An Allosteric Potentiator of the Dopamine D1 Receptor Increases Locomotor Activity in Human D1 Knock-In Mice without Causing Stereotypy or Tachyphylaxis


Received July 15, 2016; accepted October 27, 2016

ABSTRACT

Allosteric potentiators amplify the sensitivity of physiologic control circuits, a mode of action that could provide therapeutic advantages. This hypothesis was tested with the dopamine D1 receptor potentiator DETQ [2-(2,6-dichlorophenyl)-1-((1S,3R)-3-(hydroxymethyl)-5-(2-hydroxypropan-2-yl)-1-methyl-3,4-dihydroisoquinolin-2(1H)-yl)ethan-1-one]. In human embryonic kidney 293 (HEK293) cells expressing the human D1 receptor, DETQ induced a 21-fold leftward shift in the cAMP response to dopamine, with a Kᵦ of 26 nM. The maximum response to DETQ alone was ~12% of the maximum response to dopamine, suggesting weak allosteric agonist activity. DETQ was ~30-fold less potent at rat and mouse D1 receptors and was inactive at the human D5 receptor. To enable studies in rodents, an hD1 knock-in mouse was generated. DETQ (3 mg/kg orally) caused a robust (~10-fold) increase in locomotor activity (LMA) in habituated hD1 mice but was inactive in wild-type mice. The LMA response to DETQ was blocked by the D1 antagonist SCH9166 and was dependent on endogenous dopamine. LMA reached a plateau at higher doses (30–240 mg/kg) even though free brain levels of DETQ continued to increase over the entire dose range. In contrast, the D1 agonists SKF 82958, A-77636, and dihydrexidine showed bell-shaped dose-response curves with a profound reduction in LMA at higher doses; video-tracking confirmed that the reduction in LMA caused by SKF 82958 was due to competing stereotyped behaviors. When dosed daily for 4 days, DETQ continued to elicit an increase in LMA, whereas the D1 agonist A-77636 showed complete tachyphylaxis by day 2. These results confirm that allosteric potentiators may have advantages compared with direct-acting agonists.

Introduction

Allosteric receptor potentiators increase the affinity and/or efficacy of agonists by binding to a site separate from the orthosteric agonist binding site. Because the response to the potentiator is dependent on the presence of agonist, a potentiator should amplify endogenous control circuits, acting when and where the endogenous agonist is released. (We use the term “potentiator” rather than the more general “positive allosteric modulator” to emphasize that the present study focuses on the consequences of amplifying the response to...
endogenous agonist.) In contrast, an exogenously administered agonist will activate all the receptors to which it has access for as long as it is present. Given their more physiologic mode of action, allosteric potentiators may have advantages over direct-acting agonists in terms of margin of safety and side-effect profile (Bruns and Fergus, 1990; Epping-Jordan et al., 2007).

It is not difficult to envision other advantages of potentiators. For instance, potentiators may be self-limiting in their actions because they depend on the release of endogenous agonist, which can be down-regulated by the organism in the event of overstimulation. In addition, because the effect of a potentiator will only occur during intermittent release of endogenous agonist, there may be a reduced potential for desensitization. However, there are only a few cases in which these predicted advantages have been documented in vivo, a prime example being the improved safety of benzodiazepines (GABA<sub>A</sub> receptor potentiators) compared with the older barbiturates (GABA<sub>B</sub> receptor full allosteric agonists) (Harvey, 1980). Despite the proliferation of allosteric modulators of G-protein-coupled receptors in recent years (May and Christopoulos, 2003; Raddatz et al., 2007), we have been unable to identify examples in which the predicted advantages of G-protein-coupled receptor potentiators have been verified experimentally by side-to-side comparison with direct-acting agonists.

The present report describes initial in vitro and in vivo profiling of DETQ (2-(2,6-dichlorophenyl)-1-(1S,3R)-3-(hydroxymethyl)-5-(2-hydroxyprop-2-yl)-1-methyl-3,4-dihydroisoquinolin-2(1H)-yl)ethan-1-one, a novel allosteric potentiator of the dopamine D1 receptor. DETQ causes a roughly 10-fold increase in locomotor activity (LMA) in transgenic mice expressing the human D1 receptor (hD1 mice) but is inactive in wild-type mice, which have a ~30-fold lower affinity for DETQ. The response depends on endogenous dopamine tone because it is blocked by a selective D1 antagonist and is not seen in animals in which dopamine levels have been depleted by pretreatment with reserpine.

Interestingly, although D1 agonists show bell-shaped dose-response curves due to competing stereotypical behavior at higher doses, DETQ maintains a plateau in its dose-response curve even though free brain concentrations continue to rise, indicating that the response is self-limiting. In addition, unlike some D1 agonists, DETQ does not show rapid tolerance development on repeated dosing. These results provide experimental confirmation that allosteric potentiators may have clinically relevant advantages compared with orthosteric agonists. The use of DETQ to explore potential therapeutic applications for D1 potentiators will be described in a second paper in this series (Bruns et al., submitted for publication).

**Materials and Methods**

**Materials.** DETQ (referred to as example 3) was synthesized as described elsewhere (Beadle et al., 2014). The acronym DETQ is derived from key chemical groups in the structure: Dichlorophenylacetyl-5-(1-hydroxy-1-methyl-ethyl) Tetrahydroisoquinoline. Dopamine agonists and antagonists were purchased from Sigma-Aldrich (St. Louis, MO) except for A77636 (1R,3S-1-adamantyl)-1-(aminomethyl)-3,4-dihydro-1H-isochromene-5,6-diol;hydrochloride, which was purchased from Tocris (Bristol, United Kingdom). [3H]-SCH23390 ([66S,13βR]-11-chloro-7-methyl-5,6,7,8,9,13b-hexahydropyrido[1,2-a][3]benzazepin-12-ol) was obtained from PerkinElmer (Waltham, MA) (NET930; specific activity = 84.3 Ci/mM).

**Human D1 Cell Line (HER293).** A human embryonic kidney cell line (HEK293) stably expressing the human D1 receptor was generated via retroviral gene transduction using the pBABE-bleo vector and the Phoenix retroviral system. The cells were grown in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (DMEM/ F-12) (human D1; GIBCO, Waltham, MA) or DMEM (rat D1; GIBCO) supplemented with 10% fetal bovine serum (FBS), 20 mM HEPES, 2 mM glutamate, and 150 μg/ml zeatin at 37°C in 5% CO<sub>2</sub>.

**Measurement of cAMP Response in hD1 HER293 Cells.** At approximately 80% confluency, the cells were harvested using 0.25% trypsin/EDTA, suspended in FBS plus 8% dimethylsulfoxide (DMSO), and stored in liquid nitrogen. On the day of the assay, the cells were thawed and resuspended in STIM buffer (Hanks Balanced Salt Solution supplemented with 0.1% bovine serum albumin, 20 mM HEPES, 200 μM IBMX, and 100 μM ascorbic acid). For the potentiator-mode assay, DETQ was serially diluted (1:3) in DMSO and then further diluted 1:40 into STIM buffer lacking dopamine (agonist mode) or containing 2X an EC<sub>20</sub> (24 nM, or 12 nM final) concentration of dopamine (potentiator mode). (*Note:* In this dilution scheme, the test compound does not come in contact with water until it is diluted into 2X the final concentration. We adopted this scheme because we observed precipitation with compounds from the acyl-tetrahydroisoquinoline series when they were serially diluted in aqueous buffer.). A 25-μl volume of compounds stock solution was mixed with 25 μl of cell suspension (1250 cells) and dispensed into each well of 96-well, half-area plates; the final DMSO concentration was 1.25%. The plates were incubated at room temperature for a total reaction time of 60 minutes.

Cyclic AMP production was quantified using homogeneous time-resolved fluorescence detection (Ciabio, Bedford, MA) according to the vendor’s instructions: lysis buffer containing anti-cAMP cryptate (25 μl) and D2-conjugate (25 μl) was added to the wells, the plates were incubated for an additional 60–90 minutes, and time-resolved fluorescence was detected using an EnVision plate reader (PerkinElmer).

Fluorescence data were converted to cAMP concentrations using a cAMP standard curve and were analyzed using a four-parameter nonlinear logistic equation (ActivityBase v5.3.1.22; IBDSS, Guildford, United Kingdom). For potentiator-mode concentration–response curves, the results were expressed as the percentage of the window between an EC<sub>20</sub> concentration of dopamine alone and the maximum response to dopamine (defined by 5 μM final concentration).

Alpha factor (maximum leftward shift) and K<sub>e</sub> were calculated by fitting the allosteric Schild ratio EC<sub>50</sub>/EC<sub>20</sub> to the equation y = (K<sub>e</sub> + αx) / (K<sub>e</sub> + x), where y is the Schild ratio and x is the potentiator concentration (Ehlert, 1988). In the Schild plot, the x and y axes are both logarithmic.

**Measurement of cAMP Response in rD1 HER293 Cells.** A stable HER293 cell line expressing the rat D1 receptor was created as described earlier for the hD1 cell line, and the cAMP assay was performed as for the hD1 line except that the EC<sub>20</sub> concentration of dopamine was 5 nM (final) and the cell concentration was 2500 per well.

**Species Comparison in Transiently Expressed D1 Receptors.** The human, rhesus monkey, dog, or mouse D1 receptor was cloned in the Phoenix retroviral system. The cells were grown in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (DMEM/F-12) (human D1; GIBCO, Waltham, MA) or DMEM (rat D1; GIBCO) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, and 150 μg/ml zeatin at 37°C in 5% CO<sub>2</sub>.
**D2, β1, β2, β3, and 5HT6 cAMP Assays.** The D2, β1, β2, β3, and 5HT6 cAMP assays were performed at DiscoverRx using the protocols described on their website (www.discoverx.com).

**3H-SCH23390 Binding to Human D1 HEK293 Cell Membranes.** Binding studies were performed using crude membrane homogenates from HEK293 cells stably expressing human D1 receptors. All test solutions were prepared in assay buffer (50 mM Tris-HCl, pH 7.4; 120 mM NaCl, and 1 mM EDTA). Concentration–response curves for dopamine (alone and with three DETQ concentrations) as well as a curve for DETQ alone were preincubated with human D1/HEK293 receptor membranes for 45 minutes at 25°C. Subsequently, 3H-SCH23390 was added to a final concentration of 0.6 nM and a final assay volume of 500 μl, with a final DMSO concentration of 1%. Nonspecific and total binding were determined in the presence of 10 μM (+)-butaclamol or assay buffer, respectively.

The reaction was incubated for 75 minutes at 25°C, then bound radioligand was separated from free and collected on a glass fiber filter (GF/C) (PerkinElmer) presoaked in 0.3% polyethyleneimine (Sigma-Aldrich) using a TomTec cell harvester machine. The filter was embedded with Meltix A solid scintillant (PerkinElmer) and counted on a Wallac Tri-Lux Microbeta (Perkin-Elmer).

Specific binding was calculated by subtracting nonspecific binding from total binding. Specific binding in the presence of test compounds was expressed as a percentage of control specific binding. EC50 values for dopamine alone and in the presence of DETQ were calculated using GraphPad software (La Jolla, CA).

**Generation of Humanized D1 Mice by Gene Replacement.** The targeting vector was based on an approximately 11-kilobase (kb) genomic fragment of the mouse Drd1a gene locus retrieved from the C57Bl/6J RP23 BAC library. A codon-optimized human DRD1 cDNA was synthesized and used to replace the entire mouse Drd1a open reading frame in exon 2. Exon 2 itself was flanked by loxP sites to allow for the generation of a null allele after Cre-mediated recombination [this option was not used in the current studies]. Immediately downstream of the 5’ loxP site in intron 2, an Frt-flanked neomycin resistance cassette was inserted. The 3-kb upstream region of the 5’ loxP site served as a short arm of homology, while approximately 4-kb downstream of the 3’ loxP site constituted the long homology arm. A thymidine kinase cassette was inserted at the far 3’ end to allow for negative selection against random integration.

The quality-tested C57BL/6NTac embryonic stem (ES) cell line was grown on a mitotically inactivated feeder layer composed of mouse embryonic fibroblasts in DMEM High Glucose medium containing 20% FBS (PAN Biotech GmbH) and 1200 U/ml leukemia inhibitory factor (ESG 1107; Millipore, Billerica, MA). We electroporated (Gene Pulser; Bio-Rad Laboratories, Hercules, CA) 1 × 107 cells and 30 μg of linearized DNA targeting vector at 240 V and 500 μF. Positive selection with G418 (200 μg/ml) started on day 2 after electroporation. Selection with ganciclovir (2 mM) started on day 5 after transfection. G418 selection with G418 (200 μg/ml) started on day 2 after electroporation.

**D1 Potentiator Increases Locomotor Activity in hD1 Mice**

Fig. 1. Chemical structure of DETQ.
sections (20 μm) were cut and thaw-mounted on Fisherbrand Super-
frost/Plus microscope slides (Thermo Fisher Scientific, Waltham, MA).
Sections were allowed to dry at room temperature then stored
frozen at –80°C until used for autoradiography. After 15 minutes
of preincubation, binding was conducted in assay buffer (50 mM Tris-
HCl, pH 7.4, 120 mM NaCl, 1 mM MgCl2, 5 mM KCl, 2 mM CaCl2)
containing various concentrations of [3H]-SCH23390 [8-chloro-3-methyl-
5-phenyl-1,2,4,5-tetrahydro-3-benzazepine-7-ol] (0.05–10 nM, Perkin
Elmer). Nonspecific binding was determined by the addition of 10 μM
(+)-butaclamol. After 60 minutes of incubation at 22°C, the sections
were washed in cold assay buffer (2 × 5 minutes), briefly dipped in water,
and air dried. The slides were exposed to Fuji BAS-TR2025 phosphor-
imaging plates and digitized with a BAS5000 scanner (FujiFilm,
Tokyo, Japan). Quantitative autoradiogram analysis was performed using a computer-assisted image analyzer
(Imaging Research, St. Catharines, Ontario, Canada).

Locomotor Activity Measurement in Habituat Mice. Male
hD1 mice, 8–10 weeks old (weighing 18 to 25 g at the time of the
experiment), were used. Monitoring of mouse LMA took place in
end of the session, the mice were removed from the chambers, dosed

with compound (vehicles for DETQ and SKF 82958 were the same as
for LMA experiments), and immediately placed back into the cham-
bers for a 60-minute test session. All behavioral activity was moni-
tored and scored by the HomeCageScan system.

D1 Receptor Internalization. These experiments were per-
ded at DiscoverRx. The assay uses a PathHunter cell line in which
two inactive complementary portions of β-galactosidase are expres-
sed. One β-galactosidase fragment is fused to the human D1 receptor,
and the other is expressed in the endosome. Enzyme activity is only
seen when the two fragments are contiguous.

Cells were seeded in a 20-μl volume into white 384-well plates and
preincubated at 37°C for 16 hours. Test compounds were diluted to
5X final concentration in assay buffer (OptiMEM; Invitrogen) plus
0.05% bovine serum albumin) A 5-μl volume of test sample was added,
followed by incubation for 30 minutes at room temperature. The final
DMSO concentration was 1%.

Subsequently, 5 μl of 6X EC20 of dopamine (407 nM final con-
centration) was added, and the incubation continued for 180 minutes
at room temperature. The assay signal was generated by the addition
of 15 μl of PathHunter detection reagent cocktail, followed by 60 minutes
of incubation at room temperature.

The plates were read with a PerkinElmer Envision instrument
fusing chemiluminescent signal detection. The percentage of modula-
tion was calculated using the following formula with RLU (relative
light units): % Modulation = 100 × [(Mean RLU of test sample – Mean
RLU of EC20 dopamine)/(Mean RLU of max dopamine – Mean RLU of
EC20 dopamine)].

Measurement of Free Brain Concentrations of DETQ. Im-
mediately after the LMA measurements described in Fig. 8, the brains
were removed, and brain homogenate concentrations were deter-
mined by liquid chromatography with tandem mass spectrometry.
The unbound fraction in brain was determined by equilibrium dialysis

<table>
<thead>
<tr>
<th>TABLE 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative potency = potentiator mode RA divided by agonist mode RA.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mode</th>
<th>EC50 (nM)</th>
<th>Top (%)</th>
<th>RA</th>
<th>Relative Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potentiator</td>
<td>5.83</td>
<td>95.2</td>
<td>16.33</td>
<td>42.8</td>
</tr>
<tr>
<td>Agonist</td>
<td>30.4</td>
<td>11.6</td>
<td>0.38</td>
<td>—</td>
</tr>
</tbody>
</table>

Fig. 2. Increase in cAMP by DETQ in the presence and absence of an EC20 concentration of dopamine in HEK293 cells expressing the human D1 receptor. Potentiator mode (with 12 nM dopamine): relative EC50 5.83 ± 1.47 nM (S.E.), top 95.2% ± 3.2%, n = 5, data from five separate experiments. Agonist mode (without dopamine): relative EC50 30.4 ± 6.9 nM, top 11.6% ± 0.4%, n = 8, data from a single experiment. In three additional agonist-mode experiments, efficacy of DETQ in the absence of dopamine ranged from 2.7% to 13%, suggesting significant variability in this parameter.
in a mouse brain homogenate mixture after incubation of the compound for 4.5 hours (Kalvass et al., 2007).

The unbound brain concentrations were calculated from the following equation:

\[
\text{Total brain concentration} \times \text{Unbound fraction in brain} = \frac{\text{Unbound brain concentration}}{\text{Cu, brain}}
\]

**Results**

**D1 Potentiator Activity of DETQ In Vitro.** The acyl-tetrahydroisoquinoline DETQ (Fig. 1) originated from chemical modification of a high-throughput screening hit. In HEK293 cells expressing the human D1 receptor, DETQ increased cAMP with an EC50 of 5.8 nM when tested in the presence of an EC20 concentration of dopamine (Fig. 2). In the absence of dopamine, the response to DETQ was 11.6% of the maximum response to dopamine (Fig. 2), and the EC50 in the absence of dopamine was shifted 6-fold to the right, indicating that under these conditions DETQ behaved mostly as a potentiator, with marginal allosteric agonist activity.

Relative activity (RA), the efficacy divided by the EC50, is a simple measure of potency that is determined by the initial slope of the concentration–response curve (Ehlert, 2008). Comparing RA for potentiator versus agonist mode, DETQ was 43-fold more potent in potentiator mode (Table 1). Dopamine curve-shift experiments (Fig. 3) showed a 20.8-fold leftward shift of the response to dopamine, with a small (22%) increase in the maximum response. The maximum overall sensitization to dopamine, expressed as leftward shift (a) times upward shift (b), was 20.8 \( \times 1.22 = 25.4 \) fold.

Compared with the human D1 receptor, the functional affinity of DETQ for the rat D1 receptor was 28-fold less (EC50 162 \( \pm 18 \) nM, \( n = 4 \)). In addition, the maximum response relative to that of dopamine was only 39.9\% \( \pm 3.7\% \), compared with 98.6\% \( \pm 4.2\% \) for the human D1. A similar loss of potency was seen for the mouse D1 receptor (Fig. 4 and Table 2), but the rhesus monkey and dog D1 receptors showed nearly identical potency to their human counterpart.

DETQ was inactive at the human D5 receptor in potentiator mode up to 25 \( \mu \)M, and was inactive at the D2, \( \beta_1, \beta_2, \beta_3, \) and 5HT6 receptors in potentiator, agonist, and antagonist mode up to 10 \( \mu \)M (Supplemental Data, Supplemental Table 1).

DETQ was also inactive at 10 additional targets from the standard Lilly selectivity panel, but showed modest activity at the 5HT2B receptor in antagonist and potentiator mode (respectively 56% inhibition and 48% stimulation at 10 \( \mu \)M, but only 13% and 5% at 1 \( \mu \)M).

DETQ increased the affinity of dopamine to inhibit binding of the D1 receptor antagonist SCH23390 (Fig. 5), indicating that DETQ directly affects the affinity of dopamine for the orthosteric binding site of the D1 receptor.

**Generation of a Human D1 Receptor Knock-in Mouse.** Initial attempts to show in vivo activity of the acyltetrahydroisoquinolines in rat and mouse were unsuccessful, as expected given the poor affinity of these compounds for rodent D1 receptors in vitro. To overcome this problem, we commissioned the generation of a transgenic mouse in which the human D1 receptor replaces its murine counterpart. Behavior and breeding of the homozygotes was overtly normal, and the colony was maintained as the homozygote genotype. Distribution of D1 receptor binding in the brain closely resembled distribution in wild-type C57Bl/6 NTac.

**TABLE 2**

<table>
<thead>
<tr>
<th>Species</th>
<th>EC50 (nM)</th>
<th>SE</th>
<th>Top (%)</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>11.4</td>
<td>1.6</td>
<td>75.6</td>
<td>1.8</td>
</tr>
<tr>
<td>Dog</td>
<td>8.6</td>
<td>1.3</td>
<td>85.2</td>
<td>2.0</td>
</tr>
<tr>
<td>Rhesus</td>
<td>12.0</td>
<td>1.6</td>
<td>80.0</td>
<td>1.9</td>
</tr>
<tr>
<td>Mouse</td>
<td>312.0</td>
<td>47.4</td>
<td>50.0</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Fig. 3. Leftward shift by DETQ of the cAMP response to dopamine in HEK293 cells expressing the human D1 receptor. (A) Dopamine concentration–response curves at different concentration of DETQ. (B) Allosteric Schild plot for DETQ. Best-fit values: \( a \)-factor 20.8 \( \pm 1.9 \), \( K_B 26.0 \pm 10.8 \) nM.

Fig. 4. Species differences in affinity for DETQ. Human, dog, rhesus, and mouse D1 receptors were transiently expressed in HEK293 cells. Best-fit parameters from nonlinear curve-fitting are shown in Table 2.

Fig. 5. Leftward shift by DETQ of the cAMP response to dopamine in HEK293 cells expressing the human D1 receptor. (A) Dopamine concentration–response curves at different concentration of DETQ. (B) Allosteric Schild plot for DETQ. Best-fit values: \( a \)-factor 20.8 \( \pm 1.9 \), \( K_B 26.0 \pm 10.8 \) nM.
animals (Fig. 6), although D1 receptor density was about 50% lower than in wild-type mice (Fig. 7); a similar difference in receptor density was seen in striatal homogenate binding (results not shown).

The IC_{50} values of SCH23390, (+)-butaclamol, and dopamine were similar in wild-type and hD1 mice (Table 3). The concentration–response curve for dopamine in \textsuperscript{3}H-SCH23390 binding was shifted to the left by GTP (data not shown), indicating that coupling to G_s was normal. Overall there were no obvious behavioral differences between the two genotypes, and they showed similar behavioral and neurochemical responses to dopamine D1 agonists and antagonists (not shown).

**Increase in LMA Caused by DETQ in hD1 Mice.** When mice are introduced to a new environment, they show an initial burst of exploratory behavior. To achieve a lower baseline of activity, all animals were habituated for 1 hour before recording responses to treatment. Transgenic hD1 mice showed a 10-fold dose-related increase in LMA in response to DETQ given orally (Fig. 8). A statistical analysis of responses to various D1 potentiators from the same series as DETQ (W.H. Anderson, unpublished results) showed that a 4-fold increase in LMA consistently showed statistical significance versus vehicle \((P < 0.05)\); the dose causing a 4-fold increase in LMA \((ED_{4X})\) was therefore used as an index of potency. The \(ED_{4X}\) for DETQ was 7.2 mg/kg. In contrast to the robust LMA response in hD1 mice, in wild-type C57Bl/6 mice 30 mg/kg DETQ increased LMA by only 32%, failing to reach statistical significance \((P = 0.07)\) (Fig. 8).

D1 antagonists are known to lower basal LMA (Chipkin et al., 1988), indicating that LMA is dependent on ongoing dopamine release. Given that DETQ showed minimal allosteric agonist activity in vitro, it seemed likely that the LMA response to DETQ was mediated by potentiation of endogenous D1 tone. Confirming this expectation, the LMA response to DETQ was blocked by the D1/D5-selective antagonist SCH39166 (Fig. 8). In nonhabituated mice, SCH39166 lowered basal exploratory activity at 10 μg/kg and lowered DETQ-stimulated activity at 30 μg/kg. The response to DETQ was also absent in hD1 mice whose dopamine levels were profoundly depressed by treatment with reserpine (Bruns et al., submitted for publication).

**Bell-Shaped LMA Response to D1 Agonists but Not to DETQ.** D1 agonists are known to show bell-shaped (or inverted U-shaped) dose–response relationships in various behavioral tests, with activity decreasing at higher doses.
The drop-off in LMA at higher doses is typically caused by competition from hyperactivity, with intense stereotyped behaviors such as repetitive grooming (Arnsten et al., 2016) that are incompatible with translational movement. As expected, bell-shaped curves were seen in the hD1 mice with the three D1 agonists, A-77636, SKF 82958, and dihydroextidine (Fig. 9). (Note: A fourth D1 agonist, ABT-431 [adrenergic hydrochloride], could not be tested in a wide dose range because it induced seizures in both hD1 and wild-type mice at higher doses; K.A. Svensson, unpublished observation.)

In contrast, the LMA response to DETQ did not show any drop-off at higher doses up to 240 mg/kg (Fig. 8). Free brain levels of DETQ continued to rise at the highest doses (Fig. 10), indicating that the lack of a bell-shaped curve was not due to limited brain exposure. The EC\textsubscript{50} value of 9.2 nM in LMA in vivo (Fig. 10B) agrees well with the EC\textsubscript{50} of 5.8 nM for potentiation of dopamine-induced cAMP production in vitro (Fig. 2).

**Stereotyped Behavior in hD1 Mice Treated with DETQ or D1 Agonists.** Video-tracking was used to compare grooming (an index of stereotyped behavior) with horizontal locomotion and rearing (both indices of exploratory behavior). The D1 agonists SKF 82958 and A-77636 showed an increase in horizontal locomotion and rearing at lower doses; these behaviors were supplanted by grooming at higher doses (Fig. 11). In contrast, DETQ also showed an increase in horizontal locomotion and rearing at lower doses, but this increase was maintained up to the highest dose tested; grooming behavior showed only modest changes that did not reach statistical significance compared with vehicle control (Fig. 11).

**LMA Responses to DETQ and A-77636 on Repeated Dosing.** Responses to long-acting D1 receptor agonists such as A-77636 (Kebabian et al., 1992) are known to show tachyphylaxis on repeated administration (Gulwadi et al., 2001). In agreement with the literature, the LMA response to A-77636 was completely abolished by day 2 of once-a-day dosing (Fig. 12). In contrast, the LMA response to DETQ was undiminished on day 4 of once-a-day dosing. To check whether the lack of tachyphylaxis was due to the shorter half-life of DETQ, the study was repeated using twice-daily oral dosing of 240 mg/kg. The unbound brain levels of DETQ were 19.8 ± 1.5 nM (n = 3) at 6 hours and 30.8 ± 9.4 nM (n = 3) at 12 hours. Both values are substantially in excess of the in vitro EC\textsubscript{50} of 5.8 nM for DETQ in the cAMP assay. No tachyphylaxis to DETQ was seen under these conditions; instead, there was a small but statistically significant sensitization. Sensitization on repeated dosing has been previously shown for other dopaminergic agents (Turgeon et al., 1997).

**D1 Receptor Internalization in Response to DETQ and A-77636.** If DETQ potentiated the cAMP response to dopamine but not the internalization response, this could explain the lack of desensitization seen with DETQ in vivo. To resolve this question, DETQ and A-77636 were tested for effects on D1 receptor internalization. DETQ clearly potentiated the internalization response to dopamine (Fig. 13). (Note: Complexation of the D1 receptor with β-arrestin is an earlier response along the pathway that leads to internalization. Potencies of dopamine, A-77636, and DETQ in stimulating the β-arrestin response were similar to their potencies in stimulating internalization (B.A. Heinz, unpublished observation).) Although DETQ showed a lower maximum internalization rate than A-77636, it should be noted that the maximum response to a potentiator depends on the agonist concentration (for instance, see maximum responses to DETQ at various fixed dopamine concentrations in Fig. 3) and will be higher in the presence of higher agonist concentrations.

A quantitative comparison between DETQ and A-77636 in their effects on the cAMP and internalization pathways was performed using the RA ratio method (Ehlert, 2008). Both compounds were roughly an order of magnitude more potent in stimulating the cAMP pathway than in stimulating the internalization pathway (Table 4). The ratio of pathway preferences (a measure of relative degree of pathway preference between the two compounds) was 2.0 ± 1.4 (Table 4), not significantly different from the ratio of 1.0 expected if the pathway preference was the same for both compounds. These results indicate that DETQ is capable of potentiating the internalization response to dopamine, showing approximately the same degree of pathway preference as the agonist A-77636.

**Discussion**

This study describes DETQ, a novel, highly selective D1 potentiator. DETQ causes a robust increase in LMA in transgenic mice expressing the human D1 receptor; this response is
dependent on endogenous dopaminergic tone. Unlike the response to D1 agonists, the LMA response to DETQ does not drop off at higher doses and does not show tachyphylaxis. These results provide experimental confirmation of some predicted advantages of potentiators over direct-acting agonists.
Predicted Advantages of Allosteric Potentiators.

Original research papers (Bruns and Fergus, 1990; Malherbe et al., 2008) and reviews (Christopoulos and Kenakin, 2002; May and Christopoulos, 2003; Epping-Jordan et al., 2007; Arnsten et al., 2016) have emphasized the predicted therapeutic advantages of allosteric potentiators. By amplifying the effectiveness of physiologic circuits, potentiators should give a needed boost to a hypoactive system while allowing the normal feedback loops to remain in control. Because they do not disturb inactive circuits, potentiators should be better tolerated over a wider dose range and be less prone to desensitization compared with direct-acting agonists. However, direct clinical or preclinical evidence confirming these predictions has been difficult to acquire. Using the new tool DETQ, the present study investigates two of these predictions in transgenic mice expressing the human D1 receptor.

The first prediction is the concept that the actions of allosteric potentiators should be self-limiting, because an overstimulated organism can rely on negative feedback loops to reduce neurotransmitter release and de-escalate the system. The D1 system provides an ideal platform for testing this idea because D1 agonists have bell-shaped dose–response curves, stimulating motor activity and cognitive behaviors at moderate doses but inhibiting them at higher doses (Goldman-Rakic et al., 2000; Yang and Chen, 2005). The inhibition of LMA is due to recruitment of stereotyped behaviors such as intense grooming that compete with forward locomotion.

In contrast to the response to D1 agonists, our results show that the LMA response to DETQ reaches a plateau and does not show a declining phase at higher doses. The absence of a declining phase was not due to limited exposure because free brain levels of DETQ continued to rise linearly up to the

Fig. 10. Unbound brain levels of DETQ measured immediately after LMA testing. (A) Dose dependence of calculated unbound brain levels. (B) Relationship between LMA and unbound brain levels. Each point is the mean of brain levels from three animals. The curve is the best fit to a four-parameter model. Best-fit values ± S.E. are: bottom 40% ± 127% of control; top 1,590% ± 130% of control; EC₅₀ 9.2 ± 3.1 nM; Hill coefficient 0.88 ± 0.21.

Fig. 11. Horizontal motion, rearing, and grooming in response to SKF 82958 and DETQ measured by video-tracking. The values shown are mean ± S.E., n = 7–8. Results for drug-treated animals were compared with vehicle using Dunnett’s multiple comparisons test: *P < 0.05, ***P < 0.001, ****P < 0.0001.
The lack of a declining phase implies that allosteric potentiators may have a broader therapeutic window, a potential major advantage over direct agonists. (Note: An alternative interpretation of the lack of stereotypy with DETQ could be that the stereotypy is mediated by the D5 receptor, at which DETQ is inactive. We view this as a less likely explanation given the powerful stereotypy seen with D1/D5 agonists and the very low density of D5 receptors in the brain.)

The second prediction is related to the observation that endogenous control systems are often active in bursts (Paladini and Roeper, 2014), turning on when a stimulus is needed and turning off during resting periods. Many systems have mechanisms of desensitization built in to prevent chronic, continuous activation. Because agonists cause continuous activation, they may trigger these desensitization mechanisms. This is the case for long-acting D1 agonists, which show marked tachyphylaxis when dosed for multiple days (Gulwadi et al., 2001). In contrast, a D1 potentiator would be predicted to show less desensitization on chronic exposure because it would only have an effect during those transient periods when dopamine tone was active. Such was the case in our studies. The agonist A-77636 showed a complete loss of responsiveness by day 2 of daily dosing, while DETQ still showed full responsiveness at day 4.

An alternative interpretation of the lack of stereotypy with DETQ could be that the stereotypy is mediated by the D5 receptor, at which DETQ is inactive. We view this as a less likely explanation given the powerful stereotypy seen with D1/D5 agonists and the very low density of D5 receptors in the brain.)

The second prediction is related to the observation that endogenous control systems are often active in bursts (Paladini and Roeper, 2014), turning on when a stimulus is needed and turning off during resting periods. Many systems have mechanisms of desensitization built in to prevent chronic, continuous activation. Because agonists cause continuous activation, they may trigger these desensitization mechanisms. This is the case for long-acting D1 agonists, which show marked tachyphylaxis when dosed for multiple days (Gulwadi et al., 2001). In contrast, a D1 potentiator would be predicted to show less desensitization on chronic exposure because it would only have an effect during those transient periods when dopamine tone was active. Such was the case in our studies. The agonist A-77636 showed a complete loss of responsiveness by day 2 of daily dosing, while DETQ still showed full responsiveness at day 4.

The potential therapeutic advantages described here apply to allosteric potentiators and should be less applicable (or completely inapplicable) to allosteric agonists, defined as allosteric modulators that cause a full response in the absence of agonist. A pertinent clinical example is seen with positive...
allosteric modulators of the GABA<sub>A</sub> receptor. Barbiturates are full allosteric agonists at the GABA<sub>A</sub> receptor (Rho et al., 1996) and can be lethal in overdose due to respiratory depression (Harvey, 1980). In contrast, benzodiazepines are nearly pure allosteric potentiators (Barker et al., 1986) and do not cause severe respiratory depression on overdose unless combined with synergists such as alcohol (Harvey, 1980). Because of this advantage, benzodiazepines (along with structurally different agents that act at the same site) have largely replaced barbiturates in clinical practice (Harvey, 1980). With respect to the above issue, it should be noted that DETQ was 43 times more potent as a potentiator than as an allosteric agonist, indicating that under practical conditions it can be regarded as a pure potentiator. In addition, DETQ did not reverse hypokinesia in hD1 mice pretreated with a high dose of the dopamine-depleting agent reserpine (Bruns et al., submitted for publication), indicating that DETQ lacks direct-acting D1 agonist effects in vivo.

**D1 Potentiators as Pharmacologic Tools.** DETQ is the first example of a new class of agents that selectively potentiate the function of the dopamine D1 receptor in vivo. D1 receptors have a prominent role in cognition, motivation, and motor activity (Sibley, 1999; Goldman-Rakic et al., 2000; Beaulieu and Gainetdinov, 2011). Based on this role, D1 potentiators may be useful for treatment of several central nervous system disorders, including Parkinson’s disease, Alzheimer’s disease, schizophrenia, major depressive disorder, attention deficit hyperactivity disorder, and narcolepsy. In vivo results supporting these indications are described in a separate study (Bruns et al., submitted for publication).

Several features make DETQ a useful tool for studying the D1 receptor. Its >1000-fold selectivity for the D1 receptor over the closely related D5 is particularly striking, given that numerous attempts to design agonists that distinguish between the two receptors have shown limited success (Giorgioni et al., 2008). Similarly high selectivity versus other related receptors such as D2, 5HT6, and the β-adrenergic receptors provides hope that DETQ will be a highly specific pharmacologic tool.

Another advantage of DETQ is its ability to achieve high free brain levels after oral dosing, up to ~600 nM at 240 mg/kg (Fig. 10). This is nearly two orders of magnitude higher than the free brain EC<sub>50</sub> of 9.2 nM, implying ample headroom for dosing in animal studies. Furthermore, no overt adverse behavioral effects were observed at any of the doses, consistent with the lack of off-target activity seen in vitro.

A potential disadvantage of DETQ is its lower affinity for the mouse and rat D1 receptors, which necessitated the generation of a transgenic hD1 mouse to show in vivo activity. However, our results with the dog (Table 2) and rabbit (unpublished observations) D1 receptors suggest that the lower affinity is limited to rodents. Efficacy in the nonhuman primate has also been confirmed using the spontaneous eye-blink model (Bruns et al., submitted for publication).

**Unique Pharmacology of Allosteric Potentiators.** Allosteric potentiators have a unique place as pharmacologic tools (Conn et al., 2009; Canals et al., 2011; Keov et al., 2011). Whereas antagonists work by subtraction and agonists work by addition, potentiators work by multiplication. That is to say, a potentiator increases the sensitivity of its target receptor to its endogenous neurotransmitter or hormone, amplifying the physiologic role of that receptor. In contrast, an agonist will activate its receptor regardless of whether the natural ligand is present, leaving unanswered the question of what is physiologic and what is only pharmacologic. Furthermore, a potentiator provides qualitatively opposite information compared with an antagonist: with an antagonist, one can study a physiologic function by observing its disappearance whereas with a potentiator one can observe an amplification or exaggeration of the function, which can be an advantage if the purpose is to characterize the function in detail. For instance, the ability of DETQ to stimulate locomotor activity in hD1 mice shows that D1 receptors have a tonic, ongoing role in maintaining motor activity. This result was not surprising in light of prior knowledge that selective D1/D5 antagonists suppress basal motor activity (Chipkin et al., 1988). However, the wide dynamic range of the D1 system, illustrated by the ability of DETQ to increase motor activity by more than 10-fold, is something that could not have been deduced from studies of D1 antagonists.

**Desensitization and Accommodation.** In interpreting these studies, it is important to distinguish between two phenomena, which we will refer to as desensitization and accommodation. We define desensitization as refractoriness to additional input so that an input that normally would produce a response no longer does so. An example would be the tachyphylaxis to D1 agonists upon chronic dosing. In contrast, we define accommodation as the ability to adjust an input to produce the correct magnitude of output. A potential example would be the lack of stereotypy shown by DETQ, in that animals do not show signs of overstimulation even when exposed to extremely high free brain levels of DETQ. Our studies show that potentiators allow accommodation without causing desensitization, a unique profile that should confer therapeutic advantages.

### Table 4

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cyclic AMP</th>
<th>Internalization</th>
<th>Pathway Preference</th>
<th>Relative Preference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DETQ</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; = 5.83 nM</td>
<td>Top (% of Dopamine) = 95.2%</td>
<td>RA = 44.6 nM</td>
<td>Top (% of Dopamine) = 112.0%</td>
</tr>
<tr>
<td>A-77636</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; = 2.34 nM</td>
<td>Top (% of Dopamine) = 121.0%</td>
<td>RA = 14.4 nM</td>
<td>Top (% of Dopamine) = 51.6%</td>
</tr>
</tbody>
</table>

Pathway preference for D1 receptor internalization versus cAMP for DETQ and A-77636 (Table 4). RA is the top divided by the EC<sub>50</sub>. Pathway preference is the RA for cAMP divided by the RA for internalization. Relative preference is the pathway preference of DETQ divided by the pathway preference of A-77636. To ensure comparability, all experiments (including those with A-77636) were performed in the presence of an EC<sub>50</sub> concentration of dopamine (12 nM for cAMP, 407 nM for internalization). Results for DETQ are from Fig. 2 and Fig. 13. A-77636 internalization results are from Fig. 13. Statistics for A-77636 in the cAMP assay (curve not shown) are: S.E. of the EC<sub>50</sub> = ±0.86 nM, S.E. of the top = ±17.4% of dopamine max, n = 5. The overall S.E. for relative pathway preference (computed from the S.E.s of the eight components that went into the RA calculations) was ±1.4 (no units).
Conclusions. In the present studies, we have used the novel D1 potentiator DETQ to verify two predicted advantages of allosteric potentiators. In contrast to D1 agonists, DETQ increases LMA without causing stereotypy at high doses, confirming that potentiators may have broader therapeutic windows compared with agonists. In addition, unlike D1 agonists, DETQ did not cause tachyphylaxis on repeated dosing, indicating that potentiators may have better ability to maintain efficacy on chronic dosing. We recommend that these advantages of potentiators be explored in clinical studies.

Acknowledgments
The authors thank Taconic Biosciences GEM Design group for the design and generation of the humanized dopamine D1 mouse, Niles Fox and Edda Roberts (Lilly) for providing the transgenic animals support and generation of in vitro dopamine D5 data, and Mark Heiman, Kevin Burris, Chris Felder, and Ken Savin for their support and advice.

Authorship Contributions

Conducted experiments: Getman, Wang, Menezes, Maren, Falcone, Wright, Morin, Adams, Suter, Lucaites, Emkey, Delapp, Wiernicki.

Contributed new reagents or analytic tools: Schaus, Beck, Hao, Krushinski, Reinhard, Cohen, Hellman, Rogovoy, Emkey, Bruns.


Wrote or contributed to the writing of the manuscript: Svensson, Heinz, Schaus, Nelson, Yang, Bruns.

References


Schaffhauser H, and Marino MJ (2007) Allosteric approaches to the design and generation of the humanized dopamine D1 mouse, Niles Fox and Edda Roberts (Lilly) for providing the transgenic animals support and generation of in vitro dopamine D5 data, and Mark Heiman, Kevin Burris, Chris Felder, and Ken Savin for their support and advice.

Address correspondence to: Dr. Kjell A Svensson, Lilly Research Laboratories, Eli Lilly & Co., Lilly Corporate Center, Indianapolis, IN 46285. E-mail: svensson_kjell_a@lilly.com

Svensson et al.