

Characterization of the First Potent and Selective PDE9 Inhibitor Using a cGMP Reporter Cell Line

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ABSTRACT

We report here the *in vitro* characterization of 1-(2-chlorophenyl)-6-[(2*R*)-3,3,3-trifluoro-2-methylpropyl]-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidine-4-one (BAY 73-6691), the first potent and selective inhibitor of phosphodiesterase 9 (PDE9), which is currently under preclinical development for the treatment of Alzheimer's disease. This compound selectively inhibits human ($IC_{50} = 55$ nM) and murine ($IC_{50} = 100$ nM) PDE9 activity *in vitro* and shows only moderate activity against other cyclic nucleotide-specific phosphodiesterases. We also report the generation and characterization of a stably transfected PDE9 Chinese hamster ovary cell line, additionally expressing soluble guanylate cyclase (sGC), the olfactory cyclic nucleotide-gated cation channel CNGA2 and the photoprotein aequorin. In this cell line, intracellular cGMP levels can be monitored in real-time via aequorin luminescence induced by Ca^{2+} influx through CNGA2, acting as the intracellular cGMP sensor. This simple and sensitive assay system was used for

the characterization of the cellular activity of the new PDE9 inhibitor. BAY 73-6691 alone did not significantly increase basal cGMP levels in this experimental setting. However, in combination with submaximal stimulating concentrations of the sGC activator 4-[[[(4-carboxybutyl){2-[(4-phenethyl-benzyl)oxy]phenethyl]amino)methyl] benzoic acid (BAY 58-2667), the compound induced concentration-dependent luminescence signals and intracellular cGMP accumulation. The PDE9 inhibitor significantly potentiated the cGMP signals generated by sGC activating compounds such as BAY 58-2667 or 5-cyclopropyl-2-[1-(2-fluorobenzyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl]pyrimidin-4-ylamine (BAY 41-2272) and induced leftward shifts of the corresponding concentration-response curves. Using our newly generated PDE9 reporter cell line, we could show that BAY 73-6691 is able to efficiently penetrate cells and to inhibit intracellular PDE9 activity.

Cyclic nucleotide-specific phosphodiesterases (PDEs) play an essential role in signal transduction by regulating the intracellular levels of cAMP and cGMP and, therefore, are important pharmacological targets. To date, 21 mammalian PDE genes have been identified and subgrouped into 11 isoenzyme families on the basis of their substrate specificity, regulation, and pharmacology (Beavo, 1995; Soderling and Beavo, 2000; Conti and Jin, 2001; Francis et al., 2001).

Phosphodiesterase 9 (PDE9) is a novel PDE family that has been identified and characterized recently. Only one

member of this PDE family (PDE9A) is known so far. However, multiple alternatively spliced variants with differential tissue distribution and subcellular localization could be identified. High PDE9A expression was detected in various tissues, including brain, kidney, spleen, prostate, colon, and intestine. PDE9 is highly specific for cGMP and shows the highest affinity for cGMP of any PDE known to date ($K_m = 0.07$ – 0.17 μ M). PDE9 is insensitive to a variety of known PDE inhibitors such as vinpocetine, EHNA, enoximone, rolipram, dipyridamole, and tadalafil, as well as the nonselective phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (IBMX). So far, no potent and selective PDE9 inhibitors are available. Only zaprinast ($IC_{50} = 29$ – 46 μ M), sildenafil ($IC_{50} = 2.6$ – 11 μ M), SCH 51866 ($IC_{50} = 1.6$ – 3.3 μ M), and vard-

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ABBREVIATIONS: PDE, cyclic nucleotide-specific phosphodiesterase; CNG, cyclic nucleotide-gated; IBMX, 3-isobutyl-1-methylxanthin; sGC, soluble guanylate cyclase; RIA, radioimmunoassay; BSA, bovine serum albumin; CHO, Chinese hamster ovary; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; BAY 73-6691, 1-(2-chlorophenyl)-6-[(2*R*)-3,3,3-trifluoro-2-methylpropyl]-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidine-4-one; BAY 58-2667, 4-[[[(4-carboxybutyl){2-[(4-phenethyl-benzyl)oxy]phenethyl]amino)methyl] benzoic acid; BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluorobenzyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl]pyrimidin-4-ylamine; SCH 51866, *cis*-5,6a,7,8,9,9a-hexahydro-2-[4-(trifluoromethyl)phenylmethyl]-5-methylcyclopent[4,5]imidazo[2,1-*b*]purin-4(3*H*)-one; BAY 60-7550, 2-(3,4-dimethoxybenzyl)-7-[(1*R*)-1-[(1*R*)-1-hydroxyethyl]-4-phenylbutyl]-5-methylimidazo[5,1-*f*][1,2,4]triazin-4(3*H*)-one.

enafil ($IC_{50} = 0.58\text{--}3.4\ \mu\text{M}$) have been identified as weak and nonselective inhibitors of PDE9 (Fischer et al., 1998; Guipponi et al., 1998; Soderling et al., 1998; Corbin and Francis, 2002; Rentero et al., 2003; Wang et al., 2003; Bischoff, 2004).

In rodent brain, neuronal PDE9 expression has been detected in cerebellum, neocortex, caudate putamen, and hippocampus. According to the high expression of PDE9 in these brain regions, the enzyme might be involved in the regulation of neuronal cGMP levels and cGMP-mediated signal transduction (van Staveren et al., 2002). Several lines of evidence implicate a crucial involvement of cGMP in the process of learning and memory. Thus, it has been shown that activation of *N*-methyl-D-aspartate-type glutamate receptors on postsynaptic neurons activates NO synthase and stimulates the production of cGMP (Garthwaite, 1991). Blocking this activation through specific NO synthase inhibitors impairs memory performance in rodents (Yamada et al., 1996). On the other hand, it has been shown that intrahippocampal injection of the cGMP analog 8-bromo-cGMP improves learning (Bernabeu et al., 1996; Prickaerts et al., 2002). We have shown recently that blocking the degradation of cGMP with a specific inhibitor of PDE2 (which cleaves both cGMP and cAMP) results in enhanced memory performance in several rodent models of learning and memory (Boess et al., 2004). However, under physiological conditions, several PDEs are likely to simultaneously control cGMP levels in any given cell, possibly by affecting distinct subcellular cGMP pools.

Because the physiological role of PDE9 is not well-characterized, novel specific PDE9 inhibitors are highly desirable as both pharmacological tools and new potential therapeutics. We report here the *in vitro* characterization of BAY 73-6691, a novel potent and selective PDE9 inhibitor that currently is under preclinical development for the treatment of Alzheimer's disease (Hendrix, 2005).

To characterize the cellular activity of BAY 73-6691, we have generated a stably transfected PDE9 CHO cell line, additionally expressing soluble guanylate cyclase (sGC), the olfactory cyclic nucleotide-gated cation channel CNGA2, and the photoprotein aequorin. In this cGMP reporter cell line, intracellular cGMP levels can be monitored via aequorin luminescence induced by Ca^{2+} influx through CNGA2, acting as the intracellular cGMP sensor (Wunder et al., 2005).

We used the recombinant PDE9 cell line to identify and characterize the cellular effects of the selective PDE9 inhibitor BAY 73-6691 under basal and stimulated conditions. When combined with submaximal prestimulating concentrations of the soluble guanylate cyclase activator BAY 58-2667, BAY 73-6691 concentration-dependently induced luminescence signals and intracellular cGMP accumulation. BAY 73-6691 potentiated the aequorin luminescence and the cGMP levels generated by different soluble guanylate cyclase-activating compounds such as BAY 41-2272 and BAY 58-2667 (Stasch et al., 2001, 2002) and induced leftward shifts of the corresponding concentration-response curves.

Materials and Methods

Preparation of PDE Isoenzymes. Human PDEs and murine PDE9 [accession numbers NM_005020 (PDE1C), NM_002599 (PDE2A), NM_000922 (PDE3B), NM_002600 (PDE4B), NM_018945 (PDE7B), AF056490 (PDE8A), NM_002606 (PDE9A), AF031147

(mPDE9A), NM_006661 (PDE10A), and NM_016953 (PDE11A)] were recombinantly expressed in Sf9 insect cells using the pFAST-BAC baculovirus expression system (Invitrogen, Carlsbad, CA). Cells were harvested and resuspended in lysis buffer (20 ml/l cultures; 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM $MgCl_2$, 1.5 mM EDTA, and 10% glycerin). The cells were disrupted by sonication at 4°C, and cell debris was removed by centrifugation at 15,000g at 4°C for 30 min. The supernatant was designated PDE cell extract and was stored at -80°C. PDE5 was purified from human platelets by sonication followed by centrifugation and ion exchange chromatography of the supernatant on a Mono Q 10/10 column (linear NaCl gradient, elution with 0.2 to 0.3 M NaCl in buffer containing 20 mM HEPES, pH 7.2, and 2 mM $MgCl_2$).

Enzyme Inhibition. The commercially available [3H]cAMP and [3H]cGMP Scintillation Proximity Assay (GE Healthcare, Little Chalfont, Buckinghamshire, UK) system was used for enzyme inhibition studies. For the determination of the *in vitro* effect of test substances on the PDE reactions, 2 μ l of the respective test compound in dimethyl sulfoxide (serial dilutions) were placed in wells of microtiter plates (Isoplate; PerkinElmer Wallac, Weiterstadt, Germany). Fifty milliliters of a dilution of PDE cell extract (see above) in buffer A (50 mM Tris-HCl, pH 7.5, 8.3 mM $MgCl_2$, 1.7 mM EDTA, and 0.2% BSA) was added. The dilution of the PDE cell extract was chosen such that the reaction kinetics were linear, and less than 70% of the substrate was consumed. The reaction was started by the addition of 50 μ l (0.025 μ Ci) of 1:2000 in buffer A without BSA-diluted substrate, [3H]guanosine 3',5'-cyclic phosphate (PDE9 and PDE5), or [3H]adenosine 3',5'-cyclic phosphate (all other PDEs) (1 μ Ci/ μ l; GE Healthcare). The PDE1 assay additionally contained 10^{-7} M calmodulin and 3 mM $CaCl_2$, and PDE2 was stimulated by the addition of 1 μ M cGMP. After incubation at room temperature for 60 min, the reaction was stopped by the addition of 25 μ l of a suspension containing 18 mg/ml yttrium scintillation proximity beads (GE Healthcare) in water. For PDE9, 25 μ l of a PDE9 inhibitor dissolved in buffer A without BSA was added before the addition of beads (e.g., BAY 73-6691, 5 μ M final concentration). The microtiter plates were measured in a Microbeta scintillation counter (PerkinElmer Wallac). IC_{50} values were determined from sigmoidal curves by plotting the percentage of PDE activity versus log compound concentration. IC_{50} is defined as the concentration of the inhibitor required to reduce the cyclic nucleotide hydrolyzing activity of tested PDEs by 50%.

Generation of the Recombinant PDE9 Cell Line. The cGMP reporter cell line, stably overexpressing soluble guanylate cyclase, was generated as described previously (Wunder et al., 2005). This cell line was cotransfected with the murine PDE9 cDNA (Soderling et al., 1998; mPDE9A1, accession number AF031147) in PCR3.1 and a plasmid providing hygromycin resistance (pCEP4d, EBNA-1 deletion). After selection with hygromycin, positive clones with high PDE9 expression and activity were identified by quantitative TaqMan real-time reverse transcriptase-polymerase chain reaction (Applied Biosystems, Weiterstadt, Germany) and measurement of the PDE9 activity using the [3H]cGMP scintillation proximity assay (GE Healthcare). Positive clones were further purified by the limited dilution technique. Plasmid vectors were purchased from Invitrogen.

Cell Culture Conditions and Luminescence Measurements. Cells were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium/Ham's F-12 medium (1:1) with L-glutamine, 15 mM HEPES, supplemented with 10% (v/v) inactivated fetal calf serum, 1 mM sodium pyruvate, 0.9 mM sodium bicarbonate, 50 U/ml penicillin, 50 μ g/ml streptomycin, 2.5 μ g/ml amphotericin B, 1 mg/ml geneticin, 0.25 mg/ml zeocin, and 0.6 mg/ml hygromycin. Next, 1800 cells/well were plated onto 384-well microtiter plates and cultured for 2 days. After removal of the cell-culture medium, cells were loaded for 3 h with 0.8 μ g/ml coelenterazine in Ca^{2+} -free tyrode (130 mM NaCl, 5 mM KCl, 20 mM HEPES, 1 mM $MgCl_2$, and 4.8 mM $NaHCO_3$, pH 7.4) at 37°C and 5% CO_2 . Compounds were added in Ca^{2+} -free tyrode for 6 min. IBMX (0.2 mM) was used in some exper-

iments to prevent cGMP degradation by endogenous PDEs. Immediately after adding Ca^{2+} ions (final concentration, 3 mM), measurement of the aequorin luminescence was started by using a charge-coupled device camera (Hamamatsu Corporation, Shizuoka, Japan) in a light-tight box. Luminescence was monitored continuously for 40 s.

cGMP Determination by RIA. Cells were seeded on 24-well microtiter plates (20,000 cells/well) and cultured for 2 days. The medium was removed, the cells were washed once, and test compounds were added in Ca^{2+} -free tyrode for 10 min at 37°C. IBMX (0.2 mM) was used in some experiments to prevent cGMP degradation by endogenous PDEs. After removal of the supernatant, cGMP was extracted overnight with 70% EtOH at -20°C. Intracellular cGMP was measured by using a commercially available RIA kit (IBL, Hamburg, Germany). Measurements were performed in triplicate.

Compounds. BAY 73-6691 was synthesized by the chemistry department of Bayer HealthCare AG as described previously (Hendrix et al., 2004; example 37-2). BAY 58-2667 and BAY 41-2272 were synthesized as described previously (Alonso-Alija et al., 2001; Straub et al., 2001). EHNA, milrinone, trequinsin, and rolipram were purchased from Calbiochem (San Diego, CA). IBMX, dipyridamole, and vinpocetine were purchased from Sigma-Aldrich (Taufkirchen, Germany), and tadalafil was purified from commercially available tablets. BAY 60-7550 (Boess et al., 2004) was synthesized by the chemistry department of Bayer HealthCare AG.

Statistics. The data are presented as mean values with S.D. error. The GraphPad Prism Software (version 3.02; GraphPad Software Inc., San Diego, CA) was used for curve-fitting and calculation of the IC_{50} and EC_{50} values. The IC_{50} values were determined from three to six independent experiments. For the determination of EC_{50} values, three to four independent experiments were performed in quadruplicate. IC_{50} and EC_{50} values are given as means \pm S.E.M.

Results

Potency and Selectivity of BAY 73-6691 against Different PDE Isoenzymes. In the search for novel PDE9 inhibitors, we could identify BAY 73-6691 (Fig. 1A), which

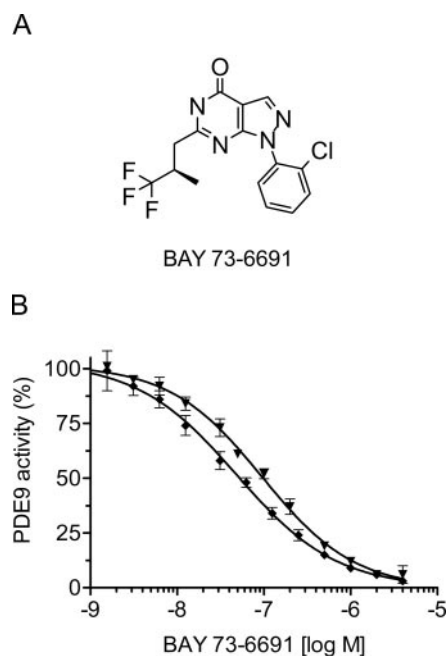


Fig. 1. BAY 73-6691 inhibits PDE9 activity in vitro. A, structure of BAY 73-6691. B, concentration-dependent inhibition of recombinant human (◆) and murine (▼) PDE9 by BAY 73-6691. Data are presented as mean values with S.D. error.

potently inhibits human recombinant PDE9 with an IC_{50} value of 55 ± 9 nM as shown in Fig. 1B and a K_i value of 45 ± 15 nM (data not shown). The murine PDE9 is inhibited by BAY 73-6691 with an IC_{50} value of 100 ± 20 nM (Fig. 1B). We also studied the effects of BAY 73-6691 on different PDE isoenzymes, the corresponding IC_{50} values are shown in Table 1. BAY 73-6691 shows only moderate inhibition of human PDE1 (PDE1C; $\text{IC}_{50} = 1400 \pm 350$ nM) and human PDE11 ($\text{IC}_{50} = 2600 \pm 400$ nM) with selectivity ratios of 25 and 47, respectively. In addition, BAY 73-6691 shows very low potency against human PDE2, PDE3, PDE4, PDE5, PDE7, PDE8, and PDE10 ($\text{IC}_{50} > 4000$ nM).

Generation of the Recombinant PDE9 Cell Line. A cGMP reporter cell line, stably overexpressing soluble guanylate cyclase, was generated as described previously (Wunder et al., 2005). This cell line was cotransfected with the murine PDE9 cDNA (Soderling et al., 1998; mPDE9A1) and a plasmid providing hygromycin resistance. After selection with hygromycin, positive clones with high PDE9 expression were identified by quantitative TaqMan real-time reverse transcriptase-polymerase chain reaction, and measurement of the PDE9 activity was performed after cell lysis by using the [^3H]cGMP Scintillation Proximity Assay (data not shown). Positive clones were further purified by the limited dilution technique, and one clonal PDE9 cell line was selected for further characterization. In this PDE9 cell line, intracellular cGMP accumulation can be monitored via Ca^{2+} influx and measurement of the aequorin luminescence (Wunder et al., 2005).

Characterization of the Cellular Activity of BAY 73-6691. We used the newly generated recombinant PDE9 cell line to characterize the cellular activity of BAY 73-6691. When applied to the cells under basal conditions without prestimulation, BAY 73-6691 did not induce significant luminescence signals (Fig. 2A) or intracellular cGMP accumulation measured by RIA (Fig. 4A). However, in combination with submaximal stimulating concentrations of the sGC activator BAY 58-2667 (Stasch et al., 2002), BAY 73-6691 induced concentration-dependent luminescence signals (Fig. 2A) and an increase of the intracellular cGMP concentration (Fig. 4A).

In the presence of 0.01, 0.03, and 0.1 μM BAY 58-2667,

TABLE 1
Selectivity profile of BAY 73-6691 against different human PDE isoenzymes

All enzymes were human recombinant, except for PDE5, which was purified from human platelets. IC_{50} values were determined from sigmoidal curves by plotting the percentage of PDE activity versus the log compound concentration. IC_{50} is defined as the concentration of the inhibitor (in nanomoles) required to reduce the cyclic nucleotide hydrolyzing activity of tested PDEs by 50%. The relative selectivity for PDE9A was determined by calculating the ratio between the IC_{50} for each PDE and the IC_{50} for PDE9.

	IC_{50}	Ratio
	nM	
PDE9A	55 ± 9	
PDE1C	1400 ± 350	25
PDE2A	$\gg 4000$	$\gg 73$
PDE3B	$\gg 4000$	$\gg 73$
PDE4B	$\gg 4000$	$\gg 73$
PDE5A	$\gg 4000$	$\gg 73$
PDE7B	$\gg 4000$	$\gg 73$
PDE8A	$\gg 4000$	$\gg 73$
PDE10A	$\gg 4000$	$\gg 73$
PDE11A	2600 ± 400	47

BAY 73-6691 stimulated the aequorin luminescence with EC_{50} values of 28.2 ± 4.4 , 4.3 ± 1.3 , and $1.3 \pm 0.1 \mu\text{M}$, respectively. In combination with $0.1 \mu\text{M}$ BAY 58-2667, BAY 73-6691 significantly stimulated the aequorin luminescence with a minimum effective concentration of $\sim 300 \text{ nM}$.

We also studied the effects of BAY 73-6691 in the absence of the nonselective phosphodiesterase inhibitor IBMX. Compared with the results obtained in the presence of IBMX (Fig. 3A), the concentration-dependent luminescence signals induced by the sGC activator BAY 58-2667 were diminished, and the corresponding concentration-response curve was shifted rightward ($EC_{50} = 2.86 \pm 0.86 \mu\text{M}$; data not shown). Therefore, higher prestimulating concentrations of BAY 58-2667 were used. Under these conditions, BAY 73-6691 was

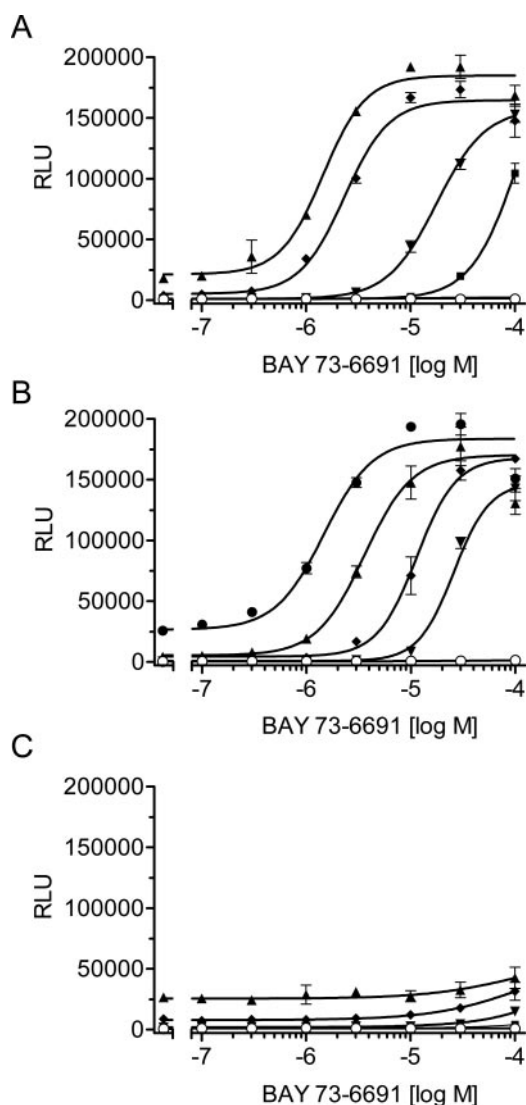


Fig. 2. Effects of the sGC activator BAY 58-2667 on BAY 73-6691 concentration-response curves. Stimulation of luminescence signals in the PDE9 cell line after 6-min incubation with BAY 73-6691 in Ca^{2+} -free tyrode with 0.2 mM IBMX (A) or without IBMX (B) in the absence (\circ) or presence of 0.003 μM (\blacksquare), 0.01 μM (\blacktriangledown), 0.03 μM (\blacklozenge), 0.1 μM (\blacktriangle), and 1 μM (\bullet) BAY 58-2667. C, stimulation of luminescence signals in the parental cell line expressing soluble guanylate cyclase but not PDE9 after 6-min incubation with BAY 73-6691 in Ca^{2+} -free tyrode with 0.2 mM IBMX in the absence or presence of BAY 58-2667 (0.003–0.1 μM). Measurements were started immediately after the addition of Ca^{2+} . Results are expressed as relative light units (RLU), and data are presented as mean values with S.D.

able to induce concentration-dependent luminescence signals (Fig. 2B). In the presence of 0.03 μM , 0.1, and 1 μM BAY 58-2667, BAY 73-6691 stimulated the aequorin luminescence with EC_{50} values of 16.5 ± 2.3 , 2.9 ± 0.4 , and $1.4 \pm 0.04 \mu\text{M}$, respectively.

The effects of BAY 73-6691 on the parental cell line, which expresses soluble guanylate cyclase but lacks PDE9 expression, were also characterized. BAY 73-6691 induced only negligible luminescence signals and no significant intracellular cGMP accumulation in the parental cell line (Figs. 2C and 4B).

We also studied the effects of BAY 73-6691 on concentration-response curves of the sGC activating compounds BAY 58-2667 and BAY 41-2272 (Fig. 3). BAY 73-6691 was able to significantly enhance the luminescence signals (Fig. 3A) and the intracellular cGMP accumulation induced by BAY 58-2667 (Fig. 4C) and induced leftward shifts of the corresponding concentration-response curves. In the absence of BAY 73-6691, BAY 58-2667 stimulated the aequorin luminescence with an EC_{50} value of $373 \pm 92 \text{ nM}$. In the presence of 0.1, 1, and 10 μM BAY 73-6691, the EC_{50} values were shifted to 296 ± 82 , 198 ± 59 , and $19.5 \pm 4.0 \text{ nM}$, respectively. The EC_{50} value for intracellular cGMP accumulation induced by BAY 58-2667 in the absence of BAY 73-6691 was $340 \pm 57 \text{ nM}$. In the presence of 1 and 10 μM BAY 73-6691, the EC_{50} values were reduced to 191 ± 49 and $30.8 \pm 4.0 \text{ nM}$, respectively.

BAY 73-6691 also enhanced the luminescence signals stimulated by BAY 41-2272, a compound that stimulates cGMP

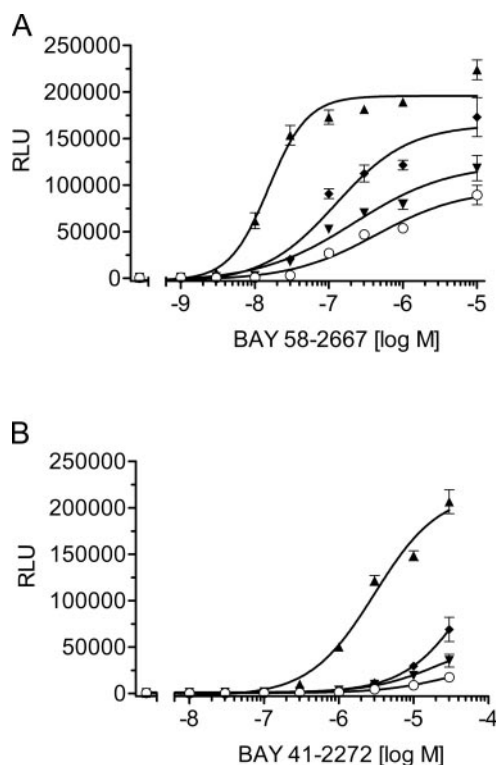


Fig. 3. Effects of the PDE9 inhibitor BAY 73-6691 on BAY 58-2667 and BAY 41-2272 concentration-response curves. Stimulation of luminescence signals in the PDE9 cell line after 6-min incubation with BAY 58-2667 (A) or BAY 41-2272 (B) in the absence (\circ) or presence of 0.1 μM (\blacktriangledown), 1 μM (\blacklozenge), and 10 μM (\blacktriangle) BAY 73-6691 in Ca^{2+} -free tyrode with 0.2 mM IBMX. Measurements were started immediately after the addition of Ca^{2+} . Data are presented as mean values with S.D.

synthesis by sGC by a different mechanism from that of BAY 58-2667 (Stasch et al., 2001, 2002) and induced a leftward shift of the corresponding concentration-response curve (Fig. 3B). The minimum effective concentration of BAY 73-6691 that significantly potentiated BAY 58-2667 and BAY 41-2272 induced luminescence signals was 100 nM.

Activities of Other PDE Inhibitors on the PDE9 Reporter Cell Line. We also tested various PDE inhibitors in combination with submaximal stimulating concentrations of the sGC activator BAY 58-2667 for their ability to stimulate luminescence signal increases in the PDE9 reporter cell line. Vinpocetine, EHNA, BAY 60-7550 (PDE2 inhibitor; Boess et al., 2004), milrinone, trequinsin, rolipram, tadalafil, and dipyrindamole were tested up to 100 μ M and did not significantly enhance the aequorin luminescence (data not shown).

In contrast, the PDE5 inhibitors sildenafil and vardenafil,

which have been reported to also weakly inhibit PDE9 (Soderling et al., 1998; Corbin and Francis, 2002; Bischoff, 2004), induced concentration-dependent luminescence signals in combination with submaximal prestimulating concentrations of BAY 58-2667 (Fig. 5).

Real-Time cGMP Measurements. To monitor changes of the intracellular cGMP levels induced by BAY 58-2667 in the absence or presence of IBMX and BAY 73-6691 in real time, compounds were added in tyrode containing 2 mM Ca^{2+} ions, and luminescence measurements were started immediately after the addition of compounds ("kinetic mode"; Wunder et al., 2005). The results are shown in Fig. 6. The addition of 0.3 μ M BAY 58-2667 induced a weak luminescence signal that started after \sim 42 s. In the presence of IBMX, an increased luminescence signal, which already started after 36 s, was observed. Additional inhibition of PDE9 by 1 μ M BAY 73-6691 further enhanced the luminescence signal and reduced the signal delay to 30 s.

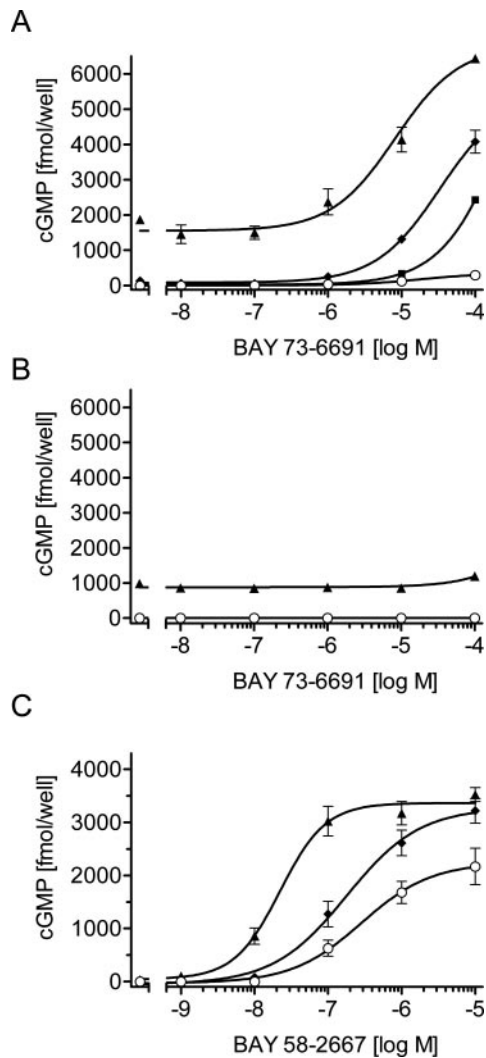


Fig. 4. Intracellular cGMP formation measured by RIA. A, concentration-dependent cGMP formation induced by BAY 73-6691 in the absence (○) or presence of 0.003 μ M (■), 0.03 μ M (◆), and 0.1 μ M (▲) BAY 58-2667 in Ca^{2+} -free tyrode with 0.2 mM IBMX. B, effects of BAY 73-6691 on cGMP formation in the parental cell line expressing soluble guanylate cyclase but not PDE9 in the absence (○) or presence of 0.1 μ M (▲) BAY 58-2667. Incubation in Ca^{2+} -free tyrode with 0.2 mM IBMX. C, stimulation of cGMP synthesis by BAY 58-2667 in the recombinant PDE9 cell line in the absence (○) or presence of 1 μ M (◆) and 10 μ M (▲) BAY 73-6691 in Ca^{2+} -free tyrode with 0.2 mM IBMX. Data are presented as mean values with S.D.

Discussion

To date, 21 mammalian cyclic nucleotide-specific phosphodiesterases grouped into 11 isoenzyme families have been isolated and characterized leading to the development of selective inhibitors for several of the PDE families, most notably for PDE3, PDE4, and PDE5. These inhibitors are being evaluated as potential therapeutics for a variety of clinical indications. PDE9 represents a new family of cGMP-specific PDEs that was identified very recently. Therefore, no selective PDE9 inhibitors have been published so far. To elucidate the physiological relevance of this specific phospho-

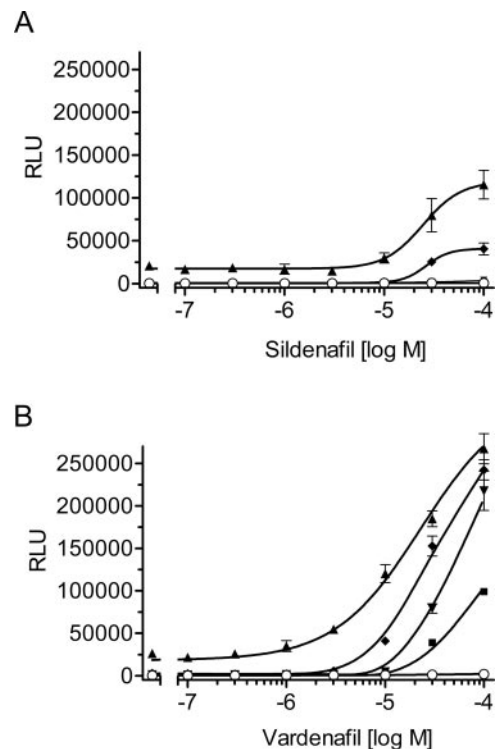


Fig. 5. Stimulation of the PDE9 cell line with sildenafil (A) and vardenafil (B) in the absence (○) or presence of 0.003 μ M (■), 0.01 μ M (▼), 0.03 μ M (◆), and 0.1 μ M (▲) BAY 58-2667. Cells were incubated for 6 min in Ca^{2+} -free tyrode with 0.2 mM IBMX. Measurements were started immediately after the addition of Ca^{2+} . Data are presented as mean values with S.D.

diesterase, potent and selective PDE9 inhibitors are necessary and highly desirable.

We report here the identification of BAY 73-6691, the first potent and selective PDE9 inhibitor, currently under preclinical development for the treatment of Alzheimer's disease. BAY 73-6691 has been shown to improve long-term potentiation and was found to be active in several animal models of cognition (Hendrix, 2005).

In this study, we have characterized the activity of BAY 73-6691 *in vitro*. BAY 73-6691 selectively inhibits isolated human and murine PDE9 activity with submicromolar IC_{50} values. In addition, BAY 73-6691 shows only moderate inhibition of other PDE isoenzymes. The closest IC_{50} values were for PDE1 and PDE11, with selectivity ratios of 25 and 47, respectively. The other PDE isoenzymes were all minimally affected by BAY 73-6691, with IC_{50} values more than 100-fold higher than for PDE9.

A discrepancy between the *in vitro* activity on the isolated enzyme and the cellular activity has been reported for some PDE inhibitors (Pon et al., 1998; Marko et al., 2002). Therefore, it is highly desirable to have a simple and sensitive method for the characterization of the cellular activity of PDE inhibitors. Therefore, we have generated and characterized a recombinant PDE9 cell line. In this cGMP reporter cell line, intracellular cGMP synthesis can be stimulated by soluble guanylate cyclase activation. Intracellular cGMP accumulation leads to the activation of the cyclic nucleotide-gated cation channel CNGA2 and influx of Ca^{2+} ions. The Ca^{2+} influx can easily be monitored by aequorin luminescence measurements. This assay system is very sensitive for intracellular cGMP accumulation, and it has proven to be very robust (Wunder et al., 2005).

PDE9 is not inhibited, even at very high concentrations, by the nonselective phosphodiesterase inhibitor IBMX (Fischer et al., 1998; Soderling et al., 1998). Therefore, it is possible to specifically monitor PDE9 activity in the presence of IBMX, which inhibits other phosphodiesterases that are endogenously expressed in the CHO cell line. Hence, this newly generated reporter cell line is a powerful tool for the characterization of the cellular activity of PDE9 inhibitors.

Under nonstimulated conditions, BAY 73-6691 did not significantly increase basal cGMP levels or luminescence signals in the PDE9 cell line. However, in combination with submaximal stimulating concentrations of the sGC activator BAY 58-2667, BAY 73-6691 induced concentration-dependent luminescence signals and intracellular cGMP accumu-

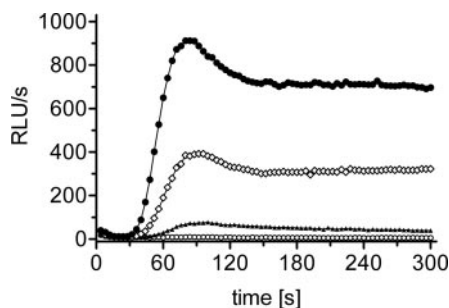


Fig. 6. Real-time detection of intracellular cGMP accumulation. Kinetics of the luminescence signals generated by $0.3 \mu\text{M}$ BAY 58-2667 in the presence of Ca^{2+} ions ("kinetic mode"): control (\circ), BAY 58-2667 (\blacktriangle), BAY 58-2667 with 0.2 mM IBMX (\diamond), and BAY 58-2667 with 0.2 mM IBMX and $1 \mu\text{M}$ BAY 73-6691 (\bullet).

lation. With increasing concentrations of BAY 58-2667, the BAY 73-6691-stimulated luminescence signals also increased. An optimal prestimulation of the cGMP synthesis was observed at $\sim 0.03 \mu\text{M}$ BAY 58-2667. Under these conditions, a maximal stimulation of the aequorin luminescence of approximately 100-fold could be induced by the addition of BAY 73-6691. Higher concentrations of BAY 58-2667 stimulated significant luminescence signals even in the absence of BAY 73-6691, and a diminished signal-to-background ratio was observed.

When the same set of experiments was repeated in the absence of the nonselective PDE inhibitor IBMX, approximately 3- to 10-fold higher concentrations of BAY 58-2667 were necessary to induce a comparable level of prestimulation compared with the experiments performed in the presence of IBMX. However, the luminescence signals induced by BAY 73-6691 in combination with BAY 58-2667 reached similar maximum levels, and the corresponding concentration-response curves were shifted rightward. This might be caused by the presence of endogenously expressed phosphodiesterases which are involved in cGMP turnover in these cells and are sensitive to inhibition by IBMX.

Recombinant expression of PDE9 in the cGMP reporter cell line reduced the ability of the sGC activator BAY 58-2667 to stimulate intracellular cGMP accumulation. In the parental cell line, BAY 58-2667 stimulated the aequorin luminescence with an EC_{50} value of $10.3 \pm 1.7 \text{ nM}$ (Wunder et al., 2005). In the recombinant PDE9 cell line, this EC_{50} value was shifted to $373 \pm 92 \text{ nM}$ in the absence of BAY 73-6691, which points to a high recombinant expression level and activity of PDE9 within this cell line. The addition of $10 \mu\text{M}$ BAY 73-6691 significantly enhanced the luminescence signals induced by BAY 58-2667 and reduced the EC_{50} value to $19.5 \pm 4.0 \text{ nM}$. These results imply that the PDE9 inhibitor BAY 73-6691 is able to inhibit most of the intracellular PDE9 activity at a concentration of $10 \mu\text{M}$, which is in good agreement with the inhibition curve using the isolated enzyme. Thus, BAY 73-6691 is able to efficiently penetrate cells and to inhibit the intracellular PDE9 activity.

In the parental CHO cell line, BAY 73-6691 did not significantly potentiate luminescence signals or intracellular cGMP accumulation induced by BAY 58-2667. These results imply that the endogenous expression of PDE9 in the CHO cell line is very low. In contrast, in the recombinant PDE9 cell line, BAY 73-6691 significantly potentiated the luminescence signals induced by different types of soluble guanylate cyclase activators, as exemplified here with BAY 58-2667 and BAY 41-2272, and induced leftward shifts of the corresponding concentration-response curves. These results were further confirmed by RIA measurements of the intracellular cGMP accumulation induced by BAY 58-2667 in the presence or absence of BAY 73-6691, which correlated very well with the luminescence measurements.

Depending on the particular experimental design, the minimum effective concentration of BAY 73-6691 that significantly enhanced the aequorin luminescence signals was in the range of 100 to 300 nM. Although the intracellular concentration of BAY 73-6691 is not known, these extracellular concentrations are comparable with or only slightly higher than the IC_{50} value of 100 nM for inhibition of the murine PDE9 *in vitro*, which suggests that the drug is rapidly and efficiently taken up by the cells.

The PDE5 inhibitors sildenafil and vardenafil have been reported as weak inhibitors of PDE9 (Corbin and Francis, 2002; Bischoff, 2004). As expected, both compounds stimulated concentration-dependent luminescence signals in our newly generated PDE9 cell line which could only be detected in the presence of submaximal stimulating concentrations of the sGC activator BAY 58-2667. The rank order of potency for the stimulation of luminescence signals by these two PDE5 inhibitors compared with BAY 73-6691 fits very well to their respective IC_{50} values for PDE9 inhibition in vitro: BAY 73-6691 > vardenafil > sildenafil. The activity of sildenafil and vardenafil on the PDE9 cell line could not be ascribed to PDE5 inhibition because tadalafil, a potent PDE5 inhibitor that does not inhibit PDE9 (Corbin and Francis, 2002), shows no activity in this experimental setting. We also tested several additional phosphodiesterase inhibitors that were also found to be inactive on our PDE9 cell line. Therefore, PDE9 activity and inhibition can be selectively monitored using this PDE9 reporter cell line.

We also determined the kinetics of cGMP accumulation induced by BAY 58-2667 in the absence or presence of the nonselective phosphodiesterase inhibitor IBMX and the PDE9 inhibitor BAY 73-6691. In the absence of any PDE inhibitor, BAY 58-2667 apparently stimulated only minor intracellular cGMP accumulation and, therefore, induced only small increases in the observed luminescence signal. Subsequent addition of IBMX and BAY 73-6691 significantly increased the luminescence signals induced by BAY 58-2667 and reduced the signal delay as well as the time to peak. It is interesting that the addition of 1 μ M BAY 73-6691, a concentration which does not fully inhibit PDE9, on top of IBMX more than doubled the observed luminescence signal, indicating that the intracellular PDE9 activity is very high.

Because intracellular PDE9 inhibition and cGMP accumulation can easily be monitored by aequorin luminescence measurements, the PDE9 reporter cell line is suitable for the search of new PDE9 inhibitors by ultra-high-throughput screening. In a similar approach, the parental cell line was used in an automated ultra-high-throughput screening assay, which ultimately led to the successful identification of a new class of soluble guanylate cyclase activators (Stasch et al., 2002; Wunder et al., 2005).

In summary, we have identified the first potent and selective PDE9 inhibitor, BAY 73-6691. Using our newly generated PDE9 reporter cell line, we show that BAY 73-6691 is able to efficiently penetrate cells and to inhibit intracellular PDE9 activity. Thereby, BAY 73-6691 is able to significantly amplify the cGMP responses elicited by stimulators of the nitric oxide/cGMP pathway.

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References

Alonso-Alija C, Heil M, Flubacher D, Naab P, Stasch JP, Wunder F, Dembowski K, Perzborn E, and Stahl E (2001), inventors; Bayer AG, assignee. Novel dicarboxylic

- acid derivatives with pharmaceutical properties. World patent WO2001018780-A. 2001 March 22.
- Beavo JA (1995) Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiol Rev* **75**:725–748.
- Bernabeu R, Schmitz P, Faillace MP, Izquierdo I, and Medina JH (1996) Hippocampal cGMP and cAMP are differentially involved in memory processing of inhibitory avoidance learning. *Neuroreport* **7**:585–588.
- Bischoff E (2004) Potency, selectivity and consequences of nonselectivity of PDE inhibition. *Int J Impot Res* **16**:S11–S14.
- Boess FG, Hendrix M, van der Staay FJ, Erb C, Schreiber R, van Staveren W, de Vente J, Prickaerts J, Blokland A, and Koenig G (2004) Inhibition of phosphodiesterase 2 increases neuronal cGMP, synaptic plasticity and memory performance. *Neuropharmacology* **47**:1081–1092.
- Conti M and Jin SL (2001) The molecular biology of cyclic nucleotide phosphodiesterases. *Prog Nucleic Acid Res Mol Biol* **63**:1–38.
- Corbin JD and Francis SH (2002) Pharmacology of phosphodiesterase-5 inhibitors. *Int J Clin Pract* **56**:453–459.
- Fischer DA, Smith JF, Pillar JS, Denis SHS, and Cheng JB (1998) Isolation and characterization of PDE9A, a novel human cGMP-specific phosphodiesterase. *J Biol Chem* **273**:15559–15564.
- Francis SH, Turko IV, and Corbin JD (2001) Cyclic nucleotide phosphodiesterases: relating structure and function. *Prog Nucleic Acid Res Mol Biol* **65**:1–52.
- Garthwaite J (1991) Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends Neurosci* **14**:60–67.
- Guipponi M, Scott HS, Kudoh J, Kawasaki K, Shibuya K, Shintani A, Asakawa S, Chen H, Lalioti MD, Rossier C, et al. (1998) Identification and characterization of a novel cyclic nucleotide phosphodiesterase gene (PDE9A) that maps to 21q22.3: alternative splicing of mRNA transcripts, genomic structure and sequence. *Hum Genet* **103**:386–392.
- Hendrix M (2005) Selective inhibitors of cGMP phosphodiesterases as procognitive agents. *BMC Pharmacol* **5** (Suppl 1):S5.
- Hendrix M, Bärfacker L, Erb C, Hafner FT, Heckroth H, Schauss D, Tersteegen A, van der Stay FJ, and van Kampen M (2004), inventors; Bayer Healthcare AG, assignee. 6-Cyclylmethyl- and 6-alkylmethyl-substituted pyrazolopyrimidines. World patent WO2004099211-A. 2004 Nov 18.
- Marko D, Merz KH, Kunz C, Muller A, Tarasova N, and Eisenbrand G (2002) Intracellular localization of 7-benzylamino-6-chloro-2-piperazino-4-pyrrolidino-pteridine in membrane structures impeding the inhibition of cytosolic cyclic AMP-specific phosphodiesterase. *Biochem Pharmacol* **63**:669–676.
- Pon DJ, Plant M, Tkach J, Boulet L, Muise E, Allen RA, and Rodger IW (1998) Characterization of CHO-K1 cells stably expressing PDE-IV enzymes. *Cell Biochem Biophys* **29**:159–178.
- Prickaerts J, de Vente J, Honig W, Steinbusch HW, and Blokland A (2002) cGMP, but not cAMP, in rat hippocampus is involved in early stages of object memory consolidation. *Eur J Pharmacol* **436**:83–87.
- Rentero C, Monfort A, and Puigdomenech P (2003) Identification and distribution of different mRNA variants produced by differential splicing in the human phosphodiesterase 9A gene. *Biochem Biophys Res Commun* **301**:686–692.
- Soderling SH, Bayuga SJ, and Beavo JA (1998) Identification and characterization of a novel family of cyclic nucleotide phosphodiesterases. *J Biol Chem* **273**:15553–15558.
- Soderling SH and Beavo JA (2000) Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions. *Curr Opin Cell Biol* **12**:174–179.
- Stasch JP, Becker EM, Alonso-Alija C, Apeler H, Dembowski K, Feurer A, Gerzer R, Minuth T, Perzborn E, Pleiß U, et al. (2001) NO-independent regulatory site on soluble guanylate cyclase. *Nature (Lond)* **410**:212–215.
- Stasch JP, Schmidt P, Alonso-Alija C, Apeler H, Dembowski K, Haerter M, Heil M, Minuth T, Perzborn E, Pleiss U, et al. (2002) NO- and haem-independent activation of soluble guanylyl cyclase: molecular basis and cardiovascular implications of a new pharmacological principle. *Br J Pharmacol* **136**:773–783.
- Straub A, Stasch JP, Alonso-Alija C, Benet-Buchholz J, Ducke B, Feurer A, and Füstner C (2001) NO-independent stimulators of soluble guanylate cyclase. *Bioorg Med Lett* **11**:781–784.
- van Staveren WC, Glick J, Markerink-van Ittersum M, Shimizu M, Beavo JA, Steinbusch HW, and de Vente J (2002) Cloning and localization of the cGMP-specific phosphodiesterase type 9 in the rat brain. *J Neurocytol* **31**:729–741.
- Wang P, Wu P, Egan RW, and Billah MM (2003) Identification and characterization of a new human type 9 cGMP-specific phosphodiesterase splice variant (PDE9A5). Differential tissue distribution and subcellular localization of PDE9A variants. *Gene* **314**:15–27.
- Wunder F, Stasch JP, Hütter J, Alonso-Alija C, Hüser J, and Lohrmann E (2005) A cell-based cGMP assay useful for ultra-high-throughput screening and identification of modulators of the NO/cGMP pathway. *Analyt Biochem* **339**:104–112.
- Yamada K, Hiramatsu M, Noda Y, Mamiya T, Murai M, Kameyama T, Komori Y, Nikai T, Sugihara H, and Nabeshima T (1996) Role of nitric oxide and cyclic GMP in the dizolcypine-induced impairment of spontaneous alternation behaviour in mice. *Neuroscience* **74**:365–374.

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