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WO03/062224 is an *in vivo* selective agonist at nicotinic β4 receptors

Clare E. Greenhalgh [a], Janice W. Smith [b], Peter G. Clifton [a,*]

[a] *Department of Psychology, University of Sussex, Brighton, BN1 9QG, UK*
[b] *Eli Lilly & Co. Ltd., Lilly Research Centre, Sunninghill Road, Windlesham, Surrey, GU20 6PH, UK*

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ABSTRACT

Pharmacological agents that increase cholinergic transmission have considerable use in cognitive disorders and evidence from both human and animal studies suggests that nicotinic acetylcholine receptors (nAChRs) represent an attractive target for treating certain neurological disorders. This investigation aimed to provide an *in vivo* verification of the *in vitro* data on WO03/062224, an agonist selective at β4 subunit-containing nicotinic receptors. The effects of WO03/062224 were tested on wildtype and β4 nAChR null mice on two behavioural paradigms; locomotor behaviour and instrumental responding for food on a second order schedule. Separate groups of wildtype and β4 nAChR subunit knockout mice were tested in each paradigm with instrumental responding and forward locomotion being measured. WO03/062224 had a greater effect in the wildtype mice than the β4 knockout mice in both locomotor activity (unconditioned behaviour) and instrumental responding (conditioned behaviour). In wildtype mice WO03/062224 caused a significant initial depression in locomotor activity followed by a significant increase in activity. The β4 knockout mice displayed no significant drug-induced alterations in locomotor activity at any time point. In wildtype mice WO03/062224 caused a significant depression in instrumental responding throughout the session at both 3 mg/kg and 10 mg/kg. The β4 knockout mice only displayed a reduction in initial responding at 10 mg/kg. The present study demonstrated that the effects of WO03/062224 at 3 mg/kg on locomotor activity and instrumental responding are likely occurring through a β4 nicotinic mechanism. This investigation has shown that at an appropriate dose WO03/062224 is a suitable *in vivo* probe for the contribution of β4-containing nAChRs to behaviour and suggests that their involvement is greater than previously recognised.

1. Introduction

Neuronal nicotinic acetylcholine receptors (nAChR) are composed of five subunits arranged in a variety of either homomeric or heteromeric complexes. 11 different subunits (α2–α10 and β2–β4) have been identified (Cooper et al., 1991; Corringer et al., 2000; Le Novere et al., 2002). The predominant nAChR complexes in the CNS are α4β2, α4α5β2 and α7 (Colquhoun and Patrick, 1997; Gotti et al., 1997; McGehee and Role, 1995). The α2–α7 and β2–β4 subtypes have been cloned in many species, including rat, chick and human, but the α8 subunit has only been found in the chick and the α9 subunit only in the rat (Elgoyhen et al., 1994; Schoepfer et al., 1990). The pharmacological response to nicotinic drugs strictly depends on the composition of subtypes in the receptor (Papke et al., 1993).

In rats, the β4 subunit is predominantly expressed in the medial habenular with significant levels also detected in the cortex, olfactory regions, hippocampus, hypothalamus, locus coeruleus, pontine nuclei and cerebellum (Dineley-Miller and Patrick, 1992; Xu et al., 1997). In the mouse, expression of the β4 subunit is more restricted than the rat, with significant levels detected only in the olfactory bulb, medial habenula, pineal gland, interpeduncular nucleus, and inferior colliculus (Salas et al., 2003; Xu et al., 1999a; Xu et al., 1999b). α3β4 receptors appear to mediate nicotine-elicited noradrenalin release (Fu et al., 1999; Luo et al., 1998). Possibly due to its restricted expression pattern and the lack of very specific pharmacological agents, the β4 subunit has not been as extensively studied as the more abundant subunits, such as α4, α7 and β2.

As yet there are very few *in vivo* studies investigating the effects of compounds selective for the β4 nAChR subunit *in vitro*. Grottick et al. (2001) investigated locomotor activity after administration of SIB-1553A, a novel ligand with agonist selectivity at nAChRs containing the β4 subunit (Bontempi et al., 2001; Rao et al., 2003). SIB-1553A induced a locomotor stimulation similar to that observed after nicotine treatment (Grottick et al., 2001). However this activity effect was insensitive to antagonism by dihydro-β-erythroidine (DHβE) and mecamylamine, suggesting a non-nicotinic action. Since the receptor binding data relating to SIB-1553A was performed *in vitro,* the locomotor effects of SIB-1553A provide a good example of the need for *in vivo* studies to confirm *in vitro* binding data.

Central cholinergic systems are involved in attention and memory processes (Bartus et al., 1982; Bierer et al., 1995; Everitt and Robbins, 1997). Pharmacological agents that increase cholinergic transmission

* Corresponding author.
E-mail address: pete@sussex.ac.uk (P.G. Clifton).

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may enhance cognition and are currently one of the main treatments for the cognitive dysfunction in Alzheimer's disease (Farlow, 2002). Evidence from both human and animal studies suggests that nAChRs represent an attractive target for treating cognitive dysfunction (Graham et al., 2002; Gray et al., 1996; Levin and Simon, 1998). However, the clinical utility of nicotine is limited by its side effect profile, presumably related to indiscriminate activation of nAChRs. In this regard, subtype-selective nAChR ligands hold considerable promise and are likely to be of use in cognitive dysfunction.

In the present investigation we provide an *in vivo* verification of the *in vitro* data on WO03/062224 (Smith et al., 2007), an agonist selective at β4-containing nAChRs. *In vitro* data from a Scintillation Proximity Assay using cloned human nicotinic receptors expressed in HEK-293 cells shows that WO03/062224 has high affinity (K_i=1.5 nM) for α3β4 nAChRs but a much lower affinity (K_i=413.4 nM) for α4β2 nAChRs in the same system (Smith et al., 2007). It is completely inactive in a functional assay using cells expressing α7-containing nAChRs (Smith et al., 2007). In the present study, the effects of WO03/062224 are tested on β4 nAChR null mice on two behavioural paradigms; locomotor behaviour and instrumental responding for food on a second order schedule.

2. Materials and methods

2.1. Animals

The instrumental responding experiment (n=12) and the locomotor experiment (n=9) used separate groups of wildtype and β4 nAChR subunit knockout mice. The β4 mutant mice were initially created as described by Xu et al. (1997) and maintained in a mixed 129/SvEv and C57BL/6J background. The β4 knockout mice obtained from Harlan UK for the present experiments were bred on a C57BL/6J background.

Animals used in the instrumental responding experiment were individually housed and were maintained at 85% of their free-feeding weight. They were given a weighed quantity (2–3 g) of standard laboratory chow at 1600–1700 h in the home cages, adjusted in relation to expected body weight gain. Water was available ad libitum. Animals used in the locomotor experiment were housed in groups of three, and had free access to food and water. All animals were housed in a holding room with a 12-h light/dark cycle (light off: 1700 h), maintained at 21–22 °C and 40–60% relative humidity. Solid bottomed cages were used with paper bedding and a cardboard tube in addition to wood chip bedding. Testing took place between 0900 and 1600 h. All procedures in this paper were conducted in accordance with the requirements of the UK Animals (Scientific Procedures) Act 1986.

2.2. Drugs

WO03/062224 (1-methyl-4(2-chloro-4-hydroxyphenylthio)-piperidine) was synthesised by Discovery Chemistry Research, Lilly Research Laboratories and was administered at 3 mg/kg and 10 mg/kg. Injections were administered subcutaneously in a volume of 10 ml/kg. WO03/062224 was dissolved in 0.9% NaCL NaCl and the pH was adjusted with 1 M NaOH. The drug solutions were freshly prepared for each test session. All doses quoted are those of the free base. WO03/062224 was administered 30 min prior to operant testing but immediately before the animals were placed in the locomotor apparatus.

2.3. Instrumental responding apparatus and procedure

The eight operant cages used were standard two-lever operant cages (Medical Associates, Vermont, USA), housed in light resistant chambers fitted with a fan for ventilation and to mask any background noise. Pellets (20 mg, Noyes Formula A/I) were delivered via activation of a pellet dispenser outside the chamber into a recessed magazine. Each chamber was fitted with a houselight located in the centre of the roof and a centre cue light located 6 cm above the magazine entry. Two, 1.5 cm-wide, retractable levers were located on either side of the magazine entry 11 cm apart and 2 cm from the grid floor. Experimental sessions were controlled and data recorded using programs written in house using MedPC IV© software.

2.3.1. Second-order operant schedule

Animals underwent a habituation period, a training period with several different schedules which gradually increased in complexity (see below), and finally the test sessions. Training sessions were initially 1 h long and were reduced in length to 30 min once animals were responding on the test session schedule. During the training sessions animals were deemed ready to move onto the subsequent schedule only when stable responding had been achieved for all subjects i.e. when there was no significant effect of day on responding over four days and where >90% instrumental responding was for the active lever relative to the inactive lever.

2.3.2. Habituation

Animals were given access to food pellets in their home cage to minimise food neophobia. The following day, animals were placed in the operant chambers where food pellets were freely delivered on a 120 s random time schedule for 1 h. Animals underwent the habituation period for 3 consecutive days. Levers were retracted during the habituation phase.

2.3.3. Training

Following habituation, animals were trained to lever press on a continuous reinforcement schedule fixed ratio 1 (FR1) for 1 h. Animals were placed in the operant boxes with both levers retracted and the houselight on, after 2 min both levers were inserted into the chamber, signalling the beginning of the session. A single press on the correct lever led to the illumination of a centrelight for 8 s and delivery of a single pellet after 4 s (reinforced lever presses). The light was illuminated prior to, during and subsequent to pellet delivery to enhance the association of the two. Lever presses on the reinforced lever recorded whilst the centrelight was illuminated, indicating that the FR requirement has been fulfilled, had no programmed consequences (non-reinforced lever presses). Lever presses on the incorrect lever also had no programmed consequences (inactive lever presses).

Once animals were making >100 reinforced lever presses on this schedule they were transferred onto an FR5 schedule. Responding under the FR5 schedule was reinforced with illumination of the centrelight and delivery of one pellet after five lever presses in a similar manner to that described for the FR1 schedule. Once animals were achieving stable responding on this schedule they were transferred to an FR5(2) schedule. Under the FR5(2) schedule rats were required to obtain two consecutive centrelight presentations to receive delivery of two pellets, i.e. ten presses in total. Again once stable responding was achieved animals were transferred to a more extended schedule, FR5(3) schedule. The subsequent training schedule remained in essence an FR5(3), however during the first 3 min of the session responding was reinforced with the illumination of the centrelight only and no pellets were delivered. Once 3 min had passed the schedule continued as usual with delivery of three pellets after every three centrelight presentations. The animals were then moved to the final training schedule, an FI5, FR5(5). In this schedule animals were required to obtain five centrelight illuminations at which point five pellets were delivered. There was a fixed interval of 5 min at the start of the session reinforced with illumination of the centrelight only. Once stable responding was achieved the length of the operant schedule was reduced by 10 min every 2 days until the FI5, FR5(5) schedule was 30 min in length. Test sessions were identical to the FI5, FR5(5) schedule used during training.

2.4. Locomotor experiment apparatus and procedure

The locomotor equipment consisted of 9 locomotor arenas placed on opaque perspex platform (125×92 cm) elevated 127 cm from the floor. The locomotor arenas were 24.5 cm in diameter with a 6.5 cm corridor. The equipment was illuminated from above, and locomotor activity was recorded from below using a digital camera. Locomotor activity was recorded and tracked using a PC running MATLAB based software (written in house by John Anderson). For the analysis of the locomotor activity the arena was separated into eight equal sized areas at 45° separation. Forward locomotor activity was assessed by counting the number of movements across a 45° separation of the arena that occurred following three consecutive movements across separations in that direction. Although technically different, this system gives data which is very similar to that reported in previous studies from our laboratory (Dalton et al., 2004).

2.5. Experimental design

In both experiments, animals were weighed, dosed and remained in their home cages for 30 min before being placed in the test equipment. The instrumental responding test sessions lasted for 30 min and the locomotor activity test sessions lasted for 90 min. The instrumental responding experiments used a mixed design with dose of WO03/062224 as a repeated measure and genotype as a between subjects measure (N=12 for each group). Doses were counterbalanced using an ascending Latin square design. The locomotor experiment used a between subjects design in which animals of either genotype received a single treatment of either vehicle or WO03/062224 (N=9 in each group) immediately before the test session. Animals were randomly assigned to the test groups.

2.6. Data analysis

In the instrumental second order task, the number of lever presses on the active lever were was analysed and presented as total lever presses, number of lever presses in the initial 5 min and the average number of lever presses per 5 min for the remaining 25 min. The instrumental data were initially analysed by a mixed design ANOVA (factors: dose, genotype) using Genstat. Significant overall effects were investigated using Dunnett's test.

Forward locomotor activity counts were analysed using the appropriate mixed design ANOVA (Genstat). Simple contrasts were used to compare the effects of drug and vehicle at a particular timepoint if the overall ANOVA achieved significance.

3. Results

3.1. Instrumental responding

There was no significant difference in the acquisition of the second order task in the wildtype and β4 knockout mice (Table 1). WO03/062224 caused a dose dependent depression in reinforced responding on a second order operant schedule in wildtype mice, with the highest dose completely abolishing all responding (Fig. 1a). The effects of WO03/062224 on reinforced responding were smaller in the β4 knockout mice (Fig. 1b), leading to significant interaction between drug dose and genotype for total responding, responding during the first 5 min and average responding over the remaining 25 min ($F(2,44)=12.025$, $p<0.001$; $F(2,44)=33.604$, $p<0.001$; $F(2,44)=27.785$, $p<0.001$, respectively).

Paired comparisons revealed that the depressant effect of WO03/062224 on responding in wildtype mice was significant at both the 3 mg/kg dose and 10 mg/kg throughout the test session. Total responding was reduced by WO03/062224 at both the 3 mg/kg dose and 10 mg/kg dose (p=0.035, $p<0.001$, respectively). There were also significant reductions in responding during the initial 5 min of the test session (p=0.050, $p<0.001$, respectively) and average responding over the remaining 25 min of the test session (p=0.042, $p<0.001$, respectively). By contrast, in β4 nAChR knockout mice paired comparisons revealed that only the highest dose of 10 mg/kg WO03/062224 decreased total responding and responding during the initial 5 min of the test session

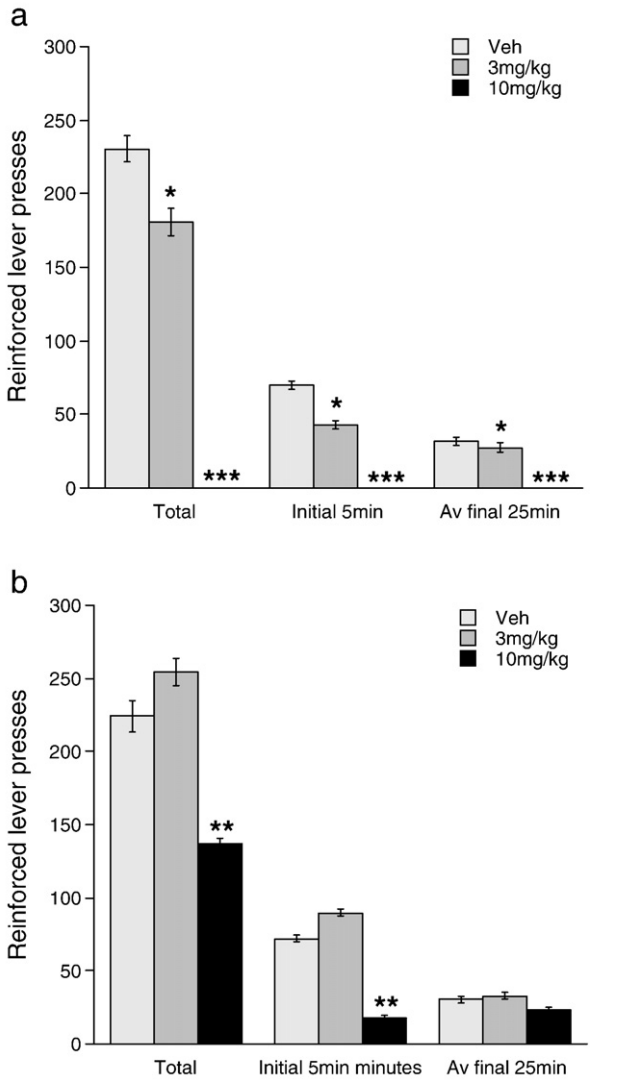

Fig. 1. a. Effects of WO03/062224 (0, 3, 10 mg/kg sc) on reinforced lever presses in a second order operant schedule on wildtype. The figure represents total lever presses for the 30 min test session, lever presses during the initial 5 min of the test session, and average lever presses per 5 min for the remaining 25 min. b. Effects of WO03/062224 (0, 3, 10 mg/kg sc) on reinforced lever presses in a second order operant schedule on β4 nAChR subunit null mice. The figure represents total lever presses for the 30 min test session, lever presses during the initial 5 min of the test session, and average lever presses per 5 min for the remaining 25 min. Data are expressed as means±SEM. ***$p<0.001$, **$p<0.01$, *$p<0.05$, denote a significant drug effect compared to relevant controls.

Table 1
Acquisition of instrumental responding in wildtype and β4 knockout mice

	Training day 1 (presses/session)		Training day 3 (presses/session)		Training day 5 (presses/session)		Training day 7 (presses/session)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Wildtype	87	14	97	12	216	24	258	27
β4 knockout	99	16	103	23	191	28	237	21

The number of reinforced lever presses made during the 1st, 3rd, 4th and 7th training days for the wildtype and β4 knockout mice. Data shown represent the mean and standard error of the mean (SEM) of the wildtype and β4 knockout groups.

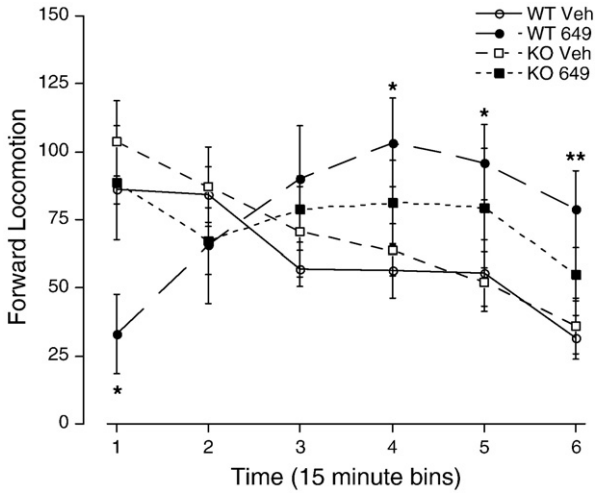

Fig. 2. The effects of acute WO03/062224 (3 mg/kg sc) on spontaneous locomotor activity in wildtype and β4 nAChR knockout mice. Data are expressed as means ± SEM. $^{**}p<0.01$, $^{*}p<0.05$, compared to vehicle. (* denotes a significant drug effect).

($p=0.005$, $p=0.002$, respectively). The lower dose of 3 mg/kg WO03/062224 had no effect on responding at either timepoint in the β4 nAChR knockout mice. WO3/062224 also significantly decreased total incorrect responses at the 10 mg/kg dose in both wildtypes and β4 knockouts ($p=0.001$ and $p=0.004$, respectively).

3.2. Locomotor activity

An initial analysis revealed no effect of WO03/062224 on total forward locomotion over the 90 min test session. However further analysis of changes in locomotor activity over time revealed a significant interaction between session time, drug dose and genotype ($F(5,90)=2.74$, $p=0.025$). When the analysis was restricted to each genotype in turn the interaction was highly significant in the wildtype mice ($p<0.001$). Paired comparisons at each timepoint (Fig. 2) revealed that the wildtype mice treated with WO03/062224 were significantly less active in the initial 15 min than the wildtype vehicle group ($p=0.018$), but that their locomotor activity increased above vehicle level towards the end of the session at 60, 75 and 90 min ($p=0.011$, 0.017, 0.007, respectively). The interaction term was also significant in the β4 nAChR knockout mice ($p<0.03$) and the pattern of change with time was similar in form, but of smaller magnitude to that in the wildtype mice at later time points (Fig. 2). However none of the paired comparisons at individual timepoints reached significance in these animals.

4. Discussion

WO03/062224 had a greater effect in the wildtype mice than the β4 knockout mice in assays of both locomotor activity (unconditioned behaviour) and instrumental responding (conditioned behaviour). Administration of WO03/062224 to wildtype and β4 knockout mice caused no significant effect on total locomotor activity over the 90 min test session, but when split into 15 min time bins the data reveal biphasic effects on the wildtype mice. In these animals, WO03/062224 caused a significant initial depression in locomotor activity followed by a significant increase in activity. In contrast, the β4 knockout mice displayed no significant alterations in locomotor activity at any time point, although the marginally significant time×drug interaction in this genotype was consistent with a similar but reduced effect to that observed in the WT mice at later time points. WO03/062224 caused a significant depression in instrumental responding at both 3 mg/kg and 10 mg/kg through out the session in wildtype mice, but the β4 knockout mice only displayed a reduction in responding at 10 mg/kg during the initial 5 min of the session. These findings suggest that at 3 mg/kg, WO03/062224 is a selective agonist at β4-containing nAChRs. Since WO03/062224 completely abolished instrumental responding at 10 mg/kg in the wildtype mice, only the 3 mg/kg was used in the locomotor activity assay.

There are very few putative selective β4 agonists, and Grottick et al. (2001) have demonstrated that the locomotor effects of SIB-1553A, the only compound with published *in vivo* data, depend on a non-nicotinic mechanism. Nevertheless, the pattern of activity observed here is similar to that observed in locomotor activity studies using nicotine, which also demonstrates an initial locomotor suppression followed by a locomotor activation (Clarke and Kumar, 1983). Picciotto et al. (2000) suggest that that the hyper- and hypo-locomotor effects of nicotine depend on different neuroanatomical substrates and nicotinic receptor subtypes. Specifically they suggest that hyperlocomotion may depend on effects at either the ventral tegmental area or nucleus accumbens and be mediated by α4β2- and α6β3-containing nAChRs. By contrast these authors suggest that α3β4- and α2β4-containing nAChRs in the medial habenula and interpeducular nucleus mediate the hypo-locomotor effect of nicotine. These structures have an inhibitory relationship to the mesolimic dopamine system. The first part of this hypothesis is consistent with the finding that mice lacking the β2 subunit do not show the enhancement of locomotor activity following chronic administration of nicotine that is observed in wildtype mice (King et al., 2004). However it is inconsistent with the finding that TC2559, an agonist selective for α4β2-containing nAChRs, produced a sustained hypolocomotion over a 90 min period (Bencherif et al., 2000). The second part of the hypothesis is consistent with the lack of nicotine-induced hypolocomotion in mice lacking the β4 subunit (Salas et al., 2004).

The present data suggests that activation of β4-containing nAChRs by WO03/062224 can both depress and stimulate locomotor activity. Gahring et al. (2004) have confirmed the localisation of β4 subunits within medial habenula, entopeduncular nucleus and also the subthalamic nucleus, all of which are important components of motor circuitry within the basal ganglia. In addition it has been shown that β4 subunit immunoreactivity is present within both the ventral tegmental area and the substantia nigra (Azam et al., 2002). In both the ventral tegmental area and substantia nigra β4 subunits were localised to non-dopaminergic cells. Taken together these data suggest multiple sites through which β4-containing nAChRs might modulate locomotor activity and the mesolimbic dopamine system in rodents. In addition it is possible that loss of β4-containing nAChRs may lead to indirect activation of behaviour through the stimulation of other nicotinic receptor subtypes. A parallel situation is well established for serotonergic modulation of locomotor activity. In this case a loss of 5-HT_{2C} receptor function allows the expression of a strong serotonergic facilitation of locomotor activity through activation of 5-HT_{2A} and 5-HT_{1B} receptors (Dalton et al., 2004).

An additional finding from the locomotor activity test was that there was no genotype difference between the two vehicle groups. This implies that the loss of the β4 subunit does not disrupt the basic processes of habituation and adaptation to the novel environment of the circular runway, although it is possible that compensatory changes during development could have replaced the normal role of β4-containing nAChRs in this regard. In addition, the rates of acquisition of the second order schedule in the two strains were similar, allowing the similar conclusion that the loss of the β4 subunit does not impair the acquisition of instrumental learning.

Acute administration of WO03/062224 at the pharmacologically selective dose of 3 mg/kg induced a substantial decrease in responding in the wildtype mice in both the initial appetitive and in the later mixed appetitive/consummatory phase of the second order schedule. The data suggest that WO03/062224 is selective for the β4 containing nAChRs at a dose of 3 mg/kg. The complete absence of responding observed in the wildtype mice at the highest dose and the significant genotype effect at this dose, suggests that the effects observed in the β4 knockout mice at the higher dose are also a result of non-specific

drug effects. However the strong suppression of responding induced by WO03/062224 at 3 mg/kg in WT mice is likely to be behaviourally specific. The drug had been administered 30 min before the test session. In the locomotor tests, where the drug was administered immediately before testing, the effects were waning after 30 min. This suggests that motor impairment is a relatively unlikely explanation for the suppression of responding in these operant tests. In rats, nicotine also suppresses responding in this schedule, but has very much more limited effects on a simpler FR5 schedule (Greenhalgh and Clifton, 2005). Interestingly, nicotine actually enhances responding in a conditioned reinforcement paradigm (Olausson et al., 2004).

In summary, the present study demonstrates that the effects of WO03/062224 at 3 mg/kg on locomotor activity and instrumental responding are likely occurring through a β4 nicotinic mechanism. Strong evidence is provided by the fact that the β4 knockout mice were relatively unaffected by administration of the drug, but to exclude the possibility of other neural systems being involved, selective antagonist studies should be conducted. In addition studies of the residual effects of WO03/062224 in β4 knockout mice with non-selective agonists would be informative. However this preliminary investigation has shown that at an appropriate dose WO03/062224 is a suitable *in vivo* probe for the involvement of β4-containing nAChRs to behaviour. It also suggests a more significant role of β4-containing nAChRs, relative to β2-containing nAChRs than has usually been accepted.

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