LONG-TERM ANTIDEPRESSANT EFFICACY OF A SELECTIVE ORGANIC CATION TRANSPORTER BLOCKER

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Running title : Antidepressant efficacy of a novel OCT-targeted prodrug

Abstract

Current antidepressants act principally by blocking monoamine reuptake by high-affinity transporters in the brain. However, these antidepressants show important shortcomings such as slow action onset and limited efficacy in nearly a third of patients with major depression disorder. Here, we report the development of a prodrug targeting organic cation transporters (OCT), atypical monoamine transporters recently implicated in the regulation of mood. Using molecular modeling, we designed a selective OCT2 blocker, which was modified to increase brain penetration. In a rodent model of chronic depression induced by corticosterone exposure, daily administration of this compound, H2-cyanome, induced positive effects on several behaviors mimicking symptoms of depression, including anhedonia, anxiety, social withdrawal, and memory impairment. Importantly, in this validated model, H2-cyanome compared favorably with the classical antidepressant fluoxetine, with a faster action on anhedonia and better anxiolytic effects. Integrated Z-scoring across these depression-like variables revealed a lower depression score for mice treated with H2-cyanome than for mice treated with fluoxetine for 3 weeks. Repeated H2-cyanome administration increased VTA dopaminergic neuron firing, which may underlie its rapid action on anhedonia. H2-cyanome also modulated in a similar way than fluoxetine several intracellular signaling pathways previously involved in antidepressant response. Our findings provide proof-of-concept of long-term antidepressant efficacy of an OCT blocker, and a mechanistic framework for the development of new classes of antidepressants and therapeutic alternatives for resistant depression and other psychiatric disturbances such as anxiety.

Keywords: organic cation transporter; prodrug; antidepressant; depression model; behavior

Introduction

Depression is a common and disabling disorder worldwide, with considerable clinical, social, and economic impact[1]. For over a half century, major depressive disorder has been managed primarily with medications modulating aminergic neurotransmission. This has provided the underlying rationale for the enduring monoamine hypothesis, which stipulates that depression results from an imbalance in serotonin (5-hydroxytryptamine, 5-HT), norepinephrine (NE), and/or dopamine (DA) signaling in the brain, which may be restored by long-term antidepressant treatment[2]. Most standard antidepressants, such as selective serotonin reuptake inhibitors (SSRIs) and norepinephrine-serotonin reuptake inhibitors (NSRIs), target high-affinity transporters for 5-HT and NE located in the plasma membrane of nerve terminals. However, these current treatments show important limitations, such as slow action onset and adverse side-effects. More importantly, they do not always lead to positive outcomes, and about a third of major depression patients do not respond satisfactorily to antidepressant therapy[3]. In this context, we speculated that targeting atypical monoamine transporters such as the organic cation transporters (OCT) could constitute a relevant strategy for the discovery and development of innovative antidepressant drugs.

OCT are facilitated diffusion transporters that participate in the absorption and clearance of various endogenous compounds and xenobiotics, by mediating their vectorial transport in renal, hepatic and placental cells[4]. These transporters can transport with low affinity (in the micromolar range) the biogenic monoamines DA, 5-HT, NE and histamine[5-7]. Two subtypes in particular, OCT2 and OCT3, are expressed in the central nervous system[6-9], where they contribute to modulate mood-related functions such as anxiety, response to stress and antidepressant efficacy[9-13]. Brain OCT have been proposed to be an alternate monoamine clearance system, prevailing in areas lacking the high-affinity transporters, or when these transporters are saturated or inhibited, for instance after antidepressant administration[9, 13, 14].

In this study, we developed a novel prodrug targeting OCT, on the assumption that it could exert antidepressant effects by engaging mechanisms differing from high-affinity transporter blockade.

Known OCT inhibitors cover a wide range of compounds, of varied efficacy and often low selectivity[4, 13]. We selected disprocynium 24 (D24), an isocyanine derivative, as lead compound for pharmacomodulation[15]. D24 is a potent and relatively selective OCT inhibitor, interacting with a putative high-affinity binding site of these transporters[16] and affinity constants ranging from 15 to 280 nM for brain OCT[17, 18]. Nevertheless, this derivative has limitations for preclinical studies or clinical use. Cyanine dyes are believed to diffuse poorly across the brain-blood barrier and have significant effects on peripheral organs[18-21]. Specifically, D24 inhibits potently α-adrenergic receptors[18], and affects OCT-mediated catecholamine clearance in sympathetically-innervated tissues[19, 21]. We addressed these issues by combining molecular modeling and a prodrug delivery approach, to generate a compound with increased selectivity for OCT2. This prodrug was evaluated for its long-term antidepressant efficacy in a validated model of chronic depression, in comparison with the classical antidepressant fluoxetine. The robust behavioral effects of the prodrug, and its action on ventral tegmental area (VTA) dopaminergic neuron firing and intracellular signaling pathways demonstrates a high therapeutic potential for treatment of depressive disorders.

Material and methods

Homology modeling and drug design

Three-dimensional (3D) OCT and adrenoceptor models were built from sequence alignments and crystallographic atomic coordinates of XylE (PDB code 4GBY) and a combination of β1 adrenoceptor (PDB codes 3ZPR and 3D4S) and 5-HT1B receptor (PDB code 4IAR) using

MODELER 9.0 (Discovery Studio 2016, BIOVIA, Dassault Systèmes, Vélizy-Villacoublay, France). The models were minimized and docking calculations were performed using CDOCKER[22]. The poses showing the lowest energy were retained and clustered according to their binding mode. 3D snapshots of the protein-ligand complexes were generated using BIOVIA DS Visualizer. Synthesis protocols, compounds characterization and full modeling and drug design procedures are detailed in supplementary materials and methods.

In vivo detection of H2-cyanome

Brain or plasma samples were homogenized in 33% acetonitrile. Analyses were performed on a high-performance liquid chromatography- mass spectrometry (HPLC-MS/MS) system consisting of an AmaZon SL ion trap mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) equipped with an ESI interface and an Ultimate 3000 device (Dionex, Thermo Fisher Scientific, Sunnyvale, CA), a thermostated autosampler and column compartment and an UV detector. Data were acquired and processed using Hystar and the extension QuantAnalysis (version 3.2, Bruker Daltonics GmbH). HPLC separation was performed with an A/B gradient (A: 10 mM ammonium acetate, pH 4.6, and B: CH_3CN/HCOOH [999:1, vol/vol]). The column was equilibrated with 20% B for 5 min, followed by a linear gradient to B over 10 min, and 4 min in B. The column was then equilibrated with a second linear gradient from 100 to 20% B over 1 min and an isocratic concentration of 20% B over 8 min. The analysis was performed in the reversed phase mode (ACE Excel 2 C18-Amide, 2 µm, 2.1 ×150 mm, Ref. EXL-1012-1502U, AIT, Houilles, France), and detection was carried out in MS mode with UV wavelength scaled at 220 nm.

Behavioral studies

Animals

Male C57BL6/J mice were used for the experiments. Most behavioral studies were performed during the inactive phase (09:00–13:00) with age-matched (8–16 weeks) mice. Animal care and experiments were conducted in accordance with the European Communities Council Directive for the Care and the Use of Laboratory Animals (2010/63/UE) and approved by the French ethical committee (#5786-2016062207032685).

Model of chronic depression

To induce a chronic depression-like state, individually housed mice were administered corticosterone in drinking water (35 µg ml-1; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) dissolved in 0.45% (wt/vol) hydroxypropyl-b-cyclodextrin (Sigma-Aldrich) as previously reported[9]. Fluoxetine (15 mg/kg/day; LKT laboratories, St Paul, MN, USA) or H2-cyanome (0.1 mg/kg/day) was administered intraperitoneally (i.p.) daily during the last 3 weeks of the corticosterone regimen. Mice were tested for sucrose consumption, social interaction, open-field, elevated O-maze and object location test, before antidepressant treatment and after 10 days and 3 weeks of treatment. The coat state was assessed weekly as a measure of motivation toward self-care. It was evaluated as the sum of the score of different parts of the body, ranging between 0 for a well-groomed coat and 1 for an unkempt coat for head, neck, dorsal/ventral coat, tail, and forepaws/hindpaws. For the sucrose preference test, singled-house mice were first habituated for 48 h to drink water from two bottles. On the following 3 days, the mice could choose between a water bottle and a 1% (wt/vol) sucrose solution bottle, with the position switched daily. Sucrose solution intake for 24 h was measured during the last 2 days and expressed as a percentage of the total amount of liquid ingested. The elevated O-maze consisted of an annular runway positioned 40 cm above the

floor and divided into two opposing 90º closed sectors and two 90º open sectors. Mice were individually placed in the closed sector and their behavior recorded over a 5-min period. The time spent in each sector and the number of sector entries (a sector entry was defined as all four paws being placed in a sector) were determined by video tracking (Viewpoint, Lyon, France). The social interaction test was performed in a white open-field (42 x 42 cm) containing an empty wire mesh cage (10 x 6.5 cm) located at an extremity of the field in a low luminosity environment (25 lux). Individual mice were allowed to explore the open-field for two consecutive sessions of 2.5 min. During the second session, an unfamiliar mouse was introduced into the wire mesh cage. Between the two sessions, the test mouse was placed back into its home cage for approximately one minute. The time spent by the test mouse in the interaction zone, defined as an 8-cm-wide region surrounding the mesh cage, was measured in both sessions by video tracking (Viewpoint). For the object location test, the mice were habituated during two successive days to an open-field containing an intra-field cue (one wall covered with black and white stripes). Each mouse was allowed to freely explore the open-field for a 30-min period on day 1 and for two 10-min sessions separated by 5 h on day 2. On the third day, the test mouse was allowed to explore for 5 min two identical objects (5 x 2.5 cm) positioned in two adjacent corners of the open-field (acquisition phase) then returned to its home cage for 1 h. For the sample phase trial, one of the two objects was displaced to the opposite corner of the open-field. The time spent exploring both objects was recorded over a 5-min session by video tracking. The open field consisted of a white Plexiglas field (42x42 cm) with the center brightly illuminated (100 lux). General locomotor activity in the center and periphery of the open field were scored for 9 min. The time and number of entries in the center zone (20x20 cm) were evaluated as an index of anxiety-related response. Integrated z-scores of depression-related variables were calculated to compare the effects of H2-cyanome with those of fluoxetine in the corticosterone depression model. Individual

depression-related z-scores were generated with data from five tests (time and activity in the center of open-field, time and latency to enter the open zone of elevated O maze, percentage of sucrose intake in the sucrose preference test, time of interaction in social interaction test, and coat state). Test z values were calculated by averaging individual Z-scores. Z-score methodology was used to combine results across the different tests and the data was normalized as previously described[23]. Integrated z-scores were calculated after 3-week treatment with fluoxetine or H2-cyanome.

Behavioral evaluation of acute treatment with H2-cyanome

For the evaluation of its acute effects in the open-field, the elevated-O maze and forced-swim tests, various doses H2-cyanome were dissolved in saline (0.9% NaCl [wt/vol]) and administered intraperitoneally at a volume of 10 ml/kg 30 min before each test. For the forced-swim test, the mice were placed individually in a glass beaker filled with 25±1 °C water to a depth of 12 cm. The duration of immobility (time during which the mice make only minimal movements to stay afloat) was recorded during the last 4 min of the 6-min testing period, after 2 min of habituation.

In vivo electrophysiological recordings of ventral tegmental area (VTA) DA neurons

Mice were anesthetized with chloral hydrate (400 mg/kg i.p.) and placed into a stereotaxic frame. Extracellular recordings of DA neurons in the VTA were performed using single-barreled glass micropipettes (Stoelting, Dublin, Ireland) preloaded with a 2 M NaCl solution to obtain an impedance ranging between 6–9 MΩ. Electrical signals were amplified by a high-impedance amplifier (Bak Electronics, Umatilla, OR, USA) and monitored visually with an oscilloscope (Tektronix, Beaverton, OR, USA) and audibly through an audio monitor (A.M. Systems Inc., Sequim, WA, USA). The signal was digitalized, sampled at 25 kHz and

recorded on a computer using Spike2 software (Cambridge Electronic Design, Cambridge, UK). To record VTA DA neurons, the glass micropipette was positioned using the following coordinates (in mm from bregma): AP: 3.5 to 3.1 mm, L: ±0.5 to 0.7 mm, V: -3.5 to -4.5 mm. Spontaneously active dopaminergic neurons were identified on the basis of previously established electrophysiological *in vivo* criteria[24]. At least 2 minutes of spontaneous baseline electrophysiological activity were recorded before systemic injection of H2-cyanome alone or in combination with cocaine (30 mg/kg in a final volume of 100 µl). The firing rate of VTA DA neurons was then determined until neuronal activity stabilization, thereby allowing the determination of the percent of change of basal firing rate.

Western blots

Whole tissue extracts were prepared from bilateral punches (1–1.5 mm diameter; Miltex, York, PA, USA) of brain regions from adult mice at basal state, treated with corticosterone or with corticosterone plus H2-cyanome or fluoxetine. Samples were homogenized by sonication in 2 vol of ice-cold phosphate-buffered saline containing 1% Triton X-100, protease inhibitors (Complete Protease Inhibitor Cocktail, Roche Diagnostics, Meylan, France) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail 3; Sigma-Aldrich). Protein concentrations were determined by Bradford's method. Protein samples (15 µg) suspended in NuPage LDS sample buffer (Invitrogen, Carlsbad, CA, USA) were separated by Bis-Tris sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% gels) and transferred onto nitrocellulose membranes (Invitrogen). Transfer efficacy was controlled by Ponceau S staining. Unspecific binding sites were blocked with Tris-buffered saline containing 0.1% Tween-20 and 5% nonfat milk and membranes were immunoprobed with antibodies against glycogen synthase kinase-3β (GSK3β, 1/2000) from Millipore (Billerica, MA, USA), phosphorylated GSK3β (p GSK3β, 1/1000), Akt (1/2000), pThr308 or Ser473 Akt (1/200), phosphorylated extracellular-signal

regulated kinase1/2 (pErk1/2, 1/1000) and p-P70S6 (1/500) from Cell Signaling (Danvers, MA, USA), Erk1/2 (1/1500) from Santa Cruz, or β-actin (1/2500) from Sigma-Aldrich. Membranes were incubated with infrared-labeled secondary antibodies (IRDye 700DX and IRDye 800CW; 1/5000; Rockland, Gilbertsvillle, PA, USA). Immunoblotting was quantified with an Odyssey Infrared Imaging System and Application Software version 3.0 (LI-COR Biosciences, Lincoln, NE, USA).

Statistics

PRISM (GraphPad Software, San Diego, CA, USA) was used for statistical calculations. For sucrose preference, social interaction, open-field, O-maze, object location test, coat state and forced swim-test data were analyzed using one-way or two-way analysis of variance (ANOVA), followed by Fisher's LSD test. Student's *t*-test was used for analysis of z-scores after H2-cyanome and fluoxetine treatment, firing of VTA neurons, and Western blot experiments. Statistical significance was set at $P < 0.05$.

Results

Development of a selective OCT prodrug

To develop a new potent and selective OCT ligand, 3D homology models of the human α2-adrenergic receptor (Figure1b), an unwanted target, and of human OCT2 (Figure 1c) were generated from selected templates (Figure b-d). The OCT2 model generated was coherent with previous models[25, 26], and interactions defined in functional studies[16, 27, 28]. By comparing positioning and interactions of D24 in both models, we modified this compound to introduce a bulky substituent hindering interaction with the α2C adrenoceptor, thereby generating the analog cyanome (Figure 1a). Cyanome showed a similar docking pattern of interaction to D24

with hOCT2 (Figure 1c). The relevance of the interactions predicted for cyanome was verified through binding and uptake experiments. Binding experiments showed that cyanome inhibited less potently the human α2C and the α2A adrenoceptors than D24 (Supplementary figure 1f). The observed inhibition constant (K_i) values were 185 ± 42 nM (mean ± s.e.m.) for the α2A adrenoceptor and 252 ± 32 nM for the α2C adrenoceptor (Supplementary figure 1f), compared to 28 nM and 66 nM for D24 for α2A and α2C adrenoceptors, respectively[18]. Cyanome also retained the ability to block the transport activity mediated by hOCT2 in transfected cells, with an IC_{50} value of 123 ± 26 nM (Supplementary figure 1f), a value comparable to that of D24 [18]. These results suggest that modification of D24 into cyanome markedly increased selectivity at hOCT2, as predicted by molecular modeling. Next, we speculated that charged cyanine analogs such as cyanome might have limited potential for use as antidepressants, due to low brain penetration. By exploiting a redox brain delivery system previously described[29], cyanome was modified into a prodrug that could more readily diffuse into the brain parenchyma and be activated therein, H2-cyanome (Figure 1a). The fate of H2-cyanome *in vivo* after systemic administration was determined by HPLC and mass spectrometry analysis of mice plasma and brain extracts, at different time points after a single i.v. injection. These analyses showed that after i.p. injection H2-cyanome diffuses into the brain and is rapidly cleared from the soluble fractions of both plasma and brain (Table 1). Furthermore, to identify the potential metabolites of H2-cyanome in a physiological context, we investigated the nature of the derivatives generated after incubation with human liver microsomal preparations. Four main metabolites were identified within the first minutes of incubation, the most abundant metabolite of which was cyanome (Supplementary figure 2).

Antidepressant effects of H2-cyanome in a chronic depression model

H2-cyanome was evaluated for its behavioral effects in mice, in particular antidepressant efficacy. In a model of chronic depression induced by corticosterone administration[9, 30], daily administration of a low dose of H2-cyanome (0.1 mg/kg, i.p.) induced rapid positive effects on several behaviors mimicking symptoms of depression, such as anhedonia, anxiety, social withdrawal, and memory impairment (Figure 2 and supplementary figure 3). The action of this compound on these parameters was as robust as those of the classical antidepressant fluoxetine (15 mg/kg). Specifically, H2-cyanome efficiently reversed the action of corticosterone on anhedonia, evaluated by sucrose preference (Figure 2b), on social interaction (Figure 2c), on anxiety level, evaluated by time spent in the anxiogenic zone of the elevated O-maze (Figure 2d), by latency to enter this zone (Figure 2e) and by time and activity in the center of the open-field (Supplementary figure 3b), and on short-term memory, evaluated by performance in the object location test (Figure 2f). This last effect, similar to that exerted by fluoxetine, was confirmed in the novel object test (Supplementary figure 3c). Importantly, H2-cyanome showed an accelerated onset of action on anhedonia compared to fluoxetine at 11 days (Figure 2b) and better effects on anxiety level at 21 days (Figure 2d-e and supplementary figure 3b). Finally, H2-cyanome also improved partially coat state (Figure 2g) and induced no weight loss over time (Supplementary figure 3d), as fluoxetine. To evaluate more finely the effects of H2-cyanome, we applied z-normalization across measures of these depression-related variables, i.e., anxiety and anhedonia-like behaviors and coat state. Interestingly, individual *z*-scores of mice treated with H2-cyanome were significantly lower than those of mice treated with fluoxetine (Figure 2h), suggestive of a better antidepressant effect of H2-cyanome.

Contrasting with the effects of repeated administration, acute injection of various doses of H2-cyanome had no effect on locomotor activity or anxiety level in the open-field (Figure

3a) and elevated O-maze (Figure 3b). However, acute injection elicited a moderate but reproducible antidepressant-like effect on immobility in the forced-swim test, which appeared to reach a maximum at the dose of 0.1 mg/kg (Figure 3c).

Effects of H2-cyanome systemic administration on the firing of VTA neurons

H2-cyanome was initially designed to influence monoamine neurotransmission in the brain through blockade of OCT. Based on its potent action on anhedonia in the sucrose preference test, we speculated that H2-cyanome might have an impact on dopaminergic signaling. Dopaminergic neurons of the VTA encode reward, motivation and learning[31], functions profoundly impaired in depressed patients. We thus investigated the effects of both acute and repeated H2-cyanome administration on the firing of this neuronal population. The acute injection of H2-cyanome alone did not alter VTA dopaminergic neuron firing, but potentiated the inhibitory effect of cocaine, a DA transport blocker (Figure 4a). Since VTA neuron firing is negatively controlled by local extracellular DA, this observation reflects an inhibitory effect of H2-cyanome on DA clearance. Remarkably, a 10-day repeated administration of H2-cyanome alone exerted an opposite action, i.e., a significant increase in the firing of VTA neurons (Figure 4b). This increased activity may underlie the antidepressant efficacy of H2-cyanome in our chronic model of depression.

Effects of H2-cyanome on intracellular signaling pathways in the brain

ERK1/2, GSK3β and mammalian target of rapamycin (mTOR) signaling have been implicated in antidepressant response in depressed patients and in animal models[32-34]. To gain further insight into the mechanisms of action of H2-cyanome, we investigated its influence on these signaling pathways in the brain, by Western blot analysis in the corticosterone depression model. We found that all three pathways (ERK1/2, GSK3β and mTOR) were

strongly modulated by chronic corticosterone treatment in mouse prefrontal cortex and hippocampus. The phosphorylation levels of ERK1/2 (Figure 5a), Akt at Ser-473 (Figure 5c) and p70S6 (Figure 5d) were significantly decreased in both brain areas after corticosterone, reflecting inhibition of ERK1/2, TORC2 and TORC1 pathways, respectively. Phosphorylation of GSK3β at Ser-9, a site controlling negatively its activity (Figure 5b), was also decreased, reflecting activation of this pathway. All these alterations in activity induced by corticosterone were reversed in both brain areas after a 21-day treatment with either fluoxetine or H2-cyanome (Figure 5a-d). Thus, H2-cyanome restored basal state ERK1/2, GSK3β and mTOR signaling in prefrontal cortex and hippocampus as efficiently as fluoxetine in this depression model.

Discussion

There is at present a pressing need for novel well-tolerated antidepressants combining better efficacy and quicker action onset. In spite of interesting leads[35], there has been no significant paradigm shift over the last years in the pharmacological management of major depression disorder in current practice. Important shortcomings in standard treatments include poor efficacy, slow action onset, adverse side effects and unpredictable resistance, with one-third of patients not responding adequately to two or more successive trials of treatment. We demonstrate here proof-of-concept that targeting the atypical monoamine transporters OCT is a promising strategy for the development of novel antidepressant drugs.

OCT have been drawing increasing attention within the last decade, as evidence accumulated that they subserve major central functions, controlling anxiety, stress response and antidepressant action[9-13]. OCT2 and OCT3 specifically have been proposed to act as a low-affinity monoamine clearance system, regulating aminergic tonus and complementing the

better-known high-affinity transporters in the brain[13, 36]. However, until now, these potential therapeutic targets have been seldom exploited for drug development[26, 37]. The present study describes a novel OCT ligand with a high therapeutic potential, developed by combining molecular modeling with a prodrug brain delivery strategy.

In a two-step process, we generated starting from the OCT inhibitor D24 a ligand with increased selectivity for OCT2, which we further modified into an analog that could efficiently penetrate the brain, H2-cyanome[29]. To evaluate its antidepressant potential, we took advantage of a rodent model of chronic depression with strong construct, face and predictive validity[9, 30, 38], which surpasses acute behavioral despair tests commonly used for antidepressant screening[9, 37, 39]. In this model, prolonged corticosterone exposure induces a panel of behavioral modifications that mimic various symptoms of depression. As with human depression, these persistent depression-like anomalies can be improved by long-term, but not acute, antidepressant treatment, triggering on a longer time-scale specific mechanisms[40, 41]. In this depression model, H2-cyanome compared positively to the classical antidepressant fluoxetine, with a faster action on anhedonia, evaluated by sucrose preference, and better anxiolytic effects at a low dose (0.1mg/kg; Figure 2). Integrated Z-scores across depression-like variables revealed a significant difference of H2-cyanome compared to fluoxetine after 3-week treatments, supporting a stronger antidepressant effect. The parameters evaluating potential toxicity were encouraging (Supplementary figures 3 and 4, and supplementary table 1). Daily H2-cyanome at 0.1 mg/kg was as well tolerated as fluoxetine, with no weight loss after prolonged administration (Supplementary figure 3d). Blood parameters after 3 weeks of treatment were comparable to those after fluoxetine, with more favorable effects on hepatic enzymes (Supplementary table 1). In the brain, no evident neurotoxicity was detected in various areas including dopaminergic pathways (Supplementary figure 4).

The precise mechanisms of action of H2-cyanome *in vivo* remain to be clarified. A main issue is the nature of the metabolites of H2-cyanome present in the brain and their full impact on central processes, in particular after prolonged exposure. OCT blockers may be suspected of acting differently than current antidepressants like SSRI and NSRI. As opposed to the high-affinity transporters gathered in aminergic terminals, OCT are highly expressed in the cell bodies and processes (neurons and occasionally astrocytes) throughout the brain, in the principal regions receiving aminergic projections. This broad distribution and the high capacity properties of OCT, which compensate for their low-affinity, may explain their importance in the removal of extracellular monoamines[9, 13, 14]. Thus, in contrast with SSRI and NSRI, molecules targeting OCT could regulate NE, 5-HT and DA tone, in a widespread manner throughout the brain[13]. In addition, as the lead compound D24 shows a modest affinity for the SERT[18], potential modulation of this transporter must also be considered. Supporting an unconventional mode of action, H2-cyanome induced few behavioral modifications when administered acutely. In particular, this compound showed only moderate antidepressant-like effects in the forced-swim test, contrarily to SSRIs and NSRIs which exert robust anti-immobility effects in behavioral despair paradigms[42].

In vivo electrophysiological recordings revealed a complex action of H2-cyanome on DA signaling, on two distinct time scales. Our experiments showed that acute H2-cyanome could potentiate the inhibitory effects of cocaine, a dopamine transporter inhibitor, on the firing of VTA neurons. Extracellular DA levels in the VTA have been shown to negatively modulate VTA DA neuron firing[43], via inhibitory somatodendritic D2 receptors[44]. Our results suggest therefore that acute H2-cyanome could dampen DA neuron activity synergistically with cocaine by increasing local DA concentrations, through the inhibition of OCT-mediated DA clearance in this brain region. To gain a full understanding of the action of the prodrug,

additional experiments *in vivo* and *ex vivo* are required to determine how H2-cyanome affects NE and 5-HT as well as DA clearance throughout the brain.

Electrophysiological recording of DA neurons of the VTA also revealed significant effects of prolonged H2-cyanome administration. Contrarily to acute injection, a 10-day H2-cyanome treatment was associated with an increased firing frequency of this neuron population, a process that may underlie its rapid action on anhedonia. VTA dopamine-releasing neurons display remarkable functional heterogeneity, with distinct subpopulations controlling motivated behaviors in a complex manner[31]. Notwithstanding, their activation has been consistently associated with reward and motivation[31], functions often altered in MDD patients. The phasic activity of VTA DA neurons was also shown previously to be inhibited by chronic stress, a classical depression paradigm, while their optogenetic activation could reverse the depression-like behaviors induced in this paradigm[45]. Thus, activation of these DA neurons could play a key role in the efficacy of H2-cyanome on behaviors reflecting anhedonia, in particular its early effects on sucrose preference at 11 days of treatment, when fluoxetine is still inactive (Figure 2).

At another level, our study discloses main effects of H2-cyanome on intracellular signaling pathways involved in the regulation of mood and in antidepressant efficacy. The MAP kinase ERK1/2 was previously shown to be inhibited in prefrontal cortex and hippocampus of depressed suicide victims[46] and activated by long-term antidepressant treatment in animal models of depression[47]. Abnormal GSK3β activity has been found in prefrontal cortex of individuals with major depressive disorder[48, 49], and inhibition of GSK3β shown to play a role in antidepressant action[33]. The mTOR intracellular pathway has been proposed to underlie the fast-acting antidepressant effects of ketamine in humans[34]. In our model, all three pathways responded to corticosterone, in parallel with the emergence of depression-related behavioral anomalies. Remarkably, the beneficial effects on these

behaviors of a 3-week treatment with either fluoxetine or H2-cyanome were associated with a return to basal activation states, similar to those before corticosterone treatment. Altogether, these experiments indicate that H2-cyanome modulates these intracellular regulatory pathways in a similar way than fluoxetine, suggesting at least in part common regulatory mechanisms within the brain, downstream of aminergic signaling.

We speculate that both limited interactions with α adrenoceptors and efficient brain penetration contribute to the robust antidepressant effects of H2-cyanome. α adrenoceptors play important roles in controlling blood pressure, heart rate and contractility[50-52]. Based on the metabolic profile of H2-cyanome in microsomal fractions, two charged metabolites of H2-cyanome, cyanome and its demethylated derivative, are plausible candidates for mediating the antidepressant effects of the prodrug. Our experiments suggest that these two compounds should interact less potently than D24[18] with α2A and 2C adrenoceptors *in vivo*, while remaining capable of blocking OCT. Central α2A adrenoceptors and α2C adrenoceptors in the adrenal medulla both exert a negative control over blood pressure and heart rate, by mediating respectively sympathoinhibition[53] and feedback control of epinephrine release[52]. Thus, restricting the antagonism at α2 adrenoceptors may be beneficial to avoid unwanted cardiovascular effects. Importantly, interactions with central α2 adrenoceptors could also play a part in the antidepressant efficacy of H2-cyanome, but it is at this stage difficult to predict to what extent α2 blockade is a disadvantage. Some previous studies suggest that restricting α2A antagonism might improve antidepressant action and anxiety[54, 55], while others suggest on the contrary that α2 adrenoceptor blockade could contribute to the pharmacological activity of antidepressants[56], or potentiate their action[57]. Finally, we also believe that choice of D24 as lead scaffold was critical for favoring interactions with central OCT and limiting potential adverse side-effects. Other cyanine dyes such as decynium 22 (D22) can also inhibit OCT, with overall less affinity than D24[4, 18, 58]. However, while both D24 and D22 inhibit

peripheral α1 adrenoceptors[59], D22 has a 50-fold higher affinity for these receptors than D24[18] or cyanome (data not shown), restricting its use *in vivo* due to its pronounced hypotensive action[59]. These mechanisms may also have a bearing on the adverse effects of D22 on locomotor activity[37]. Considering the diversity of functions attributed to α adrenoceptors both at periphery and in the brain, in-depth studies with compounds with refined selectivity are required to identify the combinations of binding properties at OCT and α adrenoceptor subtypes optimal for clinical use.

In conclusion, we have devised through molecular modeling and pharmacomodulation a novel OCT-targeted drug with improved selectivity, which can penetrate the brain. Its long-term antidepressant efficacy in a validated depression model demonstrates a high potential for therapeutic use, compared to the conventional antidepressant fluoxetine. Our findings provide a conceptual and mechanistic framework for the design of selective OCT ligands, which may open a field of discovery for new classes of antidepressants with improved properties.

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Author contributions

N.P., F.A., and S.G. conceived the study. N.P. conceived and performed molecular modeling and designed the compounds. N.P., A.H., and P.D performed microsomal fraction analysis.

N.P., L.C-B., and R.A.D.S. synthetized the compounds. A.O., S.R.A., and V.V. performed behavioral analyses. B.P.G. designed and performed the electrophysiological studies. A.O. performed Western blot analyses. A.O. and T.Z. participated in HPLC/MS analysis. B.G. provided key facilities, equipment and advice. F.L. and C.G. performed the histochemical experiments. S.G. coordinated the study. N.P., A.O., B.P.G., and S.G. wrote the manuscript. All authors reviewed and approved the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Supplementary information is available at MP's website

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Figure legends

Figure 1 Strategy for prodrug development. Design of a disprocynium (D24) analog with optimized pharmacological properties. Improvement of selectivity for OCT2 against α2 adrenergic receptor was addressed by three-dimensional homology modeling and molecular docking (**a**-**c**) Key interactions are shown in stick mode. (**a**) Chemical structure of D24 (red), cyanome (blue) and H2-cyanome (black). (**b**) Global view of D24 docked into the hα2C adrenoceptor homology model (center), characterization of chemical optimization sites on D24 (left), and selected position for steric clash introduction (right). (**c**) Global view of D24 and cyanome docked into the hOCT2 homology model (center) and key interactions of D24 (left) or cyanome (right). Ionic interactions are indicated by dashed red lines and hydrophobic interactions by dashed green lines. Cyanome shows a similar pattern of interaction than D24 with hOCT2, with two additional interactions, between its aromatic ring and W218, and between its 6-methoxy substituent and N157. Position 6 of D24 was substituted in cyanome with a bulky substituent hindering interaction with the fourth helix of the α2C adrenoceptor and cyanome was reduced in H2-cyanome to facilitate brain penetration.

Figure 2 Antidepressant efficacy of H2-cyanome in a model of chronic depression. (**a**-**h**) Experimental scheme for the chronic depression model induced by corticosterone (**a**). One-way analysis of variance (ANOVA) (n = 8-29) shows a significant effect of treatment on (**b**) sucrose preference ($F_{5,60}$ = 9.76; P < 0.0001), (**d**) time ($F_{5,52}$ = 7.67; P < 0.0001) and (**e**) latency ($F_{5,53}$ = 8.20; P < 0.001) to enter the open zone of the elevated O-maze, and (**g**) coat state ($F_{5,9}$ = 94.03; P < 0.0001). Fisher's post hoc tests indicate a significant effect of the corticosterone treatment (**P < 0.01, ***P < 0.001). H2-cyanome but not fluoxetine significantly increased sucrose preference at 11 days (**b**). Both H2-cyanome and fluoxetine

significantly increased sucrose preference (**b**), increased the time in the open zone of the elevated O-maze (**d**), decreased the latency to enter this zone (**e**) after 21 days, and improved coat state at 11 days (**g**). Fisher's post-hoc test, #P < 0.05, ##P < 0.01, ###P < 0.001. For the object location test (**f**), two-way ANOVA (n = 8-10) shows significant main effects of object on exploration time ($F_{1,70}$ = 48.42; P < 0.0001). Fisher's post-hoc test reveals significant differences before corticosterone and after both treatments (###P < 0.001). For the social interaction test (**c**), unpaired two-tailed Student's t-test (n = 9-14) shows significant effects on interaction time for corticosterone compared to basal state (**P < 0.01), for fluoxetine at 11 days of treatment, and for H2-cyanome and fluoxetine at 21 days of treatment (#P < 0.05). **(h)** Integrated z-scores across depression-related variables in the corticosterone depression paradigm show a lower depression score for H2-cyanome than for fluoxetine after a 3-week treatment (n = 9). Unpaired 2-tailed student's *t*-test, *** P< 0.001. Values are given as mean ± s.e.m.

Figure 3. Acute behavioral effects of H2-cyanome. One-way analysis of variance (ANOVA) (n = 7-9) revealed no significant effects of acute treatment with various doses of H2-cyanome on locomotor activity or on time spent in the periphery or the center of the open field (**a**), nor on time in the open zone of the elevated O-maze (**b**). In the forced-swim test (**c**), one-way ANOVA (n = 5-17) showed a significant main effect of treatment 30 min after acute injection of saline or various doses of H2-cyanome ($F_{6,65}$ = 3.09; P= 0.0101). Fisher's post-hoc test showed significant differences with doses 0.1, 0.5 and 1 mg/kg (***P < 0.001, **P < 0.01, *P < 0.05) compared to vehicle. Results are given as mean ± s.e.m.

Figure 4 Action of H2-cyanome on dopaminergic neuron firing in the ventral tegmental area (VTA). (**a**-**b**) Effects of acute and chronic H2-cyanome administration on the

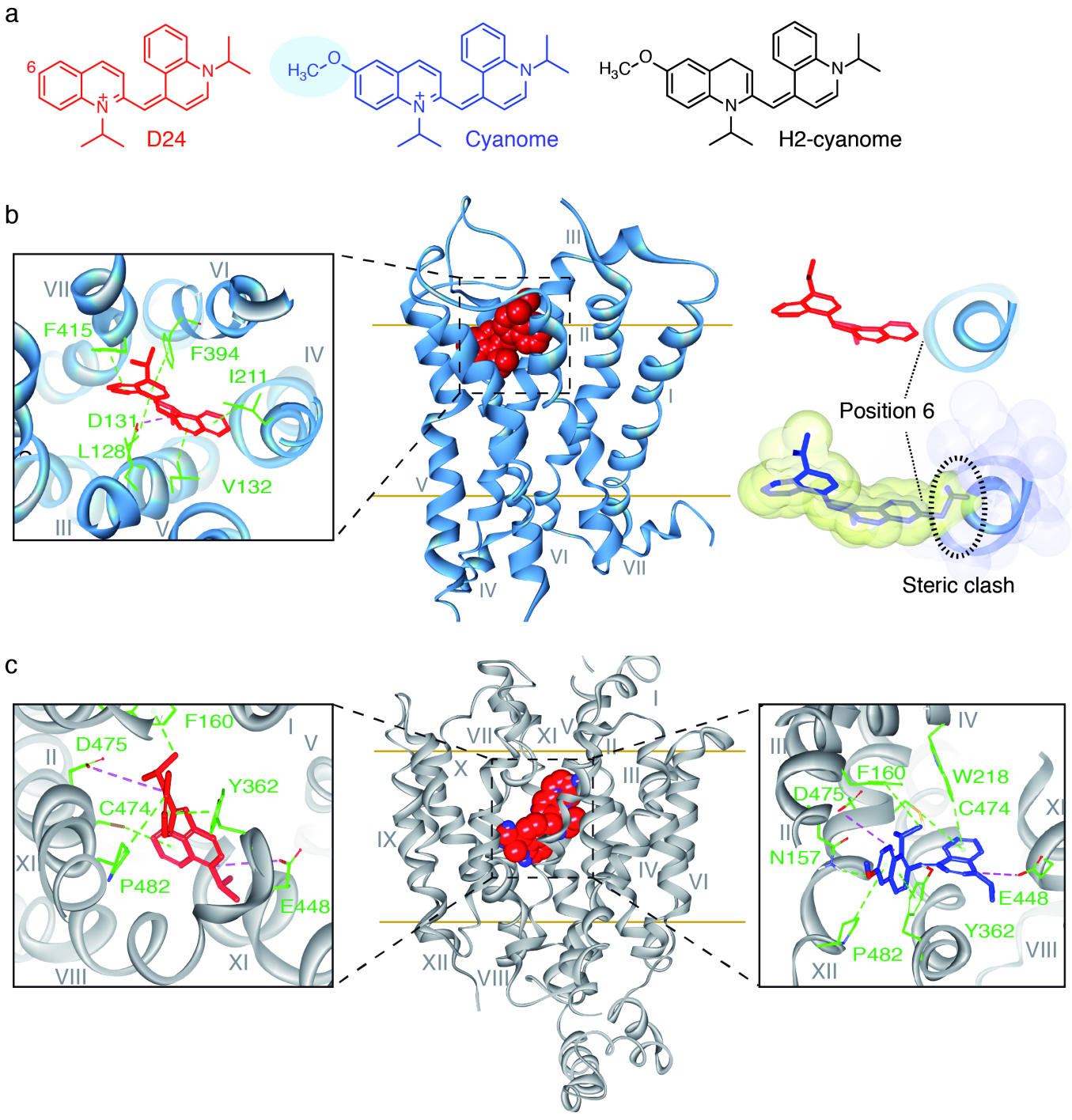
electrophysiological properties of VTA dopamine (DA) neuron activity. (**a**) Acute H2-cyanome administration accentuates the inhibitory effect of cocaine on the firing rate of VTA DA neurons. Data are means (± s.e.m.) of percent of decrease in DA neurons basal firing rate. (**b**) 10-day H2-cyanome increases VTA DA neuron activity. Data represent means ± s.e.m. Unpaired 2-tailed student's *t*-test, * P<0.05.

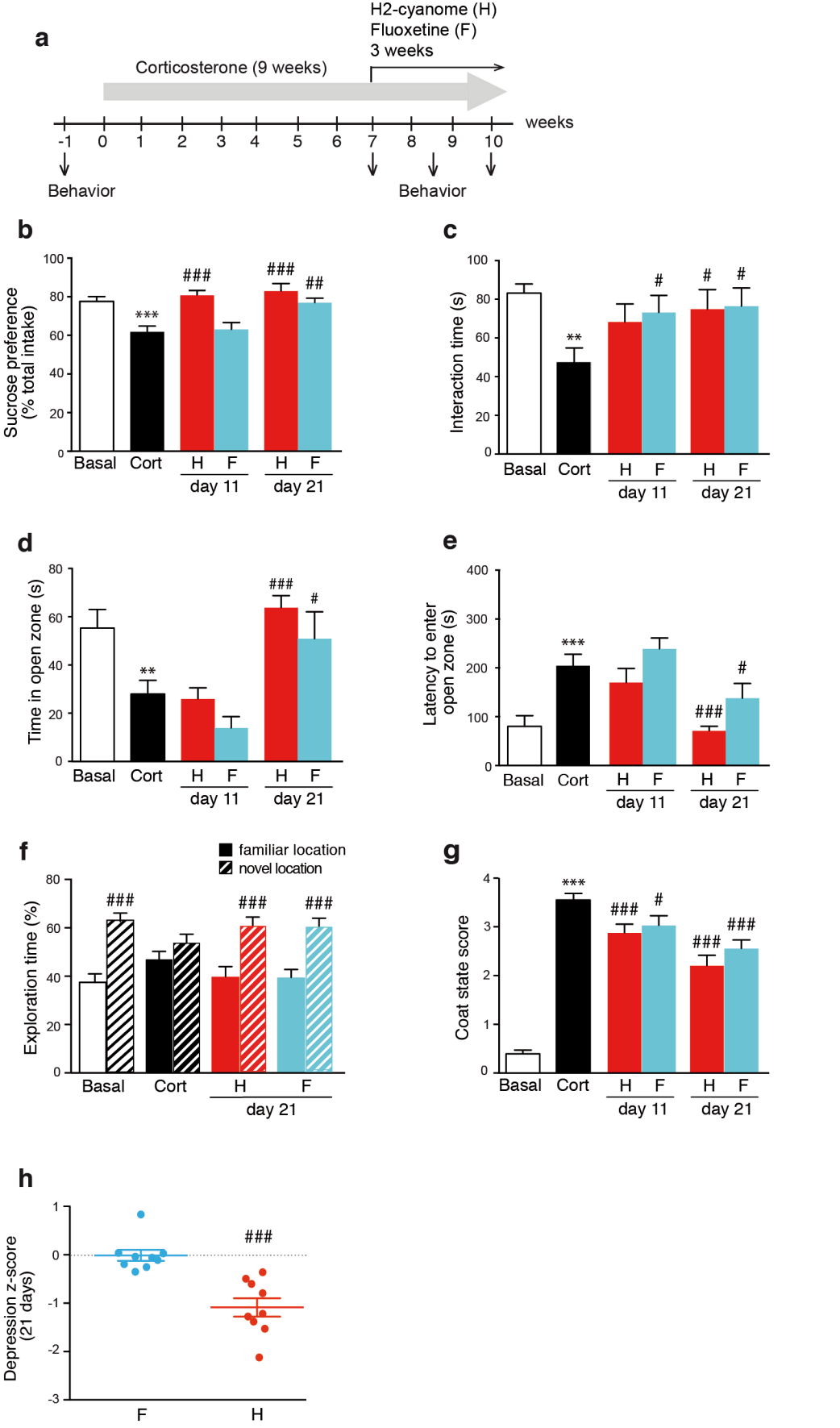
Figure 5. Action of H2-cyanome and fluoxetine on brain intracellular signaling pathways. Chronic corticosterone administration induces alterations of activity of extracellular-signal regulated kinase 1/2 *(*ERK1/2), glycogen synthase kinase-3β (GSK3β), and mammalian target of rapamycin (mTOR) signaling, which are reversed by either fluoxetine or H2-cyanome treatment. Quantitative Western blot analysis shows alterations of phosphorylation state of ERK1/2 (**a**), pSer9 GSK3β (**b**) and pSer473 Akt (**c**), and of levels of phosphorylated p70S6 (**d**) in the prefrontal cortex (PFC) and the hippocampus (HPC). Unpaired 2-tailed Student's *t*-test (n=4-7) reveals a significant effect of corticosterone treatment (*P < 0.05, **P < 0.01, ***P < 0.001), and significant increases after fluoxetine or H2-cyanome treatments compared to corticosterone-treated groups (#P < 0.05, ##P < 0.01, ###P < 0.001). Results are given as mean of phosphorylated over non-phosphorylated protein ratio ± s.e.m, or for p70S6 as mean of phosphorylated protein over β-actin ratio ± s.e.m.

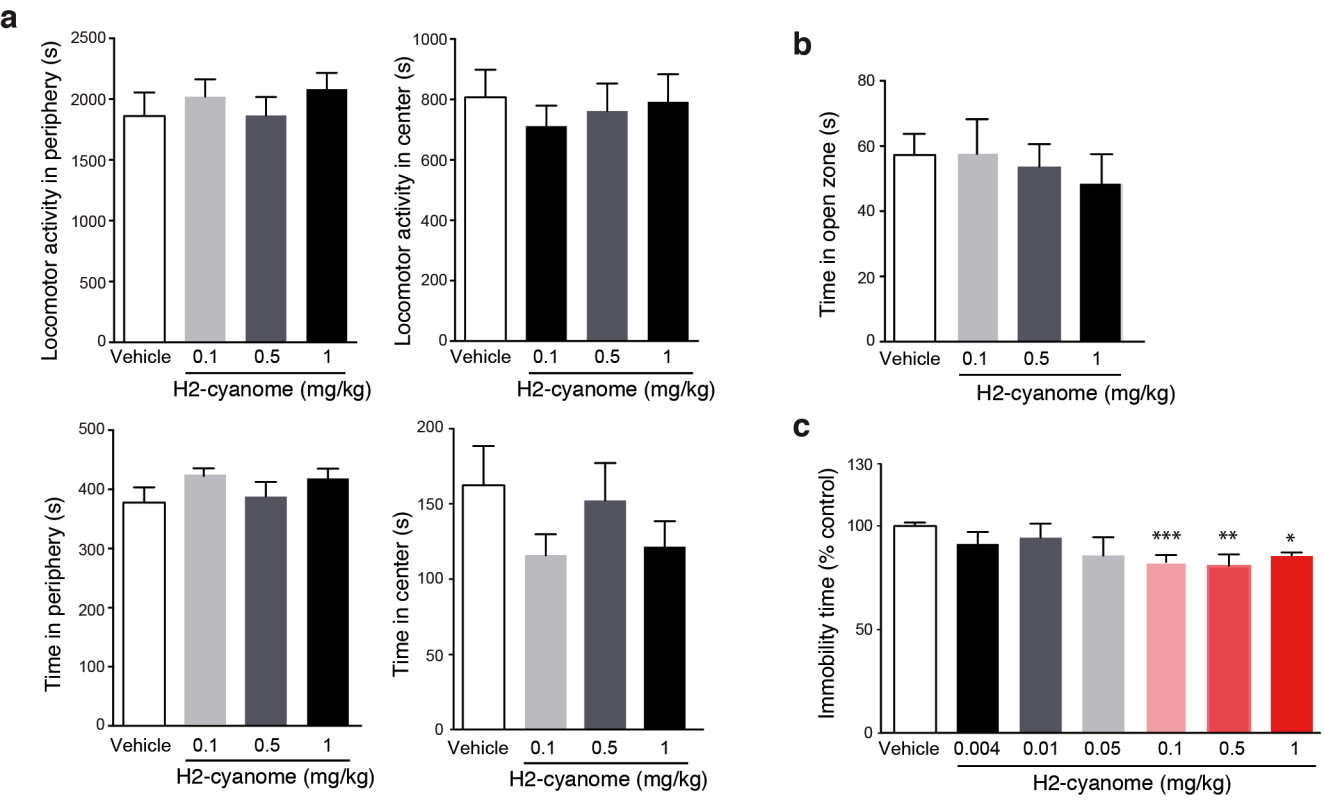
Table 1. Detection of H2-cyanome in plasma and brain

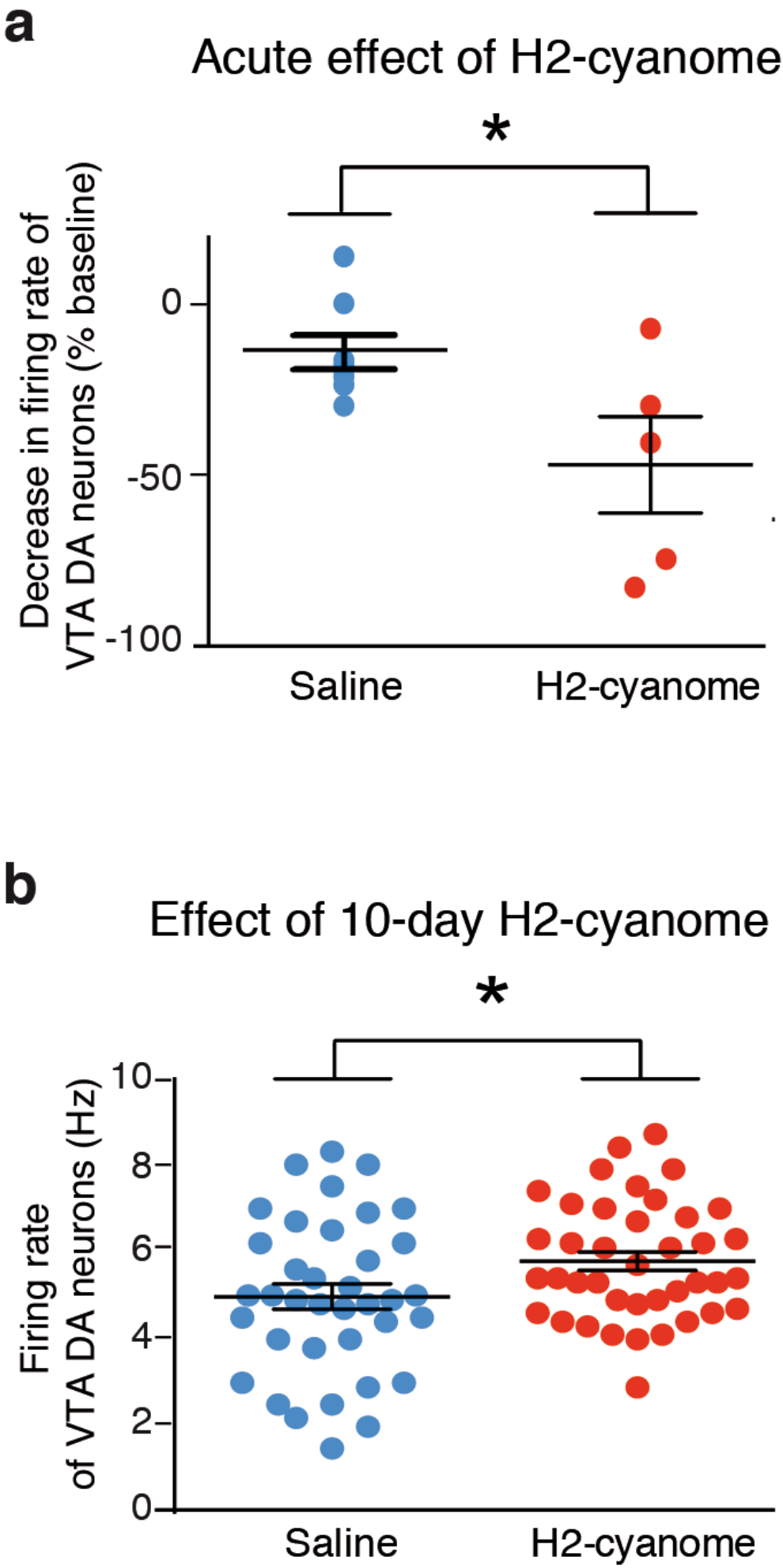
Time after Injection (i.v.)	5 min	15 min	2 h	24 h
Plasma (ng/ml)	3.65 ± 1.69	2.15 ± 0.31	< 0.5	< 0.5
Brain (ng/g)	3.97 ± 2.3	0.56 ± 0.56	< 0.5	ND

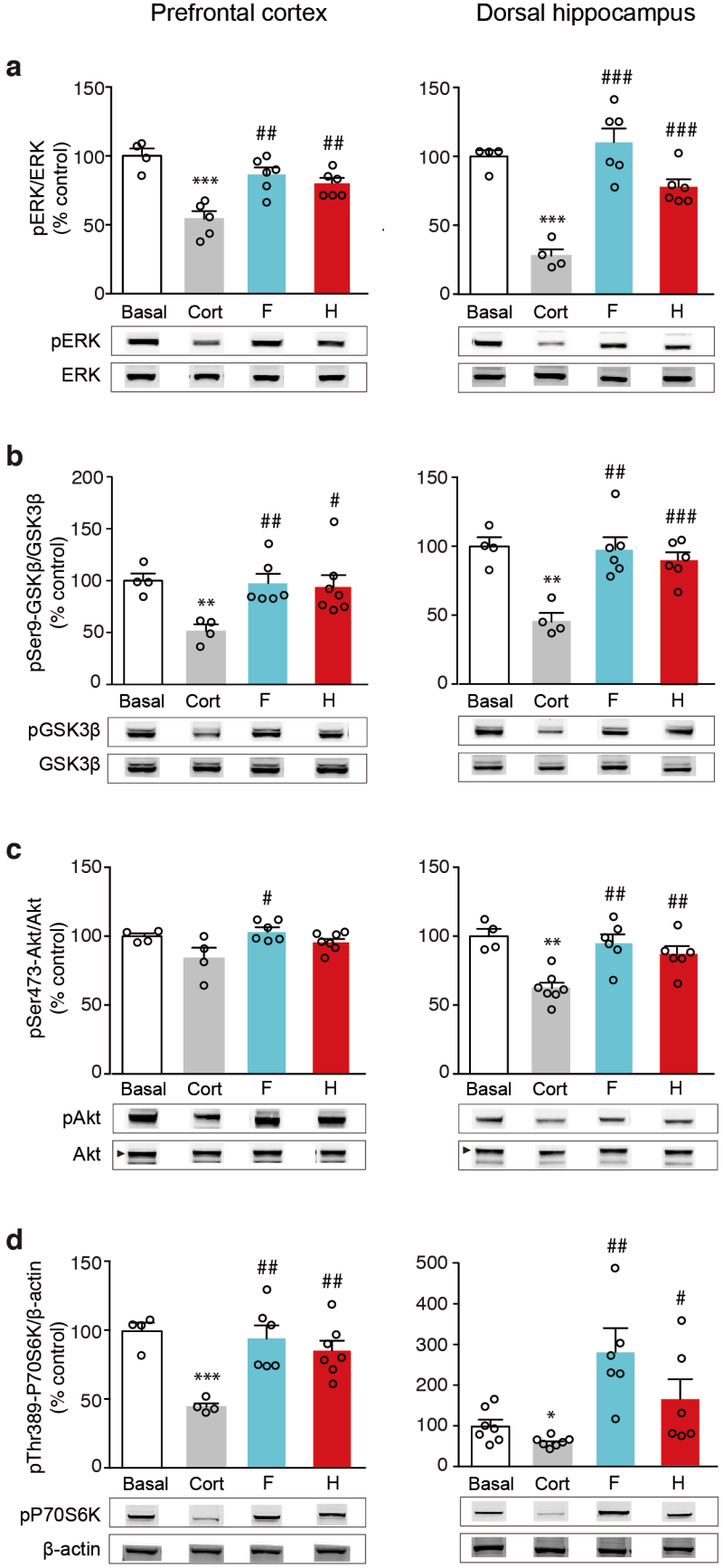
Data are expressed as means ± s.e.m. (n=6). ND: not detectable


a
6
D24
Cyanome
H2-cyanome
b
VII
VI
F415
F394
IV
I211
D131
L128
V132
III
V
II
I
VI
VII
IV
Position 6
Steric clash
c
F160
D475
II
I
V
Y362
C474
XI
P482
E448
VIII
XI
VII
XI
V
I
X
II
III
IX
IV
VI
XII
VIII
IV
III
F160
W218
D475
C474
II
N157
E448
Y362
P482
XII
VIII


a
H2-cyanome (H)
Fluoxetine (F)
3 weeks
Corticosterone (9 weeks)
weeks
-1 0 1 2 3 4 5 6 7 8 9 10
Behavior
Behavior
b
Sucrose preference (% total intake)
Basal Cort H F H F
day 11 day 21
c
Interaction time (s)
d
Time in open zone (s)
e
Latency to enter open zone (s)
f
familiar location
novel location
Exploration time (%)
g
Coat state score
h
Depression z-score (21 days)
F H


a
Locomotor activity in periphery (s)
2500
2000
1500
1000
500
0
Vehicle
0.1
0.5
1
H2-cyanome (mg/kg)
Locomotor activity in center (s)
1000
800
600
400
200
0
Vehicle
0.1
0.5
1
H2-cyanome (mg/kg)
b
Time in open zone (s)
80
60
40
20
0
Vehicle
0.1
0.5
1
H2-cyanome (mg/kg)
Time in periphery (s)
500
400
300
200
100
0
Vehicle
0.1
0.5
1
H2-cyanome (mg/kg)
Time in center (s)
200
150
100
50
0
Vehicle
0.1
0.5
1
H2-cyanome (mg/kg)
c
Immobility time (% control)
130
100
50
0
***
**
*
Vehicle
0.004
0.01
0.05
0.1
0.5
1
H2-cyanome (mg/kg)


a
Acute effect of H2-cyanome
*
Decrease in firing rate of VTA DA neurons (% baseline)
0
-50
-100
Saline
H2-cyanome
b
Effect of 10-day H2-cyanome
*
Firing rate of VTA DA neurons (Hz)
10
8
6
4
2
0
Saline
H2-cyanome


Prefrontal cortex
Dorsal hippocampus
a
pERK/ERK (% control)
Basal
Cort
F
H
pERK
ERK
b
pSer9-GSKβ/GSK3β (% control)
pGSK3β
GSK3β
c
pSer473-Akt/Akt (% control)
pAkt
Akt
d
pThr389-P70S6K/β-actin (% control)
pP70S6K
β-actin