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Dose-dependent dissociation of pro-cognitive effects of donepezil on attention and cognitive flexibility in rhesus monkeys

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ABSTRACT

BACKGROUND: Donepezil exerts pro-cognitive effects by non-selectively enhancing acetylcholine (ACh) across multiple brain systems. Two brain systems that mediate pro-cognitive effects of attentional control and cognitive flexibility are the prefrontal cortex and the anterior striatum which have different pharmacokinetic sensitivities to ACh modulation. We speculated that these area-specific ACh profiles lead to distinct optimal dose-ranges for donepezil to enhance the cognitive domains of attention and flexible learning.

METHODS: To test for dose-specific effects of donepezil on different cognitive domains we devised a multi-task paradigm for nonhuman primates (NHPs) that assessed attention and cognitive flexibility. NHPs received either vehicle or variable doses of donepezil prior to task performance. We measured donepezil intracerebral and how strong it prevented the breakdown of ACh within prefrontal cortex and anterior striatum using solid-phase-microextraction neurochemistry.

RESULTS: The highest administered donepezil dose improved attention and made subjects more robust against distractor interference, but it did not improve flexible learning. In contrast, only a lower dose range of donepezil improved flexible learning and reduced perseveration, but without distractor-dependent attentional improvement. Neurochemical measurements confirmed a dose-dependent increase of extracellular donepezil and decreases in choline within the prefrontal cortex and the striatum.

CONCLUSIONS: The donepezil dose for maximally improving attention differed from the dose range that enhanced cognitive flexibility despite the availability of the drug in two major brain systems supporting these functions. These results suggest that in our small cohort of adult monkeys donepezil traded improvements in attention for improvements in cognitive flexibility at a given dose range.

INTRODUCTION

The acetylcholinesterase (AChE) inhibitor donepezil (Aricept) is one of few FDA approved cognitive enhancers that aims to address a wide range of cognitive deficits in subjects with mild cognitive impairment or dementia (1–3). Basic research suggests that the cognitive domains that can be enhanced with AChE inhibitors range from selective attention, working memory, response inhibition, learning, and long-term memory (4–6). Consistent with these reports, clinical studies assessing donepezil at one or two doses across larger cohorts of subjects with varying stages of Alzheimer's disease have found improvements of compound scores of cognitive testing batteries (4,7–10). It is, however, not clear whether the standard doses of donepezil used in clinical studies improve multiple cognitive domains directly, or whether at a particular effective dose its major route of action is to enhance arousal, which then provides an indirect, overall cognitive advantage for attention, working memory, learning and memory processes (6,11). Assessing whether donepezil affects multiple cognitive domains simultaneously at a given dose is important for

evaluating its therapeutic efficiency and to identify cognitive domains that should be targeted in drug discovery efforts for improved future cognitive enhancers.

One potential limitation of donepezil and other AChE inhibitors is that they increase acetylcholine (ACh) concentrations non-selectively across multiple brain systems. Such a non-selective ACh increase has shortcomings when brain systems are differently sensitive to ACh action so that the same donepezil dose that is optimally affecting one brain system might over- or under-stimulate another brain system. In primates, muscarinic ACh subreceptors relevant for attention and memory functions (12–15), have enhanced densities in prefrontal cortex (PFC) (16), suggesting that PFC may be more sensitive to modulation by AChE inhibitors than posterior brain areas. Moreover, a comparison of transcription factor (CREB) activation of the PFC and the striatum to muscarinic modulation by Xanomeline has reported a 10-fold higher receptor sensitivity of the striatum (17), consistent with other studies reporting significantly higher muscarinic binding potential and higher AChE activity in the striatum than in other cortical regions (18). It is unclear how these differences affect ACh modulation of attention functions that depend on the PFC (19) and on flexible learning functions that are dependent on the striatum (20,21). One consequence of the brain area specific sensitivity to ACh levels could be that a *Best Dose* for enhancing cognitive functions supported by the striatum might not sufficiently stimulate the PFC, and that a *Best Dose* for enhancing PFC functions might overstimulate the striatum.

To test for these possible implications of brain region-specific ACh action, we devised a drug testing paradigm for monkeys that assessed the effects of three different doses of donepezil across different domains of arousal, attention, and cognitive flexibility in a single testing session. We evaluated the attention domain with a visual search task that varied the number and perceptual similarity of distracting objects and quantified the domain of cognitive flexibility with a learning task asking monkeys to flexibly adapt to new feature-reward rules and avoid perseverative responding. This assessment paradigm goes beyond existing nonhuman primate studies of donepezil that so far have found enhanced short-term memory using delayed match-to-sample tasks (4,6,10,15,22–27), enhanced arousal and non-selective speed of processing (15,27), or no consistent effect (18) (surveyed in **Table S1**), and takes into account that studies in rodents report positive donepezil effects across a wider range of domains including reversal learning (28), paired associate learning (29), object discrimination (30), novelty detection (31) and variable results on serial choice tasks indexing attention functions (32) (surveyed in **Table S2**). With our design we found that donepezil improves interference control over distractors at doses that caused an overall slower responding (i.e. reduced speed of processing) and peripheral side effects. In contrast, a lower dose of donepezil caused no clear attentional effect but improved cognitive flexibility. These findings document domain-specific dose-response effects of donepezil for attention and cognitive flexibility.

METHODS AND MATERIALS

Nonhuman Primate Testing Protocol

Three adult male rhesus macaques were separately given access to a cage-mounted Kiosk Station that provided a touchscreen interface inside the animal's housing unit to perform cognitive tasks

(**Figure 1A**) (28) (see Supplement). The behavioral tasks was controlled by the Unified Suite for Experiments (USE) (34).

Drugs and Procedures

We used donepezil (Sigma-Aldrich ,catalog number D6821; St. Louis, MO, USA) in three doses: 0.06, 0.1 and 0.3 mg/kg to operate within the dosing range of previous studies reporting pro-cognitive effects (surveyed in **Tables S1-2**). At this IM range, plasma concentrations of donepezil are roughly the same when dosing with ~10x the concentration via PO (15). Animals received saline as vehicle control, or a dose of donepezil IM injection 30 minutes prior to starting task performance taking into account its expected 1h half-life (35). Administration was double-blinded. Drug side effects were assessed 15 min following drug administration and after completion of the behavioral performance with a modified Irwin Scale (36–39) for rating autonomic nervous system functioning (salivation, etc.) and somato-motor system functioning (posture, unrest, etc.). Monkeys' behavioral status was video-monitored throughout task performance (**Figure 1A**).

Behavioral Paradigms

Monkeys performed in each experimental session a visual search (VS) task to measure attentional performance metrics and a feature-reward learning (FL) task to measure cognitive flexibility metrics. Each performance day was made up of an initial set of 100 trials of VS, a set of 21 learning blocks with 35-60 trials each of the FL task, and a second set of 100 trials of the VS task (**Figure 1Aii**). Details of both tasks are provided in the **Supplement**. The VS task required monkeys to find and touch a target object among 3, 6, 9, or 12 distracting objects in order to receive fluid reward (**Figure 1B**). The target was the object that was shown in 10 initial trials without distractors. Targets and distractors were multidimensional, 3D rendered Quaddle objects (34) that shared few or many features of different features dimensions (colors, shapes, arms, body patterns), which rendered search easier when there was no or few similarities among features of targets and distractors, or more difficult if the target-distractor (T-D) similarity was high (**Figure 2A**). The FL-task required monkeys to learn through trial-and-error which object feature is rewarded in blocks of ~35-60 trials (**Figure 1C**). The rewarded feature changed un-cued and switched to a new feature of the same or different feature dimensions, which makes the task similar to conceptual set shifting tasks (e.g. 40,41), but different by using a larger set of features that varied within and across sessions in order to vary task difficulty. In each trial three objects were shown that varied either in features of one feature dimension (e.g. having different colors or body shapes), or that varied in features of two feature dimensions (e.g. having different colors and body shapes). Choosing the object with the correct feature was rewarded with a probability of 0.8. Blocks where only 1 feature dimension varied (*1D blocks*) were easier as there was lower attentional load than in blocks with 2 varying feature dimensions (*2D blocks*).

Neurochemical Confirmation of Drug Effect

To evaluate the levels of donepezil in brain structures that are necessary for successful attention and learning performance, we measured the ACh metabolite choline and donepezil concentrations in the prefrontal cortex and the anterior striatum (caudate nucleus) 15 min after administering a low and high dose of donepezil (0.06 and 0.3 mg/kg, IM) in a separate experiment. Measures of

donepezil were made at the time when we observed dose-limiting side effects at the 0.3 mg/kg dose and the two tested doses were accompanied by pro-cognitive effects in our task (see results). We used microprobes that sampled the local neurochemical milieu with the principles of solid phase micro-extraction (SPME) (42) followed by quantification of the concentrations with liquid chromatography and mass spectrometry (42). The detailed procedures used here are described in (43) and in the **Supplement**.

Statistical Analysis

Data were analyzed with standard nonparametric and parametric tests as described in the **Supplement**.

Results

Each monkey was assessed in 38 sessions total including 17 vehicle days and 7 days with each dose (0.06, 0.1 and 0.3 mg/kg). Drug side effects were noted following IM injections of the 0.3 mg/kg dose in the first 30 min post injection as changes in posture, sedation, vasoconstriction and paleness of skin, but no adverse effects persisted beyond 30 min. (**Table S3**). First, we confirmed that monkeys performed the visual search (VS) task at high 84.4% (± 0.54) accuracy (monkeys Ig: 85.2% ±0.81; Wo: 88.3% ±0.94; Si: 79.8% ±0.97) and showed the expected set-size effect evident in decreased accuracy and slower reaction times with increasing numbers of distractors (**Figure 1D, Figure S1** and **S2**, **Supplemental**). When targets were more similar to distractors (high T-D similarity) VS performance decreased from 92.9% (±0.4) to 85.5% (±0.3) and 81.6% (±1.0) for low, medium and high T-D similarity, respectively ($H(2) = 169.48$, $p < .001$) (**Figure 2B**). In the feature learning (FL) task, the monkeys reached learning criterion faster in the easier 1D (low distractor load) condition (avg. trials to ≥80% criterion: 12.5 ± 0.2 SE), than in the 2D (high distractor load) condition (avg. trials to ≥80% criterion: 15.6±0.2) (**Figure 3A**, **Supplemental**).

Dose-dependent improvement of visual search accuracy and slowing of choice reaction times

Donepezil significantly improved accuracy of the visual search task ($F(1,1722) = 18.95$, $p < .001$)(**Figure 1D**), but on average slowed search reaction times ($F(1,1722) = 4.83$, $p = .028$)(**Figure S1B**). The slower choice reaction times were evident already to the single target object in the 10 target familiarization trials (**Figure S1A**). These main behavioral drug effects were evident prominently in the first visual search block (**Figure 1D**, **Figure S1A**). We therefore focused our further analysis on the first search block.

The improved accuracy of visual search was dose-dependent ($F(3,896) = 10.77$, $p < .001$). The 0.06 mg/kg dose enhanced performance by 2.5% ±1.0, 4.4% ±1.3, 6.1% ±1.4 and 6.3% ±1.6 (mean ±SD) for 3/6/9/12 distractor trials, respectively (Tukey's, $p = .005$). The 0.3 mg/kg dose enhanced performance by 2.7% ±1.0, 6.3% ±1.2, 8.5% ±1.3 and 11.0% ±1.4 (mean ±SD) for 3/6/9/12 distractor trials respectively (Tukey's, $p < .001$) (**Figure 1E**). Thus, we found larger improvement the more distractors interfered with the target search. We confirmed this by fitting a regression line across performance at different number of distractors, which revealed overall significantly shallower slopes with donepezil (slopes: -0.013 ±0.001, -0.009 ±0.002, -0.015 ±0.003 and -0.005

±0.002 for vehicle, 0.06, 0.1, and 0.3 mg/kg of donepezil respectively (H(3) = 11.46, p = .01)). Pairwise comparison showed that the 0.3 mg/kg drug dose and the vehicle condition showed significantly different slopes (Tukey's, p = .013)(**Figure 1F**).

In contrast to improving visual search accuracy, donepezil slowed down reaction times across all distractor conditions at the 0.3mg/kg dose relative to vehicle by on average 100 ms ±40, 238 ms ±79, 208 ms ±99, 264 ms ±102 (mean ± SD) for 3/6/9/12 distractors respectively (F(3, 896) = 15.15, p < .001, Tukey's, p < .001) (**Figure S1C**). The slope of the regression over different number of distractors did not differ between 0.3 mg/kg dose and vehicle which shows the reaction time effect is a non-selective effect that is independent of distractors (regression slope on RTs: 0.061 ±0.002, 0.065 ±0.007, 0.067 ±0.007 and 0.076 ±0.009 (H(3) = 3.37, n.s.) for vehicle, 0.06, 0.1, 0.3 mg/kg of donepezil respectively (**Figure S1D**).

Across sessions visual search accuracy was correlated with reaction times only for the vehicle (Pearson, ρ: -0.30, p < .001) and 0.1 mg/kg donepezil dose condition (Pearson, ρ: -0.46, p = .034), but not for the 0.06 and 0.3 mg/kg dose conditions in which monkeys showed improved accuracy, which suggests the accuracy improvement is independent from a slowing of reaction speed (**Figure S2A-B**).

We next tested whether improved interference control over increasing number of distractor objects was likewise evident when increasing the similarity of distractor and target features (**Figure 2A**). First, we confirmed that higher target-distractor similarity overall reduced performance (F(2,672) = 16.17, p < .001, **Supplemental**). Donepezil significantly counteracted this similarity effect and improved performance at the 0.06 and 0.3 mg/kg doses (F(3,672) = 7.75, p < .001, Tukey's, p = .034 and p < .001 respectively). This finding shows that the beneficial effect of donepezil significantly increased when there was higher demand to control perceptual interference from distracting objects (**Figure 2B**). This was also evident as a statistical trend of a shallower regression slope at 0.06 and 0.3 mg/kg doses of donepezil, which indicates less interference from distracting features when they were similar to the target (**Figure 2C**) (H(3) = 2.79, n.s.; slope changes relative to vehicle for 0.06, 0.1 and 0.3 mg/kg doses were: +0.0357 ±0.0236, -0.0289 ±0.0334, -0.0656 ±0.0197). The improved search performance with donepezil for visual search with higher target-distractor similarity and with a higher number of distractors was evident in significant main effects, but there was no interaction, suggesting they improved performance independently of each other (F(2, 2615) = 64.59, p < .001; F(3,2615) = 28.85, p < .001; F(6,2615) = 0.69, n.s. respectively)(**Figure 2D**). This independence was also suggested by the absence of a correlation of the target-distractor similarity effect and the number-of-distractor effect (Pearson, n.s.) (**Figure S3**).

Dose-dependent improvement of flexible learning performance

Donepezil also improved feature learning performance, but only at the 0.06 mg/kg dose (**Figure 3B**) and most pronounced for the first third of the behavioral session (F(3,602) = 3.3, p = .020; **Figure 3C**). We therefore focused further analysis on the first third of the learning blocks, which revealed that the learning improvement at the 0.06 mg/kg dose was significant for the low distractor load condition (significant interaction effect of drug condition and distractor load (Condition x Distractor Load F(3, 1052) = 3.59, p = .013); and for vehicle, 0.06, 0.1 and 0.3 mg/kg

donepezil doses the trials to criterion were 11.3 ±0.4, 7.7 ±0.9, 12.3 ±1.3 and 11.0 ±1.2 trials long with the 0.06 mg/kg dose and vehicle being significantly different (p = .020, Bonferroni correction)(**Figure 3D**). There was no change in learning speed with other doses at low or high distractor load**.**

Beyond learning speed, we found overall slower choice reaction times at the 0.3 mg/kg donepezil dose (**Figure 3E**) (main effect of drug condition: ($F(3,1052) = 12.29$, $p < .001$). While reaction times were overall slower at high distractor load ($F(1,1052) = 7.18$, $p = .008$) there was no interaction with drug dose ($F(3,1052) = 0.26$, n.s.). After visually inspecting the results we separately tested the 0.3 mg/kg dose of donepezil and found it led to significantly slower choice reaction time than vehicle (Tukey's, $p < .001$)(**Figure 3E**). The changes in choice reaction times did not correlate with changes in learning performance (number of trials to criterion) at any drug condition, indicating they were independently modulated (Pearson, all n.s.)(**Figure S2D**).

We predicted that the faster learning at the 0.06 mg/kg donepezil dose could be due to a more efficient exploration of objects during learning, which would be reflected in reduced perseverative choices of unrewarded objects. Overall, perseverative errors (defined as consecutive unrewarded choices to objects with the same feature dimension) made up 20% of all errors. As expected, we found significantly shorter sequences of perseveration of choosing objects within distractor feature dimensions at the 0.06 mg/kg dose of donepezil (**Figure 3F**). For 0.06, 0.1 and 0.3 mg/kg doses the average length of perseverations in the distractor dimension was: 2.1 ±0.1, 1.8 ±0, 1.9 ±0.1 and 1.9 ±0.1 trials with the difference between vehicle and the 0.06 dose being significant (p = .021). Perseverative choices in the target feature dimension were not different between conditions (for 0.06, 0.1 and 0.3 mg/kg donepezil doses the avg. perseveration length in the target dimension was: 1.7 ±0, 1.7 ±0, 1.6 ±0, and 1.7 ±0 trials (n.s.).

Dissociation of attention and learning improvements, but slowing is correlated

The effects of donepezil on feature learning and visual search might be related, but we found that learning speed and search accuracy was not correlated at those doses at which the drug improved learning and search (0.06 mg/kg dose) or improved only visual search (0.3 mg/kg dose) (Pearson, all n.s.). A significant correlation was found only for the 0.1 mg/kg dose (Pearson, ρ: -0.54; $p = .012$) (**Figure 4A**). Learning at low or high distractor load and the set size (slope) effects in the visual search task was uncorrelated (Pearson, all n.s.). However, at the 0.3 mg/kg donepezil dose the target-distractor similarity effect (i.e. the search slope change) in the visual search task positively correlated with the difference of the learning speed at high versus low distractor load in the learning task (Pearson, ρ: 0.60; $p = .008$). This effect signifies that better attentional search of a target among similar distractors is associated with poorer flexible learning of new targets when there are multiple object features to search through (high distractor load).

In contrast to accuracy, choice reaction times in the learning task and visual search were significantly correlated for the 0.1 mg/kg donepezil dose (Pearson, ρ: 0.52; $p = .016$), the 0.3 mg/kg dose (Pearson, ρ: 0.66; $p = .002$), and the vehicle control condition (Pearson, ρ: 0.60; $p < .001$)(**Figure 4B**).

Determination of extracellular donepezil and choline levels in the prefrontal cortex and anterior striatum

Visual search and flexible learning are realized by partly independent brain systems, including the PFC and anterior striatum (44). To determine whether extracellular levels of donepezil were increased to a similar magnitude in the PFC and anterior striatum, we measured its concentration after administering doses of either 0.06 and 0.3 mg/kg donepezil IM in the PFC, assumed to be necessary for efficient interference control during visual search (19), and in the head of the caudate nucleus which is necessary for flexible learning of object values (20,21). We used a recently developed microprobe that samples chemicals in neural tissue based on the principles of solid-phase microextraction (SPME) (42,43). We found that donepezil was available in both brain areas and its extracellular concentration more than doubled after injecting 0.3 mg/kg than 0.06 mg/kg in both areas ($F(1,16) = 9.69$, $p = .007$), with no significant difference between PFC and caudate ($F(1,16) = 1.44$, n.s.)(**Figure 5A**). Donepezil should cause a depletion of the ACh metabolite choline (45). Using HPLC/MS analysis of the SPME samples we found in the PFC that 0.06 and 0.3 mg/kg donepezil reduced choline concentrations by 74.2% ±14.9 ($p = .005$) and 85.7% ± 26.9 ($p = .007$) of their baseline concentrations, and in the caudate, it reduced choline by 68.4% ±13.8 ($p = .022$) and 81.0% ±12.9 ($p = .009$) of respective baseline concentrations (**Figure 5B**). The 11.5% and 12.6% stronger reduction of choline at the 0.3 versus 0.06 mg/kg dose in PFC or caudate was not significant (n.s.).

To obtain an independent physiological marker of dose-dependent effects we quantified during actual task performance how donepezil changed the heart rate (HR) before versus after drug administration (**Supplemental**). HR showed a transient peak ~20 min after donepezil injection relative to baseline, which was significant for the 0.3 mg/kg dose (pre-injection 102.3 ±7.1 to post-injection 121.6 ±2.6; $p = .021$), but not for the 0.06 mg/kg dose (pre-injection: 90.3 ±4.2 to post-injection: 94.8 ±5.4; n.s.). The 0.3 mg/kg dose caused a significantly higher HR peak than the 0.06 mg/kg dose ($p = .006$) (**Figure 5C**).

Discussion

Here, we dissociated donepezil's improvement of attentional control of interference during visual search from improvements of cognitive flexibility during feature reward learning. At the highest dose tested donepezil reduced interference during visual search particularly when there were many distractors and high similarity of distractors to the target, while concomitantly slowing down overall reaction times and inducing temporary peripheral side effects. In contrast, the lowest dose donepezil did not affect target detection times during visual search, but improved adapting to new feature-reward rules and reduced perseverative responding. These findings document a dose-dependent dissociation of the best dose of donepezil for improving attention and for improving cognitive flexibility.

Different donepezil dose-ranges for improving interference control and flexible learning

Using a behavioral assessment paradigm with two tasks allowed us to discern differences of the donepezil dose that maximally improved interference control (in the visual search task) versus the

dose that maximally improved flexible learning (in the reward learning task). In both tasks, donepezil modulated performance early within the session (first of two VS blocks and first third of learning blocks) consistent with its short half-life and rapid time to peak concentration with IM delivery (15,35). Our results focused therefore on these early time windows. We do not expect different conclusions if we had altered the task sequence (see **Supplemental Discussion**). At the 0.06 mg/kg dose donepezil facilitated flexible learning of a new feature reward rule and reduced the length of perseverative errors (**Figure 3C,F**). These behavioral effects are indicators of improved cognitive flexibility across reward learning and set-shifting tasks (46–48). At the same 0.06 mg/kg dose visual search response times were unaffected (**Figure S1**) and visual search accuracy was overall improved but independent of the number of distractors, i.e. independent of the degree of interference (**Figure 1E,F**). In contrast, at the higher donepezil doses flexible learning behavior was indistinguishable from the no-drug vehicle control condition showing that improving flexibility required donepezil at a lower dose.

This conclusion is opposite to the drug effects on visual search performance, which was maximally improved at the 0.3 mg/kg dose. At this dose, subjects not only showed improved resistance to interference when there were more distracting objects (**Figure 1E,F**), but also improved resistance to distracting objects that were visually similar to the searched-for target (**Figure 2B-D**). These findings document that donepezil enhances the robustness to distraction (49,50), which critically extends insights from existing primate studies with donepezil that mostly used simpler tasks to infer pro-cognitive effects on working memory or arousal (*see* **Table S1**). The process of attentional control of interference also goes beyond a short-term memory effect measured with delayed match-to-sample tasks. In the visual search paradigm we used, short-term memory of the target object is already necessary for performing the easier trials with 3 or 6 distractors, while an attention specific effect can be inferred when there is greater improvement in performance with increased attentional demands in trials with 9 or 12 distractors. Thus, our study provides strong evidence that donepezil causes specific attentional improvement at higher doses, which supports the neuro-genetic model of cholinergic modulation of attention (51) that has received recently functional support in studies reporting enhanced distractor suppression in nonhuman primates with nicotine receptor specific ACh modulation (52–54), and improved suppression of perceptually distracting flankers in human subjects tested with a single dose (55). We should note, however, that donepezil caused at the high dose already a non-selective slowing of reaction times indicative of peripheral side effects (see **Supplementary Discussion**).

The finding that different dose ranges improved flexible learning and visual search distractor filtering suggests that these processes have partially independent Yerkes-Dodson style inverted-U dose-response curves (**Figure 6**). One reason supporting this suggestion is that flexible learning and distractor filtering are supported by partially different brain networks, which likely have differential sensitivity to cholinergic modulation. Lesion studies in nonhuman primates have shown that flexible reward learning is closely associated with the medial and orbito-frontal prefrontal cortex and the striatum where lesions impair learning visual reward associations (46,56). In contrast, visual search distractor filtering in primates depend on the dorsolateral prefrontal cortex (dlPFC) and its connections with posterior parietal cortices, with bilateral dlPFC lesions impairing filtering distraction (57). Brain areas within these partly segregated networks for learning and distractor filtering might be differently sensitive to cholinergic modulation. For example, primate dlPFC has been documented to be uniquely sensitive to neuromodulation by

catecholamines and ACh for spatial working memory and switching between distracting features (5,58), with ACh depletion in PFC causing deficits in attention, but not learning (59). During cognitive processes ACh modulates synaptic efficacy, post-synaptically, in an inverted-U manner through both alpha 7 nicotinic receptors (60) and M1 muscarinic receptors (61). Such inverted-U curves for different receptors are not likely to be homogenous or fully overlapping when taking into consideration variable task demands within a cognitive domain or when considering different cognitive domains entirely (62). This is supported by studies showing the disruption of rule-selective activity with iontophoretic M1 overstimulation of dlPFC neurons (63), while at lower doses, delay-cell firing and spatial tuning were enhanced (61). Our results may thus reflect different inverted-U curves along a construct of flexible attention shifting, required for optimal performance in our feature learning task, and stable filtering of distractors required for optimal performance in our visual search task (**Figure 6**).

Quantifying extracellular levels of donepezil and choline in prefrontal cortex and striatum

We confirmed the presence of extracellular donepezil in the PFC and the anterior striatum at the doses tested (**Figure 5A**) and that it prevented ACh metabolism as evident in 68-86% reduced choline levels (**Figure 5B**). To our knowledge this is the first quantification of donepezil's action on the breakdown of ACh in two major brain regions in the primate. The observed reduction of choline is higher than reductions of AChE activity (of ~25- 70%) reported with positron emission tomography or in brain homogenate (64,65). Previous studies suggest that evaluating blood plasma levels or cerebrospinal concentrations may not predict how effectively AChE drugs influence behavioral outcomes (66). One likely reason is that intracerebral concentrations can be multifold higher than extracerebral concentration levels (64,67) and do not reflect the actual bioactive concentration available in target neural circuits. By confirming that donepezil prevented ACh breakdown in the PFC and striatum, we thus established a direct link of behavioral outcomes and local drug bioavailability in two brain structures that causally contributes to attention and learning (*see* above) (46,56,57,59,68). While our study showed that donepezil has a similar effect on ACh breakdown in both areas, it leaves open whether or how choline concentrations in either brain area relate to finer performance variations across tasks as we measured choline only during one task and with insufficient statistical power to establish such a link at this stage.

The neurochemical measurements of donepezil in PFC and striatum were achieved with a recently developed microprobe that samples neurochemicals through principles of solid phase microextraction (SPME) (42,43,69–71), and so far was used for testing the consequence of drugs only in rodents (70,72,73). We believe that leveraging this technique in primate drug studies will be important for clarifying whether systemically administered drugs reach the desired target brain systems in which they are supposed to exert their pro-cognitive effects.

In our study, confirming donepezil's action in PFC and striatum critically constrains the interpretation of the behavioral results, suggesting that different behavioral outcome profiles are not due an uneven drug availability. Rather, the different '*Best Doses*' for visual search and flexible learning performance will likely be due to brain area specific pharmacokinetic profiles of receptor densities, drug clearance profiles, or auto-receptor mechanisms that intrinsically downregulate local drug actions (74–76). One prediction from the specific distribution and kinetics of nicotinic or muscarinic receptors in PFC and striatum is that donepezil might at lower doses act

predominantly in the striatum via activation of muscarinic sub-receptors as they have a particularly high binding potential (18) and respond stronger to muscarinic ACh receptor activation compared with the PFC (17) (*see* **Supplementary Discussion**). However, it might also be possible that donepezil recruits nicotinic receptors which are upregulated with chronic donepezil use (77). It will be important to disentangle in future studies the role of nicotinic and muscarinic sub-receptors in PFC and striatum to optimize the clinical potential to improve learning and attention functions in conditions with cognitive impairment and particularly in dementia (see **Supplementary Discussion**).

In summary, our results provide rare quantitative evidence that a prominent ACh enhancing drug exerts domain specific cognitive improvements of attentional control and cognitive flexibility at a distinct dose range. A major implication of this finding is that for understanding the strength and limitations of pro-cognitive drug compounds it will be essential to test their dose-response efficacy at multiple cognitive domains.

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Appendix A. Supplementary Information

Figure Legends

Figure 1. Task design, meta-structure and visual search performance as a function of distractor number. (**A**) **i**. Picture of one of the subjects working in the custom-built kiosk, interacting with the touchscreen and receiving fluid reward. **ii.** The meta structure of the Multi-task. Each experimental session consists of 3 super-blocks of VS, FL and VS respectively. Each VS block is preceded by 10 familiarization trials which is identical to a VS trial but without any distractors. Each VS block contains trials with 3/6/9/12 distractors randomly selected and counterbalanced over the block. In contrast, each FL block will contain 0/1 irrelevant feature dimensions in addition to the relevant feature dimension (the dimension with the rewarded feature value) counterbalanced over the session. (**B**) **i**. From the grand pool of quaddles which includes four feature dimensions and a variable number of feature values (9 shapes, 9 patterns, 8 colors, and 11 arms), three feature values from three feature dimensions are chosen. This 3x3 pool is then counterbalanced for dimension presentation and feature reward association and is utilized for 2 weeks of data collection where all presented quaddles are selected from this 3x3 pool. **ii.** Example trials. Two example VS trials (top) within the same block with 3 distractors (left) and 9 distractors (right). Each VS block will contain one of 5 backgrounds, with the VS blocks in the same day never having the same background. All distractors and target objects in VS blocks are three dimensional objects and distractors may be duplicated in each trial. Quaddles are spatially randomly presented at the intersections of a 5x4 virtual grid pattern on screen. The red box highlights the rewarded target object, which is invariable within the VS block, in these examples. Two example FL trials (bottom) within the same block containing 2D quaddles (1 distracting dimension plus the relevant dimension). The rewarded feature value in this block is the checkered pattern independent of what color feature value it is paired with. Quaddles may be presented in 8 possible locations in a circle each being 17 degrees of visual radius away from the center of the screen. The red box signifies the rewarded target object, which is a variable combination of the rewarded feature value (the checkered pattern in this example) with a random feature value of the distractor dimension (color in this example). (**C**) The trial structure for both the FL (top) and VS (bottom) blocks of the task are very similar. A trial is initiated by a 0.3-0.5s touch and hold of a blue square (3° visual radius wide) after which the blue square disappears for 0.3-0.5s before task objects, which are 2.5° visual radius wide, are presented on screen. Once the task objects are on screen, the subject is given 5s to visually explore and select an object via a 0.2s touch and hold. A failure to make a choice within the allotted 5s results in an aborted trial and did not count towards the trial count. Brief auditory feedback and visual feedback (a halo around the selected object) are provided upon object selection, with any earned fluid reward being provided 0.2s following object selection and lasting 0.5s along with the visual feedback. Non-rewarded trials had a different auditory tone and a light blue halo around the selected, non-rewarded object. Rewarded objects had a higher pitch auditory tone, a light yellow halo around the selected rewarded object and had an accompanying fluid (water) reward. (**D**) Average VS performance by distractor number for vehicle and all donepezil doses combined, both separated by the first vs second VS block. VS performance was significantly different for block number ($F(1,1722) = 22.19$, $p < .001$) as well as condition ($F(1,1722) = 19.0$, $p < .001$). The inlet shows individual monkey average VS performance linear fits. **(E**) Average VS performance by distractor number between vehicle and 0.06, 0.1, and 0.3 mg/kg donepezil doses for the first VS block ($F(3,896) = 10.77$, $p < .001$). Both the 0.06 and 0.3 mg/kg doses were significantly different from vehicle (Tukey's, $p = .005$ & $p < .001$ respectively). Error bars here reflect standard deviation in this panel. (**F**) The set size effect of VS performance by distractor

number for each condition. The 0.3 mg/kg dose set size effect was significantly shallower from the vehicle set size effect ($H(3) = 11,46$, $p = .010$; Tukey's, $p = .013$).

Figure 2. Visual search task performance and change in difficulty through increasing distractor numbers and target-distractor similarity. (**A**) A visual description of the target-distractor similarity measure in the VS task. For an example target in the red square, 3 example distractors are presented with 0, 1 and 2 features in common respectively from left to right. The cartoon plot below shows the impact of the average target-distractor similarity of an individual trial on performance. (**B**) Similar to **Figure 1D**, but here we plot average VS performance by T-D similarity. There was a significant effect of T-D similarity on performance ($F(2,627) = 16.17$, $p < .001$) as well as condition with both the 0.06 and 0.3 mg/kg donepezil doses being significantly different from vehicle ($F(3,267) = 7.75$, $p < .001$; Tukey's $p = .034$ and $p < .001$ respectively). (**C**) The change in the slope of VS performance with 0.06, 0.1 and 0.3 mg/kg donepezil relative to vehicle. The change in slope by distractor number is plotted on the left y axis (same data as **Figure 1F**) ($H(3) = 11.46$, $p = .010$) while the change in slope by T-D similarity is plotted on the right y-axis ($H(3) = 2.8$, n.s.). (**D**) A visualization of the combined effect of distractor number and T-D similarity on performance. From left to right, each cluster of lines represents increasing distractor numbers while data within each line represents low, medium and high T-D similarity from left to right respectivel. Both distractor number ($F(3,2615) = 28.85$, $p < .001$) and T-D similarity ($F(2,2615) = 64.59$, $p < .001$) impact VS performance with no significant interaction ($F(6,2615) = 0.69$, n.s.).

Figure 3. Feature learning task learning curves and performance. (**A**) Average learning curves of each monkey and all monkeys combined for both low and high distractor load conditions. In all instances, monkeys learned faster and with higher plateau performance in low distractor load blocks relative to high distractor load blocks. (**B**) All monkey average learning curves for vehicle, 0.06, 0.1 and 0.3 mg/kg donepezil doses for both low and high distractor load conditions. (**C**) Temporal progression of learning speed (LP) for vehicle, 0.06, 0.1 and 0.3 mg/kg donepezil doses for the low distractor load condition only. At the 0.06 dose, donepezil allows for faster learning in the low attentional load blocks ($F(3,602) = 3.3$, $p = .020$). Similar to the VS task, donepezil's enhancement is only visible early on and relatively close to its i.m. administration. (**D**) Average learning speed of vehicle and donepezil doses for low and high distractor load blocks across sessions reveals an interaction between drug condition and distractor load ($F(3,1052) = 3.59$, $p = .013$). (**E**) The same as D but for choice RTs instead of learning speed. The 0.3 mg/kg donepezil dose slows choice reaction times in both low and high distractor load blocks (Condition $F(3,1052) = 12.3$, $p < .001$; Tukey's, $p < .001$). (**F**) Change in the length of perseverative errors from vehicle, where feature values in the distracting dimension were the target of the perseverations. Error bars reflect SEMean for inter-monkey variability. Donepezil at the 0.06 mg/kg dose significantly reduces perseveration length in the distracting dimension ($p = .021$); other donepezil doses trends towards this as well.

Figure 4. The relationship between the visual search task and the feature learning task. (**A**). Correlation coefficients between FL learning speed (LP) and VS performance for vehicle, 0.06, 0.1 and 0.3 mg/kg donepezil doses. Only the 0.1 mg/kg donepezil dose had a significant correlation between FL and VS task performance (Pearson, ρ: -0.54; $p = 0.012$). No doses showed a significant change in correlation from vehicle. (**B**) Same as figure A but for FL choice RTs and VS search

RTs. Although vehicle, 0.1 and 0.3 mg/kg donepezil doses had a significant correlation between choice and search RTs, we found no significant change in correlation relative to vehicle.

Figure 5. In-vivo extracellular measurements of choline, donepezil as well as donepezil's effect on heart rate. (**A**) Quantified concentration of extracellular unbound donepezil with 0.06 and 0.3 mg/kg donepezil administration in the PFC and CD. We are able to reliably detect higher donepezil concentrations with 0.3 mg/kg dosing relative to 0.06 mg/kg dosing (Condition $F(1,16) = 9.69$, $p = .007$) with SPME. We also see a trend towards higher detectable donepezil in the caudate relative to the PFC at the 0.3 mg/kg dose tested, however, we found neither significant group or interaction effects. (**B**) We used choline concentrations as a metric for donepezil bio-activity as it de-activates AChE and prevents acetylcholine's degradation into choline. We extracted average session-wise change in choline from baseline with 0.06 and 0.3 mg/kg donepezil doses within the PFC and CD. Although we find significant decreases in choline by up to >80% of baseline concentrations, we found no significant effect of dosing in either the PFC or CD. (**C**) The heart rate of our fourth monkey was monitored during the neurochemical experiments. This revealed a sharp and transient increase in HR post administration of donepezil at 0.3 mg/kg dose (**Supplemental**) which lead to a higher average bpm. We found that we can significantly distinguish 0.06 and 0.3 donepezil administration via HR ($p = .006$).

Figure 6. Each task will have its own specific demands based on the cognitive domain(s) involved that may be best met with some cholinergic tone which may be endogenously, or in this case pharmacologically, shifted to reach optimal performance. Here, the 'feature learning task' and 'visual search task' have varying demands in terms of attentional flexibility and therefore different inverted-U curves for optimal performance. These curves may be shifted by changing the attentional flexibility or a given subject may travel along the x-axis due to pharmacological intervention, aging-related changes and other mechanisms that may change their effective cholinergic tone.

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