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Selective α7 nicotinic receptor activation by AZD0328 enhances cortical dopamine release and improves learning and attentional processes

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ABSTRACT

AZD0328, a novel spirofuropyridine neuronal nicotinic receptor partial agonist, was used to investigate the role of α7 neuronal nicotinic receptor (NNR) activation in the modulation of midbrain dopamine neuron function, cortical dopamine release and on two behavioral tasks known to be dependent on optimal levels of cortical dopamine. *In vivo* recordings from area 10 (ventral tegmental area) in rat brain showed an increased firing of putative dopamine neurons in response to low (0.00138 mg/kg) doses of AZD0328. Bursting patterns of dopamine neuron activity remained largely unchanged by application of AZD0328. *In vivo* microdialysis in awake rats showed an increase in extracellular prefrontal cortical dopamine in response to low doses of AZD0328. Compound-stimulated dopamine release showed an inverted dose effect relation that was maximal at the lowest dose tested (0.00178 mg/kg). Peak extracellular dopamine levels were reached 2 h after dosing with AZD0328. Acquisition of operant responding with delayed reinforcement in rats was dose dependently enhanced by AZD0328 with a plateau effect measured at 0.003 mg/kg. This effect was blocked by pre-treatment of animals with the selective α7 antagonist methyllycaconitine. AZD0328 improved novel object recognition in mice over a broad range of doses (0.00178–1.78 mg/kg) and the compound effect was found to be absent in homozygous α7 KO animals. Together, these data indicate that selective interaction with α7 NNRs by AZD0328 selectively enhances midbrain dopaminergic neuronal activity causing an enhancement of cortical dopamine levels; these neurochemical changes likely, underlie the positive behavioral responses observed in two different animal models. Our results suggest selective α7 NNR agonists may have significant therapeutic utility in neurologic and psychiatric indications where cognitive deficits and dopamine neuron dysfunction co-exist.

1. Introduction

Nicotine and selective NNR agonists activate central neuronal nicotinic receptors and have been shown to enhance memory and attention. Activation of NNRs has been shown to modulate neurotransmitter release and function throughout the central nervous system, specifically this cognition enhancement is thought to be, at least in part, mediated through modulation of dopaminergic and glutamatergic transmission [1]. NNR subtypes composed of pentameric arrangements of α2, α4, α6, β2, and β4 subunits as well as α7 subunits show distinct neuro-anatomical distributions in mammalian brain consistent with involvement in the modulation of excitatory and inhibitory synaptic networks. Particularly, these subtypes of NNRs are expressed in the mesostriatal and mesolimbic dopamine pathways and have been shown to modulate the firing patterns of these dopaminergic neurons [2–5]. Appropriate activation of midbrain dopaminergic neurons is crucial to many of the motivational aspects of goal directed behavior and decision-making. These aspects of behavior include the allocation of attentional resources and processes involved in reward evaluation, prediction, and outcome expectancy. In both human and animal models, the functional properties of the dopamine system influence an organism's ability to alter behavioral choices based upon recent experience and have been shown to correlate with improvements in learning and memory [3,21]. While dopaminergic dysfunction is implicated in the motivational and cognitive abnormalities associated with a number of neurological and psychiatric disorders, neuronal mechanisms regulating dopaminergic function *in vivo* are not well established.

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Despite an incomplete understanding of dopamine's regulation *in vivo*, converging molecular, pharmacological, and physiological evidence indicates a role for cholinergic inputs in the regulation of the midbrain dopaminergic system. Of particular importance in the control of dopamine release is a diverse set of nicotinic receptors located both directly on dopamine-producing cells within the ventral tegmental area (VTA) as well as on local inhibitory interneurons. Nicotine directly activates VTA neurons causing a number of time-dependent changes in dopamine release. Dopamine system activation is thought to account for the rewarding properties of nicotine and may also partially account for nicotine-associated improvements in attention and memory [3]. Nevertheless, the functional roles played by distinct subtypes of nicotinic receptors relative to the dopamine system remain to be fully elucidated.

Here we show that administration of low doses of a selective α7 NNR agonist, AZD0328, leads to a significant increase in the excitability of midbrain dopaminergic neurons with no independent change in the spike patterns produced by these neurons. Within the same dose range, AZD0328 led to a significant increase in cortical dopamine release in awake rodents and improved both conditioned response learning and memory retention in an object recognition task. The selective α7-receptor antagonist, methyllycaconitine (MLA), blocked the dopamine releasing and memory improving functions of AZD0328. Interestingly, during the training phase of the object recognition test, AZD0328 administration led to a robust increase in the targeted and selective exploration of objects without an overall increase in locomotor activity. To confirm the primary receptor requirement for AZD0328's mechanism of action, we tested the effect of AZD0328 on novel object recognition (NOR) in Chrna7 knockout mice and their non-transgenic littermates. This novel finding suggests a role for α7 NNRs in the selective allocation of attentional resources mediated, in part, by an enhancement in dopamine release. Together, these data provide mechanistic insight into how highly selective α7 agonists can be used to treat attentional and motivational abnormalities associated with a number of neurological and psychiatric conditions including: Alzheimer's disease, schizophrenia, bipolar disorder and attention deficit hyperactivity disorder (ADHD).

2. Materials and methods

2.1. Test compounds

AZD0328 ((2′*R*)-spiro-[1-azabicyclo[2.2.2]octane-3,2′(3′H)-furo[2,3-b]pyridine] D-tartrate) (structure shown in Fig. 1) was prepared by AstraZeneca Wilmington CNS Discovery Chemistry [6]. For *in vitro* studies, AZD0328 was dissolved in distilled water or DMSO before use. For *in vivo* studies, AZD0328 was dissolved in 0.9% saline or phosphate buffered saline before use. Chloral hydrate, ketamine, methyllycaconitine, xylazine and all other reagents were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Receptor binding

All receptor binding determinations were performed using standard techniques as described in Macor et al. [8] and Gordon et al. [31]. Binding affinity for AZD0328 was determined using a filter binding assay with membranes derived from a human α7 receptor expressing HEK293 cell line. Either ^{125}I-α-bungarotoxin or a proprietary tritiated α7 radioligand, AZ11637326, was used to label α7 receptor sites on the membranes. Briefly, membranes were homogenized in an assay/wash buffer containing 120 NaCl, 5 mM KCl 1 mM MgCl2, 2 mM CaCl2 and 50 mM TrisHCl pH 7.4 and kept on ice. GF/C filter plates (Packard GF/C Plates 500 μL well polypropylene u-bottom) pre-soaked in wash buffer with 1% BSA, 0.02% polyethyleneimine. Nicotine 100 μM was used to determine non-specific binding at each compound concentration. Test compound ± nicotine was added first followed by membranes to each well. Radioligand was added to each well and incubated for 2 h at room temperature with gentle shaking. Membranes were collected onto filter plates using a cell harvester and washed 5 times with wash buffer. Plates were dried, scintillation fluid added and counted on a topcount scintillation counter. Data were calculated as an IC_{50} and the Cheng–Prusoff conversion was used for estimation of the binding K_i [32].

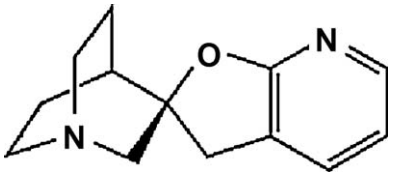

Fig. 1. Structure of AZD0328.

2.3. Xenopus oocyte recordings

Two-microelectrode voltage-clamp recordings were carried out using standard techniques similar to Macor et al. [8]. Briefly, *Xenopus* laevis frogs (Xenopus I, Kalamazoo, MI) were anesthetized using 0.15% tricaine. Oocytes were removed to OR2 solution: (in mM) 82 NaCl, 2.5 KCl, 5 HEPES, 1.5 NaH_2PO_4, 1 $MgCl_2$, 0.1 EDTA, pH 7.4. The oocytes were defolliculated by incubation in 25 mL OR2 containing 0.2% collagenase 1A (SIGMA) two times for 60 min on a platform vibrating at 1 Hz and stored in Leibovitz's L-15 medium. Oocytes were injected the following day. Leibovitz's L-15 medium contained 50 mg/mL gentamicin, 100 units/mL penicillin, and 100 mg/mL streptomycin. The external recording solution consisted of (in mM) 90 NaCl, 1 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 HEPES, pH 7.4. Two-electrode voltage-clamp recording was carried out using an OpusXpress™ (model 6000A, Molecular Devices - Axon Instruments, Union City, CA). Oocytes were impaled with two electrodes of 1–2 M(tip resistance when filled with 3 M KCl. Recordings were begun when membrane potential became stable at potentials negative to −40 mV (V_{hold} = −60 mV). Analysis was performed using PCLAMP 9 (Axon Instruments, Union City, CA). Signal traces were acquired at a rate of 0.2 kHz. Pre-application assays were run with an incubation time of 40 s. All agonist applications were 15 s at a flow rate of 2 mL/min. Calculation of current amplitude and curve fitting Current amplitude, measured from baseline to peak or by net charge analysis, was perfomed using Clampfit (MDC - Axon Instruments). EC_{50}'s, maximal effect, and Hill slopes were estimated by fitting the data to the logistic equation using GraphPad Prism (GraphPad Software Inc., San Diego, CA. The human nAChR subunit was cloned from a complementary DNA library. The cDNAs were subcloned into the pBSTA vectors. Messenger RNA was transcribed *in vitro* using the mMessage mMachine® T7 kit (Ambion, Austin, TX) and analyzed using a bioanalyzer (AgilentTechnologies, Palo Alto, CA).

2.4. Anesthetized VTA recordings

Male Sprague Dawley rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and surgically implanted with a femoral vein cannula for compound administration. Putative dopaminergic neurons within VTA (Lambda: 2.0–2.2 mm anterior, 1.6–1.8 mm lateral, 12° angle toward the midline, 6.5–8.0 ventral from the cortical surface) were targeted using a combination of spike shape and spontaneous firing rate. Following a 10-min baseline recording period, rats were administered a single dose (0.00138 mg/kg (3.72 nmol/kg), iv) of AZD0328, and neural activity was monitored for a minimum of 20-min post injection.

Individual neurons identified offline using M-clust software (AD Redish) running in MATLAB environment based on a combination of spike features including: spike height, spike width, energy, wave FFT, and the first three principle components derived from all recorded waveforms within a session. To be included in the analysis, putative VTA neurons had to produce tri-phasic action potentials with an average duration >2.0 ms and a baseline firing rate <15 Hz. In addition, the recording had to be maintained at a >3:1 signal to noise ratio for a minimum of 30 min.

2.4.1. Firing rate analysis

Sorted spikes were binned into 3-min bins and each bin was normalized by the average of the last two bins of the pre-injection period. Based on this time course, pre-injection and post-injection epochs were identified for subsequent statistical analysis using non-parametric statistics.

Spike Train Variability Measurements: $CV2 = 2^{*}| \Delta t_i + 1 - \Delta t_i |/(\Delta t_i + 1 + \Delta t_i)$ where Δt_i and $\Delta t_i + 1$ are adjacent interspike intervals (isi).

2.4.2. Surprise method of burst detection

As implemented in *NeuroExplorer* and applied here, the surprise algorithm [6], detected putative burst events by first locating two consecutive ISIs (i.e. 3 spikes arbitrarily designated as minimum # in a burst) that were less than half the mean ISI sampled for the designated treatment period. It then calculated the probability of finding these ISIs within a Poisson distribution generated around that measured mean firing rate. The negative natural logarithm of this calculated probability was taken as the observed surprise value. Adjacent ISIs were then added until a continuous grouping of ISIs with a maximal surprise value was reached—this grouping was then identified as a burst if the surprise value ≥2. Following the detection of all burst events within a spike train by this procedure, the fraction of spikes within bursts was determined by dividing the number of spikes within bursts by the total number of spikes within the treatment epoch.

2.5. In vivo microdialysis and HPLC

2.5.1. Animals

Experimentally naive male Sprague Dawley rats (90–120 days old) were housed in individual cages in a temperature- and humidity-controlled, AAALAC-accredited animal care facility on reversed 12-h light, 12-h dark cycle (lights on at 19:00 h) until the start of the experimental procedures. The animals had free access to water and food throughout the experiment. All procedures were approved by the AstraZeneca animal care and use committee and were carried out in accordance with the NIH "Principles of Laboratory Animal Care" (NIH publication No. 85-23).

2.5.2. Surgery

Under Ketamine (60 mg/kg) and Xylazine (8 mg/kg) anesthesia, animals were implanted with a guide cannula with a dummy insert (CMA/12) aimed into PFC using the following coordinates: AP, +4.0 mm; L, ±1.2 mm; V, 1.0 mm. The guide cannulas were anchored to the skull with screws and dental cement. Animals were allowed to recover from surgery for 6 days. Microdialysis probes (CMA/12, 4 mm membrane length) were implanted in the brain 18 h before each experiment and were perfused with artificial CSF (aCSF, CMA Microdialysis AB) at a flow rate of 1.1 mL/min. In each experiment three 20-min samples were collected to define the baseline followed by dosing with vehicle or compounds. After dosing, samples were collected every 20 min over the next 4 h. Concentrations of dopamine in 3 samples collected before administration of compounds/vehicle were averaged and defined as baseline (100%). Concentrations of neurotransmitters in the subsequent microdialysates were then expressed as percentage of baseline levels.

During the microdialysis procedure, animals had free access to food and tap water; all studies were performed during the light cycle.

2.5.3. HPLC analysis

The high performance liquid chromatography (HPLC) system consisted of an EICOM HTEC–500 stand-alone HPLC-ECD system, Eicom PP-ODS (4.6 mm ID × 30 mm) column, Eicom WE-3G working electrode, and PC-based integration system (all from EICOM Corp., Japan). The mobile phase was: 0.1 M sodium phosphate buffer (pH 6.0, 1% Methanol, 500 mg/L SDS, and 50 mg/L EDTA). Potential was set at +0.4 V and flow rate was maintained at 500 μL/min at 25 °C. Data were collected, using a PC-based acquisition/analysis system, integrated, and transferred into spreadsheet and graphic software for further analysis. Microdialysates from each animal were collected directly into the online autoinjector and then injected on HPLC for the analysis. Analysis time for each sample was ~5 min.

2.5.4. Data analyses

The mean and the standard error of the mean (SEM) were used as measures of central tendency and variation in the experiment. Analysis of variance was made by 1-way ANOVA followed by Dunnett's post hoc test. Statistical analyses for microdialysis studies were performed using repeated measures MANOVA followed by Dunnett's post hoc test.

2.6. Operant training

2.6.1. Animals

Groups of 15 male, Long-Evans rats were used for each treatment level. Animals were experimentally naïve at the beginning of the study, and were placed on a restricted diet 24 h prior to testing by removing all food from the cage, except for 20 g, which served to limit variability in the deprivational state of the animals entering the study.

2.6.2. Apparatus

Standard 2-lever operant chambers were used. 16 of the chambers were Med Associates model # 007 (Med Associates; Georgia, VT). 8 of the chambers were older Gerbrands chambers, which are no longer commercially available.

2.6.3. Procedure

Animals were placed into standard two-lever operant chambers for a single 14-h, overnight session. The training procedure used was a modification of one previously described [16]. The operant sessions were scheduled such that delivery of a food pellet was initially contingent upon a single lever press on either of the levers in the chamber (FR1, continuous reinforcement). Following delivery of each 20 reinforcers, the response requirement was increased in the following series: FR2 (achieved following 20 reinforcers), FR3 (40 reinforcers), FR5 (60 reinforcers), FR7 (80 reinforcers), and FR10 (100 reinforcers). Delivery of the food pellet was delayed by 30 s following completion of any FR requirement.

2.6.4. Compounds

In studies in which compound was administered, injections were given immediately prior to placement into the operant chambers. All compounds were dissolved in 0.9% saline, and administered s.c., with the exception of MLA, which was administered i.p. Finally, the effect of co-administration of the α7 antagonist, MLA (10 mg/kg) on the effect of 1 mg/kg AZD0328 was studied.

Additional studies were conducted to systematically assess the effects of the delay of the reinforcer on the operant leaning baseline, and to this end, separate groups of rats were submitted to the operant paradigm under conditions of reinforcer delay that varied from 0 s to 30 s. In another study, the effects of an otherwise active dose of AZD0328 (1 mg/kg) were studied in a group in which no delay of reinforcement was instituted. Finally, operant learning in the Long-Evans strain was compared to that in the Sprague Dawley strain following a 6 s delay of reinforcement.

2.6.5. Statistics

The primary dependent variable was the number of reinforcers earned during the 14-h session. Data are expressed as the percentage of each group's control values, and the means of those values plotted. 95% confidence limits were generated about control means, and plotted. ANOVA were performed on these parameters, and where the omnibus ANOVA were significant, Dunnett's post hoc comparisons were made.

2.7. Novel object recognition

We used a novel object recognition (NOR) test [30] developed for mice where test object pairings, identical objects during the acquisition trial and different during the recall trial were presented in opposing quadrants of a divided circular arena (see Fig. 5A). In this environment the mouse cannot see both objects at the same time and must continuously explore the entire arena in order to place itself in the context of the test objects. This test environment engenders a rapid forgetting curve between a familiar object acquisition trial and subsequent novel object testing relative to a typical open style object recognition arena. Typically C57/Bl6 mice will not discriminate a novel object from familiar after a 15-min interval following an acquisition trial.

For the object recognition task, male C57/Bl6 mice (22–25 g) were habituated to an empty divided, circular environment 24 h prior to testing for 60 min and again for 9 min immediately prior to the acquisition trial. During testing the mice were presented with 2 identical objects for 9 min(acquisition) followed by a 15-min rest period prior to exposure to a familiar/novel object pairing for another 9-min period. Mice were randomly assigned to receive vehicle or AZD0328 at 0.00178 mg/kg, 0.0891 mg/kg or 1.78 mg/kg (4.8 nmol/kg, 240 nmol/kg and 4800 nmol/kg) and were dosed 30 m prior to acquisition via the s.c. route at 10 mL/kg. A blinded observer scored object interaction from video recordings made of the test procedure. Groups were analyzed using ANOVA followed by Tukey's post hoc testing where appropriate to determine if the ratio of novel: total (novel + familiar) object interaction was different in drug versus vehicle treatment groups.

To measure the effect of AZD0328 on object interaction during the acquisition phase the above protocol was followed (up to and including the acquisition point) however the animals were observed using Noldus EthoVision XT (Noldus; Wageningen, Netherlands) during the acquisition test. We used this video measurement software to track locomotor activity, total time interacting with both identical objects and the frequency of individual interactions (having defined "off time" breaks in between). We used a feature of EthoVision XT (when the software was used), which allowed us to determine the relative proximity of the animal's nose to a donut shaped ring that extended from 0.75 to 1.5*x* radius of the object. Time when the nose alone was further away than 1.5*x* radius or over the center of the object was not scored as interaction. This automated measurement was the closest to that employed by a human observer counting interaction times.

Mice with a null mutation in the alpha7 neuronal nicotinic receptor gene (Chrna7) generated by deleting the last three exons (8–10) of the gene were used to assess whether the intact alpha7 gene is required for novel object recognition. These mice were produced and characterized by Orr-Urtreger et al. [12]. and obtained as heterozygotes from The Jackson Laboratory (Stock number: 003232) and back-crossed for two generations onto C57BL/6J mice. Homozygous null mutant (KO) mice and their non-transgenic littermates (WT) were produced by a random breeding of non-sibling heterozygous mice. They were genotyped using the PCR protocol recommended by The Jackson Laboratory. Male and female mice 2–3 months old were used.

3. Results

The *in vitro* binding characteristics of AZD0328 were determined on nicotinic receptor subtypes, and the structurally related $5HT_3$ receptor, using a wide variety of radioligands and membrane preparations (Table 1). AZD0328 exhibited high affinity for the native α7 NNR of rat hippocampal membranes using the classical α7 antagonist radioligand [^{125}I]α-bungarotoxin. AZD0328 exhibited similar high affinity for the human α7 NNR stably expressed in HEK-293 cell membranes as assessed with [^{125}I]α-BTX and the novel α7-selective agonist radioligand [^{3}H]AZ11637326 [9]. AZD0328 also exhibited high affinity for both the native $5HT_3R$ of rat small bowel and the human $5HT_{3A}R$ expressed in HEK-293 cell membranes. In contrast, AZD0328 exhibited only moderate affinity for α4β2 NNR of rat cortical membranes and very low affinity for the native "ganglionic" α3* nAChR of NGF-differentiated PC12 cell (rat) membranes and the embryonic muscle α1β1γδ nAChR of differentiated BC_3H1 (mouse) cell membranes. Functional activity of AZD0328 was determined using two-electrode voltage-clamp (TEVC) of xenopus oocytes expressing nAChRs and $5HT_3Rs$ from rodent and human (Table 2). Superfusion of AZD0328 elicited inward currents in voltage-clamped oocytes expressing the α7 subtype. Analyzing peak currents, maximal intrinsic activity relative to ACh (1 mM) was 89% and 58% for rat and human respectively. EC_{50} by peak current analysis was 1052 nM and 1041 nM for rat and human, respectively. Analyzing by net charge transfer, maximal intrinsic activity relative to ACh

Table 1
AZD0328 binding affinities at nicotinic and 5-HT_3 receptor subtypes.

Membrane source	Radioligand	Receptor	$K_i \pm$ SEM	Fold rat α7 K_i
Rat hippocampus	[^{125}I]α-BTX	r α7	4.7 ± 1.4 (3)	1
HEK-293 cells	[^{125}I]α-BTX	h α7	3.0 ± 0.2 (4)	0.6
HEK-293 cells	[^{3}H]AZ11637326	h α7	6.4 ± 0.6 (4)	1.4
Rat small bowel	[^{3}H](S)Zacopride	r $5HT_3$	25 ± 5 (3)	5.3
HEK-293 cells	[^{3}H]GR65630	h $5HT_{3A}$	12 ± 2 (7)	2.6
Rat cortex	[^{3}H](-)Nicotine	r α4β2	140 ± 20 (3)	30
PC12 cells	[^{3}H]Epibatidine	r α3*	2500 ± 700 (3)	530
BC_3H1 cells	[^{125}I]α-BTX	m α1β1γδ	20000 ± 1000 (3)	4300

K_i, dissociation constant of AZD0328 in radioligand competition assays; SEM, standard error of the mean of the number of determinations in parenthesis; Protocols and reference compound K_i values reported in Macor et al. [8] and Gordon et al. [31]. α3* = α3 subunit-containing NNR; α-BTX = α-bungarotoxin; h = human; r = rat; m = mouse.

Table 2
AZD0328 intrinsic activity and potency at nicotinic and 5-HT_3 receptor subtypes.

	EC_{50} (nM) ± SEM	% of Max ± SEM
Rat α7	150.0 ± 40	61. ± 2.8%
Human α7	338.0 ± 80	64.7 ± 3.1%
Rat α4β2	10,280 ± 4200	3.4 ± 0.6%
Human α4β2	>10,000	3.9 ± 0.7%
Human α3β4	Not active	Not active
Mouse 5HT3A	474 ± 173	12.4 ± 0.7%

All values are the mean and std. error of measurements from a minimum of six oocytes.

(1 mM) was 62% and 65% for rat and human, respectively. EC_{50} by net charge transfer was 152 nM and 338 nM for rat and human, respectively. AZD0328 also elicited inward currents in oocytes expressing human and mouse $5HT_{3A}$Rs. Maximal intrinsic activity relative to serotonin was 12% and 56% for mouse and human $5HT_{3A}$R, respectively. EC_{50} was 475 nM and 135 nM for mouse and human $5HT_{3A}$R, respectively. Intrinsic activity of AZD0328 was 3% and 6% relative to ACh at rat and human nAChR α4β2, respectively. AZD0328 had no activity on the nAChR α3β4 subtype (see Table 2). Together, these data indicate that AZD0328 is a potent NNR agonist with a nicotinic subtype affinity profile markedly distinct from nicotine.

3.1. Acute activation of α7 NNR by AZD0328 increases the excitability of VTA dopaminergic neurons

To investigate the effects of AZD0328 on midbrain dopamine neurons, electrophysiological data were obtained from 10 VTA neurons in 10 anesthetized rats. Fig. 2A shows the mean time course of normalized firing rates for these 10 putative dopaminergic neurons. Across the population, there was a ~50% increase in firing rate during the post-injection period. Changes in firing rate became evident within 5 min following injection but did not become significant until ~10–15 min post-injection (1-way ANOVA: $p < 0.01$, followed by paired comparison (Wilcoxon signed-rank test for individual time points: $p < 0.05$).

Based on this time course analysis, two periods were extracted for comparison: a baseline period encompassing the last five minutes of the pre-injection period, and a post-injection period extending from 15 min post-injection to 20 min post-injection. For

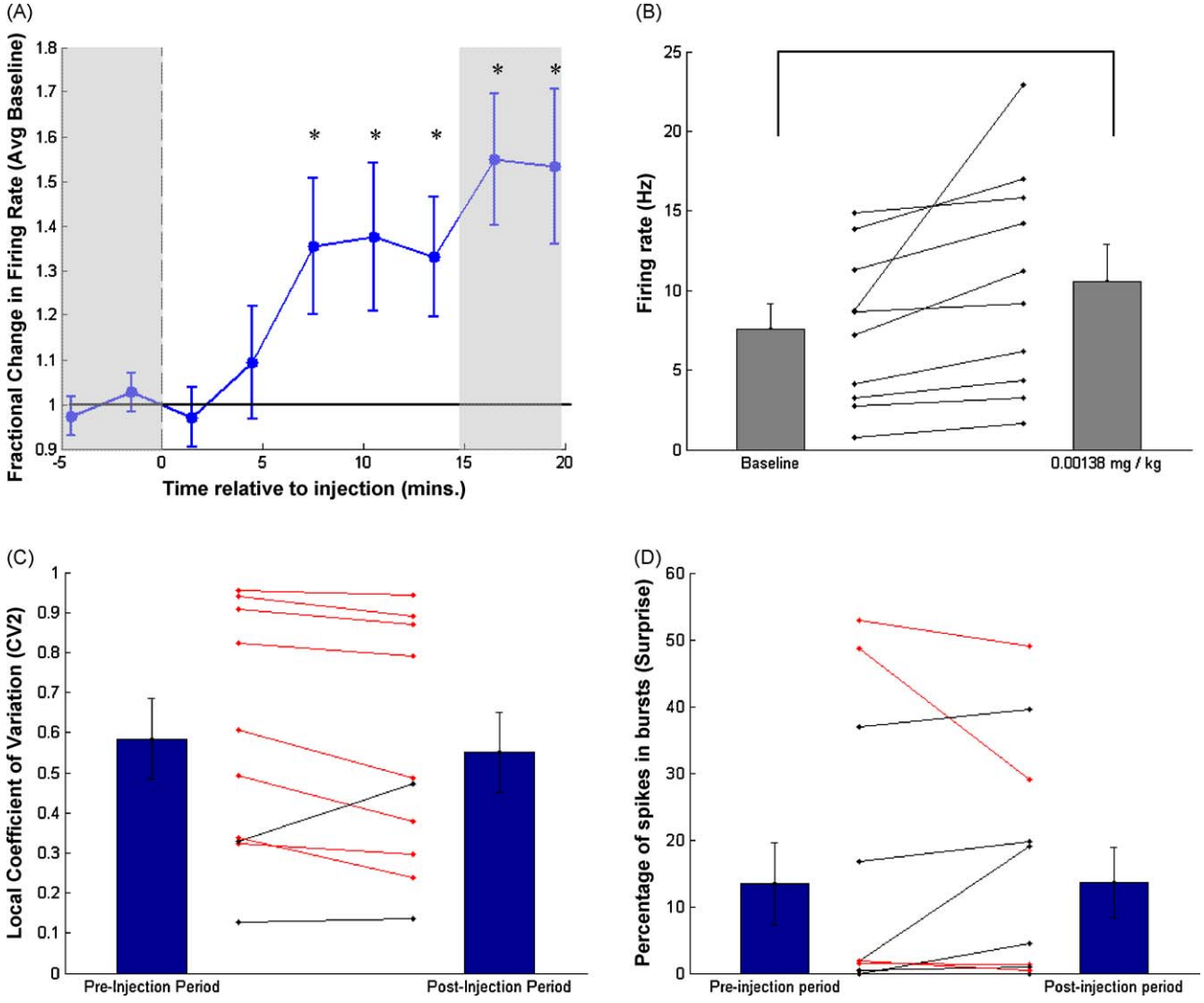

Fig. 2. Effect of AZD0328 on VTA activity *in vivo*. (A) Effect of low dose (0.00138 mg/kg, *iv.*) AZD0328 injection on the spontaneous firing of putative dopaminergic neurons within VTA. Mean (±SEM; n = 10 rats) fractional change in pre-dose firing rate as a function of time relative to AZD0328 injection. (B) Firing rate of individual dopaminergic neurons during the last five minutes of the pre-dose period vs. 15–20 min post low dose (0.00138 mg/kg, *iv.*) injection of AZD0328. Bars represent mean ± SEM for the population. (C) Although AZD0328 dosing produced a significant increase in firing rate, it did not significantly affect firing regularity, as measured by CV2. (D) No significant change in percentage of spikes within bursts across individual neuron as measured using the "surprise" method of burst detection.

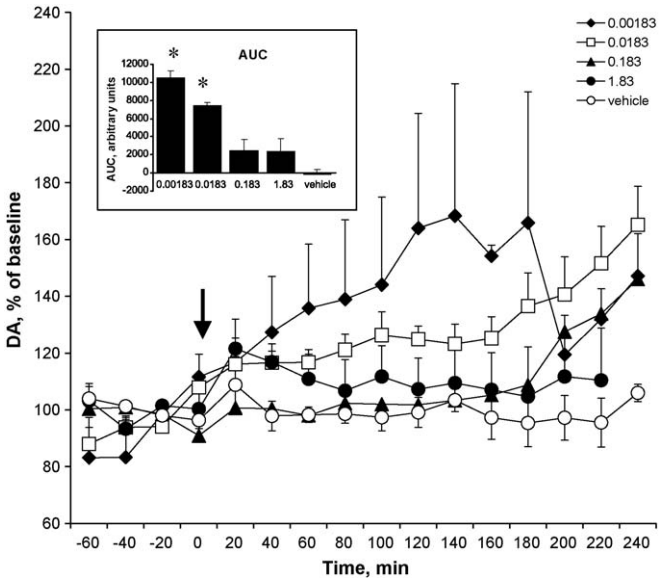

Fig. 3. Effect of AZD0328 on extracellular levels of dopamine in the prefrontal cortex of rats. Data are expressed as mean ± SEM (n = 6–9 per group) and expressed as percentage of pre-drug administration. An arrow denotes time of injection. AZD0328 at 0.00183 and 0.0183 mg/kg produced a significant effect on dopamine release ($F_{(4,61)}$ = 10.3, $p < 0.05$ at 0.00183 mg/kg and $F_{(4,61)}$ = 5.04, $p < 0.05$ at 0.0183 mg/kg). The figure inset represents a mean total dopamine efflux (i.e. AUC) ± SEM (n = 6–9 per group). Significant differences versus vehicle treated group denoted by $^{*}p < 0.05$, 1-way ANOVA.

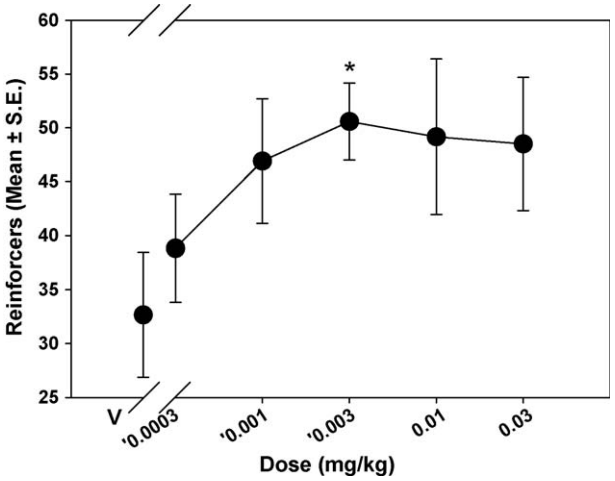

Fig. 4. Effects of administration of doses of AZD0238 before the overnight operant session on the total number of reinforcers earned by fasted, naive rats. Each data point represents a Mean and S.E. for an independent group of 15 rats. *Indicates $p < 0.05$, Dunnett's post hoc statistical analysis.

each of these periods the mean firing rate of each cell was calculated. As can be seen from Fig. 2B, 10/10 neurons ($p < 0.05$; chi-squared test) exhibited an increase in firing rate. On average, firing rates increased from a mean of 8.36 ± 1.39 to a mean of 11.43 ± 2.14 Hz. Due to the high degree of across cell variability, a non-parametic Wilcoxon signed-rank test for differences in medians was used to determine statistical significance—yielding a p-value of < 0.001.

Previous studies have demonstrated that dopamine release within efferent target structures is a function of both the rate and pattern of firing by VTA neurons. For a given mean firing rate, action potentials produce more efficient dopamine release if they are clustered in bursts rather than equally spread out in time [9,10]. Therefore, quantification of effects of AZD0328 on burst firing represented an additional assessment of the mechanism by which α7 NNR activation affects the function of this dopaminergic system. Previous studies examining the discharge patterns of VTA neurons in anesthetized rats have distinguished between bursting and non-bursting spike events using a set of fixed interspike interval criteria for burst detection [9,11]. Given the potential interaction between fixed interval criteria for burst detection and changes in firing rate (Quirk et al.; unpublished observations), patterns of spike activity were measured using both the surprise algorithm for burst detection [7], and local coefficient of variation (CV2)–two metrics with less sensitivity to changes in mean firing rate (Quirk et al.; unpublished observations). Based on these two metrics, acute activation of α7 NNR by AZD0328 had no appreciable effects on the discharge patterns of putative dopaminergic neurons within the VTA of anesthetized rats (Fig. 2C and D). Together these data suggest that α7 NNR activation acutely increases the excitability of dopaminergic neurons without significantly altering their spike patterns.

3.2. Activation of α7 NNR leads to a dose dependent increase in cortical dopamine release

Given the ability of AZD0328 to increase the excitability of VTA-dopaminergic neurons in anesthetized rats, we next evaluated whether α7 NNR activation translated into an increased cortical dopamine release in awake rats. To determine whether AZD0328 modulates dopamine release in the rat PFC, microdialysis studies were conducted. In the microdialysis experiment, basal levels of dopamine in rat PFC were 160.1 ± 4.7 pM. Administration of AZD0328 in a dose range of 0.00183–1.83 mg/kg, sc produced a dose-dependent increase in dopamine concentrations in microdialysates collected from the PFC (Fig. 3). The response peaked 100–120 min after the injection, reaching 160–170% of the baseline level. The dose-response curve for AZD0328 is inverted with lower doses of compound (0.00183 and 0.0183 mg/kg (5 and 50 nmol/kg)) producing a significant effect on dopamine release ($F_{(4, 61)}$ = 10.3, $p < 0.05$ at 0.00183 mg/kg and $F_{(4, 61)}$ = 5.04, $p < 0.05$ at 0.0183 mg/kg). There was no statistically significant effect on dopamine concentrations in microdialysates from PFC at 0.183 and 1.83 mg/kg (500 and 5000 nmol/kg). The data suggest that AZD0328 is a potent activator of dopaminergic system in rats, causing the increase in extracellular concentrations of dopamine in the PFC most likely through the activation of dopamine release.

3.3. Effects of AZD0328 upon acquisition of operant responding with delayed reinforcement

Successful acquisition of reinforced tasks involves dopaminergic input into frontal cortex in order to consolidate the association between reinforcer delivery and the preceding response [13]. Delaying reinforcer delivery profoundly impairs animals' ability to acquire operant tasks [14,15], and decreasing dopaminergic input into cortex in the absence of delay of reinforcement similarly impairs performance [16]. To investigate the role of α7 NNRs in these processes, naïve rats were trained on an operant response task in a single, 14-h overnight session [17] modified such that the

Table 3
Antagonism of the effect of AZD0328 by MLA.

Treatment (mg/kg)	# of reinforcers earned ± S.E.M.
Vehicle (saline)	34 ± 13
AZD0328 1.0	67 ± 24[a]
MLA 10.0	39 ± 18
MLA 10.0 + AZD0328 1.0	42 ± 16

[a] Indicates value is significantly different from vehicle by the Student's t-test.

reinforcer delivery was delayed by 30 s. Under these conditions, subjects typically earned fewer than 40 reinforcers during the course of a session (Table 4), contrasting with >140 reinforcers if no delay is imposed. Under conditions of delayed reinforcement, AZD0328 increased the number of reinforcers earned relative to vehicle administration (Fig. 4) in a dose-dependent manner, achieving asymptote at 0.003 mg/kg (8 nmol/kg). At doses above 0.03 mg/kg (80 nmol/kg) up to and including 10 mg/kg (26,700 nmol/kg) AZD0328 retained activity in the delayed reinforcement operant training protocol (data not shown, except for 1 mg/kg in Table 3). When animals were pretreated with the α7 antagonist MLA, the delay-dependent increase in reinforcers associated with AZD0328 was reversed (Table 3), a result consistent with the hypothesis that increased behavioral performance was directly related to α7 activation. Administration of AZD0328 did not change the number of reinforcers obtained when no delay was interposed between response and reinforcement, nor when no reinforcers were delivered (Table 4), indicating that the compound effect was not due to a non-specific increase in responding.

3.4. Increased object exploration and improved memory following activation of α7 NNRs

To assess the effects of α7 NNR activation on memory retention, we used a novel object recognition-testing environment for male C57/BL6 mice in which learning (or acquisition) object pairings were presented in opposing quadrants of a divided circular arena [18]. The test mice were allowed to explore the objects for 9 min before being returned to their home cages. The mice were reintroduced to the test arena 15 min following acquisition and allowed to explore one familiar and one substituted novel object and were scored on the ratio of novel:total interaction times. Mice that were pre-treated with subcutaneous AZD0328 (0.00178 mg/kg, 0.089 mg/kg or 1.78 mg/kg) 30 min prior to acquisition demonstrated significantly increased novel object interaction during the subsequent test phase relative to vehicle (PBS) treated mice ($p < 0.001$, $p < 0.01$ and $p < 0.05$ respectively (ANOVA with Tukey's post hoc analysis) (Fig. 5B).

To gain further insight into the possible mechanisms underlying AZD0328's memory enhancing abilities, we examined the behavior of AZD0328 treated mice during the initial acquisition phase of the object recognition task. Relative to vehicle treated animals, AZD0328 (0.00178 mg/kg) treated animals exhibited a significant increase in both the duration and frequency of object interaction(s) ($p < 0.05$ Student's *t*-test) (Fig. 5C and D). This directed exploration was made towards both objects in the arena and was measured by the cumulative time spent interacting and the number of separate interactions in a 9-min period. In contrast, AZD0328 had no effect on locomotor activity within the test environment when the total distance traveled was measured (Fig. 5E). These data are consistent with the hypothesis that the memory enhancing effects of AZD0328 are mediated in part by increases in selective object attention during the encoding phase of the object recognition paradigm; a result consistent with the putative role of dopamine in novel exploration.

To confirm the receptor requirement for AZD0328's mechanism of action, we have compared the effect of AZD0328 on NOR in α7 NNR knockout mice (−/−) and their non-transgenic (+/+) littermates. A 30-min pre-treatment with AZD0328 at 0.00178 mg/kg significantly increased novel object interaction in wild type but not α7 knockout mice (Fig. 5F). This result demonstrates the requirement of an intact α7 NNR for AZD0328 to convey it's cognitive effects.

Table 4
Effects of a dose of AZD0328 following different reinforcement conditions.

Treatment (mg/kg)	# of responses emitted/session ± S.E.M.
No reinforcer delivered	
Vehicle (saline)	149 ± 14.7
AZD0328 0.3 mg/kg	113 ± 18.8
No delay of reinforcement	
Vehicle	142 ± 28
AZD0328 1 mg/kg	144 ± 38

Data for each condition were obtained from a group of 15 animals during a 14-h session.

4. Discussion

Numerous α7 NNR agonists have been reported in the literature [19] and a few have now begun to yield clinical data in early trials with normal subjects, Alzheimer's and schizophrenia patients [20]. AZD0328 is unique in that its exceptional drug-like properties allow the attainment of efficacious brain exposure with low doses in animal models (Table 5). The consistency of these low dose effects across multiple preclinical models has permitted us to efficiently translate effects on dopamine neuron function in an electrophysiological model to those seen in a neurochemical assay to animal models of cognition known to be modulated by cortical dopamine function. Hence, the current studies add to the converging molecular, pharmacological, and physiological evidence indicating a role for cholinergic inputs in the regulation of the midbrain dopaminergic system [5,21]. Our working hypothesis is that selective activation of α7 NNR receptors enhances the excitability of midbrain dopaminergic neurons making them more sensitive to naturally occurring inputs. Consistent with this hypothesis, AZD0328 increased the firing rate of VTA neurons without causing independent changes in either bursting or regularity of firing. Furthermore, even though α7 NNR activation increased cortical dopamine release, the behavioral effects of AZD0328 were different than those typically associated with non-specific activation of dopamine neurotransmission. In particular, AZD0328 treated animals exhibited neither significant increases in bar pressing under optimal learning conditions in the operant training task nor increases in general locomotor activity. Rather, AZD0328 seems to enhance "natural" performance properties such as targeted object exploration and improved response acquisition under conditions of non-optimal learning (i.e. delayed reinforcement). The behavioral effects of AZD0328 were blocked by pharmacological antagonism of α7 NNR function or genetic deletion of the gene encoding the α7 subunit, demonstrating that these actions of AZD0328 are most likely mediated through α7 neuronal nicotinic receptors. Together, these data indicate that selective interaction with α7 NNRs by AZD0328 selectively enhances midbrain dopaminergic neuronal activity causing an enhancement of cortical dopamine levels; these neurochemical changes likely, underlie the positive behavioral responses observed in two different animal models.

Dopaminergic neurons within the VTA exhibit two prominent firing modes: burst firing and non-burst firing. Recent genetic studies suggest a role for distinct subtypes of nicotinic receptors in mediating transitions between these different firing modes [11]. Here we show that the pharmacological activation of α7 NNRs increases the spontaneous firing rate of VTA neurons without appreciably affecting their discharge patterns. Similar increases in spontaneous activity have been recently shown to scale behavioral performance without appreciably altering learning rates [22]. Several factors need to be considered to reconcile this apparent contradiction. First, in our operant training task, AZD0328 administration had no effect on response acquisition under optimal learning conditions; AZD0328 was effective only when

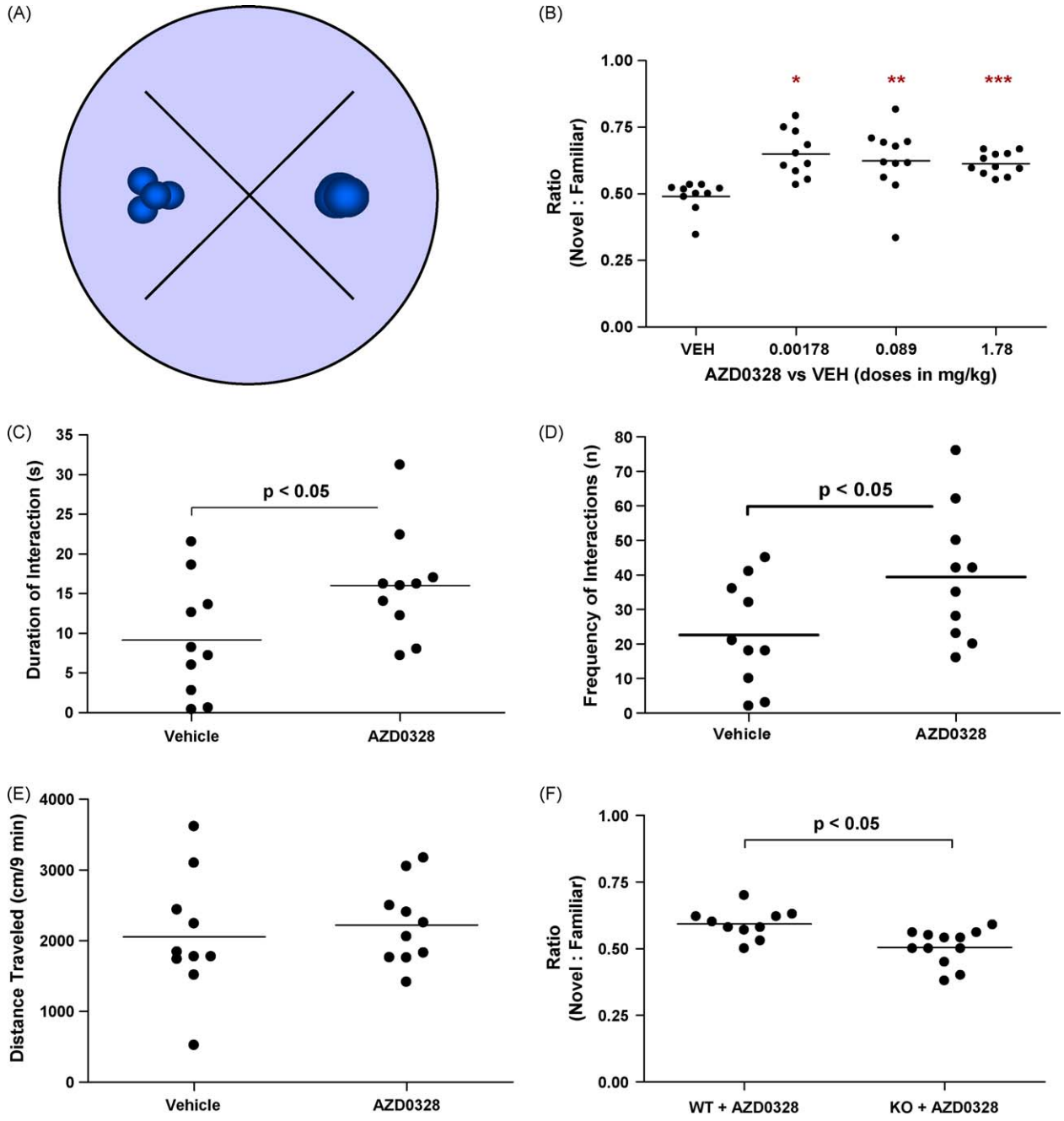

Fig. 5. (A) Novel Object Recognition test environment. A circular chamber is subdivided into quadrants with opaque dividers leaving an open perimeter. Objects comprised of either stacked glass discs or a pyramid of glass marbles are presented in opposite quadrants as a similar pair for acquisition or as a different pair for recall testing. Analysis of object interaction was made using recorded video images and Noldus EthoVision XT4. (B) Object selection ratio in the mouse novel object recognition test. AZD0328 was dosed 30 min prior to acquisition of the learning object set at doses of 0.00178 mg/kg, 0.089 mg/kg and 1.78 mg/kg SC. AZD0328 significantly increased novel object recognition assessed 15 min after the end of acquisition $p < 0.001$, 0.01 and 0.05 respectively. NOR was measured over a 9-min period and analyzed using ANOVA + Tukey's post hoc testing. (C) Duration of interaction with the learning object pairing in the mouse novel object recognition test. AZD0328 was dosed 30 min prior to the test at a dose of 0.00178 mg/kg SC. AZD0328 significantly increased the duration of time mice spent invetigating (interacting) with the learning objects $p < 0.05$. Duration of interaction was measured over a 9-min period using Noldus EthoVision XT4and analyzed using the Student's *t*-test. (D) Frequency of interactions with the learning objects in the mouse novel object recognition test. AZD0328 was dosed 30 min prior to the test at a dose of 0.00178 mg/kg SC. AZD0328 significantly increased the frequency of distinct (unique) interactions with the learning objects $p < 0.05$. Frequency of interactions were measured over a 9-min period using Noldus EthoVision XT4and analyzed using the Student's *t*-test. (E) Locomotor activity during exploration of the learning object pairing in the mouse novel object recognition test. AZD0328 was dosed 30 min prior to the test at a dose of 0.00178 mg/kg SC. AZD0328 had no effect on locomotor activity during the 9-min acquisition period. Locomotor activity was measured using Noldus Ethovision XT4 and analyzed using the Studnet's *t*-test. (F) Object selection ratio in α7 NNR KO mice in the novel object recognition test. AZD0328 was dosed 30 min prior to acquisition of the learning object set at a dose of 0.00178 mg/kg SC. AZD0328 significantly increased novel object recognition assessed 15 min after the end of acquisition in wild type $p < 0.05$ but not α7KO mice. NOR was measured over a 9-min period and analyzed using Student's *t*-test.

normal learning/performance was disrupted by the imposition of a delay between response and reinforcement. Second, it is unclear, whether the memory enhancing effects of AZD0328, in the context of current studies, are due to improved memory storage capabilities *per se*, or are due to enhanced encoding a result consistent with the selective enhancement of object exploration during the sampling period. In fact, at the low doses tested, AZD0328 does not significantly increase hippocampal long-term

Table 5
AZD0328 estimated exposure (Mean ± SD) in preclinical models.

Model	Efficacious dose (mg/kg)	$AUC^{\#}_{(0\text{-}t)}$ (ng/mL*h)	$C_{max}(T_{max})$ (ng/mL)	C_{av} (ng/mL)	$AUC_{(0-24)\ (ng/mL*h)}$
Mouse NOR SC (0–53 min post dose)	0.00178 mg/kg	0.112	0.177 (0.5 h)	0.127	0.437
Rat Operant Training SC (0–14 h post dose)	0.001–0.003 mg/kg	0.361 ± 0.052	0.252 ± 0.035	0.0258 ± 0.0037	0.360 ± 0.05
		1.08 ± 0.16	0.755 ± 0.105 (~10 min)	0.0773 ± 0.0111	1.08 ± 0.16

The exposure values were estimated with a linear PK assumption based on the actual observations from different studies.
AUC = area under curve, C_{max} = maximal concentration reached, C_{av} = average plasma exposure, T_{max} = time at which C_{max} is observed.

potentiation (unpublished observations) a putative cellular mechanism of long-term memory storage. Nevertheless, while non-dopaminergic mechanisms cannot be excluded, the effects of AZD0328 on behavioral performance are most consistent with the effects of dopamine on learning and motivation. Dopamine release is enhanced by novelty [23], and a number of polymorphic gene variants associated with the dopamine system have been linked with novelty-seeking behavior [24]. Furthermore, computational and experimental studies suggest that dopamine can enhance the eligibility trace of conditioned behaviors [25,26]—a potential mechanistic necessity for response learning under conditions of delayed reinforcement.

The ability of α7 NNR agonists to substantially increase dopamine release and improve behavioral performance has clinical relevance because dopamine dysfunction is implicated in a number of the attentional, motivational and learning abnormalities associated with disorders such as schizophrenia, bipolar disorder, and ADHD. Schizophrenia in particular is associated with deficits in delay discounting and both children with ADHD and animal models of impulsiveness and ADHD show dramatic impairments in delayed reinforcement learning [13,27–29]. To the extent that α7 NNR agonists enhance the normal function the brain's dopamine system, compounds targeting α7 NNRs are likely to provide novel therapeutic avenues for the treatment of these and related psychiatric disorders.

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