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Phosphodiesterase Inhibitors Enhance Object Memory Independent of Cerebral Blood Flow and Glucose Utilization in Rats

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Phosphodiesterase (PDE) inhibitors prevent the breakdown of the second messengers, cyclic AMP (cAMP) and cyclic GMP (cGMP), and are currently studied as possible targets for cognitive enhancement. Earlier studies indicated beneficial effects of PDE inhibitors in object recognition. In this study we tested the effects of three PDE inhibitors on spatial memory as assessed in a place and object recognition task. Furthermore, as both cAMP and cGMP are known vasodilators, the effects of PDE inhibition on cognitive functions could be explained by enhancement of cerebrovascular function. We examined this possibility by measuring the effects of PDE5 and PDE4 inhibitor treatment on local cerebral blood flow and glucose utilization in rats using [^{14}C]-iodoantipyrine and [^{14}C]-2-deoxyglucose quantitative autoradiography, respectively. In the spatial location task, PDE5 inhibition (cGMP) with vardenafil enhanced only early phase consolidation, PDE4 inhibition (cAMP) with rolipram enhanced only late phase consolidation, and PDE2 inhibition (cAMP and cGMP) with Bay 60–7550 enhanced both consolidation processes. Furthermore, PDE5 inhibition had no cerebrovascular effects in hippocampal or rhinal areas. PDE4 inhibition increased rhinal, but not hippocampal blood flow, whereas it decreased glucose utilization in both areas. In general, PDE5 inhibition decreased the ratio between blood flow and glucose utilization, indicative of general oligaemia; whereas PDE4 inhibition increased this ratio, indicative of general hyperemia. Both oligaemic and hyperemic conditions are detrimental for brain function and do not explain memory enhancement. These results underscore the specific effects of cAMP and cGMP on memory consolidation (object and spatial memory) and provide evidence that the underlying mechanisms of PDE inhibition on cognition are independent of cerebrovascular effects.

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INTRODUCTION

Phosphodiesterases (PDEs) are enzymes that hydrolyze cyclic AMP (cAMP) and/or cyclic GMP (cGMP) throughout the body, including the brain. PDE inhibitors present a novel therapeutic approach with which to arrest cognitive decline (Gong *et al*, 2004; Vitolo *et al*, 2002) or possibly reverse the decline with cognition enhancement (Blokland *et al*, 2006; Halene and Siegel 2007; Menniti *et al*, 2006; Rutten *et al*, 2008). Three promising targets through which memory improvement may be effected are cAMP-selective PDE4 (Barad *et al*, 1998; Rose *et al*, 2005; Rutten *et al*, 2007a; Zhang *et al*, 2005); cGMP-selective PDE5 (Prickaerts *et al*, 2004; Rutten *et al*, 2005); and PDE2, which hydrolyzes both cAMP and cGMP (Boess *et al*, 2004; Rutten *et al*, 2007b; Van Donkelaar *et al*, 2008). Evidence is accumulating that second messenger molecules, cGMP and cAMP, are important in memory processes in general and long-term potentiation in particular (Bach *et al*, 1999; Bernabeu *et al*, 1996; Bourtchouladze *et al*, 1998; Frey *et al*, 1993; Lu *et al*, 1999; Prickaerts *et al*, 2002a; Son *et al*, 1998). We have previously shown that PDE4, PDE5, and PDE2 inhibitors all improved the performance of rodents in an object recognition task (ORT) (Blokland *et al*, 2006) and the time-dependent effects of cGMP- or cAMP-selective PDE inhibitors suggested that cGMP was mainly involved in early phase consolidation processes and cAMP in late phase consolidation processes (Rutten *et al*, 2007b).

The beneficial effects of systemically applied PDE inhibitors on memory are thought to be related to activation

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of cAMP/protein kinase A (PKA)/cAMP responsive element-binding protein (CREB) and/or cGMP/protein kinase G (PKG)/CREB signaling pathways (Blokland *et al*, 2006; Rutten *et al*, 2007b), which are also associated with long-term potentiation (Barad *et al*, 1998; Frey *et al*, 1993; Lu *et al*, 1999; Son *et al*, 1998). However, PDE4 and PDE5 inhibitors also cause peripheral vasodilatation by elevating cAMP and cGMP, respectively (Dundore *et al*, 1993; Dundore *et al*, 1992; Paterno *et al*, 1996). Therefore, an alternative explanation could be that these drugs improve memory performance by enhancing cerebral blood flow and the delivery of glucose and oxygen to the brain, although such a mechanism is more likely to be important when the cognitive impairment arises from vascular insufficiency in the first place (eg, vascular dementia). Certainly, earlier studies by Prickaerts *et al* (1997) have shown that the PDE5 inhibitor zaprinast (10 mg/kg i.p.) clearly improved performance in an ORT but did not appear to have any peripheral vascular effect. This does not preclude the possibility of a cerebrovascular effect of PDE inhibition, however, and in this study, we investigated both ORT performance and cerebrovascular reactivity in parallel groups of rats following optimal doses of rolipram (0.03 mg/kg) or vardenafil (1.0 mg/kg).

Considerable debate exists about the role of the hippocampus in object memory, and it has been argued that in particular the perirhinal cortex plays a prominent role in object recognition (Mumby 2001; Winters and Bussey, 2005). However, recent studies in rats have pointed out a role for the hippocampus in consolidation of object memory as, for instance, it was demonstrated that after an ORT extracellular signal-regulated kinase (ERK) phosphorylation was increased in the hippocampus (DG and CA1 area) (Kelly *et al*, 2003). In addition, intrahippocampal injections of protein synthesis inhibitors disrupted consolidation of object recognition memory, again suggesting a crucial role in object memory for the hippocampus as well (Rossato *et al*, 2007). In this study we used the ORT, which may be hippocampus and rhinal cortex dependent, and additionally we used the object location task (OLT), a spatial memory test that is predominantly dependent on the hippocampus (Ennaceur *et al*, 1997). PDE inhibitors, rolipram (PDE4), vardenafil (PDE5), or Bay 60–7550 (PDE2), were given either immediately after OLT training or after 3 h, to investigate early *vs* late phase consolidation processes, respectively. This study investigates for the first time (i) the effects of PDE inhibitors on early and late phase consolidation in a spatial recognition task (OLT), and (ii) the effects of PDE inhibitors on cerebral blood flow and glucose metabolism.

MATERIALS AND METHODS

Animals

All studies were subjected to local ethical review processes and were performed in full accordance with national legislation and associated governmental guidelines in The Netherlands and United Kingdom as well as European Communities Directive 86/609.

Male Wistar rats (Charles River, The Netherlands, total number = 70), weighing between 225 and 400 g were allocated to the OLT and ORT behavioral studies, and they were divided at random into experimental groups ($n = 16$–18/group). The animals were housed individually in standard type 3 Makrolon cages on sawdust bedding in an air-conditioned room (20°C). They were kept under a reversed 12/12-h light/dark cycle (lights on from 1800–0600 h) and had free access to food and water. Rats were housed in the room where they were tested. A radio that played softly provided background noise in all rooms. All behavioral testing was carried out between 0900 and 1700 h under red light conditions.

An analysis of cerebrovascular reactivity in response to drug treatments, and the relationship between cerebral perfusion and the metabolic demands of brain tissue (flow-metabolism coupling, FMC) was conducted in parallel with the behavioral studies. Local cerebral blood flow (LCBF) and local cerebral glucose metabolism (LCMRglu) utilization were measured in groups of male Wistar rats (Charles River, UK; total number = 47) weighing between 230 and 300 g. Animals were group housed, but otherwise husbandry conditions were similar to those experienced by animals used in behavioral studies.

Drug Treatments

All substances were dissolved in appropriate vehicles, made up fresh when required on the day of the experiment, and were administered at one of two time points per substance. On the basis of previous findings in the ORT (Rutten *et al*, 2007b), the time points of administration for each substance were immediately after, or 3 h after the first trial (T1). The order of treatment conditions was allocated at random, and the doses of the drugs used were based upon our previously published studies (Prickaerts *et al*, 2002b; Rutten *et al*, 2006, 2007b).

The PDE5-I vardenafil was dissolved in 0.5% tylose (methyl-cellulose) and 99.5% distilled water and delivered p.o. in a volume of 2 ml/kg in all experiments. In the OLT studies, vardenafil was injected at one of three different doses; 0.3, 1.0, and 3.0 mg/kg, whereas in ORT and FMC studies a single dose was used (1.0 mg/kg). Control animals received vehicle alone.

The PDE4–I rolipram was suspended in 5% ethanol, 1% tylose, and 94% distilled water and delivered i.p. in a volume of 2 ml/kg. In the OLT studies, rolipram was injected at one of three different doses; 0.03, 0.1, and 0.3 mg/kg, whereas in ORT and FMC studies a single dose was used (0.03 mg/kg). Control animals received vehicle alone.

The PDE2–I Bay 60–7550 was dissolved in 5% ethanol, 1% tylose, and 94% distilled water and delivered p.o. in a volume of 2 ml/kg. In the OLT studies, Bay 60–7550 was injected at one of three different doses; 0.3, 1.0, and 3.0 mg/kg. Control animals received vehicle alone.

Object Location Test and Object Recognition Test

Object location test was performed as described elsewhere (Ennaceur *et al*, 1997). The specific details about the apparatus, the objects, and training procedures are identical to those described in previous study (Rutten *et al*, 2007b). A testing session comprised two 3 min trials. During T1, the apparatus contained two identical objects (samples). A rat

was always placed in the apparatus facing the wall in the center of the segment with the transparent front. After the first exploration period, the rat was put back into its home cage. Subsequently, after a delay interval, the rat was put back into the apparatus for T2, but while one object remained in its previous position, the other was placed in a novel location. The novel position of the object could be either 20 cm toward the front or 25 cm toward the back of the arena for both objects (see Figure 1). Of note, in the ORT, instead of moving an object to a novel location in T2, the original object was replaced by a novel object (Ennaceur and Delacour, 1988). In addition, all combinations and locations of objects were used in a balanced design to reduce potential bias due to any preferences for particular locations or objects. The time spent exploring each object during T1 and T2 were recorded manually using a computer.

Exploration was defined as follows: directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose. Sitting on the object was not considered exploratory behavior. To avoid the presence of olfactory trails, the objects were thoroughly cleaned after each trial. Moreover, each object was available in triplicate, so neither of the two objects from the T1 had to be reused as the familiar object in T2. Two testing sessions were run each week, one session comprised Monday (T1) and Tuesday (T2) and the other one comprised Thursday (T1) and Friday (T2).

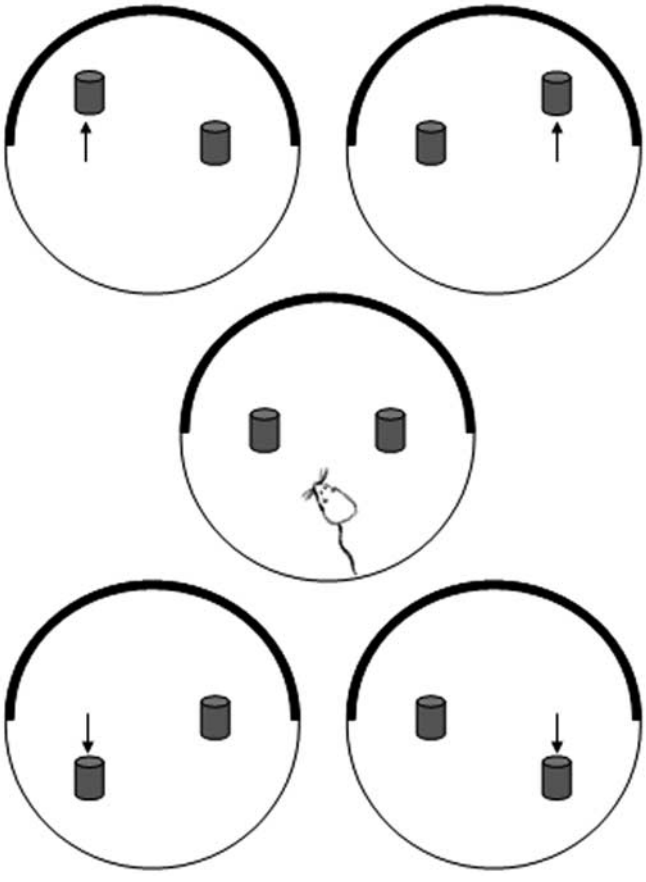

Figure 1 Possible novel locations for the second trial of objects in the object location task. All possible novel object locations were presented in a counter balanced manner to eliminate potential biases due to preferences for particular orientations.

As we expected drug treatment to improve long-term memory performance, the experimental design required a delay interval after which rats would normally be unable to discriminate between the objects at levels above chance. An appropriate delay was determined from preliminary studies in which we tested a number of different inter-trial intervals (ITI), ie, 1, 3, 6, or 24 h, and from the results of these studies (see below), we opted for an ITI of 24 h for all subsequent investigations of the effects of different PDE inhibitors on spatial memory.

Surgical Preparation for FMC Studies

Surgical procedures were carried out as described previously (Ferrington *et al*, 2006; Kelly *et al*, 1995). On the day of the experiment, animals were anesthetized with halothane (maintained at 1% in a mixture of 70% nitrous oxide and 30% oxygen), and polythene cannulae were inserted into both femoral arteries, to allow sampling of arterial blood and monitoring of arterial blood pressure, and both femoral veins, for the injection of radiolabelled tracers and barbiturate at the time of killing. A loose-fitting, individually molded plaster cast was applied around the hindquarters and pelvis and secured to a weighted platform. While lightly restrained and supported, a rectal temperature probe was inserted and anesthesia was discontinued. Animals were allowed to recover from anesthesia for at least 1 h before further experimental manipulation, and mean arterial blood pressure and rectal temperature were monitored throughout the entire experiment.

Measurement of Local Cerebral Glucose Utilization

The LCMRglu was measured in a total of 24 rats using the fully quantitative [^{14}C]-2-deoxyglucose autoradiographic technique (Sokoloff *et al*, 1977). Animals were treated with vardenafil (vehicle, $n=6$; vardenafil, $n=6$) or rolipram (vehicle, $n=6$; rolipram, $n=6$) by the routes, and at the doses, described above. At 30 min after drug administration, an intravenous injection of [^{14}C]-2-deoxyglucose (1.3 MBq per rat in 0.75 ml saline; Sigma-Aldrich, UK) was injected at a constant rate over 30 s. Over the subsequent 45 min, a total of 14 timed arterial blood samples was collected at predetermined intervals (ie, at 0, 15, 30, 45, and 60 s, then at 2, 3, 5, 7.5, 10, 15, 25, 35, and 45 min) and centrifuged to separate the plasma. Aliquots of each plasma sample were taken for the determination of glucose levels (10 μl) and ^{14}C concentrations (20 μl) by semiautomated glucose oxidase assay (Beckman Glucose Analyzer) and liquid scintillation analysis (Packard, Tricarb 2900TL), respectively. At 45 min, the animals were killed by rapid intravenous injection of sodium pentobarbitone, and the brains were immediately dissected out intact, rapidly frozen in pre-cooled 2-methylbutane (-45°C) and mounted onto specimen holders with embedding medium (Lipshaw/Shanon M-1; Thermo Electron Corp., Pittsburgh, PA) and stored at -80°C.

Measurement of LCBF

Local cerebral blood flow was measured in a parallel group of 23 rats using the quantitative [^{14}C]-iodoantipyrine autoradiographic technique described previously (Sakurada

et al, 1978). Animals were treated with vardenafil (vehicle, $n=6$; vardenafil, $n=5$) or rolipram (vehicle, $n=6$; rolipram, $n=6$) by the routes, and at the doses, described above. The [^{14}C]-iodoantipyrine (1.5 MBq per rat in 0.6 ml saline; ARC (UK) Ltd, Cardiff, UK) was infused intravenously at a constantly increasing rate over 45 s, at the end of which the animals were killed by decapitation and the brains were dissected out and prepared as in the LCMRglu experiments. During the tracer infusion, a maximum of 18 timed arterial blood samples taken at approximately equal intervals over the period of infusion were collected onto pre-weighted filter paper discs from the free flowing femoral arterial cannula. The discs were re-weighed at the end of the measurement period to determine the weight of each sample. Sample volume was calculated from weight assuming a specific gravity of 1.01, and tracer concentrations were measured by liquid scintillation analysis.

Preparation and Analysis of Autoradiograms

Frozen brains were sectioned (20 μm) in the coronal plane in a cryostat (−22°C). A series of three sections were retained from every 200 μm, thaw mounted onto glass coverslips and rapidly dried on a hot plate (75°C). Autoradiograms were prepared by applying these sections to X-ray film (Kodak, SB-5), together with a series of eight precalibrated ^{14}C standards (40–1069 nCi/g tissue equivalents: Amersham International, UK), and stored at 4°C for 7 days. Analysis of the autoradiograms was performed using a computer-based image analysis system (MCID/M5 + Imaging Research Inc., Ontario, Canada). Local tissue isotope concentrations were derived from the optical density of autoradiographic brain images relative to the ^{14}C standards, and LCMRglu and LCBF were calculated from tissue tracer concentrations and arterial tracer profiles using the appropriate operational equations. Measurements were made in a total of 27 anatomically distinct and functionally diverse brain regions.

Statistical Analysis

The basic measures were the times spent by rats in exploring an object during T1 and T2.

The values *e*1 and *e2* are measures of the total exploration time spent at both objects during T1 and T2, respectively. *e1* is the measure of the time spent in exploring both identical objects (*a1* and *a2*) in T1 ($e1 = a1 + a2$), *e2* is the measure of the time spent in exploring both the familiar (*a*) and relocated/novel object (*b*) in T2 ($e2 = a + b$). The value *d2* was considered as the index measure of discrimination between the relocated, or novel, and the familiar objects. In fact, *d2* is a relative measure of discrimination, which corrects the difference between exploring the old and the relocated/novel object for exploration activity ($d2 = b - a/e2$).

The determination of the appropriate ITI was analyzed with a one-way ANOVA. For each of the three drug groups, the effects of the different doses were analyzed using one-way ANOVA, for both time points of drug administration. Main effects were analyzed in more detail using Dunnett *post hoc* (two-way) comparisons with vehicle condition ($P<0.05$).

Local cerebral blood flow and LCMRglu data were evaluated statistically using Student's grouped *t*-test ($P<0.05$). To determine whether there were differences in the overall relationship between LCBF and LCMRglu between groups, the LCBF/LCMRglu ratios were calculated and subjected to Mann–Whitney analysis for each drug *vs* vehicle treatment, with Grubb's test applied to detect outliers in the data sets.

RESULTS

Delay-Dependent Forgetting in the OLT

Mean exploration times in the OLT when different ITI were applied are described in Table 1. The length of ITI had no effect upon the level of exploration in T1 (*e1*: $F(3,71)=0.46$; NS) or T2 (*e2*: $F(3,71)=0.41$; NS). When comparing the discrimination performance between the familiar and the relocated object, differences were observed between ITI conditions (*d2*: $F(3,71)=3.29$; $P<0.05$). Discrimination performance in OLT was found to decrease as a function of the increased delay between T1 and T2 (Figure 2). Animals were found to perform better after an ITI of 1 h than after 6 or 24 h, and furthermore, spatial performance after 3 h was better than performance after 24 h.

Table 1 Delay Dependent Forgetting in the Object Location Test: The Effects of Different Inter Trial Intervals (ITI) on the Measures of Exploration

	ITI: delay between T1 and T2			
	1 h	3 h	6 h	24 h
e1	23.67 (2.06)	24.96 (2.03)	25.70 (3.33)	21.96 (2.00)
e2	25.29 (2.38)	23.29 (2.54)	26.70 (2.43)	24.33 (1.56)

Wistar rats ($n=18$ per ITI) were tested in the OLT with an ITI of either 1, 3, 6, or 24 h. Mean values (±SEM) of total exploration time (s) during the first (*e1*) and second trial (e2).

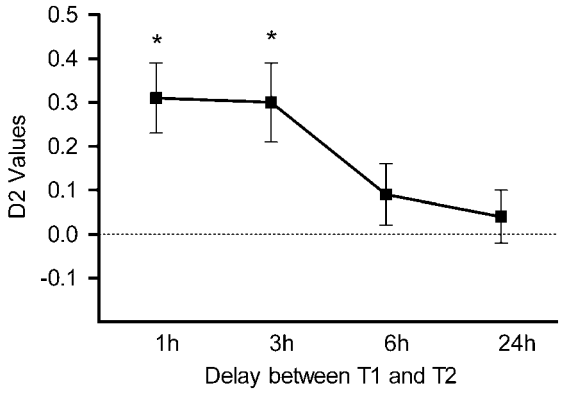

Figure 2 Delay dependent forgetting in the object location task. Memory, that is, *d2* values, decline when longer delays are interposed between the first trial (T1) and the second trial (T2). *Asterisks depict significant differences from zero ($P<0.05$).

PDE5 Inhibition in OLT

The effects of treatment with the PDE5 inhibitor vardenafil on exploration times are shown in Table 2A. When vardenafil was administered immediately after T1 in the OLT, no differences in exploration times were observed between treatment conditions in T1 (*e1*: $F(3,71) = 1.06$; NS) and T2 (*e2*: $F(3,71) = 0.26$; NS). The time-dependent effects of vardenafil treatment on object location performance are shown in Figure 3 (left panel). Significant differences were found between treatment conditions in the discrimination index, *d2* (*d2*: $F(3,71) = 6.92$; $P < 0.01$), and *post hoc* analysis revealed that vardenafil administered immediately after the first trial improved object location performance at a dose of 3 mg/kg when compared with vehicle-treated animals (Dunnett).

When vardenafil was administered 3 h after the first trial, no differences in exploration times were observed between dose conditions in T1 (*e1*: $F(3,71) = 0.43$; NS) and T2 (*e2*: $F(3,71) = 2.37$; NS). Furthermore, vardenafil treatment had no effect on discrimination performance when administered 3 h after T1 (*d2*: $F(3,68) = 0.81$; NS).

Table 2 The Effects of Treatment with PDE Inhibitors on the Exploration Measures of the Object Location Test

	Vehicle	0.3 mg/kg	1 mg/kg	3 mg/kg
(a) Vardenafil				
Injection immediately after T1				
e1	23.24 (2.15)	22.50 (1.69)	22.42 (2.22)	18.43 (2.34)
e2	22.38 (2.38)	22.78 (2.16)	24.82 (1.92)	22.76 (2.24)
Injection 3 h after T1				
e1	22.44 (2.59)	20.36 (1.79)	20.86 (3.07)	24.22 (2.98)
e2	23.09 (2.30)	25.44 (1.29)	18.23 (1.97)	22.68 (2.12)
(B) Rolipram				
	Vehicle	0.03 mg/kg	0.1 mg/kg	0.3 mg/kg
Injection immediately after T1				
e1	21.83 (2.17)	20.44 (1.87)	18.86 (2.20)	21.35 (3.54)
e2	23.44 (2.71)	19.58 (2.26)	19.09 (1.72)	16.78 (1.97)
Injection 3 h after T1				
e1	19.22 (1.75)	18.34 (1.98)	21.27 (1.77)	22.04 (2.07)
e2	23.00 (1.74)	20.96 (1.64)	25.73 (2.02)	23.23 (2.31)
(C) Bay 60–7550				
	Vehicle	0.3 mg/kg	1 mg/kg	3 mg/kg
Injection immediately after T1				
e1	19.37 (2.11)	20.04 (2.00)	18.58 (2.36)	24.64 (2.36)
e2	19.24 (1.78)	24.31 (1.92)	25.75 (1.71)	23.70 (1.94)
Injection 3 h after T1				
e1	19.83 (2.33)	19.76 (2.34)	20.41 (2.47)	20.94 (1.61)
e2	25.37 (1.50)	19.75 (1.94)	22.29 (2.05)	20.73 (1.70)

Wistar rats ($n = 18$ per dose/drug) were administered vardenafil (p.o. 0, 0.3, 1, or 3 mg/kg), rolipram (i.p. 0, 0.03, 0.1, or 0.3 mg/kg) or Bay 60–7550 (p.o. 0, 0.3, 1, 3 mg/kg). The delay between the first and the second interval was 24 h. Mean values (± SEM) of total exploration time (s) during the first (*e1*) and second trial (e2).

PDE4 Inhibition in OLT

The effects of treatment with the PDE4 inhibitor rolipram on exploration times are shown in Table 2b. When rolipram was administered immediately after T1 in the OLT, no differences in exploration times were observed between treatment conditions in T1 (*e1*: $F(3,71) = 0.27$; NS) and T2 (*e2*: $F(3,71) = 1.58$; NS). The time-dependent effects of rolipram treatment on object location performance are shown in Figure 3 (middle panel). No differences were found between treatment conditions in the discrimination index, *d2,* when rolipram was administered immediately after T1 (*d2*: $F(3,71) = 0.10$; NS). Similarly, when rolipram was administered 3 h after T1, no significant difference in exploration times were observed between dose conditions in T1 (*e1*: $F(3,71) = 0.83$; NS) and T2 (*e2*: $F(3,71) = 1.01$; NS). However, rolipram treatment did alter discrimination performance when administered 3 h after T1 (*d2*: $F(3,68) = 6.07$; $P < 0.01$), and further *post hoc* analysis (Dunnett) showed that rolipram improved object location performance at a dose of 0.1 mg/kg when compared with vehicle-treated animals.

PDE2 Inhibition in OLT

The effects on exploration times of treatment with the PDE2 inhibitor Bay 60–7550 are shown in Table 2c. When Bay 60–7550 was administered immediately after T1 in the OLT, no significant differences in exploration times were observed between treatment conditions in T1 (*e1*: $F(3,71) = 1.64$; NS) and T2 (*e2*: $F(3,71) = 2.32$; NS). The time-dependent effects of Bay 60–7550 treatment on object location performance are shown in Figure 3 (right panel). Significant differences were found between treatment conditions in the discrimination index (*d2*: $F(3,68) = 5.94$; $P < 0.01$), and *post hoc* analysis (Dunnett) showed that Bay 60–7550 administered immediately after T1 improved object location performance at a dose of 1.0 mg/kg when compared with vehicle-treated animals.

When Bay 60–7550 was administered 3 h after T1, no differences in exploration times were observed between dose conditions in T1 (*e1*: $F(3,71) = 0.06$; NS) and T2 (*e2*: $F(3,71) = 1.84$; NS). However, Bay 60–7550 treatment did have a significant effect on discrimination performance when administered 3 h after T1 (*d2*: $F(3,30) = 6.07$; $P < 0.05$), and further *post hoc* analysis (Dunnett) showed that Bay 60–7550 improved object location memory at a dose of 1 mg/kg when compared with vehicle-treated animals.

PDE5 and PDE4 in ORT

The effects of treatment with vardenafil or rolipram on exploration times are shown in Table 3. Significant differences in exploration times were observed between conditions in T1 (*e1*: $F(3,63) = 3.92$; $P < 0.05$), and *post hoc* analysis showed that exploration in T1 was significantly higher in vardenafil-treated subjects than in vehicle-treated

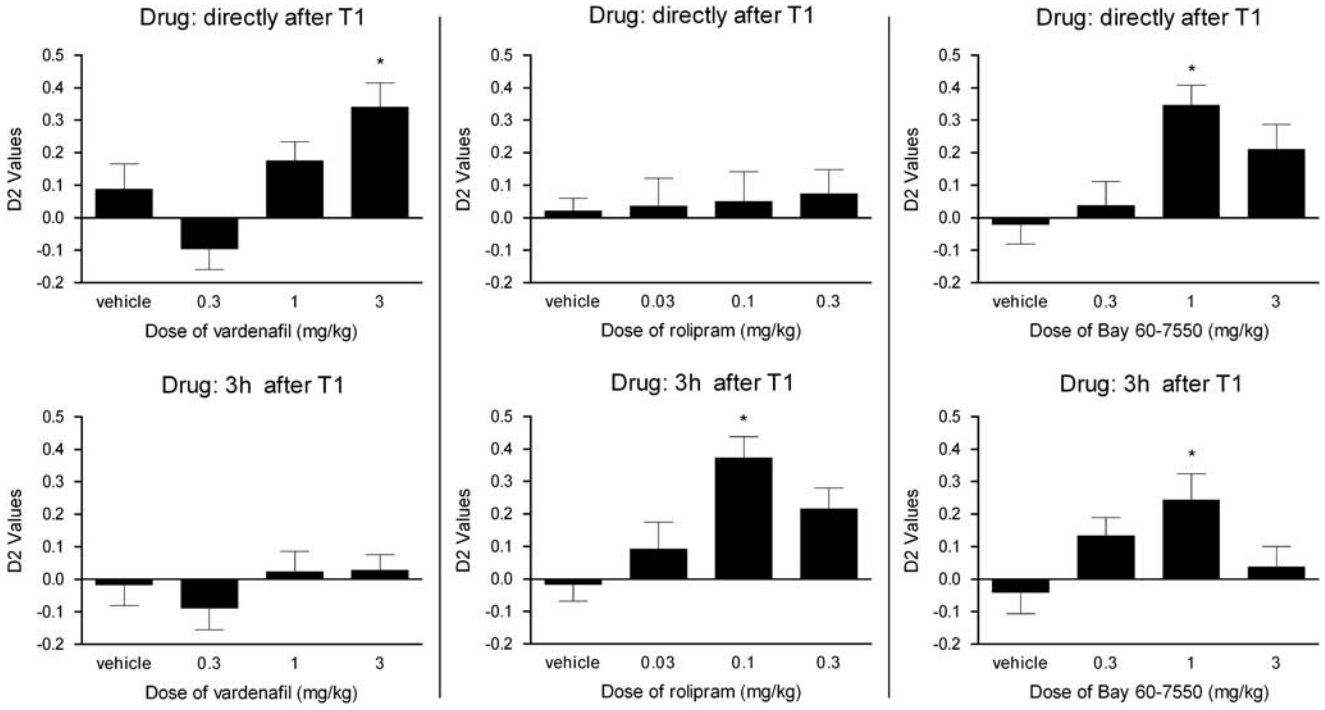

Figure 3 The effects of three PDE inhibitors on OLT memory performance with a 24-h delay between trial 1 (T1) and trial 2 (T2). The figures with black bars (top) demonstrate the effects of PDE inhibitors when administered immediately after T1. The figures with gray bars (bottom) show the effects of PDE inhibitors when administered 3 h after T1. The PDE5 inhibitor, vardenafil (left), the PDE4 inhibitor rolipram (middle) and the PDE2 inhibitor BAY 60–7550 (right) can enhance memory performance in the OLT, but this is dependent on the time of administration. *Asterisks indicate significant differences from the vehicle group (Dunnett; $P<0.05$).

Table 3 The Effects of Treatment with PDE Inhibitors on the Exploration Measures of the Object Recognition Test

	Injection directly after T1		Injection 3 h after T1	
	Vehicle	**Vardenafil**	**Vehicle**	**Rolipram**
e1	29.52 (2.44)	38.59 (2.62)*	39.16 (1.93)	34.66 (1.91)
e2	33.44 (2.07)	41.01 (2.16)*	35.74 (2.76)	41.11 (2.95)

Wistar rats ($n=16$ per dose/drug) were administered vardenafil (p.o. 0 or 1 mg/kg), immediately after T1, or rolipram (i.p. 0 or 0.03 mg/kg) 3 h after T1. The delay between the first and the second interval was 24 h. Mean values (±SEM) of total exploration time (s) during the first (*e1*) and second trial (*e2*).

controls. No significant differences in exploration times were observed in T2 (*e2*: $F(3,63)=2.35$; NS).

Figure 4 shows the effects of vardenafil and rolipram treatment on object recognition performance. Differences between treatment conditions on the discrimination index, *d2,* were observed (*d2*: $F(3,63)=5.50$; $P<0.01$). *Post hoc* analysis (Dunnett) showed both 1 mg/kg vardenafil and 0.03 mg/kg rolipram, administered immediately and 3 h after T1 respectively, improved object recognition performance when compared with vehicle-treated animals. These data corroborate findings observed previously (Rutten *et al*, 2007b).

Physiological Parameters and Behavioral Responses

Mean arterial blood pressure, rectal temperature, blood gases and blood pH were all within the normal physiological range for every animal used in this study, both before and after drug treatment. There were no significant effects of drug treatment upon any of these physiological variables. Drug treatment had no observable effect upon the overt behavior of the animals.

Local Cerebral Glucose Utilization

No significant differences in LCMRglu were found in any of the 27 brain areas investigated when the two vehicle-treated control groups were compared. Although vardenafil treatment (1 mg/kg, p.o.) produced a trend toward a generalized increase in LCMRglu, the coefficient of variation was higher in the drug-treated group than in the appropriate controls, and thus only in the septal nucleus did this increase (26%) reach statistical significance (Table 4). In contrast, rolipram treatment (0.03 mg/kg, i.p.) resulted in a trend toward generalized decreases in LCMRglu, which reached statistical significance in 12 of the 27 brain areas investigated (Table 5). However, the effects were quite modest, ranging from −12% in anterior cingulate cortex to −25% in ventral subiculum. Of particular interest were the significant effects measured in those areas thought to be involved in object recognition performance, namely rhinal cortex (−17%), dentate gyrus (−17%), and CA1 (−19%) of the hippocampus.

Local Cerebral Blood Flow

No significant differences in LCBF were found in any of the 27 brain areas investigated when the two vehicle-treated

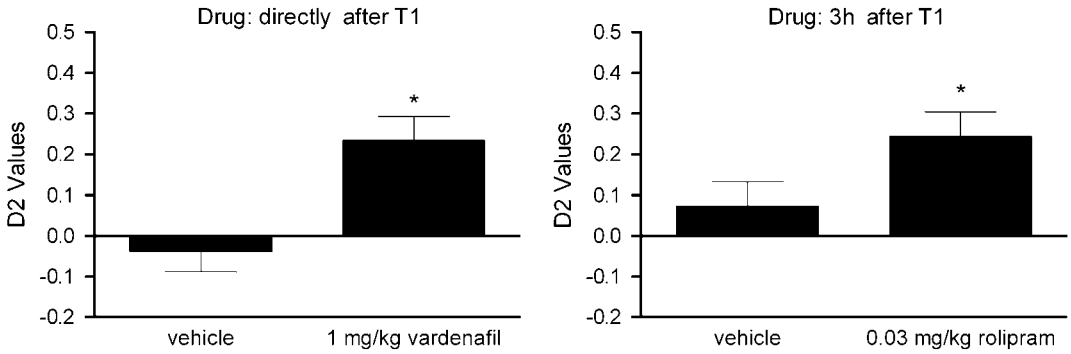

Figure 4 The effects of vardenafil and rolipram treatment on object recognition performance. Left: vardenafil 1 mg/kg administered immediately after the first trial improved memory in a 24 h. Right: rolipram 0.03 mg/kg administered 3 h after the first trial improved memory performance.

control groups were compared. Vardenafil treatment resulted in significant reductions in LCBF in eight brain areas under investigation. The effect was most marked in some neocortical areas (−39% in orbitofrontal; −30% in temperoparietal), but significant reductions were also found in septal nucleus (−24%), medial striatum (−27%), the bed nucleus stria terminalis (−25%), and the central and medial nuclei of the amygdala (−23 and −25%, respectively) (Table 5). In contrast to vardenafil-induced decreases in LCBF, rolipram treatment resulted in significant increases in LCBF in six of the brain areas investigated (Table 5). Although increased tissue perfusion was found in basolateral amygdala (18%) and cerebellum (24%), the most marked drug-induced increases were found in neocortical areas of the brain, including perirhinal (31%), temperoparietal (26%), cingulate (25%), and prefrontal cortices (24%). Interestingly, neither drug had any effect upon hippocampal blood flow.

Relationship Between LCBF and LCMRGlu

Under normal circumstances, LCBF is closely coupled to the metabolic demands of brain tissues (LCMRglu). However, the divergent effects of rolipram upon LCMRglu (decreases) and LCBF (increases) indicates that the drug treatment has altered the normal flow-metabolism relationship in the brain. Mann–Whitney analysis of the ratios derived from mean LCBF and mean LCMRglu in each brain region from vehicle and rolipram-treated animals, indicated a highly significant increase ($P<0.001$). This observation, together with the absence of any evidence for outliers in the data set, suggests a generalized drug-induced cerebral hyperemia. A similar analysis of the data from vardenafil-treated animals indicated a significant decrease in flow-metabolism ratios when compared with control ($P< 0.001$). Thus, while vardenafil had little effect upon LCMRglu (and when effects were evident, metabolism was increased), the effect of the drug upon LCBF (decreases) produced a relative oligaemia in which tissue perfusion was lower than would normally be expected to match metabolic demand.

DISCUSSION

Phosphodiesterase enzymes may be involved in the etiology of a number of CNS diseases, including Alzheimer's disease, schizophrenia, and affective disorders and have recently been proposed as potential targets for therapeutic intervention (Gong *et al*, 2004; Halene and Siegel 2007; Maxwell *et al*, 2004; Menniti *et al*, 2007; Reyes-Irisarri *et al*, 2007; Wong *et al*, 2006). In addition, PDEs may be targeted for cognitive enhancement, and inhibitors of PDEs have proven to be useful experimental tools in exploring mechanisms of learning and memory (Blokland *et al*, 2006; Prickaerts *et al*, 2004). Selective PDE inhibitors of at least five types, inhibitors of PDE2 (Boess *et al*, 2004; Rutten *et al*, 2007b), PDE4 (Barad *et al*, 1998; Rutten *et al*, 2006), PDE5 (Devan *et al*, 2004; Rutten *et al*, 2005), and PDE9 (van der Staay *et al*, 2008) have all been shown to enhance memory in different behavioral paradigms and in different species (for review see Reneerkens *et al*, 2009).

This study investigated the effects of PDE inhibition on spatial memory in the OLT. If the location of an object is changed in relation to the training context, a rat will spend more time exploring the relocated object. The same holds true for the replacement of a training object with a novel object in the ORT. Both ORT and OLT may represent different aspects of episodic memory, that is, the aspect of 'what' and the aspect of 'where,' respectively (Dere *et al*, 2005). The most important brain areas are the hippocampus and the perirhinal cortex, which appear to be both involved in both tests, although their precise contribution to episodic memory remains a matter of debate (Mumby 2001; Winters *et al*, 2006). As PDE inhibitors enhance performance in both tasks, one could argue that PDE inhibitors are suited for the enhancement of episodic memory. Of note, episodic memory deficits are among the first signs of cognitive decline in patients suffering from Alzheimer's disease (Small *et al*, 2003). However, this study cannot draw direct conclusions about the precise role of the different brain structures, for example, hippocampus and perirhinal cortex but also postrhinal cortex or medial prefrontal cortex in the memory-enhancing effects of PDE inhibitors in rodents. Future studies applying local application of drugs into these brain structures are required to elucidate the exact underlying brain systems and mechanisms.

In this study, PDE inhibitors showed memory-enhancing effects in ORT and in OLT, and spatial consolidation processes were improved after treatment in a time-dependent manner. More particularly, our results showed that PDE5 inhibition (vardenafil) enhanced only early phase consolidation processes, whereas PDE4 inhibition (rolipram) enhanced only late phase consolidation

Table 4 The effects of vehicle on vardenafil treatment on local cerebral blood flow or glucose metabolism in the brain.

	Vehicle			Vardenafil (1 mg/kg, p.o.)		
	LCBF	**$LCMR_{glu}$**	**Ratio**	**LCBF**	**$LCMR_{glu}$**	**Ratio**
Septal nucleus	83 ± 2	80 ± 4	1.03	63 ± 6*↓	101 ± 6*↑	0.62
Hippocampus						
Molecular Layer	85 ± 6	132 ± 5	0.64	75 ± 5	147 ± 11	0.51
CA1 (Dorsal)	66 ± 4	98 ± 4	0.67	60 ± 3	104 ± 8	0.57
CA2 (Dorsal)	76 ± 5	94 ± 4	0.80	69 ± 4	108 ± 8	0.64
CA3 (Dorsal)	81 ± 5	112 ± 2	0.72	76 ± 5	123 ± 8	0.61
Dendate p.o.	66 ± 4	85 ± 3	0.77	57 ± 3	90 ± 6	0.64
CA1 ventral	90 ± 7	102 ± 5	0.88	77 ± 3	116 ± 7	0.67
Subbiculum						
Dorsal	80 ± 5	132 ± 4	0.61	67 ± 3	145 ± 9	0.46
Ventral	87 ± 8	90 ± 5	0.96	72 ± 6	108 ± 6	0.76
Rhinal areas						
Enthorhinal cortex	65 ± 6	98 ± 2	0.66	51 ± 2	108 ± 10	0.47
Perirhinal cortex	109 ± 15	134 ± 6	0.81	85 ± 5	131 ± 8	0.65
Postrhinal cortex	88 ± 7	107 ± 4	0.82	76 ± 6	118 ± 10	0.65
Cerebellum Cop	78 ± 6	89 ± 5	0.88	67 ± 4	112 ± 10	0.60
Cortices						
Temperoparietal	169 ± 13	173 ± 12	0.97	118 ± 14*↓	214 ± 20	0.55
Medial PFC						
Dorsal	134 ± 11	176 ± 15	0.76	104 ± 10	202 ± 17	0.51
Ventral	89 ± 4	113 ± 6	0.78	72 ± 8	129 ± 8	0.55
Orbitofrontal	192 ± 7	213 ± 11	0.90	117 ± 13**↓	243 ± 25	0.48
Cingulate						
Posterior	120 ± 5	148 ± 10	0.81	97 ± 9	182 ± 17	0.53
Anterior	137 ± 3	166 ± 9	0.82	106 ± 12	192 ± 15	0.55
Amygdaloid nuclei						
Basolateral	83 ± 5	116 ± 6	0.72	64 ± 7	134 ± 12	0.48
Central	67 ± 4	65 ± 3	1.03	51 ± 5*↓	78 ± 5	0.65
Medial	75 ± 5	66 ± 3	1.15	58 ± 4*↓	80 ± 6	0.72
Bed nucleus Stria terminalis	56 ± 3	64 ± 3	0.88	42 ± 4*↓	75 ± 5	0.56
Caudate						
Medial	106 ± 5	149 ± 10	0.71	77 ± 9*↓	166 ± 17	0.46
Lateral	114 ± 3	170 ± 13	0.67	91 ± 11	187 ± 17	0.49
Nucleus Accumbens	131 ± 9	123 ± 9	1.06	92 ± 14*↓	150 ± 17	0.61
Corpus Callosum	32 ± 2	36 ± 1	0.94	28 ± 2	44 ± 4	0.63
Averaged ratios			0.83			0.58

LCBF (ml/100 g per min); $LCMR_{glu}$ (μmol/100 g per min); ratio ($LCBF/LCMR_{glu}$); values are mean ± SEM.
*$P<0.05$; **$P<0.01$; LCBF, local cerebral blood flow; LCMRglu, local cerebral glucose metabolism.
↓, ↑ decreased or increased compared to vehicle.

processes and PDE2 (BAY 60–7550) inhibition enhanced both early and late phase consolidation processes of spatial object memory. Exactly the same has previously been observed by us with these three PDE inhibitors in the ORT (Rutten *et al*, 2007b). These memory enhancements may be related to the subsequent increases in intracellular cGMP and/or cAMP levels after PDE inhibition, particularly, as both cGMP and cAMP are important intracellular second messenger molecules that have been found to be involved in consolidation processes (Izquierdo *et al*,, 2002) Interestingly, our distinct

Table 5 The effects of vehicle or rolipram treatment on local cerebral blood flow or glucose metabolism in the brain.

	Vehicle			Rolipram (0.03 mg/kg, i.p.)		
	LCBF	**$LCMR_{glu}$**	**Ratio**	**LCBF**	**$LCMR_{glu}$**	**Ratio**
Septal nucleus	63 ± 6	83 ± 4	0.76	72 ± 3	78 ± 5	0.93
Hippocampus						
Molecular Layer	79 ± 4	132 ± 6	0.60	94 ± 9	111 ± 8	0.85
CA1 (Dorsal)	64 ± 4	97 ± 5	0.67	68 ± 4	79 ± 5*↓	0.85
CA2 (Dorsal)	72 ± 6	94 ± 6	0.77	82 ± 6	79 ± 7	1.03
CA3 (Dorsal)	76 ± 4	111 ± 8	0.69	89 ± 8	91 ± 7	0.99
Dendate p.o.	63 ± 4	86 ± 5	0.73	67 ± 5	71 ± 4*↓	0.94
CA1 ventral	84 ± 5	110 ± 5	0.84	99 ± 9	84 ± 8	1.18
Subiculum						
Dorsal	79 ± 6	132 ± 4	0.60	90 ± 7	103 ± 4**↓	0.87
Ventral	78 ± 15	97 ± 6	0.81	101 ± 9	73 ± 6*↓	1.38
Rhinal areas						
Enthorhinal cortex	57 ± 6	104 ± 5	0.55	67 ± 5	88 ± 7	0.76
Perirhinal cortex	89 ± 7	138 ± 8	0.64	117 ± 12*↑	104 ± 8**↓	1.13
Postrhinal cortex	75 ± 8	109 ± 5	0.69	94 ± 7	90 ± 5*↓	1.04
Cerebellum Cop	68 ± 5	90 ± 7	0.76	84 ± 5*↑	85 ± 7	0.98
Cortices						
Temperoparietal	133 ± 12	184 ± 7	0.71	167 ± 9*↑	155 ± 6*↓	1.07
Medial PFC						
Dorsal	97 ± 8	170 ± 8	0.57	109 ± 4	141 ± 11	0.78
Ventral	67 ± 7	108 ± 5	0.62	83 ± 3*↑	95 ± 9	0.86
Orbitofrontal	129 ± 6	200 ± 13	0.64	151 ± 9	177 ± 11	0.86
Cingulate						
Posterior	105 ± 10	164 ± 8	0.64	131 ± 6*↑	125 ± 7**↓	1.05
Anterior	101 ± 9	156 ± 3	0.65	112 ± 3	137 ± 6*↓	0.81
Amygdaloid nuclei						
Basolateral	71 ± 5	123 ± 6	0.58	84 ± 1*↑	103 ± 5*↓	0.82
Central	58 ± 3	84 ± 5	0.69	64 ± 2	74 ± 4	0.87
Medial	66 ± 6	78 ± 4	0.85	73 ± 2	63 ± 3*↓	1.15
Bed nucleus stria terminalis	45 ± 4	72 ± 4	0.63	54 ± 2	64 ± 3	0.84
Caudate						
Medial	86 ± 7	143 ± 4	0.60	96 ± 2	124 ± 8	0.77
Lateral	97 ± 7	158 ± 5	0.61	109 ± 1	134 ± 8*↓	0.81
Nucleus Accumbens	87 ± 9	134 ± 5	0.65	105 ± 2	118 ± 7	0.89
Corpus callosum	31 ± 1	40 ± 2	0.80	33 ± 2	36 ± 4	0.92
Averaged ratios			0.68			0.94

LCBF (ml/100 g per min); $LCMR_{glu}$ (μmol/100 g per min); ratio ($LCBF/LCMR_{glu}$); values are mean ± SEM.
*$P<0.05$; **$P<0.01$; LCBF, local cerebral blood flow; LCMRglu, local cerebral glucose metabolism.
↓, ↑ decreased or increased compared to vehicle.

behavioral findings after selective PDE inhibitor treatment are in line with the sequence of molecular changes taking place in the hippocampus during memory consolidation, as recently described by Izquierdo *et al* (2006). Possible underlying mechanisms of action for memory enhancement after PDE inhibition are closely related to electrophysiological theories of learning and memory, and thus the cAMP/PKA/CREB pathway, as well as the cGMP/PKG/CREB pathway, are key candidates in providing the biochemical substrate of the long-term memory effects. Activation of both pathways may lead to CREB phosphorylation and consequently *de novo* protein synthesis.

A recent study, very delicately demonstrated in a 24 h object recognition memory task similar to ours, that memory consolidation could be disrupted by hippocampal infusions of the protein synthesis inhibitor anisomycin immediately after and 3 h after, but not 6 h after learning (Rossato *et al*, 2007). These findings support the notion that specific crucial time windows for protein synthesis in memory consolidation processes exist. Thus, we propose that PDE inhibitors can differentially modulate object memory performance (i) dependent on their substrate, that is, cGMP and/or cAMP, and (ii) and dependent on their time of administration, that is, early or late phase consolidation phase.

The 2-deoxyglucose method provides a robust approach with which to measure rates of glucose utilization (LCMRglu) in various regions of the brain (Sokoloff 1981), and numerous studies have established that functional activation of neural tissues activates energy metabolism (Sokoloff 1999). In this study, the significant decreases in LCMRglu following rolipram treatment indicate a drug-induced decrease in functional activity in a number of neocortical areas. Similar decreases in LCMRglu have been described previously by others (Ishikawa *et al*, 2002) but were interpreted as a reduction in tracer uptake secondary to the rolipram-induced inhibition of cAMP catabolism rather than a change in functional activity. Although we cannot exclude the possibility that a reduced uptake of 2-deoxyglucose might contribute to the apparent reductions in LCMRglu in our study, it is unlikely that such a nonspecific effect would be limited to so few (mainly cortical) brain areas. Moreover, a systemic reduction in 2-deoxyglucose uptake would result in reduced clearance of the tracer from the blood and relatively high residual plasma levels at the end of the experiment. In fact, a comparison of residual 2-deoxyglucose levels as a percentage of the integrated precursor specific activity derived from plasma samples showed no systematic differences between rolipram- and vehicle-treated rats. It is likely therefore that the measure of LCMRglu is indeed an index of rolipram-induced decreases in functional activation of neocortex. In contrast, there was very little effect of vardenafil treatment upon LCMRglu, and certainly no evidence of any nonspecific reductions that could be associated with reduced cGMP catabolism.

Although no change in LCMRglu in response to vardenafil may be explained by the fact that the measure was taken from sedentary animals outside of any behavioral paradigm, it is tempting to speculate that the rolipram-induced cortical depression could represent an acute increase in cognitive reserve, which allows the animal to perform more efficiently when faced with an appropriate behavioral challenge. A number of studies have found a negative correlation between cerebral metabolic activity and human cognitive performance (Berent *et al*, 1988; Haier *et al*, 1992; Parks *et al*, 1988). However, these findings reflect a constitutive state indicative of an innate capacity for more efficient information processing (Deary and Caryl, 1997) and there is no evidence that this state can be induced acutely by therapeutic, or other, intervention. Further studies are required to determine whether rolipram can indeed induce a state in which the brain functions with greater efficiency.

As both cAMP and cGMP are known vasodilators (Dundore *et al*, 1993; Dundore *et al*, 1992; Fertel and Weiss 1976; Paterno *et al*, 1996), it is possible that the cognitive enhancement evident in our studies following treatment with PDE inhibitors could be explained by increased delivery of energy substrates to the brain as a consequence of decrease cerebrovascular resistance. Certainly, rolipram did increase blood flow in the perirhinal cortex, although it had no effect in hippocampus. In contrast, glucose use was decreased by 17 and 18% in both rhinal cortex and hippocampus, respectively. Thus in both of these regions, the divergence of LCBF and LCMRglu indicates a hyperemic state that may be due to a direct vasodilator effect of the drug. This was supported by global analysis of all 27 brain areas showing that the overall ratio between LCBF and LCMRglu was increased after rolipram treatment. The effects of vardenafil on LCBF were totally unexpected. With the metabolic effects of the drug (tendency toward increased LCMRglu) and the direct vascular effects mediated through cGMP both likely to affect cerebrovascular dilatation and thus increase flow, the reductions in flow that were actually observed are difficult to explain. However, both rhinal cortex and hippocampus were spared any significant oligaemia, and so it is less surprising that vardenafil animals were able to perform appropriately in the behavioral paradigms.

Taken together, the rolipram-induced increases in rhinal blood flow could potentially have contributed to the object memory improving effects of the drug, but the object memory improvement after vardenafil treatment cannot be explained by cerebrovascular effects. As we observed (i) no effects on hippocampal levels of LCBF after vardenafil and rolipram treatment, (ii) no effect on hippocampal LCMRglu after vardenafil, and (iii) a decrease of hippocampal LCMRglu after rolipram, the effects on OLT are not likely to be explained by cerebrovascular effects. Certainly the subtle, and at times contradictory effects of the PDE inhibitors on FMC cannot explain their robust cognition enhancing effects following specific administration time points that we have repeatedly observed in the ORT and OLT and also in higher animals that is, primate object retrieval performance (Rutten *et al*, 2008). Thus, we postulate that PDE inhibitors exert their effects on memory function through mechanisms that are independent of cerebrovascular functioning.

In conclusion, this study shows that PDE inhibitors enhance both memory for objects and memory for locations and suggests that PDEs could be a possible therapeutic target for memory pathologies. Two core aspects of episodic memory have been found to be improved by PDE treatment, namely, the 'what' and the 'where' aspects of episodic memory, suggesting an important role for PDE signaling in episodic memory. Furthermore, our findings corroborate previous time-dependent effects of PDE inhibitors in the ORT and suggest a role for cGMP in early phase consolidation processes and cAMP in late phase consolidation processes. Finally, the present findings demonstrate and underscore that the memory-enhancing effects of PDE inhibitors are independent of cerebral blood flow and glucose metabolism.

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DISCLOSURE/CONFLICT OF INTEREST

We herby declare that there is no conflict of interest for any of the contributing authors of the present manuscript. Dr K Rutten, Dr L Ferrington, E van Donkelaar, E Bollen, Prof. Dr H Steinbusch, and Dr P Kelly hve not received, and do not expect to receive in the near future any compensation for professional services. Dr A Blokland has received research funds from Roche Palo Alto, Sepracor Inc. and Boehringer Ingelheim. Dr J Prickaerts has received research funds from Johnson & Johnson PRD, Solvay Pharmaceuticals and Envivo.

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