sessions, placebo-appropriate responses have no consequences; during vehicle-training sessions, nicotine-appropriate

responses have no consequences. After a period of training-sessions like these, the animal becomes trained to press only the lever associated with appropriate injection condition. It is assumed that what drives an animal in making the correct lever choice are the interoceptive effects of nicotine or its vehicle, and the interoceptive effects of drugs in humans are thought related to their subjective effects. Training completion will be followed by dose-response studies for each nicotine dose in order to assess the relationship between dose and drug stimulus properties. Once nicotine discrimination training has been completed, the so-called generalization or substitution tests can be initiated and other drugs can be tested to determine if the occasion drug- or vehicle-lever selection occurs. Such tests are designed to determine whether other drugs will generalize to, or substitute for, nicotine (they can induce nicotine- or vehicle-lever response). It should be noted that although cross-generalisation between two drugs implies that they have similar discriminative properties, it does not prove they have identical stimulus properties. Rats are tested for cross-generalization between nicotine at the training dose and four doses of test drug (including vehicle, i.e. 0 mg/kg), and each subject receives all doses in a Latin square design, with washout days in between.

1.5.2. Self-administration

Drug S/A has been widely characterized for all the drugs abused by humans, under different modes of administration. The paradigm has a high analogy to the pathological condition; it allows studying of the underlying neurobiological mechanisms, as well as having a high predictive validity for the identification of novel anti-addiction therapies. The experiments performed in this research project aim to investigate the effects of AChE-Is pre-treatment on nicotine S/A behaviour in rat by using a fixed-ratio schedule.

This conditioning protocol allows training animals to self-administer nicotine intravenously (i.v.) as a consequence of responding for a fixed number of presses on an active lever, – on a fixed-ratio (FR) of 2– in order to get an i.v. infusion from the infusion pump placed outside the Skinner box. Initially, animals are trained to lever press for a food reinforcer. They then have an i.v. cannula surgically implanted. After a period of recovery from surgery, rats start a training period that consists of a daily i.v. nicotine S/A session (lasting 1 hour) for a period of about two weeks in order to meet criteria of stable responding performance. Once the animals at FR1 schedule achieve 25 infusions per session, they will advance to the following stages of training (FR2). The stable performance required to advance to the next stage is defined as similar number of infusions or similar number of active lever presses per session +/- 20% on at least three consecutive days. Performance is measured as number of active lever presses/hour or as number of infusions/hour. The number of inactive lever presses is also monitored, as a measure of non-specific responding and of possible adverse drug effects on rat's motor activity. When the criteria of stability are reached (nicotine training), rats are pre-treated with AChE-Is or vehicle. Each subject receives all doses in a Latin square design, with washout days in between.

1.5.3. Extinction and Relapse

The reinstatement model is currently used in many laboratories to investigate mechanisms underlying relapse to drug seeking. Extinction procedure can provide measures of the incentive-motivational properties of drugs by assessing the persistence of drug-seeking behaviour in the absence of response contingent drug availability. In the extinction paradigm, animals are first trained to nicotine S/A until stable S/A patterns

are exhibited for several consecutive days. This procedure ensures that, before extinction testing, the animals have developed a strong drug S/A habit and thus exhibit resistance to extinction compared to undertrained subjects. Extinction testing sessions are identical to training sessions except that no drug is delivered after completion of the response requirements. Extinction training continues until rats reach a predetermined extinction criterion (e.g. 20% or less responding during the last extinction session as compared with the first extinction session). In subsequent test sessions, reinstatement of lever-pressing behaviour is defined as significantly higher responding on the lever previously paired with nicotine infusions (typically referred to as the active lever) following exposure to the experimental manipulations (drug cues or drug-priming) as compared with the control manipulations.

2. Materials and Methods

All animal procedures were carried out in accordance with the Principles of laboratory animal care (NIH publication No.85/23, revised 1985), the European Communities Council Directive of 24 November 1986 (86/609/EEC). The inter-departmental Centre has approved these procedures for Laboratory Animal Service and Research of the Verona University, according to art.7 D.L. 116/92 of the Italian Legislation. All efforts were made to minimise animal suffering and to keep the number of animals used as low as possible.

2.1. Drug Discrimination

2.1.1. Subjects

Experimentally naïve Sprague Dawley male rats (n=11, 175-200 g, Charles River, Italy) were individually-housed in a temperature- and humidity-controlled colony room under a 12 h light/dark cycle (lights on at 6 am) and were food deprived to maintain 85% of their free-feeding weight with water ad libitum.

2.1.2. Apparatus

Experimental sessions were performed in eight identical operant conditioning chambers (Med Associated Inc., St Albans, VT). The front panel contained two response levers, a stimulus light over each response lever, and an aperture between the levers for delivery of sugar pellets (Bilaney Consultants Ltd., UK). A house light was located on the back

panel near the chamber ceiling to provide ambient illumination. Each chamber was enclosed in a sound-isolating box equipped with an exhaust fan that provided masking noise.

2.1.3. Discrimination training

The procedure was similar to those described by Solinas (2006). Rats were initially trained to lever press for food pellets during daily 20-min sessions. During this phase, the active lever was randomly changed every day, with response on active lever producing a single sugar pellet delivery. Once responding was stable (15 reinforcers/session for at least two consecutive sessions), the fixed ratio (FR) requirement to obtain food was gradually increased from FR1 to FR10. When FR10 schedule was established and stable performance was maintained (50 reinforcers/session for at least two consecutive sessions), the discrimination training began. Rats were trained to respond on one lever (right lever for half of the rats, left lever for the other rats) following an intraperitoneal (i.p.) injection of 0.2 mg/kg nicotine (Nic; training dose) and on the other lever following vehicle (0.9% saline; Sal). In this phase, one lever was active when training drug was injected and the other lever when vehicle was injected. Pressing on the correct active lever resulted in the food delivery. Rats were injected in the home cage and, after 10 minutes were placed in the operant chamber for the session start. Nicotine and vehicle daily treatments were semi-randomly alternated (Nic-Sal-Nic-Sal-Sal-Nic-Sal-Nic-Nic-Sal-Sal-Nic-Sal-Nic-Nic-Sal). Criteria of discrimination performance were achieved when responding during the first trial was >80% and less than 5 lever presses respectively on injection-appropriate and incorrect lever, for 8 consecutive sessions. Sessions were performed 5 days a week.

2.1.4. Nicotine dose-response curve

Once the criteria of discrimination performance were achieved, different doses of nicotine were tested in order to assess a dose-response relationship. On Tuesdays and Fridays, different doses of nicotine (0.025, 0.05 or 0.1 mg/kg) were administered in order to assess the generalization level for the nicotine training dose. Intervening sessions consist of baseline session with nicotine training dose or vehicle to maintain discrimination performance.

2.1.5. Generalization test

Generalization test sessions occurred twice a week similarly to the nicotine dose-response curve sessions. Tacrine (0.625, 1.25 or 2.5 mg/kg; 60 minutes prior session), physostigmine (0.05, 0.1 or 0.2 mg/kg; 60 minutes prior session) and galantamine (1, 3 or 5 mg/kg, 30 minutes prior session) were administered as single dose administration, on separate test days. Tacrine and physostigmine were injected subcutaneously (s.c.), whereas galantamine was injected i.p. Pre-treatment time was chosen based on literature data. Brain levels of galantamine transiently increase with a maximum between 15 and 30 minutes after s.c. injection (Geerts et al., 2005). This study also suggested that the optimal conditions – in terms of brain concentration - for the APL effect of galantamine can be achieved by doses ranging from 1.5 and 5 mg/kg in the rats. The onset of action of physostigmine (0.125-0.25 mg/kg) and tacrine (0.625-2.5 mg/kg) ranged between 60 and 100 minutes after administration (Liu et al., 2000). Each drug was administered at different doses within-subject according to a Latin-square design.

2.1.6. Drugs

(-)-Nicotine hydrogen tartrate, tacrine hydrochloride, physostigmine hemisulphate and galantamine hydrobromide (Sigma-Aldrich, Italy) were dissolved in saline and pH adjusted to 7.4. Nicotine and tacrine doses were expressed as free base, while physostigmine and galantamine doses were expressed as salt in order to compare to literature data (Yamamoto et al., 1993; Shoaib et al., 1997; Le Sage et al., 2009). All doses were administered in a volume of 1 mL/kg.

2.1.7. Data analysis

All generalization data were expressed as percentage value of drug-paired lever presses acquired during the first trial of session (i.e. before receiving the first reinforcement) compared to the total number of lever presses (active and inactive). Total number of responses (presses/minute) was assessed as a dependent variable for overall response rate effect. Rate of responding was analyzed by one-way ANOVA followed by Dunnett's post-hoc tests comparing total number of lever presses/minute during generalization test sessions vs. vehicle or nicotine training dose sessions. Full generalization was defined as % Nicotine Lever Response (NLR) greater than or equal to 80%, while partial generalisation was defined as %NLR values ranging from 20% to 80% (Solinas et al., 2006).

2.2. Self-administration

2.2.1 Subjects

Experimentally naïve Sprague Dawley and Lister Hooded male rats (Harlan, Italy) were individually-housed in a temperature- and humidity-controlled colony room under a 12 h light/dark cycle (lights on at 7 am). Animals were food restricted to maintain their body weight range between 240 and 260 g (daily checked): food diet (3-4 pellets, for a total of 15-20 g/day) was made available after each experimental session. The maximum amount of sugar food pellets intake during training session was 5.4 g/day for those subjects meeting the criteria (see below 'training to lever press'). Animals had ad libitum access to water except during experimental session (3h/day). Rats were trained or tested once daily.

2.2.2. Apparatus

Behavioural testing was conducted in eight identical operant conditioning chambers (Coulbourn Instruments, Lehigh Valley, Whitehall, PA, USA) encased in sound-insulated cubicles, equipped with ventilation fans (Ugo Basile, Comerio, Italy). Each chamber was equipped with tow levers, symmetrically centred on the frontal panel, and located 12.5 cm apart, 2 cm above the grid floor. The food magazine was situated in an opening in a panel between the two levers, 1 cm above the floor. This opening was closed during nicotine S/A training. A 2 W white house light was located 26 cm above the food magazine and activated during the entire session duration, except during the Time-Out (TO= 60 seconds interval after each reinforcement in which levers are inactive). Right lever presses corresponding to FR values, required the schedule of reinforcement, produced the delivery of 45-mg sugar food pellet (Bioserv, USA) or the

activation of the infusion pump (model A-99Z, Razel Scientific Instruments Inc., Stamford, CT, USA). Nicotine or saline solutions were administered via the infusion pump at the volume of 0.027 mL during a 1-s period. Sugar food pellet delivery was signalled by the 1-s illumination of a 4 W white stimulus light located in the same hole of the food magazine only during the training for lever press. During nicotine or and reinstatement sessions, reinforcer delivery (nicotine infusion) was signalled by 1-s illumination of one yellow and one green light emitting diode (LED) centrally placed above the food magazine. During food S/A reinforcer delivery (food infusion) was signalled by 1-s sounding of a 2.9 Hz, 60 dB Sonalert device (defined as nicotine or food 'cues'). Left lever presses ('inactive lever presses') did not have any consequence. All types of lever presses, sugar food pellet and infusion deliveries was recorded. Data acquisition and schedule parameters were controlled by a med-PC software (Med Associates Inc, Georgia, USA) running on a PC-computer interfaced with the chambers via interface modules (Med Associates Inc.).

2.2.3. Training to lever press

Following a 24-h food deprivation period, all rats were trained to lever press for a food as reinforcement. The final training schedule of reinforcement was a FR 2, session duration up to 120 reinforcements were delivered or 20 min were elapsed. Once training to lever press for food reinforcement (it required approximately 2 weeks), rats underwent surgery for implant an i.v. cannula.

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2.2.4. Surgical procedure

Rats were anaesthetized with 0.5 mg/kg/0.5 mL medetomidine (Domitor®, Pfizer, Italy), 10 mg/kg tiletamine + 10 mg/kg zolazepam (Zoletil 100®, Virbac, Italy; 0.2 mL/kg intramuscular), and then implanted with a Silicon catheter (inner diameter 0.30 mm, outer diameter 0.63 mm, Cam Caths, Cambridgeshire, UK) in the right jugular vein. Immediately after surgery, animals were medicated with 5mg/kg/1 mL subcutaneous carprofen (Rymadyl®, Pfizer, Italy) and 25,000,000 IU benzylpenicilline + 1 g/kg dihydrostreptomycin (Rubrocillina Forte®, Intervet, Italy; 1 mL/kg subcutaneous), 0.5 mg/kg/0.1 mL intramuscular atipamezole (Antisedan®, Pfizer, Italy). Each day after recovery, animals received 0.1 mL of one i.v. injection of heparin solution (30 IU/mL heparin sodium, Sigma, Italy) before and after the experimental session.

2.2.5. Training in the nicotine self-administration procedure

After the recovery period, rats were trained to intravenously S/A nicotine (FR 1: nicotine 0.03 mg/kg/infusion; TO session 60s; session duration up to 25 infusions were delivered or 3 h elapsed, no priming injection). Adjustment of nicotine concentration to changes in rat body weight was not needed because rats' body weight was kept stable at 250 g (± 10g). Lever pressing during the TO period was also recorded, although it did not have any consequences. If the animals met the criterion of 25 infusions within the end of daily session, the FR value was increased to FR 2 with session duration lasting up to 3 h. Rats were considered to reach a stable responding on nicotine S/A under a FR 2 schedule of reinforcement when the value of reinforcements/session did not vary more

than 20% between three consecutive sessions. For non food-shaped rats after reaching stable responding at the FR 2, the FR value was increased to FR 3.

2.2.6. Drugs

Nicotine hydrogen tartrate (Sigma, Italy) was dissolved in heparinised bacteriostatic saline (0.9% NaCl + 0.9% benzylalcohol + 1 IU/mL heparin) and pH adjusted to 7.4 with NaOH. Nicotine unit doses are expressed as mg of free base/kg of body weight/infusion. Physostigmine hemisulphate (Sigma, Italy) was dissolved on the test day in saline solution and administered s.c. 30 minutes before session start at doses of 0.05, 0.1 or 0.2 mg/kg. Tacrine hydrochloride (Sigma, Italy) was dissolved in heparinised bacteriostatic saline on the test day and administered i.v. 20 minutes before the session start at doses of 0.032, 0.1 or 0.32 mg/kg. All doses were administered in a volume of 1 mL/kg. Each drug was administered at different doses within-subject according to a Latin-square design with at least two daily nicotine S/A sessions between the three testing session.

2.2.7. Data analysis

Data are expressed throughout the study as mean ± SEM. Comparison among groups was performed by analysis of variance (ANOVA) with a number of factors as indicated case by case in the Results section, followed by Bonferroni's or Dunnett's post-hoc test for individual comparisons between groups. Statistical analysis was performed using Prism 4 (Graph Pad, La Jolla, CA, USA).

2.3. Extinction and relapse

2.3.1. Subjects

Experimentally naïve Sprague Dawley and Lister Hooded (Harlan, Italy) male rats were individually-housed in a temperature- and humidity-controlled colony room under a 12 h light/dark cycle (lights on at 7 am). Animals were food restricted to maintain their body weight range between 240 and 260 g (daily checked): food diet (3-4 pellets, for a total of 15-20 g/day) was made available after each experimental session. The maximum amount of sugar food pellets intake during training session was 5.4 g/day for those subjects meeting the criteria (see below 'training to lever press'). Animals had ad libitum access to water except during experimental session (3h/day). Rats were trained or tested once daily.

2.3.2. Apparatus

Behavioural testing was conducted in eight identical operant conditioning chambers (Coulbourn Instruments, Lehigh Valley, Whitehall, PA, USA) encased in sound-insulated cubicles, equipped with ventilation fans (Ugo Basile, Comerio, Italy). Each chamber was equipped with tow levers, symmetrically centred on the frontal panel, and located 12.5 cm apart, 2 cm above the grid floor. The food magazine was situated in an opening in a panel between the two levers, 1 cm above the floor. This opening was closed during nicotine S/A training, extinction and reinstatement sessions. A 2 W white house light was located 26 cm above the food magazine and activated during the entire session duration, except during the TO (60 seconds interval after each reinforcement in which levers are inactive). Right lever presses corresponding to FR values, required the schedule of reinforcement, produced the delivery of 45-mg sugar food pellet (Bioser,

USA) or the activation of the infusion pump (model A-99Z, Razel Scientific Instruments Inc., Stamford, CT, USA), except during the extinction and reinstatement sessions. Nicotine or saline solutions were administered via the infusion pump at the volume of 0.04638 mL during a 1-s period. Sugar food pellet delivery was signalled by the 1-s illumination of a 4 W white stimulus light located in the same hole of the food magazine only during the training for lever press. During nicotine or and reinstatement sessions, reinforcer delivery (nicotine infusion) was signalled by 1-s illumination of one yellow and one green light emitting diode (LED) centrally placed above the food magazine. During food S/A reinforcer delivery (food infusion) was signalled by 1-s sounding of a 2.9 Hz, 60 dB Sonalert device (defined as nicotine or food 'cues'). Left lever presses ('inactive lever presses') did not have any consequence. All types of lever presses, sugar food pellet and infusion deliveries was recorded. Data acquisition and schedule parameters were controlled by a med-PC software (Med Associates Inc, Georgia, USA) running on a PC-computer interfaced with the chambers via interface modules (Med Associates Inc.).

2.3.3. Training to lever press

Following a 24-h food deprivation period, all rats were trained to lever press for a food as reinforcement. The final training schedule of reinforcement was a FR 2, session duration up to 60 min. Once training to lever press for food reinforcement (it required approximately 2 weeks), rats underwent surgery to implant an i.v. cannula.

2.3.4. Surgical procedure and training to nicotine self-administration

The surgical procedure and training to nicotine S/A were the same as those described in the Self-administration section 2.2.4 and 2.2.5, respectively.

2.3.5. Extinction, nicotine cues and priming reinstatement sessions

Extinction of responding for nicotine infusion started when responding was stable (see training in the nicotine self-administration procedure). The extinction schedule consisted of a 1-h daily session where the 1-s infusion of saline - but not nicotine cues (1 s illumination of yellow LED, green LED and tone sounding) - was obtained by animals upon responding on previously nicotine-paired lever. Animals, randomized into two groups, were pre-treated i.v. with 0.032 mg/kg/mL tacrine or saline 1 mL/kg, 20 minutes prior the session start. The substitution of nicotine with saline (after 10 sessions S/A) induced a gradual decrease of responding. Extinction criterion was defined when nicotine-paired lever pressing during the first hour of the session reached a value <50% of nicotine-paired lever value at the first extinction session.

Rats were tested for the effect of tacrine (0.032 mg/kg/mL, i.v., 20 min pre-treatment) on nicotine cue-induced reinstatement of responding. During the reinstatement session, nicotine cues were contingently presented upon responding: FR 2 = 1 s illumination of yellow and one green LED centrally placed above the food magazine, and 1-s sounding of a 2.9 Hz, 60dB Sonalert device, infusion of saline; TO 60-s period, session duration 1 h.

Twenty-four hours after the cue reinstatement session, animals underwent two days of extinction sessions (as described above) and subsequently the rats were tested for the

effect of tacrine (0.032 mg/kg/mL, i.v., 20 min pre-treatment) on nicotine priming-induced reinstatement of responding. During the priming-induced reinstatement session, animals were administered with a s.c. injection of nicotine 0.15 mg/kg/mL immediately before the session start. During the session, the nicotine-associated cues (e.g. yellow and green LED and sounding) were not presented upon responding on the nicotine active lever.

2.3.6. Drugs

Nicotine hydrogen tartrate (Sigma, Italy) in heparinised bacteriostatic saline (0.9% NaCl + 0.9% benzylalcohol + 1 IU/mL heparin) and pH adjusted to 7.4 with NaOH. Nicotine hydrogen tartrate for the priming-reinstatement test was administered s.c. at 0.15 mg/kg. Nicotine unit doses are expressed as mg of free base/kg of body weight/infusion.

Tacrine hydrochloride (Sigma, Italy) was dissolved in heparinised bacteriostatic saline on the test day and administered i.v. 20 minutes before the session start at dose 0.32 mg/kg. All doses were administered in a volume of 1 mL/kg. Chronic treatment of tacrine or saline on the cue- or priming-induced reinstatement of responding has been tested in one group of randomized rats.

2.3.7. Data analysis

Data are expressed throughout the study as mean ± SEM. Comparison among groups was performed by analysis of variance (ANOVA) with a number of factors as indicated case by case in the Results section. Mann Whitney non-parametric test was applied to compare the number of active lever presses and inactive lever presses between different

treatment groups in the cue-induced reinstatement sessions. Welch non-parametric test was applied to compare the number of active lever presses and inactive lever presses between different treatment groups in the priming-induced reinstatement sessions Statistical analysis was performed using Prism 4 (Graph Pad, La Jolla, CA, USA).

3. Results

3.1. Drug Discrimination

In rats trained to discriminate nicotine from vehicle, 0.025, 0.05 and 0.1 mg/kg nicotine produced dose-related generalization of 52.7%, 69.9% and 80.4% of total response on the nicotine-associated lever, respectively (Figure 3, panel A). Nicotine tested at 0.1 and 0.2 mg/kg induced a statistically significant ($p<0.05$) increase in rate of responding compared to vehicle (78.2 ± 8.7 and 77.1 ± 8.6 vs. 57.4 ± 6 lever/presses, mean ± S.E.M.) (Figure 3, panel B).

Tacrine failed to generalize for nicotine at the dose of 1.25 mg/kg, and partially generalized at 0.065 and 2.5 mg/kg, producing 18.3%, 29.5% or 43.6% of NLR, respectively (Figure 4, panel A). Tacrine did not affect rate of responding compared to vehicle at all doses tested. However, when comparing tacrine to nicotine there was a significant decrease ($p<0.05$) of the responding rate at 1.25 and 2.5 mg/kg tacrine compared to nicotine 0.2 mg/kg (59.9 ± 6.1 and 60.9 ± 8.6 vs 77.1 ± 8.6 lever presses/min; mean ± S.E.M.) (Figure 4, panel B).
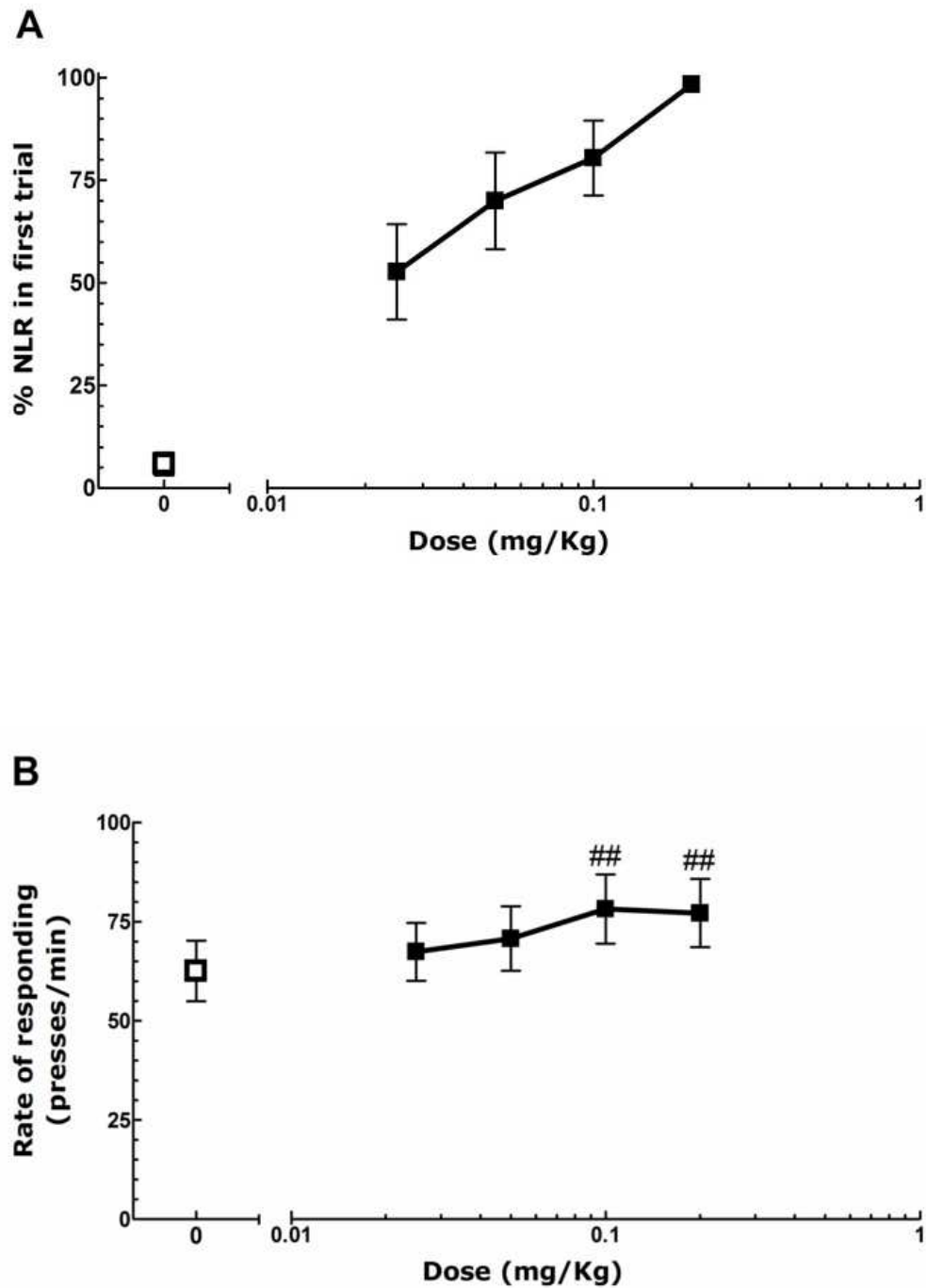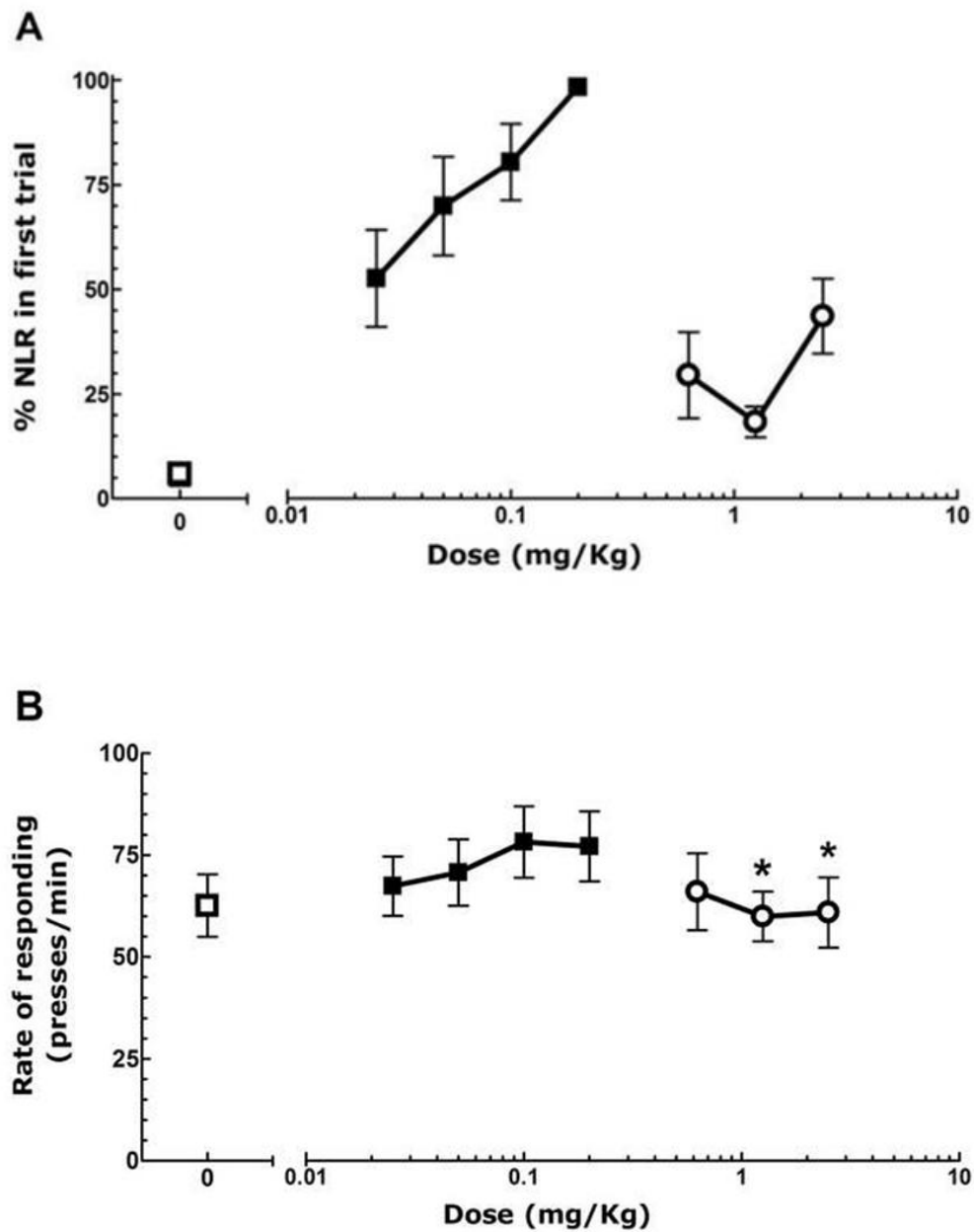
Figure 3. Nicotine discrimination dose-response curve.

Panel A. Nicotine discrimination is expressed as percentage (mean ± S.E.M.) of nicotine lever responses (NLR; ordinates) at nicotine doses 0.025, 0.05, 0.1, 0.2 (solid squares) or vehicle (open square) (mg/kg plotted in a logarithmic scale; i.p.; abscissa). Panel B. Rate of responding is expressed as number of lever presses/min (mean ± S.E.M.; ordinates) at the same nicotine doses as in Panel A (mg/Kg i.p.; abscissa). $^{##}$ = $P \leq 0.01$ vs. vehicle group, Dunnett's test, n = 11 subjects.

Figure 4. Tacrine discrimination dose-response curve.

Panel A. Tacrine discrimination is expressed as percentage (mean ± S.E.M.) of nicotine lever responses (NLR; ordinates) at tacrine doses 0.625, 1.25, 2.5 (open circle) compared to nicotine discrimination dose-response curve (solid square) and vehicle (open square) (mg/kg plotted in a logarithmic scale; s.c.; abscissa). Panel B. Rate of responding is expressed as number of lever presses/min (mean ± S.E.M.; ordinates) at the same tacrine and nicotine doses as in Panel A (mg/Kg s.c.; abscissa). *= P ≤ 0.05 vs. nicotine, Dunnett's test, n = 11 subjects.

Physostigmine partially generalized for nicotine at all doses tested (0.05, 0.1 or 0.2 mg/kg), producing 40.1%, 55.1% or 43.7% of NLR, respectively (Figure 5, panel A). The rate of responding at the highest tested dose of physostigmine (0.2 mg/kg) was significantly decreased ($p<0.01$) compared to vehicle (39.2 ± 9.3 vs. 57.4 ± 6, lever presses/min; mean $\pm$ S.E.M.) and all doses induced a significant decrease ($p<0.05$ 0.05 mg/Kg; $p<0.01$ 0.1 and 0.2 mg/kg) compared to nicotine training dose (57.0 ± 6.3, 49.7 ± 6.2 and 39.2 ± 9.3 vs 77.1 ± 8.6 lever presses/min; mean $\pm$ S.E.M.) (Figure 5, panel B).

Galantamine partially generalized for nicotine at all doses tested (1, 3 or 5 mg/Kg), producing 34.6%, 60.6% or 62.2% of NLR, respectively (Figure 6, panel A). Galantamine (5 mg/kg) induced a lower rate of responding ($p<0.01$) compared to vehicle (34.2 ± 6.9 vs. 57.4 ± 6 lever presses/min; mean $\pm$ S.E.M.) and all doses tested induced a statistically significant decrease in rate of responding ($p<0.05$ 1 mg/kg; $p<0.01$ 3 and 5 mg/kg) compared to nicotine training dose (60.9 ± 7.9, 53.4 ± 8.4 and 34.2 ± 6.9 vs 77.1 ± 8.6 lever presses/min; mean $\pm$ S.E.M.) (Figure 6, panel B).

Animals were also observed in the home cage after administration of all drugs for the assessment of gross behavioural changes. Following administration of 5 mg/kg galantamine, 0.2 mg/kg physostigmine and 2.5 mg/kg tacrine, slight yawing and limb tremors were observed in few animals. All other doses were without overt behavioural effects.
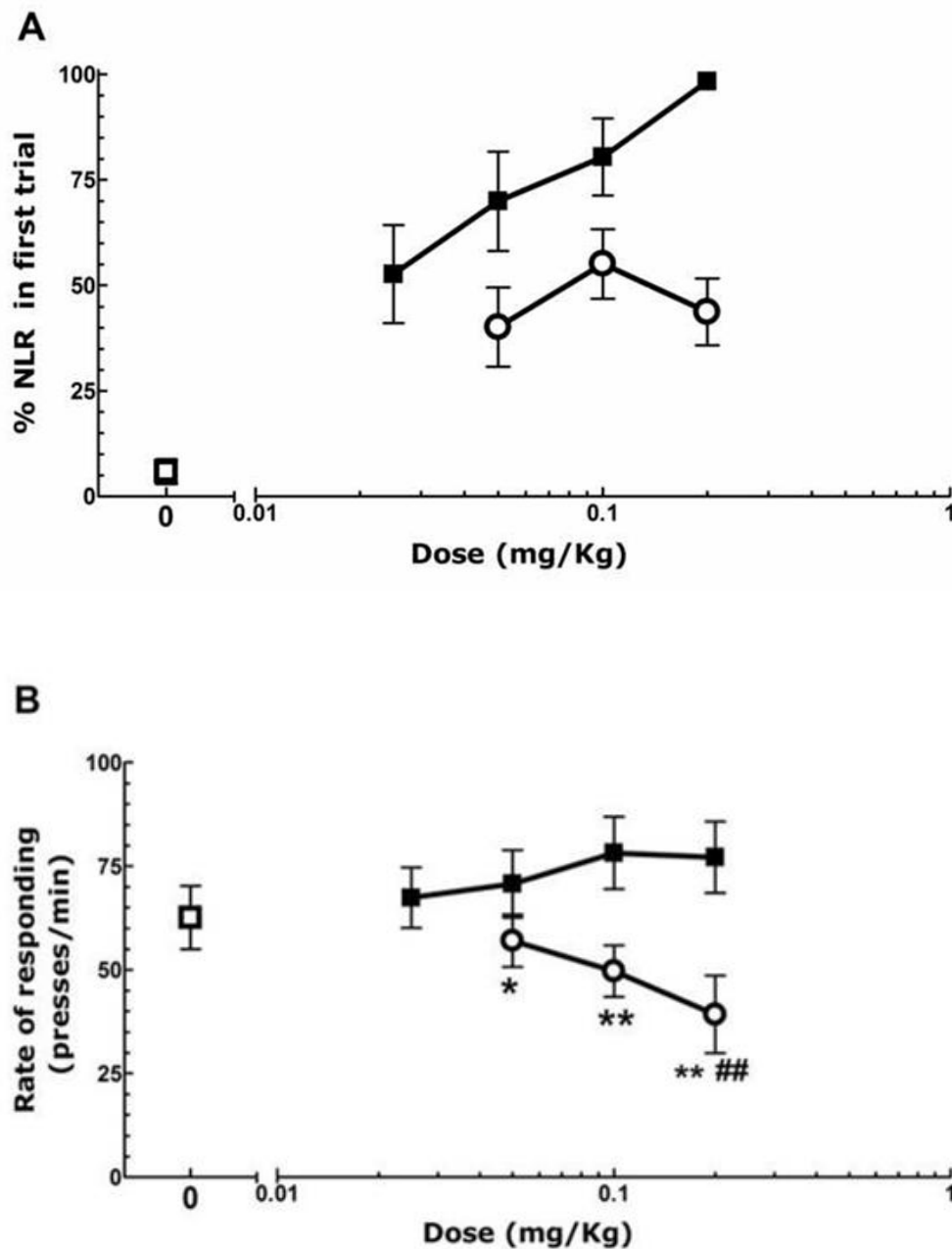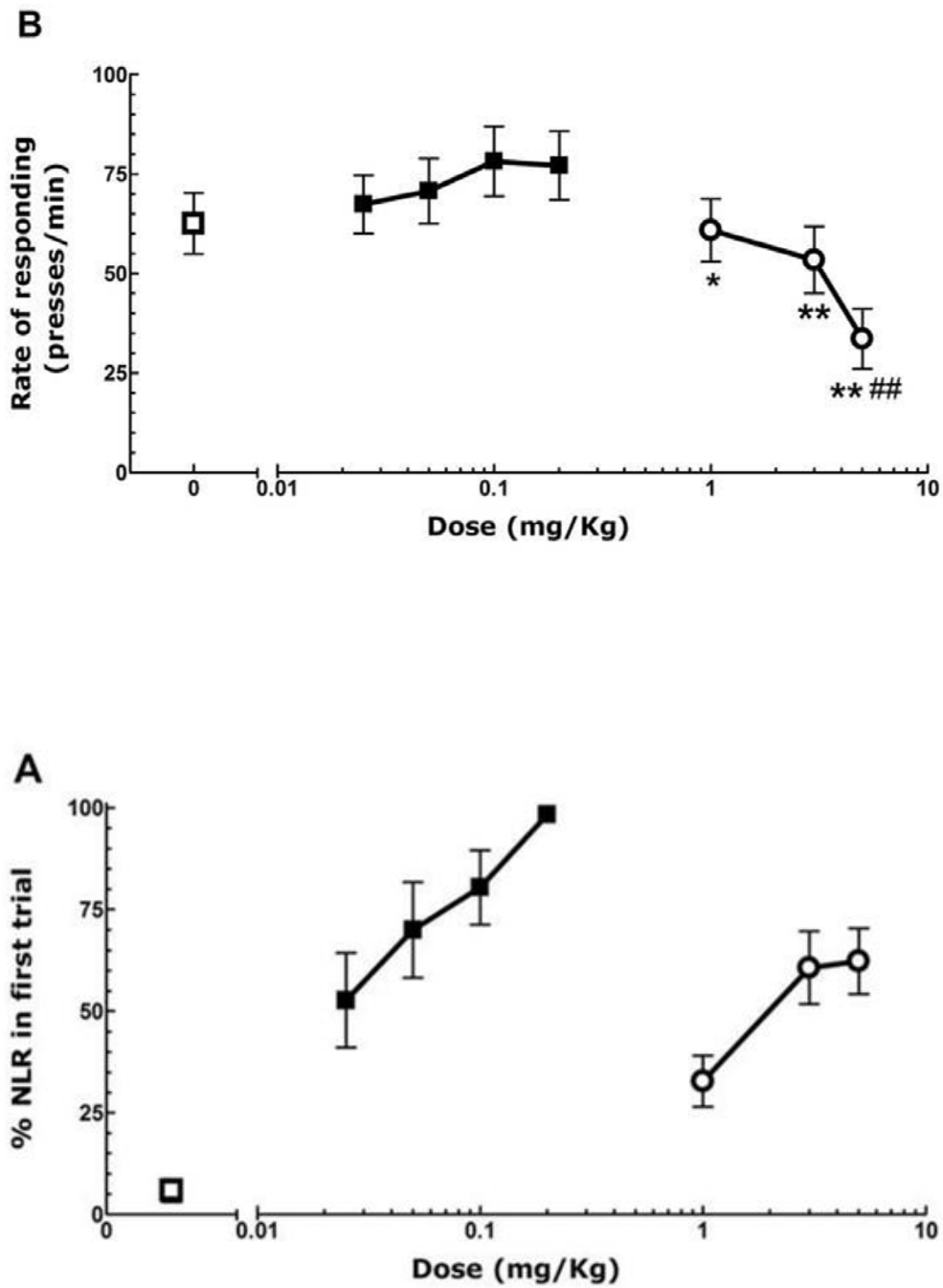
Figure 5. Physostigmine discrimination dose-response curve.

Panel A. Physostigmine discrimination is expressed as percentage (mean ± S.E.M.) of nicotine lever responses (NLR; ordinates) at physostigmine doses 0.05, 0.1, 0.2 (open circle) compared to nicotine discrimination dose-response curve (solid square) and vehicle (open square) (mg/kg plotted in a logarithmic scale; s.c.; abscissa). Panel B. Rate of responding is expressed as number of lever presses/min (mean ± S.E.M.; ordinates) at the same physostigmine and nicotine doses as in Panel A (mg/kg s.c.; abscissa). ## = P ≤ 0.01 vs. vehicle; *= P ≤ 0.05 and ** = P ≤ 0.01 vs. nicotine 0.2 mg/Kg dose; Dunnett's test, n = 11 subjects.

Figure 6. Galantamine discrimination dose-response curve.

Panel A. Physostigmine discrimination is expressed as percentage (mean ± S.E.M.) of nicotine lever responses (NLR; ordinates) at galantamine doses 1, 3, 5 (open circle) compared to nicotine discrimination dose-response curve (solid square) and vehicle (open square) (mg/kg plotted in a logarithmic scale; s.c.; abscissa). Panel B. Rate of responding is expressed as number of lever presses/min (mean ± S.E.M.; ordinates) at the same galantamine and nicotine doses as in Panel A (mg/Kg s.c.; abscissa). ## = $P \leq 0.01$ vs. vehicle; * = $P \leq 0.05$ ** = $P \leq 0.01$ vs. nicotine 0.2 mg/kg dose; Dunnett's test, n = 11 subjects

3.2. Self-administration

3.2.1. Effect of physostigmine on nicotine self-administration

Physostigmine at 0.05, 0.1 or 0.2 mg/kg has been tested in rats trained to self-administered nicotine. ANOVA analysis for 1-factor [Pre-treatment – 4 levels] shows that physostigmine pre-treatment did not induced any significant changes on the number of nicotine infusion during 60 minutes session (ANOVA, $F_{[3,24]}=2.183$; $P=0.067$; n=9). However a trend of increase and decrease in the number of nicotine infusion is noted after the administration of physostigmine at 0.05 and 0.2 mg/kg, respectively.

The mean nicotine infusion/1h values measured during test sessions were 9.3±2.7 with saline, 12.3±2.8 with physostigmine 0.05 mg/kg, 8.1±3.5 with physostigmine 0.1 mg/kg and 2.9±1.2 with physostigmine 0.2 mg/kg (Figure 7)
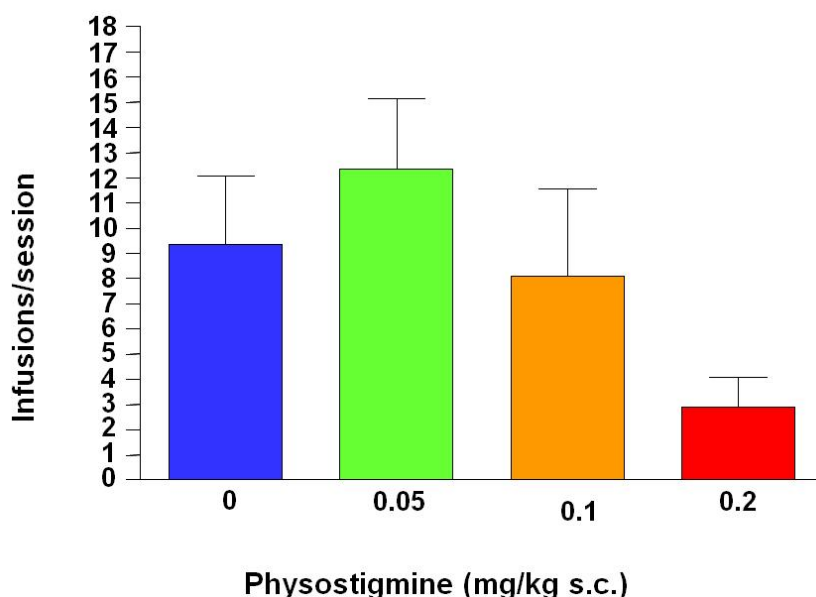

Figure 7. Effects of physostigmine pre-treatment on number of responding for nicotine infusion/session.

Physostigmine (0.05, 0.1 or 0.2 mg/kg s.c.) or vehicle (0 mg/kg) was given 30 minutes before the self-administration session start. Data represent the number of nicotine infision /session (mean±SEM; n=9).

The number of active lever presses during test session was 35.9±10.6 for saline, 53.2±11.7 for physostigmine 0.05 mg/kg, 30.0±12.9 with physostigmine 0.1 mg/kg, 11.3±4.5 with physostigmine 0.2 mg/kg. The number of lever presses on the inactive lever during test session was 2.2±0.8 for saline, 12.1±4.7 for physostigmine 0.05 mg/kg, 1.2±1.0 for physostigmine 0.1 mg/kg and 0.6±0.2 for physostigmine 0.2 mg/kg. (Figure 8). The increase of the number of inactive lever presses after 0.05 mg/kg physostigmine administration is associated with an increased locomotor activity
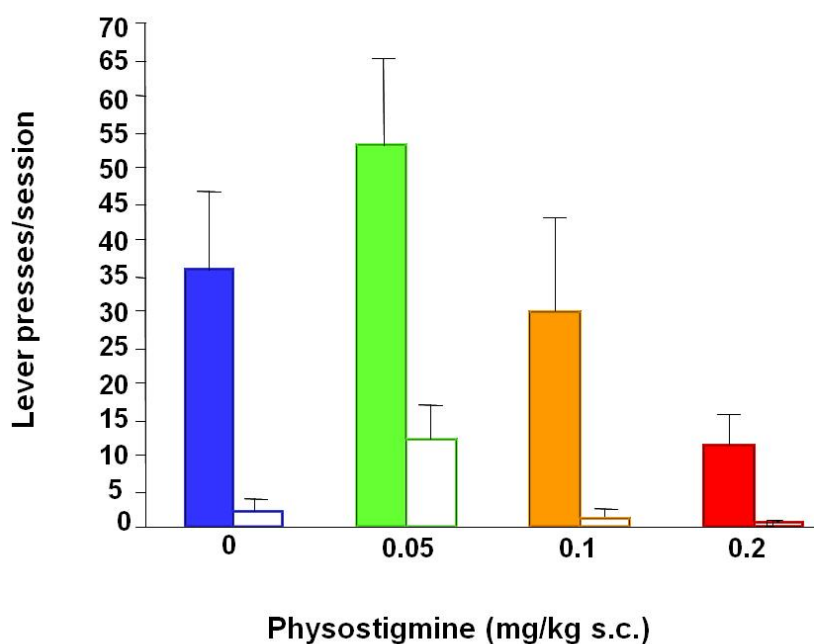

Figure 8. Effect of physostigmine on active and inactive lever presses.

Physostigmine (0.05, 0.1 or 0.2 mg/kg s.c.) or vehicle (0 mg/kg) was given 30 minutes before the self-administration session start. Solid bars represent the number of presses on active lever (nicotine-paired lever), open bars represent the number of presses on inactive lever (mean±SEM; n=9).

Test sessions have been performed when each animal reached the stability criterion, which required different number of training session between test sessions. To evaluate the effect of physostigmine pre-treatment the difference in the number of infusions during pre-test days needs to be taken into account (Figure 9).
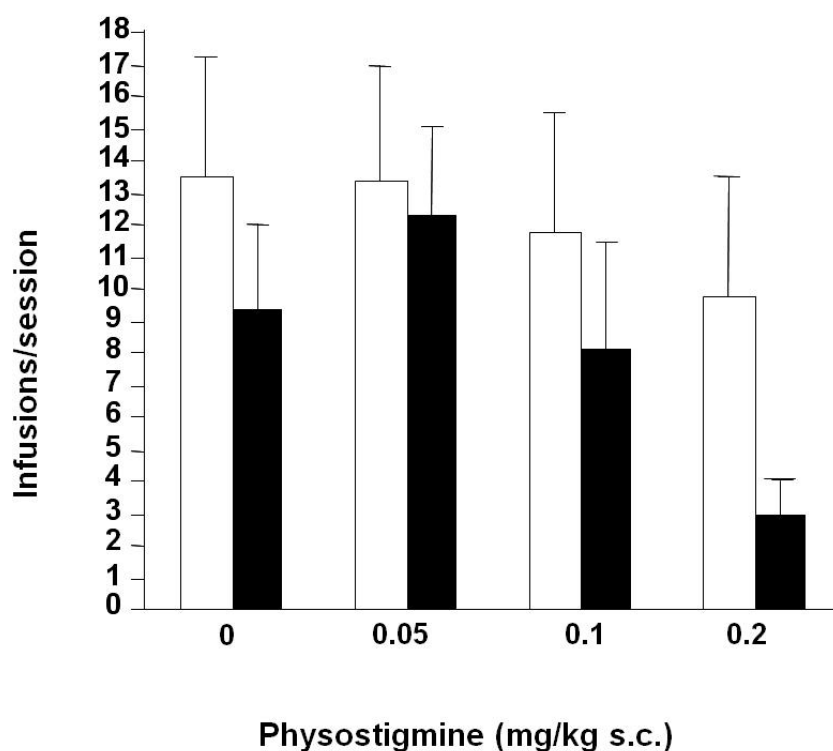

Figure 9. Effect of physostigmine on the number of infusion during pre-test and test days.

Physostigmine (0.05, 0.1 or 0.2 mg/kg s.c.) or vehicle (0 mg/kg) was given 30 minutes before the self-administration session start. White open bars represent the number of nicotine infusions on the pre-test sessions, black solid bars represent the number of nicotine infusions on the test days (mean±SEM; n=9).

Being this difference not statistically significant, the mean values of the difference between the number of infusions during pre-test and test days for each physostigmine doses was considered (delta; Figure 10).

The graph of difference depending on physostigmine dose regimen assumed a bell-shape profile with a trend of non statistically significant increase and decrease in the mean number of nicotine infusions after physostigmine administration of 0.05 mg/kg and 0.2 mg/kg, respectively (ANOVA, $F_{[3,24]}=0.569$; P=NS; n=9).
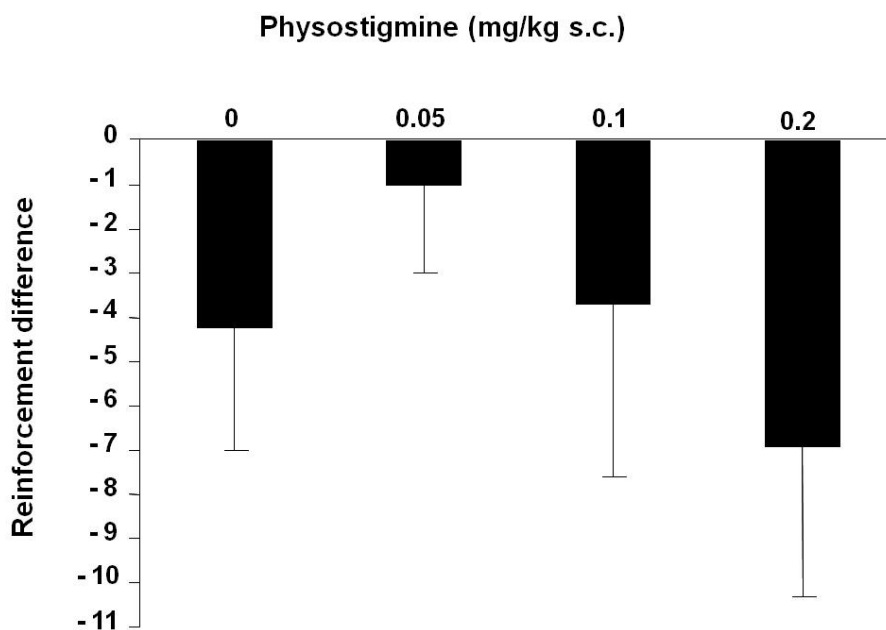
Physostigmine (mg/kg s.c.)

Figure 10. Effect of physostigmine on the nicotine reinforcements difference between pre-test and test session.

Physostigmine (0.05, 0.1 or 0.2 mg/kg s.c.) or vehicle (0 mg/kg) was given 30 minutes before the self-administration session start. Solid black columns represent thrmaen values of difference between number of nicotine infusion received during pre-test and test sessions (mean±SEM; n=9).

When the cumulative curve of nicotine number of infusions was analysed for the entire session, an effect compared to pre-test at 10 and 20 minutes was observed. Consequentially, the time-course of the mean number of infusion at different time point was analysed (Figure 11). ANOVA analysis for 2-factor [Pre-treatment – 4 levels X time 3 levels] shows a non significant interaction (F[6,32]=1.587; NS) but a statistically significant effect of pre-treatment (F[3.32]=3.106; P=0.040). Considering the delta values time-course, the ANOVA analysis for 2-factor [Pre-treatment – 4 levels X time 3 levels] does not show statistical significance compared to pre-treatment factor (F[3,32]=0.802; P=0.50), but a trend of decrease in the number of infusion with physostigmine 0.2 mg/kg and a trend of increase with physostigmine 0.05 mg/kg is observed (Figure 12).
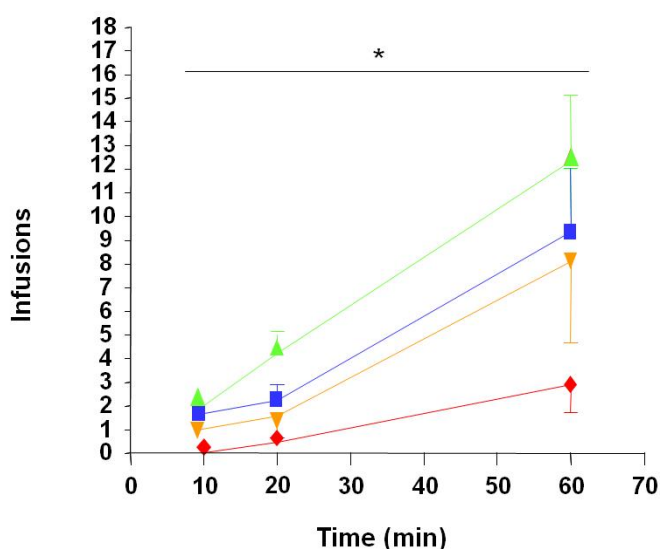

Figure 11. Time couse of physostigmine pre-treatment effects on number of nicotine infusions.

Physostigmine was given 30 minutes before the self-administration session start. Data in ordinates represent the number of cumulative nicotine infusions (mean±SEM; n=9) at different time bins during nicotine self-administration session after pre-treatment with vehicle (blue square) or physostigmine at 0.05 mg/kg (green triangle), 0.1 mgk/kg (orange triangle) and 0.2 mg/kg (red diamond). Data in abscissa represent the session time duration expressed in minutes. (* = P ≤ 0.05 ANOVA vs main effect Pre-treatment factor).
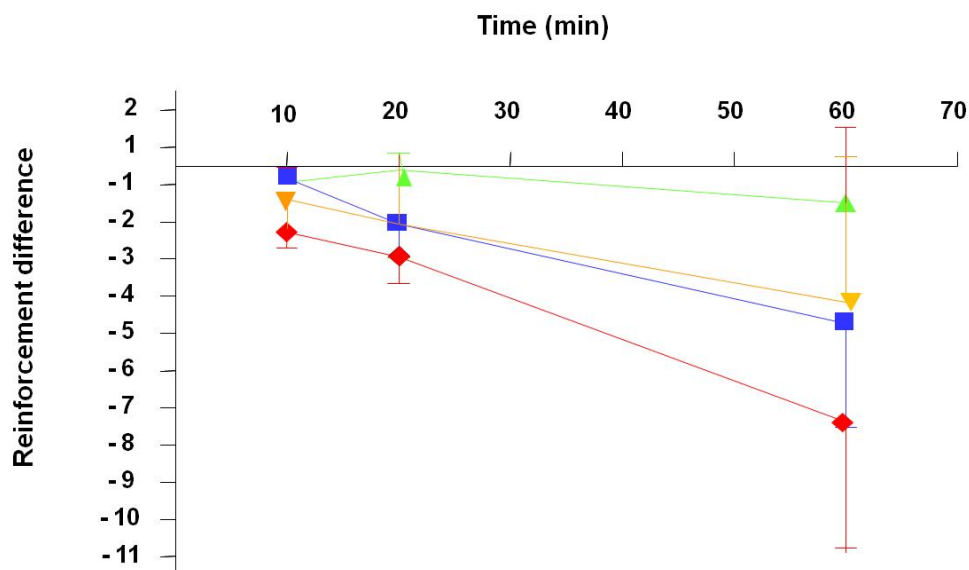
Figure 12. Time couse of physostigmine pre-treatment effects on reinforcement difference between pre-test and test sessions.

Physostigmine was given 30 minutes before the self-administration session start. Data in ordinates represent the difference in number of nicotine infusion between pre-test and test session (mean±SEM; n=9) at different time bins during nicotine self-administration session after pre-treatment with vehicle (blue square) or physostigmine at 0.05 mg/kg (green triangle), 0.1 mgk/kg (orange triangle) and 0.2 mg/kg (red diamond). Data in represent the session time duration expressed in minutes.

Animal administered with physostigmine 0.2 mg/kg, showed some side effect as limbs tremors and mandibular vertical movements.

3.2.2. Effect of tacrine on nicotine self-administration

Tacrine at 0.032, 0.1 or 0.32 mg/kg has been tested in rats trained to self-administered nicotine. ANOVA analysis for 1-factor [Pre-treatment – 4 levels] shows that tacrine pre-treatment did not induced any significant changes on the number of nicotine infusion during 60 minutes session (ANOVA, $F_{[3,16]}=0.633$; $P=0.605$; $n=5$). However a trend of increase in the number of nicotine infusion is noted after the administration of tacrine at 0.1mg/kg.

The mean nicotine-paired lever/1h values measured during test sessions were 11.0±1.6 with saline, 10.6±1.7 with tacrine 0.032 mg/kg, 14.2±2.1 with tacrine 0.1 mg/kg and 12.4±2.6 with tacrine 0.32 mg/kg (Figure 13)
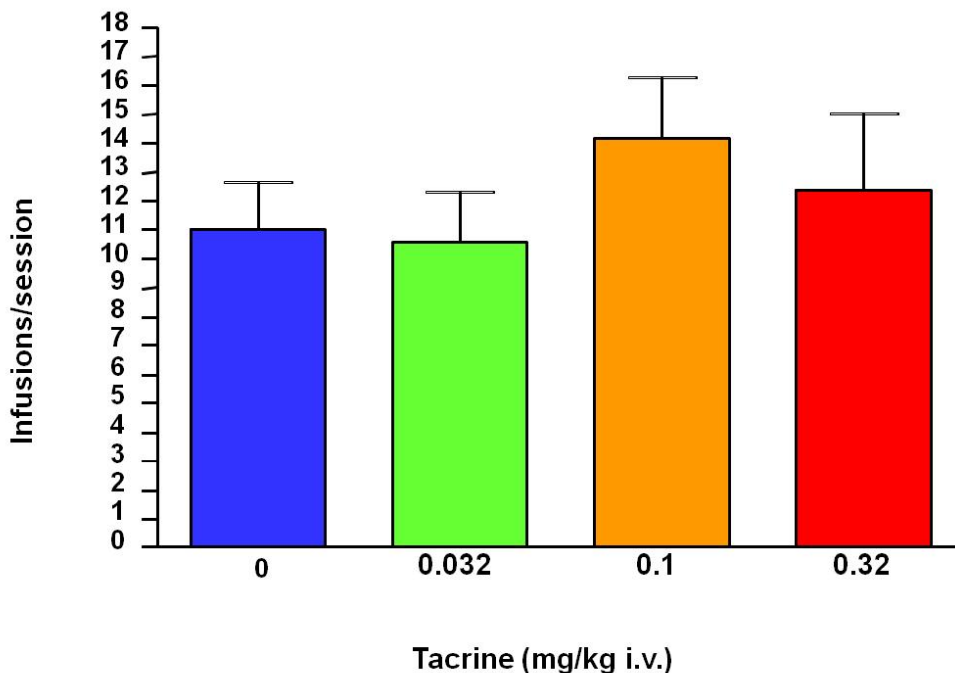

Figure 13. Effects of tacrine pre-treatment on number of nicotine infusion/session.

Tacrine (0.032, 0.1 or 0.32 mg/kg i.v.) or vehicle (0 mg/kg) was given 20 minutes before the self-administration session start. Data represent the number of nicotine infusions/session (mean±SEM; n=5).

The number of active lever presses during test session was 45.2±9.8 for saline, 46.0±11.6 for tacrine 0.032 mg/kg, 81.4±17.7 with tacrine 0.1 mg/kg, 49.8±13.3 with tacrine 0.32 mg/kg. (Figure 14).The number of lever presses on inactive lever during test session was 4.4±1.6 for saline, 6.8±3.3 for tacrine 0.032 mg/kg, 4.4±1.8 for tacrine 0.1 mg/kg and 3.2±1.7 for tacrine 0.32 mg/kg.
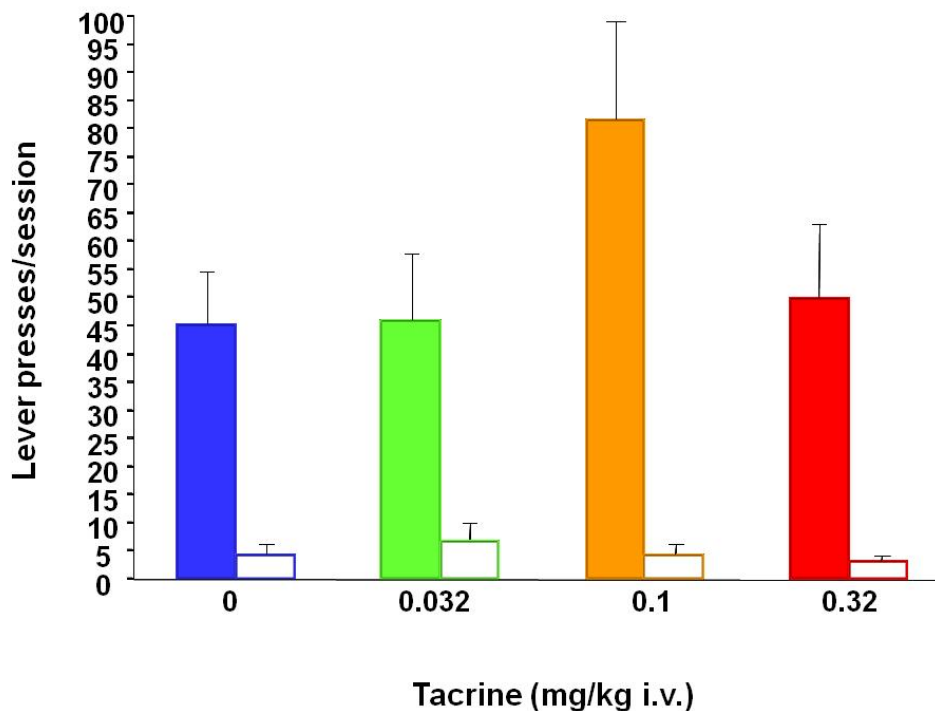

Figure 14. Effect of tacrine on active and incative lever presses.

Tacrine (0.032, 0.1 or 0.32 mg/kg i.v.) or vehicle (0 mg/kg) was given 20 minutes before the self-administration session start. Solid bars represent the number of presses on active lever (nicotine-paired lever), open bars represent the number of presses on inactive lever (mean±SEM; n=5).

Figure 15 shows the mean values of the number of infusions during pre-test compared

to test for each tacrine doses.
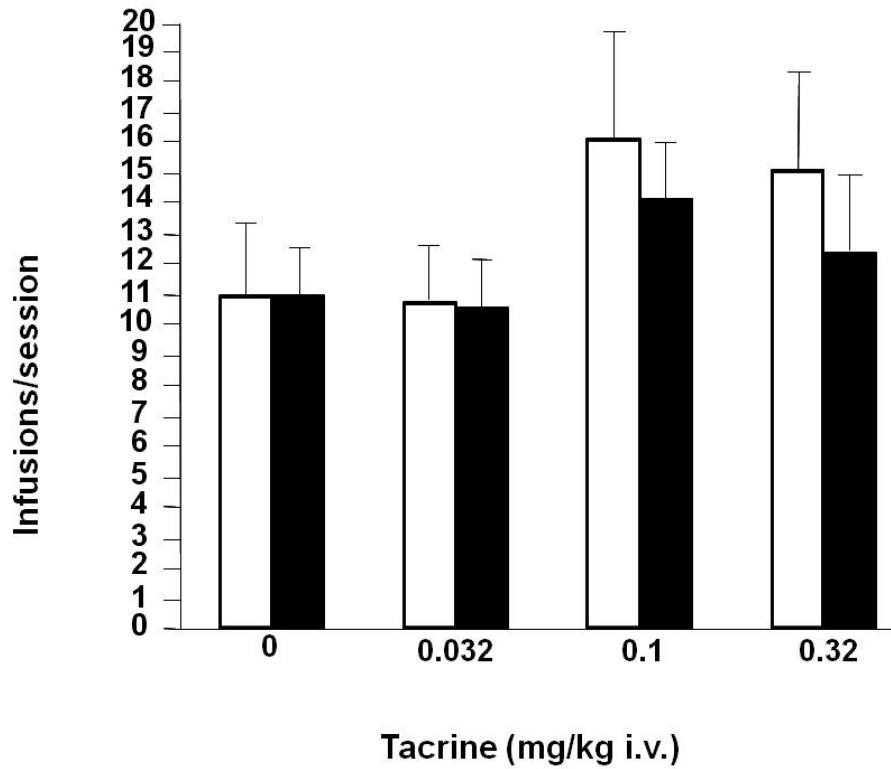

Figure 15. Effect tacrine on the number of infusion during pre-test and test days.

Tacrine (0.032, 0.1 or 0.32 mg/kg i.v.) or vehicle (0 mg/kg) was given 30 minutes before the self-administration session start. White open bars represent the number of nicotine infusions on the pre-test sessions, black solid bars represent the number of nicotine infusions on the test days (mean±SEM; n=5).

The mean values of the difference between the number of infusions during pre-test and test days for each tacrine doses (delta; Figure 16) does not show any significant difference (ANOVA, $F_{[3,16]}=0.447$; $P=0.723$; n=5).
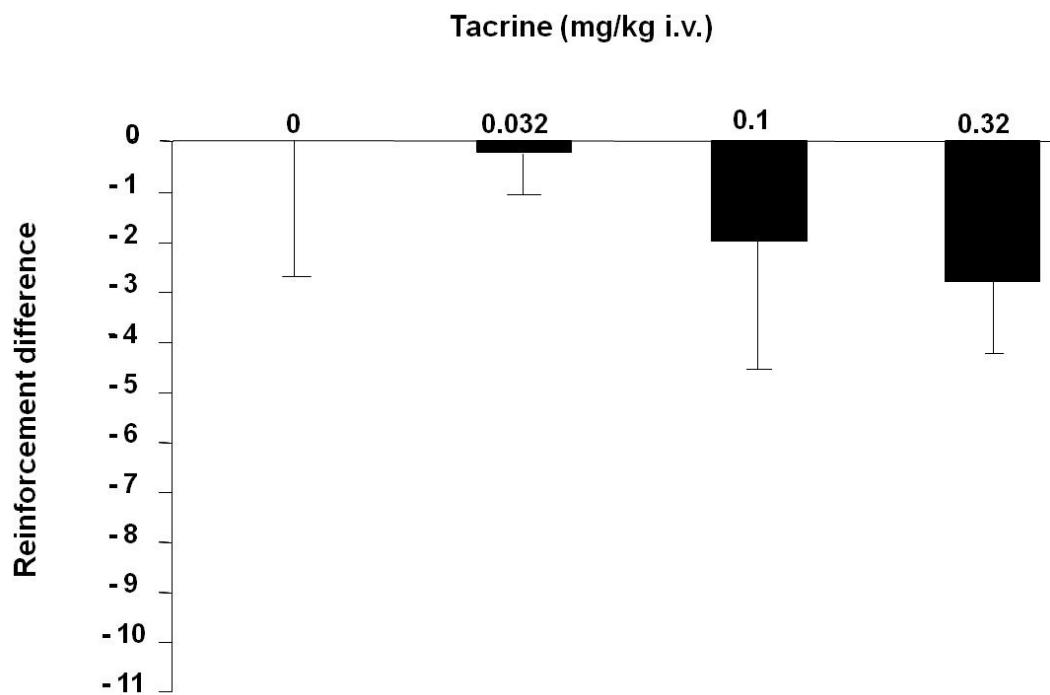

Figure 16. Effect of tacrine on the nicotine reinforcements difference between pre-test and test session.

Tacrine (0.032, 0.1 or 0.32 mg/kg i.v.) or vehicle (0 mg/kg) was given 20 minutes before the self-administration session start. Solid black columns represent the mean values of difference between number of nicotine infusion received during pre-test and test sessions (mean±SEM; n=5).

When cumulative curves of the number of nicotine infusions were analysed for the entire session, an effect compared to pre-test at 5, 10 or 30 minutes was observed. Consequentially, the time-curse of the mean number of infusion at different time point was analysed (Figure 17).
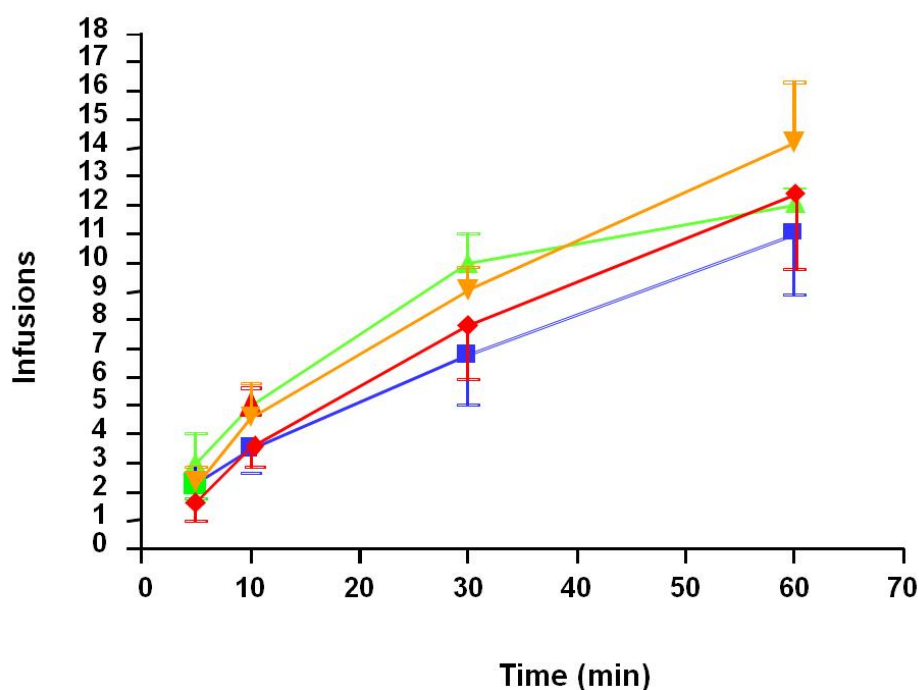
Figure 17. Time couse of tacrine pre-treatment effects on number of nicotine infusions.

Tacrine was given i.v. 20 minutes before the self-administration session start. Data in ordinates represent the number of cumulative nicotine infusions (mean±SEM; n=5) at different time bins during nicotine self-administration session after pre-treatment with vehicle (blue square) or tacrine at 0.032 mg/kg (green triangle), 0.1 mgk/kg (orange trianglee) and 0.32 mg/kg (red diamond). Data in abscissa represent the session time duration expressed in minutes.

ANOVA analysis for 2-factor [Pre-treatment – 4 levels X time 4 levels] shows neither a non significant interaction ($F[9,13]=0.519$; $P=0.852$) nor a significant effect of pre-treatment ($F[3,13]=0.495$; $P=0.692$). ANOVA analysis for 1-factor [Pre-treatment – 4 levels] at 5, 10 and 30 minutes also does not show any significant effect: ANOVA $F[3,16]=0.195$; $P=0.898$, ANOVA $F[3,16]=0.546$; $P=0.659$ and ANOVA $F[3,16]=0.529$; $P=0.669$, respectively. Considering the delta values time-course, the ANOVA analysis for 2-factor [Pre-treatment – 4 levels X time 4 levels] does not show statistical significance compared to pre-treatment factor ($F[3,16]=0.720$; $P=0.555$), but a trend of increase in the number of infusion after tacrine 0.032 administration at 5, 10 and 30 minutes mg/kg is observed (Figure 18).
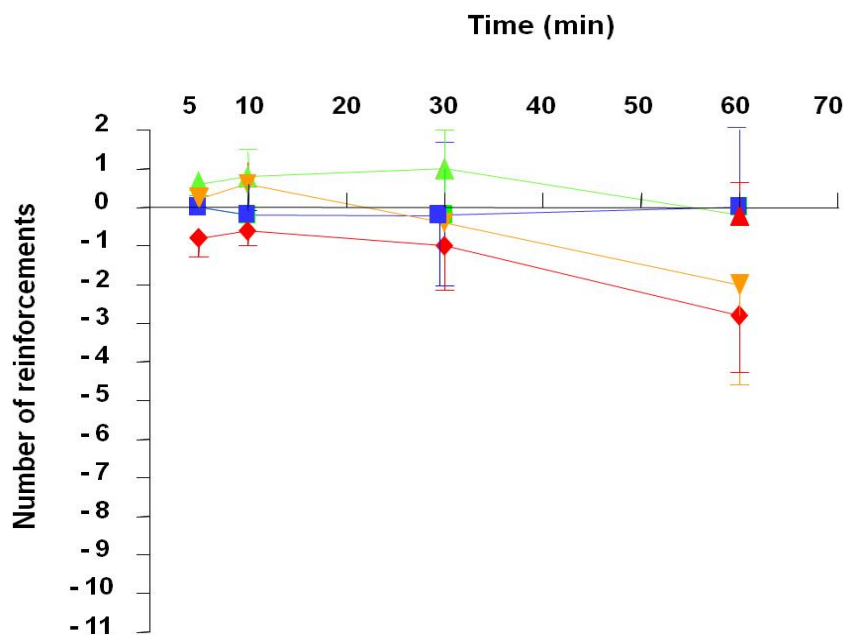
Figure 18. Time couse of tacrine pre-treatment effects on reinforcement difference between pre-test and test sessions.

Tacrine was given i.v. 20 minutes before the self-administration session start. Data in ordinates represent the difference in number of nicotine infusion between pre-test and test session (mean±SEM; n=5) at different time bins during nicotine self-administration session after pre-treatment with vehicle (blue square) or tacrine at 0.032 mg/kg (green triangle), 0.1 mgk/kg (orange triangle) and 0.32 mg/kg (red diamond). Data in abscissa represent the session time duration expressed in minutes.

3.3. Extinction and relapse

3.3.1. Effect of chronic tacrine treatment on extinction of responding for nicotine self-administration

Chronic tacrine treatment has been tested in two separated rat groups that underwent 10 sessions of extinction of responding for nicotine self-administration. As showed in Figure 19 the mean number of active lever pressing is reduced between the first and the last day of extinction in both treatment groups. The mean number of active lever pressing in saline group was 18.9±3.61 at first day, 8±1.65; 15.45±3.63; 12.18±2.64; 11.36V3.41; 7.63±1.14; 10.18±2.65; 9.27±1.66; 10.81±1.61; 8.27±0.98 at the next days from the second to the tenth, respectively.
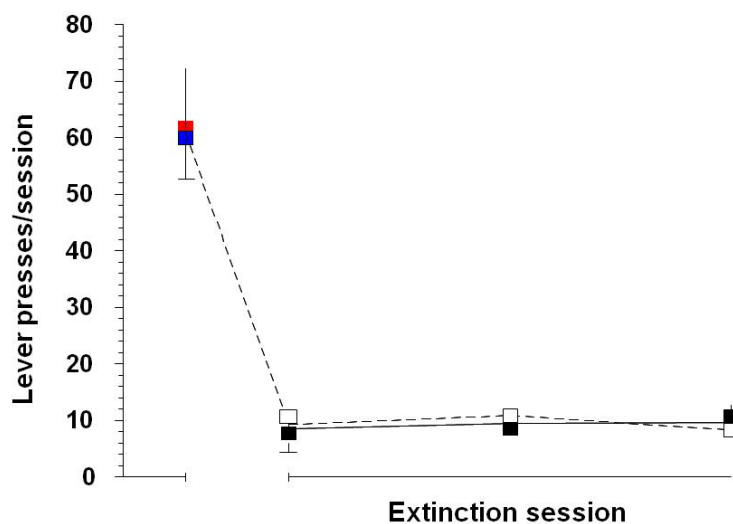
Figure 19. Tacrine effect on extinction of nicotine self-administration behaviour.

Data in ordinates represent the number of cumulative active nicotine-related lever presses (mean±SEM; n=11) during the last nicotine self-administration session for vehicle group (blue square) or tacrine group (0.32 mg/kg i.v.; red square) and the last three extinction sessions for vehicle group (white square) or tacrine group (0.32 mg/kg i.v.; black square).

The comparison of the mean number of active lever pressing between saline and tacrine treatment group during the 10 extinction sessions does not show any statistically significant difference for treatment factor (ANOVA analysis for 2-factor; F [1;20]= 1; P=0.436) (Figure 20).
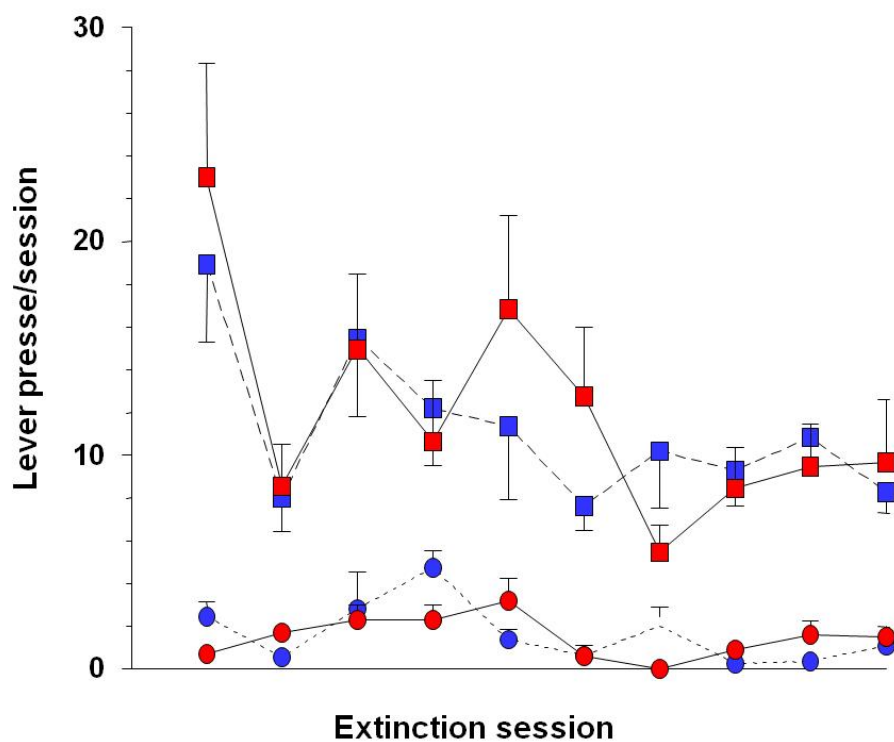

Figure 20. Tacrine effect on active or inactive lever presses during extinction.

Data in ordinates represent the number of lever presses (mean±SEM; n=11) on active nicotine-related lever (saline group blue circle; tacrine group 0.32 mg/kg i.v. red circle) or inactive lever (saline group blue square; tacrine group 0.32 mg/kg i.v. red square) during ten consecutive 1 hour-extinction session.

3.3.2. Effect of tacrine on nicotine cue-induced reinstatement of responding

The rats were tested for prevention of nicotine-cue induced reinstatement of responding after tacrine or saline treatment. Pre-treatment with tacrine 0.32 mg/kg did not induce any significant changes of nicotine cue-induced reinstatement of responding (Figure 21). The mean nicotine-paired lever/1-h values measured during reinstatement session were, respectively, 19.54 ± 3.52 after saline and 27.09 ± 6.62 after tacrine 0.32 mg/kg (Mann Whitney test, P=0.346). The number of inactive lever presses during reinstatement session was 3.27 ± 1.28 after saline and 1.27 ± 0.46 after tacrine group (Mann Whitney test, P=0.157).

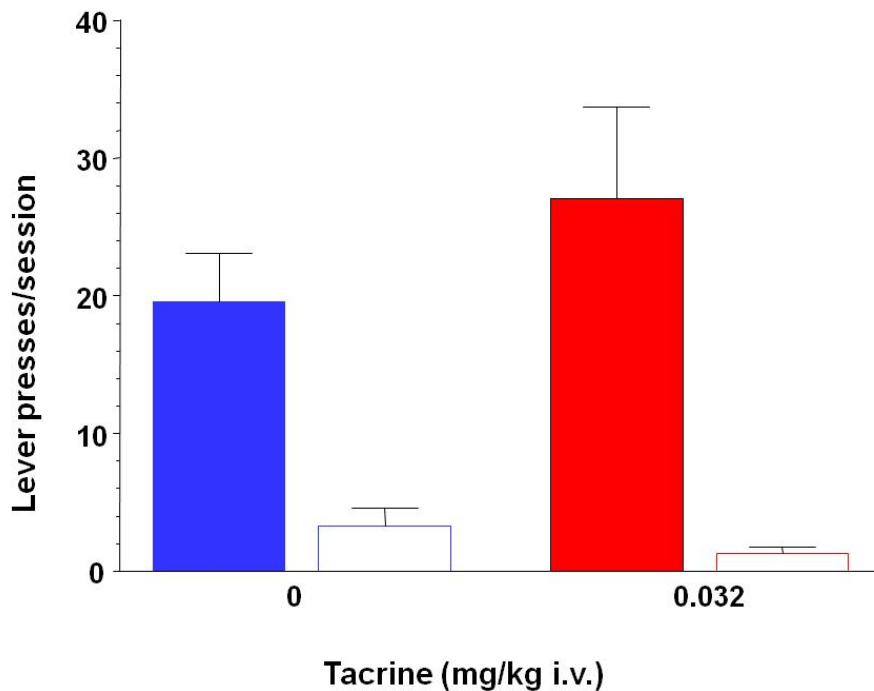Figure 21. Tacrine effect on nicotine cue-induced reinstatement of responding.

Data in ordinates represent the number of lever presses (mean±SEM; n=11) on active nicotine-related lever (saline group solid blue bar; tacrine group 0.32 mg/kg i.v. solid red bar) or inactive lever (saline group open blue bar; tacrine group 0.32 mg/kg i.v. open red bar) during 1 hour-cue-induced reinstatement session.

3.3.3. Effect of tacrine on nicotine priming-induced reinstatement of responding

The rats were tested for prevention of priming induced reinstatement of responding after tacrine or saline treatment. Pre-treatment with tacrine 0.32 mg/kg did not induce any significant changes of nicotine priming-induced reinstatement of responding (Figure 22). The mean nicotine-paired lever/1-h values measured during reinstatement session were, respectively, 19.7±4.4 after saline and 25±7.91 after tacrine 0.32 mg/kg (Welch t test, P=0.283). The number of inactive lever presses during reinstatement session was 0.7±0.49 after saline and 2.18±0.65 after tacrine group (Mann Whitney test, P=0.086).

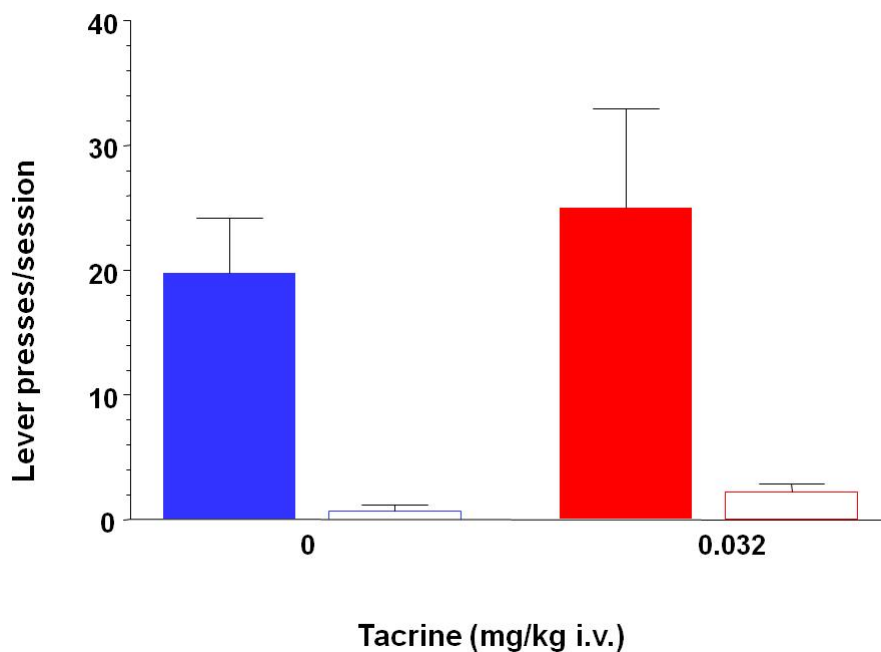Figure 22. Tacrine effect on nicotine priming-induced reinstatement of responding.

Data in ordinates represent the number of lever presses (mean±SEM; n=11) on active nicotine-related lever (saline group solid blue bar; tacrine group 0.32 mg/kg i.v. solid red bar) or inactive lever (saline group open blue bar; tacrine group 0.32 mg/kg i.v. open red bar) during 1 hour-priming-induced reinstatement session.

4. Discussion

In this research project three AChE-Is have been tested in three different animal models of nicotine dependence. The results can be summarised as follows: i) the two AChE-Is with APL properties, physostigmine and galantamine, induced partial generalization for nicotine discriminative stimulus; tacrine, a non-APL AChE-I, did not generalize for nicotine to nicotine, except at the highest tested dose; ii) physostigmine and tacrine administered pre-treatment did not exert any effect on nicotine S/A; iii) chronic tacrine treatment did not induced any significant changes on extinction of responding for nicotine S/A model and on nicotine cue and priming-induced reinstatement of responding paradigm.

Drug Discrimination

The first study of this research was designed to investigate whether AChE-Is with APL properties at nAChRs generalize to nicotine interoceptive stimulus and the operant behavioural model applied is the DD. DD methodology provides an approach for objective, quantitative study of the perception of psychoactive drug effects that can be applied to substances across numerous pharmacological classes in either human or animal subjects (Smith and Stolerman, 2009). The strengths of this model are the molecular specificity of the discriminative-stimulus effect of a drug, the correspondence between neurochemical and molecular mechanisms of action and behavioural measures, and the high predictive validity for subjective effects in man (Solinas, 2006). Nicotine produces an interoceptive stimulus that has been extensively studied pharmacologically

in the rat. Agonists acting at nAChRs, but not mAChRs, generalize to the nicotine cue (Pratt et al., 1983; Wiley 1996; Chandler and Stolerman 1997; Smith and Stolerman 2009). This DD paradigm was thus used in the current study as *in vivo* model for testing the effect the nicotine-like effects of AChE-Is on nAChRs.

In the current study, the two AChE-Is with APL properties on nAChRs, physostigmine and galantamine, induced partial generalization for nicotine discriminative stimulus; the highest degree of generalization was induced by 5 mg/kg galantamine. The degree of generalization observed with physostigmine and galantamine was greater than that induced by tacrine. Tacrine, a non-APL AChE-I, did not generalize for nicotine except for the highest dose tested which showed a partial generalisation. Galantamine dose-dependently induced nicotine-appropriate lever responding, but this effect was associated with a decrease in rate of responding. Physostigmine partially generalized for nicotine discriminative stimulus, with no clear dose-response relationship, however the decrease in the rate of responding was directly proportional to the dose. Therefore, it is possible that the reduced rate of responding could mask higher nicotine-appropriate lever presses, thus the degree of generalization observed was underestimated. Neurobehavioural side-effects (e.g., tremors, decreased locomotor activity) associated with galantamine and physostigmine administration has been reported in the literature (Sweeney et al., 1990; Mach et al., 2004; Myher et al., 2010). The behavioural signs observed at higher doses of both physostigmine and tacrine were oral movements (tongue protrusion and repeated chewing) and in some cases fasciculation. Tacrine (5 mg/kg) produced motor side effects which disrupt operant performance in the study performed by Liu et al. (2000). It is interesting to note that tacrine was reported to have

an opposite effect on ACh release at higher doses (Svensson et al., 1996). Furthermore, in vivo studies showed an inverted U-shaped response curves with rivastigmine as well as with other AChE-Is such as tacrine and galantamine (Yoshida and Suzuki 1993; Sweeney et al., 1990). Is it possible that this AChE-Is pharmacodynamic feature in vivo may explain the lack of dose-response generalization.

Both Chandler and Stolerman (1997) and Rollema et al. (2007) have demonstrated that the nAChRs partial agonist cytisine exhibited a full dose-related generalization to nicotine. Other studies have shown that the partial agonists cytisine and varenicline only partially generalize to nicotine (Smith et al., 2007). In particular, LeSage et al., (2009) demonstrated that varenicline and cytisine generalized to the nicotine stimulus to different extents, with varenicline showing greater generalization that cytisine.

This study demonstrated that AChE-Is induced partially nicotine-like effect, but the main limitation was the lack of assessment whether the dose-relationship of the generalization effect of AChE-Is vs. nicotine stimulus was dependent upon ACh levels. Systemic AChE-Is -increased ACh levels may activate mAChRs and nAChRs, both at pre-synaptic and post-synaptic level, thereby enhancing and prolonging the action of ACh on both classes of AChRs. One hypothesis is that treatment with AChE-Is could alter the spatio-temporal pattern of ACh levels maintained by AChE. Inhibition of AChE would allow for a wider spatial diffusion of ACh over a longer period of time, with the prediction that it would result in a larger and prolonged endogenous agonist binding at synaptic and non-synaptic AChRs. This effect could have a greater impact on the activation of nAChRs than mAChRs. In fact, ACh-induced activation of nAChRs

for a prolonged period of time would increase the probability of nAChRs transition to a desensitized state.

Self-administration

Following the DD experiments that allowed a pharmacological characterisation of AChE-Is, physostigmine and tacrine have been selected as tool compound for the nicotine S/A study. Intravenous S/A model is generally considered to be the most direct measure of drug's reinforcing effect (Le Foll and Goldberg, 2009). Although some of the early attempts to establish nicotine as a reinforcer met with limited success, it has now been demonstrated that animals do work to obtain nicotine. When delivered intravenously, nicotine maintains S/A behaviour in a variety of animal species, including primates (Goldberg et al., 1981; Sannerud et al., 1994) and rodents (Corrigall and Coen, 1989; Donny et al., 1995; Tessari et al., 1995; Shoaib et al., 1997). A review of methodological details of nicotine S/A can be found in Corrigall (1999).

Physostigmine has been selected as representative of the AChE-Is with APL properties, and tacrine as representative of AChE-Is with no APL properties. In this study rats have been trained to self-administered nicotine and after a training period they received pre-treatment with physostigmine or tacrine. Physostigmine pre-treatment at 0.05, 0.1 and 0.2 mg/kg did not induce any significant changes on the number of nicotine infusion during 60 minutes session. However a trend of increase and decrease in the number of nicotine infusion is noted after the administration of physostigmine at 0.05 and 0.2 mg/kg, respectively. The effect of increase in number of nicotine injection after

administration of physostigmine at 0.05 mg/kg is associated with an increase in number of lever presses and with an increase in locomotor activity. It cannot be excluded that this effect could be due to increase changes of pressing the lever as consequence of increased activity of the animals in the box. The decreasing in number of nicotine injections that appeared with physostigmine 0.2 mg/kg is associated with a decreased number of lever pressing. It is important to note that the rate reducing effect of high systemic dose (0.2 mg/kg) of physostigmine may result in part from nonspecific effects of systemic cholinergic activation, as described by Wilson and Schuster (1973) in primates and as reported by Zhou et al. (2007).

Within the same nicotine S/A experiment, tacrine at 0.032, 0.1 or 0.32 mg/kg has been administered as a pre-treatment. Results showed that tacrine pre-treatment did not induce any significant changes on the number of nicotine infusion during a 60 minutes session. However a trend of increase in the number of nicotine infusion is noted after administration of tacrine at 0.1mg/kg.

 In the VTA, carbachol and cytosine, a nicotine agonist, induced conditioned place preference (CPP) which is a commonly used paradigm to assess the strength of stimulus associated with drugs (Yeomans et al., 1985; Museo & Wise, 1994). Furthermore, lesions of the pedunculo-pontine nucleus inhibit morphine and amphetamine CPP (Bechara and van der Kooy, 1989; Olmstead & Franklin, 1993). These results indicate that VTA stimulation of both mAChRs and nAChRs are critical for the expression of conditioned reinforcement. In contrast, administration of AChE-Is into the NA suppressed cocaine- and morphine-induced CPP (Hikida et al., 2003) and also reinstatement of heroin seeking (Zhou et al., 2007). Consistently with these results, a

lower dose of cocaine was needed for example to induce CPP in mice in which NA was ablated with an immunotoxin (Hikida et al., 2001). It is important to note that the effect of AChE-Is in NA are similar to those obtained when they are systemically administered.

Systemic administration of physostigmine reduced cocaine S/A in rhesus monkeys (De La Garza & Johanson, 1982). Kameda et al. (2000) showed that drinking tacrine for six days enhanced nicotine preference and intake in the following six days in a 2-bottle choice paradigm. Since similar enhancing effects were observed after treatment with oral mecamylamine under the same conditions, the authors concluded that the increased nicotine consumption was due to a compensatory intake for the reduced reward induced by blocking (with mecamylamine) or by indirectly activating (with tacrine) nAChRs. Grasing et al. (2008) reported that tacrine induced a dose-related inhibition of cocaine and food S/A in rats, and confirmed previous results from Liu et al. (2000) on water S/A. Systemic donepezil reduced morphine and cocaine CPP and hyperlocomotion in mice (Hikida et al., 2003), and these effects were reversed by selective immunotoxin cholinergic lesion in the NA (Kaneko et al., 2000; Hikida et al., 2001). Interesting data were collected when the effects of donepezil on methamphetamine-seeking behaviour were investigated both systemically and intracranially (Hiranita et al., 2006). Overall, these data strongly indicated that the effects of AChE-Is resulted from an increase of nAChR-ergic transmission and they were consistent with the hypothesis that chronic methamphetamine decreases choline acetyltransferase (ChAT) and increases vescicular ACh transporter (VAChT) in humans (Kish et al., 1999; Siegal et al., 2004). In fact, Siegal et al. showed that chronic administration of large doses of methamphetamine, but

not cocaine and heroin, decreased brain ChAT activity, which was presumably compensated by over-expressed VAChT. Kish et al. (1999) found a severe decrease of ChAT (up to 94%) only in post-mortem brains of methamphetamine users who had the highest drug levels, whereas no significant changes were observed in post-mortem brains of cocaine dependent individuals. Hiranita et al. (2006) speculated that this cholinergic imbalance may result from down-regulated nAChR-ergic transmission in the striatum. This would explain why donepezil and nicotine, by enhancing nAChR-ergic transmission, were effective against methamphetamine reinstatement. Unfortunatly, the study lacks information about the effects of donepezil in the VTA, which would have been interesting considering the differential outcomes generated when AChE-Is are microinjected into the VTA compared to the NA. Also, the data in methamphetamine users (Kish et al., 1999; Siegal et al., 2004) indicated a general cholinergic down-regulation and therefore did not exclude an involvement of mAChRs. Opposite results were obtained however by Takamatsu et al. (2006), that showed divergent findings on the effects of systemic administration of donepezil (at doses approximately ten-fold greater than those used by Hiranita et al., 2006) on cocaine but not methamphetamine reward in mice. For example, donepezil reduced cocaine-induced CPP, hyperlocomotion and motor sensitization, but this inhibition was not observed in methamphetamine treated mice. More recent studies did not improve the unclear scenario. In fact, discrepancies are still present on the effects of AChE inhibition, depending on the AChE-I used, the route of administration, and the type of drug of abuse investigated. The most conservative hypothetical framework is to consider a different role of cholinergic modulation of DA transmission at the VTA cell bodies and

at the NA terminals levels, with a more relevant role of nAChRs at the former and of mAChRs at the latter. According to this hypothesis, Zhou et al (2007) showed that systemic administration of physostigmine reduced heroin S/A and seeking behaviour in rats. This effect was likely the result of mAChRs stimulation since it was reversed by pre-treatment with scopolamine.

The schematic hypothetical framework based on the scientific evidence, suggest that: i), increase in ACh levels in the VTA increases dopaminergic neuronal activity and, on the other hand, ii), increased ACh levels in the NA decrease the effects of released dopamine. According to pharmacological evidence, it may be also 'schematically' concluded that ACh effects in NA are mainly mediated by mAChRs whereas in the VTA are mediated by nAChRs. These simplistic assumptions have several exceptions, depending on dopaminergic system activation and experimental manipulation (basal release, acute or chronic stimulation), cholinergic tools used (agonists, antagonists, lesions), molecular or behavioural dependent measures.

Using the AChE-Is as pharmacological tool to increase brain ACh levels, we tried to reply to the question: what is the net system effect of increased ACh on nicotine addictive behaviour? Is it a VTA-based potentiation or NA-based inhibition? Accordingly to general pharmacological principles, AChE-Is effects could be reasonably predicted on the basis of a bell-shaped dose-response relationship, where the choice of an appropriate dose regimen may avoid dopamine-stimulating doses. Secondly, preclinical studies showed different results depending on the type of i), symptomatic dimensions, ii), drugs of abuse, iii), or AChE-I tested.

Extinction and relapse

AChE-Is have been pharmacologically characterized using the DD model and tested in the S/A paradigm to clarify the role of ACh in mesolimbic dopamingergic pathway. As reviewed in the S/A section, nicotine is effective as a reinforcer when different schedules of reinforcement are used and different acquisition conditions prevail across species and strains. Studies in rats have shown that non-contingent administration of nicotine during extinction of nicotine S/A behaviour reinstates responding previously reinforced by nicotine (Andreoli et al., 2003; Chiamulera et al., 1996). Exposure to drug-paired stimuli also appear effective in reinstating extinguished nicotine-seeking behaviour (Dravolina et al., 2007; Lesage et al., 2004; Liu et al., 2006; Liu et al., 2007).

AChE-Is are used in the clinic for treating cognitive deficits and they act as cognitive enhancers. In fact, cholinomimetic drugs, including AChE-Is, significantly improved clinical endpoints such as sustained attention, working memory, visual detection, and verbal fluency in patients (MacEwan et al., 2001; Kirrane et al., 2001; Lenzi et al., 2003; Buccafusco, 2004; Chiamulera & Fumagalli, 2007; Chouinard et al., 2007). Long-lasting cognitive impairment may act as a determinant factor of relapse to drug use (Block et al., 2002). These finding suggested the importance of studying the potential therapeutic value of 'cognitive enhancers' for prevention of relapse (Vocci et al., 2005). According to this rationale, the AChE-I tacrine has been tested on animal models that recreate the same clinical conditions of nicotine assumption extinction and relapse. In particular, rats have been trained to self-administered nicotine and then underwent an extinction session and subsequently to cue- or priming-induced relapse.

Tacrine has been chosen as a test compound as it lacks APL properties and had the lower degree of generalization with nicotine. With this choice we can avoid a greater impact on the activation of nAChRs that would increase the probability of nAChRs transition to a desensitised state. Tacrine 0.32 mg/kg administered chronically did not exert any effect either on extinction of nicotine S/A behaviour, or on drug cues or nicotine priming reinstatement.

Intraperitoneal administration of donepezil attenuated reinstatement of responding for the methamphetamine-associated lever induced by exposure to methamphetamine cues or by administration of methamphetamine priming in rats. Furthermore, physostigmine reduced heroin S/A to a less extent compared to heroin-seeking behaviour (Zhou et al., 2007). In fact, while the effect of daily treatment with physostigmine only slightly attenuated the acquisition of heroin S/A, this pre-treatment markedly reduced subsequent cue-induced reinstatement 14 days after the last administration of physostigmine and heroin. A different scenario was obtained when the effects of physostigmine were evaluated on heroin-seeking behaviour during cue-induced reinstatement or during extinction after intra NA or VTA injection. Physostigmine reduced reinstatement but not extinction when injected into NA. On the other hand, when injected into the VTA, physostigmine enhanced responding for both reinstatement and extinction.

Conclusion

This overall project has a number of limitations. First, administration of non-specific cholinomimetic agents, often through a systemic route, made it difficult to assess which brain site was targeted by the pharmacological modulation. Second, the use of different doses and route of administration did not allow a direct correlation between different models. Third, different nicotine doses could have been tested. Moreover, the lack of specific neuroanatomical localization of AChE inhibition might be a limitation for understanding the mechanism of action of AChE-Is (Takamatsu et al., 2006).

Based on the results obtained from this research we can conclude that: i), Physostigmine and tacrine partially shared a nicotine-like effects, ii) physostigmine and tacrine have no effect on nicotine S/A, therefore the net effect of ACh in NA has not been clarified, iii) tacrine has no effect on nicotine S/A reinstatement and relapse, excluding that ACh can facilitate the learning of extinction behaviour and prevent to cue- or priming-induced relapse.

This data coming evidence on animal models can be integrated with clinical data into a more comprehensive picture for the potential use of AChE-Is in nicotine addiction. Few studies assessed the efficacy of this class of drugs in subjects with substance abuse and dependence problems. The rationale and experimental design for these studies was mainly based on the evidence that drug dependent subjects have significant cognitive deficits which may act as a determining factor in drug relapse (Block et al., 2002; Vocci et al., 2005). However, the results of these clinical studies have not always been consistent. For example, donepezil improved cognitive performance for up to three

months after discontinuation of methamphetamine in ex-users (Jovanovsky and Zakzanis, 2003). In contrast, a recent placebo-controlled study conducted in cocaine-dependent individuals did not show any significant change in cocaine use during an 8-week donepezil treatment (Winhusen et al., 2005). Note that in the latter study, subjects were currently taking cocaine, whereas subjects were drug abstinent in the former study with methamphetamine. In addition, rivastigmine did not change total choices for amphetamine and methamphetamine S/A in a study conducted in non-treatment seeking methamphetamine users; however, it significantly attenuated methamphetamine-induced self-reports of "anxiety" and "desire" (De La Garza et al., 2008). Thus, it appears that treatment with AChE-Is induced some beneficial effects on cognitive and affective dimensions, but not on addiction-related measures (i.e., cocaine use, total amphetamine and methamphetamine choices). Similarly, a randomized placebo-controlled study on prevention of relapse was conducted in recently detoxified alcoholics but it did not show a significant difference in cessation rates between placebo and a 24-week galantamine treatment group (Mann et al., 2006; Diehl et al., 2006). Interestingly, daily alcohol consumption and smoking behaviour (i.e. number of smoked cigarette per day and number of smoking days) was significantly reduced. This specific effect of galantamine on drug-taking behaviour, but not on prevention of relapse, suggest a possible therapeutic effect on psychological dimensions that control drug reinforcement but not drug-seeking relapse. To date, the clinical data supporting the therapeutic value of AChE-Is for the treatment of drug addiction are not consistent. It is possible that such inconsistency may depend on several factors including the different classes of drugs of abuse and types and severity of drug addiction symptoms, the

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different types and dimensions of clinical endpoints, and the different types and dosing regimen of AChE-Is tested.

Considering that clinical data coming from large clinical trials are fragmented and that pre-clinical data are inconsistent, the use of human laboratory analogues of smoking behaviour can provide an efficient, cost-effective mechanistic evaluation of a medical signal on smoking behaviour. There has been much discussion among the scientific community about the need to develop human laboratory models to provide a translation between pre-clinical studies and more costly clinical trials. A number of available human laboratory models have been designed to investigate the various aspects of smoking behaviour and nicotine-dependence phenomena including DD, nicotine reinforcement and tolerance and S/A behaviour (see Lerman et al., 2007 and McKee, 2009 for review).

In conclusion, testing potential medicinal effects of AChE-Is in animals models of nicotine addiction has some limitations but has the potential to indicate some predictive elements. The translation of this knowledge to the human laboratory models can clarify the real effect of AChE-Is in treating nicotine addiction.

5. References

Albuquerque, E.X. et al. (2009) Mammalian nicotinic acetylcholine receptors: from structure to function. *Physiol Rev* 89:73-120

Alcantara, A.A. et al. (2003) Localization of dopamine D2 receptors on cholinergic interneurons of the dorsal striatum and nucleus accumbens of the rat. *Brain Res* 986:22-29

Alderson, H.L. et al. (2006) Intravenous self-administration of nicotine is altered by lesions of the posterior, but not anterior, pedunculopontine tegmental nucleus. *Eur J Neurosci* 23:2169-75

Andreoli, M. et al. (2003) Selective antagonism at dopamine D3 receptors prevents nicotine-triggered relapse to nicotine seeking behaviour. *Neuropsychopharmacology* 28:1272-80

Ator, N.A. and Griffiths, R.R. (2003) Principle of drug abuse liability assessment in laboratory animals. *DAD* 70:S55-S72

Bechara, A. and van der Kooy, D. (1989) The tegmental pedunculopontine nucleus: a brain-stem output of the limbic system critical for the conditioned place preferences produced by morphine and amphetamine. *J Neurosci* 9:3400-09

Benowitz, N.L. (2010) Nicotine addiction. *N Engl J Med* 362:2295-03

Bhat, R.V. et al. (1990) Selective changes in sensitivity to cholinergic agonists and receptor changes elicited by continuous physostigmine infusion. *J Pharmacol Exp Ther* 255:187-96

Blaha, C.D. and Winn, P. (1993) Modulation of dopamine efflux in the striatum following cholinergic stimulation of the substantia nigra in intact and pedunculopontine tegmental nucleus-lesioned rats. *J Neurosci* 13:1035-44

Blaha, C.D. et al. (1996) Modulation of dopamine efflux in the nucleus accumbens after cholinergic stimulation of the ventral tegmental area in intact, pedunculopontine tegmental nucleus-lesioned, and laterodorsal tegmental nucleus-lesioned rats. *J Neurosci* 16:714-22

Block, R.I. et al. (2002) Chronic drug use and cognitive impairments. *Pharmacol Biochem Behav* 73:491-504

Breese, C.R. et al. (2000) Abnormal regulation of high affinity nicotinic receptors in subjects with schizophrenia. *Neuropsychopharmacology* 23:351-64

Buccafusco, J.J. (2004) Neuronal nicotinic receptor subtypes: defining therapeutic targets. *Mol Interv* 4:285-95

Calabresi, P. et al. (1989) Nicotinic excitation of rat ventral tegmental neurones in vitro studied by intracellular recording. *Br J Pharmacol* 98(1):135-40.

Cerbai, F. et al. (2007) N1phenethyl-norcymserine, a selective butyrylcholinesterase inhibitor, increases acetylcholine release in rat cerebral cortex: a comparison with donepezil and rivastigmine. *Eur J Pharmacol* 572:142-50

Chandler, C.J. and Stolerman, I.P. (1997) Discriminative stimulus properties of the nicotinic agonist cytisine. *Psychopharmacology* 129:257-64

Chiamulera, C. and Fumagalli, G. (2007) Nicotinic receptors and the treatment of attentional and cognitive deficits in neuropsychiatric disorders: focus on the α7 nicotinic acetylcholine receptor as a promising drug target for schizophrenia. *Cent Nerv Syst Agents Med Chem* 7:269-88

Chiamulera, C. et al. (1996) Nicotine reinstatement of nicotine self-administration after long-term extinction. *Psychopharmacology (Berl)* 127:102-7

Chouinard, S. et al. (2007) Rivastigmine treatment as an add-on to antipsychotics in patients with schizophrenia and cognitive deficits. *Curr Med Res Opin* 23:575-83

Clarke, P.B. and Pert, A. (1985) Autoradiographic evidence for nicotine receptors on nigrostriatal and mesolimbic dopaminergic neurons. *Brain Res* 348:355-58

Consolo, S. et al. (1999) Different sensitivity of in vivo acetylcholine transmission to D1 receptor stimulation in shell and core of nucleus accumbens. *Neuroscience* 89:1209-17

Corrigall, W.A. (1999) Nicotine self-administration in animals as a dependence model. *Nicotine Tob Res* 1:11-20

Corrigall, W.A. and Coen, K.M. (1989) Nicotine maintains robust self-administration in rats on a limited-access schedule. *Psychopharmacology* 99:473-78

Corrigall, W.A. et al. (1994) Self-administered nicotine activates the mesolimbic dopamine system through the ventral tegmental area. *Brain Res* 653:278-84

Corrigall, W.A. et al. (2002) Pharmacological manipulations of the pedunculopontine tegmental nucleus in the rat reduce self-administration of both nicotine and cocaine. *Psychopharmacology* 160:198-205

Crespo, J.A. et al. (2006) Activation of muscarinic and nicotinic acetylcholine receptors in the nucleus accumbens core is necessary for the acquisition of drug reinforcement. *J Neurosci* 26:6004-10

Cummings, J.L. (2003) Use of cholinesterase inhibitors in clinical practice: evidence-based recommendations. *Am J Geriatr Psychiatry* 11:131-45

Davis, K.L. and Powchick, P. (1995) Tacrine. *Lancet* 345:625-30

De La Garza, R. and Johanson, C.E. (1982) Effects of haloperidol and physostigmine on self-administration of local anesthetics. Pharmacol. Biochem. Behav. 17:1295-99

De La Garza, R. et al. (2008) The acetylcholinesterase inhibitor rivastigmine does not alter total choices for methamphetamine, but may reduce positive subjective effects, in a laboratory model of intravenous self-administration in human volunteers. *Pharmacol Biochem Behav* 89:200-08

Diehl, A. et al. (2006) Galantamine reduces smoking in alcohol-dependent patients: a randomized, placebo-controlled trial. *Int J Clin Pharmacol Ther* 44:614-22

disorder. *Schizophr Res* 48:1-5

Donny, E.C. et al. (1995) Nicotine self-administration in rats. *Psychopharmacology* 122:390-94

Dravolina, O.A. et al. (2007) mGlu1 receptor blockade attenuates cue- and nicotine-induced reinstatement of extinguished nicotine self-administration behaviour in rats. *Neuropharmacology* 52:263-69

Everitt, B.J. and Robbins, T.W. (2005) Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. *Nat Neurosci* 8:1481-89

Freedman, R. et al. (2000) The α7-nicotinic acetylcholine receptor and the pathology of hippocampal interneurons in schizophrenia. *J Chem Neuroanat* 2:299-306

Geerts, H. et al. (2005) Brain levels and acetylcholinesterase inhibition with galantamine and donepezil in rats, mice and rabbits. *Brain Res* 1033(2):186-93

Giuliano, C. et al. (2008) Increases in cholinergic neurotransmission measured by using choline-sensitive microelectrodes: enhanced detection by hydrolysis of acetylcholine on recording sites? *Neurochem Int* 52(7):1343-50

Goldberg, S.R. et al. (1981) Persistent behaviour at high rates maintained by intravenous self-administration of nicotine. *Science* 214:573-75

Grasing, K. et al. (2008) Dose-related effects of the acetylcholinesterase inhibitor tacrine on cocaine and food self-administration in rats. *Psychopharmacology* 196:133-142

Groenewegen, H.J. et al. (1996) The nucleus accumbens: gateway for limbic structures to reach the motor system? *Prog Brain* Res. 107:485-511

Gronier, B. et al. (2000) Activation of the mesocorticolimbic dopaminergic system by stimulation of muscarinic cholinergic receptors in the ventral tegmental area. *Psychopharmacology* 147:347-355

Hikida, T. et al. (2001) Increased sensitivity to cocaine by cholinergic cell ablation in nucleus accumbens. *PNAS* 98(23):13351-54

Hikida, T. et al. (2003) Acetylcholine enhancement in the nucleus accumbens prevents addictive behaviours of cocaine and morphine. *PNAS* 100(10):6169-73

Hiranita, T. et al. (2006) Suppression of methamphetamine-seeking behaviour by nicotinic agonist. *PNAS* 103(22):8523-8527

Hughes, J.R. et al. (2004) Shape of the relapse curve and long-term abstinence among untreated smokers. *Addiction* 99:29-38

Hurd, Y.L. et al. (1990) The influence of cocaine self-administration on in vivo dopamine and acetylcholine neurotransmission in rat caudate-putamen. *Neurosci Lett* 109:227-33

Ikemoto, S. and Wise, R.A. (2002) Rewarding effects of the cholinergic agents carbachol and neostigmine in the posterior ventral tegmental area. *J Neurosci* 22:9895-9904

Imperato, A. et al. (1993)[1] Effects of cocaine and amphetamine on acetylcholine release in the hippocampus and caudate nucleus. *Eur J Pharmacol* 238:377-81

Imperato, A. et al. (1993)[2] Stimulation of both dopamine D_1 and D_2 receptors facilitates in vivo acetylcholine release in the hippocampus. *Brain Res* 618:341-45

Jha, P. et al. (2006) Tobacco Addiction. In: Jaminson DT et al., editors. Disease Control Priorities in Developing countries. 2nd Edition. Washington (DC): World Bank; Chapter 46.

Jovanovski, D. and Zakzanis, K.K. (2003) Donepezil in a chronic drug user: a potential treatment? *Hum Psychopharmacology* 18:561-64

Kameda, G. et al. (2000) Influence of various drugs on the voluntary intake of nicotine by rats. *Neuropsychobiology* 41(4):205-9

Kaneko, S. et al. (2000) Synaptic integration mediated by striatal cholinergic interneurons in basal ganglia function. *Science* 289:633-37

Kirrane, R.M. et al. (2001) Physostigmine and cognition in schizotypal personality

Kish, S.J. et al. (1999) Brain choline acetyltransferase activity in chronic human users of cocaine, methamphetamine, and heroin. *Mol Psychiatry* 4:26-32

Kume, T. et al. (2005) Up-regulation of nicotinic acetylcholine receptors by central-type acetylcholinesterase inhibitors in rat cortical neurons. *Eur J Pharmacol* 527:77-85

Lança, A.J. et al. (2000) The pedunculopontine tegmental nucleus and the role of cholinergic neurons in nicotine self-administration in the rat: a correlative neuroanatomical and behavioral study. *Neuroscience* 96:735-42

Le Foll, B. and Goldberg, S.R. (2009) Effects of nicotine in experimental animals and humans: an update on addictive properties. *Handb Exp Pharmacol* 192:335-67

Lenzi, A. et al. (2003) Effects of rivastigmine on cognitive function and quality of life in patients with schizophrenia. *Clin Neuropharmacol* 26:317-21

Lerman, C. et al. (2007) Translational research in medication development for nicotine dependence. *Nat Rev Drug Discov* 6:746-62

LeSage, M.G. et al. (2004) Reinstatement of nicotine self-administration in rats by presentation of nicotine-paired stimuli, but not nicotine priming. *Pharmacol Biochem Behav* 79:507-13

LeSage, M.G. et al. (2009). Effects of nicotine receptor partial agonist varenicline and cytisine on the discriminative stimulus effects of nicotine in rats. *Pharmacol Biochem Behav* 91:461-67

Levin, E.V. et al. (2006) Nicotinic effects on cognitive function: behavioural characterization, pharmacological specification and anatomic localization. *Psychopharmacology (Berl)* 184:523-39

Liu, W. (2000) Effects of cholinesterase inhibitors on a two-component chained schedule performance in rats. *Neurotox Teratol* 22:389-96

Liu, X. et al. (2006) Reinstatement of nicotine-seeking behaviour by drug-associated stimuli after extinction in rats. *Psychopharmacology (Berl)* 184:417-25

Liu, X. et al. (2007) Mecamylamine attenuates cue-induced reinstatement of nicotine-seeking behaviour in rats. *Neuropsychopharmacology* 32:710-18

Luttmann, E. et al. (2009) Structural model for the binding sites of allosterically potentiating ligands on nicotinic acetylcholine receptors. *Chem Med Chem* 4:1874-82

Lyvers, M. (2000) "Loss of control" in alcoholism and drug addiction: a neuroscientific interpretation. *Exp Clin Psychopharmacol* 8:225-49

MacEwan, G.W. et al. (2001) Donepezil in schizophrenia--is it helpful? An experimental design case study. *Acta Psychiatr Scand* 104:469-72

Mach, M. et al. (2004) Behavioural changes after acetylcholinesterase inhibition with physostigmine in mice. *Pharmacol Biochem Behav* 79:533-540

Maelicke, A. and Albuquerque, E.X. (2000) Allosteric modulation of nicotinic acetylcholine receptors as a treatment strategy for Alzheimer's disease. *Eur J Pharmacol* 393:165-70

Mann, K. et al. (2006) Galantamine: a cholinergic patch in the treatment of alcoholism: a randomized, placebo-controlled trial. *Psychopharmacology* 184:115-21

Mansvelder, H.D. et al. (2006) Nicotinic modulation of neuronal networks: from receptors to cognition. *Psychopharmacology (Berl)* 184:292-305

McKee, S. (2009) Developing human laboratory models of smoking lapse behaviour for medication screening. *Addict Biol* 14(1):99-107

Militante, J. et al (2008). Activation and block of the adult muscle-type nicotinic receptor by physostigmine: single-channel studies. *Mol Pharmacol* 74:764-76

Museo, E. and Wise, R.A. (1994) Place preference conditioning with ventral tegmental injections of cytisine. *Life Sci* 55:1179-86

Myhrer, T. et al. (2010) Behavioral side effects in rats treated with acetylcholinesterase inhibitors suggested used as prophylactics against nerve agents. *Pharmacol Biochem Behav* 95(3):338-43

Olmstead, M.C. and Franklin, K.B. (1993) Effects of pedunculopontine tegmental nucleus lesions on morphine-induced conditioned place preference and analgesia in the formalin test. *Neuroscience* 57:411-18

Pelosa, R. and Benowitz, N.L. (2011) Treatment of nicotine addiction: present therapeutic options and pipeline developments. *TIPS* Jan 20 [*Epub ahead of print*]

Pepeu, G. and Giovannini, M.G. (2004) Changes in acetylcholine extracellular levels during cognitive processes. *Learn Mem* 11:21-27

Pereira, E.F.R. et al. (1994) Physostigmine and galanthamine: probes for a novel binding site on the α4β2 subtype of neuronal nicotinic acetylcholine receptors stably expressed in fibroblast cells. *J Pharmacol Exp Ther* 270:768-78

Pontieri, F.E. et al. (1995) Intravenous cocaine, morphine, and amphetamine preferentially increase extracellular dopamine in the "shell" as compared with the "core" of the rat nucleus accumbens. *PNAS USA* 92:12304-308

Pratt, J.A. et al. (1983) Discriminative stimulus properties of nicotine: further evidence for mediation at a cholinergic receptor. *Psychopharmacology* 81:54-60

Pratt, W.E. and Kelley, A.E. (2004) Nucleus accumbens acetylcholine regulates appetitive learning and motivation for food via activation of muscarinic receptors. *Behav Neurosci* 118:730-39

Rada, P.V. et al. (1996) Morphine and naloxone, i.p. or locally, affect extracellular acetylcholine in the accumbens and prefrontal cortex. *Pharmacol Biochem Behav* 53:809-16

Redgrave, P. and Horrell, R.I. (1976) Potentiation of central reward by localised perfusion of acetylcholine and 5-hydroxytryptamine. *Nature* 262:305-7

Reid, R. and Sabbagh, M. (2008) Effects of cholinesterase inhibitors on rat nicotinic receptor levels in vivo and in vitro. *J Neural Transm* 115:1437-44

Ritchie, C.W. et al. (2004) Metaanalysis of randomized trials of the efficacy and safety of donepezil, galantamine, and rivastigmine for the treatment of Alzheimer disease. *Am J Geriatr Psychiatry* 12:358-69

Rogers, R.D. and Robbins, T.W. (2001) Investigating the neurocognitive deficits associated with chronic drug misuse. *Curr Opin Neurobiol* 11:250-57

Rollema, H. et al. (2007) Pharmacological profile of the α4β2 nicotinic acetylcholine receptor partial agonist varenicline, an effective smoking cessation aid. *Neuropharmacology* 52:985-94

Samochocki, M. et al. (2003) Galantamine is an allosterically potentiating ligand of neuronal nicotinic but not of muscarinic acetylcholine receptors. *J Pharmacol Exp Ther* 305:1024-36

Sanchis-Segura, C. and Spanagel, R. (2006) Behavioural assessment of drug reinforcement and addictive features in rodents: an overview. *Addiction Biol* 11:2-38

Sannerud, C.A. et al. (1994) The effects of sertraline on nicotine self-administration and food-maintained responding in squirrel monkeys. *Eur J Pharmacol* 217:461-69

Schrattenholz, A. et al. (1993) Biochemical characterization of a novel channel-activating site on nicotinic acetylcholine receptors. *J Recept Res* 13:393-12

Schroeder, J.P. and Packard, M.G. (2004) Facilitation of memory for extinction of drug-induced conditioned reward: role of amygdala and acetylcholine. *Learn Mem* 11:641-47

Schuster, C.R. and Johanson, C.E. (1988) Relationship between the discriminative stimulus properties and subjective effects of drugs. *Psychopharmacol Ser* 4:161-75

See, R.E. (2005) Neural substrates of cocaine-cue associations that trigger relapse. *Eur J Pharmacol* 526:140-46

Shoaib, M. et al. (1997) Nicotine self-administration in rats: strain and nicotine pre-exposure effects on acquisition. *Psychopharmacology* 129:35-43

Siegal, D. et al. (2004) Brain vesicular acetylcholine transporter in human users of drugs of abuse. *Synapse* 52(4):223-32

Smith, J.E. et al. (2004) Acetylcholine turnover rates in rat brain regions during cocaine self-administration. *J Neurochem* 88:502-12

Smith, J.W. and Stolerman, I.P. (2009) Recognising nicotine: the neurobiological basis of nicotine discrimination. In: Nicotine Psychopharmacology; Handbook of Experimental Pharmacology 192. J.E. Henningfield et al. editors. Springer-Verlag Berlin Heidelberg; pp.295-333

Smith, J.W. et al. (2007). Ligands selective for α4β2 but not α3β4 or α7 nicotinic receptors generalize to the nicotine discriminative stimulus in the rat. *Psychopharmacology* 190:157-70

Sofuoglu, M. and Mooney, M. (2009) Cholinergic functioning in stimulant addiction. *CNS Drugs* 23(11):939-52

Solinas, M. et al. (2006). Using drug-discrimination techniques to study the abuse-related effects of psychoactive drugs in rats. *Nat Protoc* 1:1194-1206

Storch, A. et al. (1995) Physostigmine, galanthamine and codeine act as 'noncompetitive nicotinic receptor agonists' on clonal rat pheochromocytoma cells. *Eur J Pharmacol* 290:207-19

Svensson, A.L. et al. (1996) Biphasic effect of tacrine on acetylcholine release in rat brain via M1 and M2 receptors. *Brain Res* 726:207-12

Svobodova, L. et al. (2005) Allosteric modulation of the nicotinic acetylcholine receptor by physostigmine. *Ann NY Acad Sci* 1048:355-58

Sweeney, J. et al. (1990) Effects of different doses of galanthamine, a long-acting acetylcholinesterase inhibitor, on memory in mice. *Psychopharmacology (Berl)* 102:191-200

Sziráki, I. et al. (1998) Receptor systems participating in nicotine-specific effects. *Neurochem Int* 33:445-57

Takada-Takatori, Y. et al. (2008) Mechanisms of [α]7-nicotinic receptor up-regulation and sensitization to donepezil induced by chronic donepezil treatment. *Eur J Pharmacol* 590:150-56

Takamatsu, Y. et al. (2006) Differential effects of donepezil on methamphetamine and cocaine dependencies. *Ann N Y Acad Sci* 1074:418-26

Tessari, M. et al.(1995) Nicotine reinforcement in rats with histories of cocaine self-administration. *Psychopharmacology (Berl)* 121(2):282-83

Texido, L. et al. (2005) Effects of galantamine on the human α7 neuronal acetylcholine receptor, the Torpedo nicotinic acetylcholine receptor and spontaneous cholinergic synaptic activity. *Br J Pharmacol* 145:672-78

Villarroya, M. et al. (2004) New classes of AChE inhibitors with additional pharmacological effects of interest for the treatment of Alzheimer's disease. *Curr Pharm Des* 10:3177-84

Vocci, F.J. et al. (2005) Medication Development for Addictive Disorders: The State of the Science. *Am J Psychiatry* 162:1432-40

Wiley, J.L. et al. (1996) Discriminative stimulus properties of nicotine: approaches to evaluating potential nicotinic receptor agonists and antagonists. *Drug Dev Res* 38:222-30

Williams, M.J. and Adinoff, B. (2008) The role of acetylcholine in cocaine addiction. *Neuropsychopharmacology* 33:1779-97

Wilson, M.C. and Schuster, C.R. (1973) Cholinergic influence on intravenous cocaine self-administration by rhesus monkeys. *Pharmacol Biochem Behav* 1:643-49

Winhusen, T.M. et al. (2005) A placebo-controlled screening trial of tiagabine, sertraline and donepezil as cocaine dependence treatments. *Addiction* 100:68-77

Woolf, N.J. (1991) Cholinergic systems in mammalian brain and spinal cord. *Prog Neurobiol* 37:475-524

Yamamoto, T. Et al. (1993) Discriminative stimulus properties of NIK-247 and tetrahydroaminoacridine, centrally active cholinesterase inhibitors, in rats. *Pharmacol Biochem Behav* 44:769-75

Yeomans, J.S. and Baptista, M. (1997) Both nicotinic and muscarinic receptors in ventral tegmental area contribute to brain-stimulation reward. *Pharmacol Biochem Behav* 57:915-21

Yeomans, J.S. et al. (1985) Cholinergic involvement in lateral hypothalamic rewarding brain stimulation. *Brain Res* 329:19-26

Yoshida S, Suzuki N (1993). Antiamnestic and cholinomimetic side-effects of the cholinesterase inhibitors, physostigmine, tacrine and NIK-247 in rats. *Eur J Pharmacol* 250:117-24

Zhou, W. et al. (2007) Role of acetylcholine transmission in nucleus accumbens and ventral tegmental area in heroin-seeking induced by conditioned cues. *Neuroscience* 144:1209-18

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