Brain organic cation transporter 2 controls response and vulnerability to stress and GSK3β signaling

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BRAIN ORGANIC CATION TRANSPORTER 2 CONTROLS RESPONSE AND VULNERABILITY TO STRESS AND GSK3β SIGNALING

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Running title: OCT2 controls stress response and vulnerability

ABSTRACT

Interactions between genetic and environmental factors like exposure to stress play an important role in the pathogenesis of mood-related psychiatric disorders such as major depressive disorder. The polyspecific organic cation transporters (OCTs) were shown previously to be sensitive to the stress hormone corticosterone *in vitro*, suggesting these transporters might play a physiological role in the response to stress. Here, we report that organic cation transporter 2 (OCT2) is expressed in several stress-related circuits in the brain and along the hypothalamic-pituitary-adrenocortical (HPA) axis. Genetic deletion of OCT2 in mice enhanced hormonal response to acute stress and impaired HPA function without altering adrenal sensitivity to adrenocorticotropic hormone (ACTH). As a consequence, $OCT2^{-/-}$ mice were potently more sensitive to the action of unpredictable chronic mild stress on depression-related behaviors involving self-care, spatial memory, social interaction and stress-sensitive spontaneous behavior. The functional state of the glycogen synthase kinase-3β (GSK3β) signaling pathway, highly responsive to acute stress, was altered in the hippocampus of $OCT2^{-/-}$ mice. *In vivo* pharmacology and Western blot experiments argue for increased serotonin tonus as a main mechanism for impaired GSK3β signaling in $OCT2^{-/-}$ mice brain during acute response to stress. Our findings identify OCT2 as an important determinant of the response to stress in the brain, suggesting that in man OCT2 mutations or blockade by certain therapeutic drugs could interfere with HPA axis function and enhance vulnerability to repeated adverse events leading to stress-related disorders.

Keywords: organic cation transporter; stress; HPA axis; vulnerability; UCMS; depression

Introduction

The etiology of depression and other mood-related disorders is still poorly understood, yet it is increasingly evident that interactions between genetic and experience-based risks factors play a decisive role in the development of these disorders.[1-3] A fundamental physiological process at the crossroads of these genetic-environment interactions is the activation in stressful conditions of the hypothalamic-pituitary-adrenocortical (HPA) axis, which engenders a spectrum of neurochemical and behavioral modifications over different time scales. In higher organisms, the response to stress ensures an important function in optimizing the reactions and behavioral strategies in the face of potential threats, in terms of perception, risk assessment and decision-making. However, the repeated solicitation or disruption of the underlying biological systems also imposes a negative burden on mental health by promoting disorders such as major depressive disorder (MDD) or post-traumatic stress disorder (PTSD).[4-6]

At the central level, the response to stress solicit several interconnected circuits implicating the paraventricular nucleus (PVN) of hypothalamus as well as upstream neural pathways in the forebrain and hindbrain.[7] Among these circuits, specific monoaminergic pathways are activated within minutes after stress, participating in the early steps driving the synaptic and hormonal responses.[8, 9] Previous findings have revealed the role of low-affinity organic cation transporters (OCTs) in the brain as important postsynaptic determinants of aminergic tonus and mood-related behaviors, in complement to the classical high-affinity monoamine reuptake transporters.[10-12] Contrasting with the high-affinity reuptake transporters, OCT transport activity can be inhibited by micromolar concentrations of the stress hormone corticosterone *in vitro*.[13, 14] Recent evidence also suggests that their activity in

the brain can be impaired directly or indirectly by corticosterone,[15, 16] an effect which could mediate some of the biological effects of stress.

In this paper, we addressed whether organic cation transporter 2 (OCT2) controlled the hormonal response to stress. We identify OCT2 as a central modulator of the hormonal response to acute stress in mice and explore the underlying neuroendocrine and molecular mechanisms. Importantly, we show that OCT2 activity contributes as a genetic determinant which attenuates the extent of the noxious consequences of repeated stress on the onset of stress-related behaviors.

Material and methods

Animals

$OCT2^{-/-}$ mice were generated previously by homologous recombination.[17] Heterozygous animals with 10 backcross generations into C56BL6/J were bred to generate wild-type and knockout littermates, which were genotyped as described previously.[11] Mice were maintained on a 12:12 h light/dark cycle (lights on at 0800) and 8–16 weeks-old animals were used for the experiments. Animal care and experiments were conducted in accordance with the European Communities Council Directive for the Care and the Use of Laboratory Animals (86/609/EEC) and approved by local ethical committees.

Immunohistochemistry

Adult male mice were anesthetized and perfused intracardially with 4% (wt/vol) paraformaldehyde/phosphate-buffered saline (PBS). Brains were removed, post-fixed by immersion overnight in 4% paraformaldehyde/PBS and coronal sections (20 μm) were cut on a vibratome and processed for free-floating immunohistochemistry. For immunofluorescent

immunohistochemistry, brain sections were preincubated in a blocking buffer containing 0.3% (wt/vol) triton and 4% (wt/vol) bovine serum albumin. OCT2 was detected using affinity-purified rabbit polyclonal antibodies previously validated.[11] After washing, sections were incubated with Alexa 488-conjugated secondary antibodies (Molecular Probes, Eugene, Or, USA). For double labeling experiments, OCT2 antibodies were incubated with antibodies against either glial fibrillary acidic protein (GFAP, 1/500) from Abcam (Cambridge, UK) or adrenocorticotropic hormone (ACTH, 1/1000) from Sigma-Aldrich (St Louis, MO, USA) and revealed with Alexa 555-conjugated secondary antibodies (Molecular Probes).

Swim stress, subchronic corticosterone treatment and corticosterone dosage

Naive aged-matched male mice were left undisturbed several days before the experiment. For swim stress, the mice were placed individually in a glass beaker filled with tap water at 25 ± 1°C to a depth of 12 cm for 15 min. For subchronic corticosterone treatment, mice were administered corticosterone (35 µg/ml; Sigma-Aldrich) dissolved in 0.45% (wt/vol) hydroxypropyl-β-cyclodextrin (Sigma-Aldrich) or vehicle (0.45% hydroxypropyl-β-cyclodextrin) in drinking water during 4 days. For ACTH stimulation, mice were injected with dexamethasone (100 µg/kg i.p.; Sigma-Aldrich) 2 h prior to saline or cosyntropin injection (50 µg/kg i.p.; Synacthen, Sigma-Tau, Issy-Les-Moulineaux, France). Tail blood samples were collected into EDTA-containing tubes (Microvette, Sarstedt, Numbrecht, Germany) in the morning (0800–1100) before and at different time points after swim stress or ACTH injection, centrifuged and plasma was stored at -80°C until assayed. Plasma corticosterone concentrations were determined using radioimmune assay kits (MP Biomedicals, Illkirch, France) and expressed as ng/ml of plasma. For the dexamethasone suppression test, mice were injected with dexamethasone (100 µg/kg i.p.; Sigma-Aldrich) 30 min prior to swim stress.

Unpredictable chronic mild stress (UCMS) and behavioral assessment

Mice were subjected to various unpredictable stressors for 8 weeks as described earlier.[18] Alterations of the bedding (repeated sawdust changing, removal of sawdust, damp sawdust, substitution of sawdust with 21°C water), cage-tilting (45°), predator sounds (15 min), cage shift (mice placed in the empty cage of another male), food deprivation and alterations of the light/dark cycle were used as stressors. Several parameters were evaluated before the start and at different time points during the UCMS procedure. The coat state of each animal was assessed weekly as a measure of UCMS-induced alterations in motivation towards self-care behaviors such as grooming behaviors. The total score of the coat state resulted from the sum of scores from eight different body parts: head, ears, neck, dorsal and ventral coat, tail, forepaws and hindpaws. For each area, a score of 0 was given for a well-groomed coat and 1 for an unkempt coat.[19] 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). Each test mouse was 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 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, Lyon, France). For the object location test (OLT), 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 2 objects was displaced to the opposite corner of the open-field and the time spent exploring displaced and non-displaced objects was measured over a 5-min session by video tracking. For nest building, a square cotton nestlet (5 x 5 cm, 2–3 g; Serlab, Montataire, France) was placed in the cage 1 h before the dark phase. The nests were assessed 5 h later using the following scoring system: 1, the cotton nestlet was intact; 2, the cotton nestlet was partially scattered; 3, the cotton nestlet was scattered but there is no form of nest; 4, the cotton was gathered but there is no nest ("flat nest"); 5, the cotton nestlet was gathered into a hollow sphere with one opening for entry. 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, 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.

Iontophoretic electrophysiology

Extracellular recordings of pyramidal neurons in the CA3 region of the hippocampus were carried out using multi-barreled glass micropipettes as previously described.[20] The central barrel used for extracellular unitary recording and one side barrel used for automatic current balancing were filled with a 2 M NaCl solution. The three other side barrels were filled with serotonin (5-HT, 25 mM in 0.2 M NaCl; pH 4) or norepinephrine (NE, L-NE-HCl, 25 mM in 0.2 M NaCl; pH 4) and quisqualate (1.5 mM in 0.2 M NaCl; pH 8). 5-HT and NE were ejected as cations and retained with currents of – 10 to – 8 nA. Quisqualate was ejected as an anion and retained with a current of + 1 to + 3 nA. The impedance of the central barrel was 2–5 MΩ and those of the balance barrel and side barrels were 20–30 MΩ and 50–100 MΩ, respectively. The five-barreled glass micropipettes were positioned in mm relative from bregma: A/P, – 2.5 to – 3; M/L, + 2.3 to 2.8; D/V, – 2 to – 3. Quisqualate stimulated

pyramidal neurons were identified by their high amplitude (0.5–1.2 mV), high frequency and long duration (0.6–1.0 ms) action potential and by their characteristic "complex spike" discharge. Venlafaxine was used as a dual inhibitor to block both 5-HT and NE reuptake.[21] In agreement with this action, venlafaxine at the dose of 16 mg/kg, subcutaneous, produced a reduction of the firing rate of CA3 pyramidal neurons in wild-type and $OCT2^{-/-}$ mice. The excitatory current of quisqualate was thus increased for both groups to maintain a firing activity rate similar to that before venlafaxine injection. The time required for the firing activity to recover 50% of the initial firing rate after microiontophoretic application (recovery time (RT50) value) was used as an index of the capacity of neurons of the dorsal hippocampus to remove 5-HT or NE from the synaptic cleft in the presence or absence of venlafaxine.[21]

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 or submitted to swim stress or 8-week UCMS. 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, Basel, Switzerland) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail 3, Sigma Aldrich). Protein concentrations were determined by Bradford's method. Proteins samples (15 to 50 μg) suspended in NuPage LDS sample buffer (Invitrogen, Carlsbad, CA) were separated by Bis-Tris SDS–PAGE (10% gels) and transferred onto nitrocellulose membranes (Invitrogen). Transfer efficacy was controlled by Ponceau S staining. Unspecific binding sites were blocked in 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),

pGSK3β (1/1000), pAkt (1/200), Akt (1/2000), pTrkB (1/200) from Cell Signaling (Danvers, MA, USA), tyrosine kinase receptor B (TrkB, 1/2000) from BD Biosciences (San Diego, CA, USA) or β-actin (1/5000) 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 the Odyssey Infrared Imaging System and Application Software version 3.0 (LI-COR, Biosciences, Lincoln, NE, USA). For analysis of the effect of WAY100635 on Akt/GSK3β signaling, this compound was injected (1 mg/kg i.p.) 3.5 h prior to sacrifice.

[^{35}S]GTP γS binding

The functional activity of 5-HT receptors was assessed as described previously[22] with the following modifications. Cryosections were preincubated 30 min at 20°C in HEPES buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 0.2 mM DTT and 2 mM GDP) for 5-HT1A receptors and in Tris buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 0.2 mM DTT and 2 mM GDP) for 5-HT2A receptors, then incubated with 25 nM [35S]GTPγS in the same buffers for 2 h in presence of selective agonists. 8-OHDPAT (10 μM) and DOI (5 nM) were used to stimulate [35S]GTPγS binding at 5-HT1A and 5-HT2A receptors, respectively. Receptor subtype-specificity of stimulated [35S]GTPγS binding was verified by the addition of WAY100635 (1 mM) for 5-HT1A receptors and ketanserin (10 μM) for 5-HT2A receptors. Basal binding was determined in the absence of agonists. The sections were washed in cold HEPES buffer for 2 min, briefly dipped in ice-cold water, dried and exposed to Kodak BioMax MR autoradiographic film (VWR, Rochester, NY, USA). Quantification of autoradiograms were carried out with MCID software (Imaging Research Inc, St. Catherines, Ontario, Canada).

Statistics

PRISM (GraphPad Software, San Diego, CA, USA) and Statview 5.0 (SAS Institute Inc., Cary, NC, USA) were used for statistical calculations. Student's *t*-test was used for comparisons of mean values for the two genotypes in the GTP-γS binding experiments and nest building. For corticosterone dosage, coat state, object location test, social interaction, sucrose preference, microiontophoretic electrophysiology and Western blot experiments, data were analyzed using two-way or three-way analysis of variance (ANOVA) followed by Fisher's Protected LSD test. Statistical significance was set at $P < 0.05$.

Results

OCT2 is expressed in stress-related circuits in the brain

In a previous study, frontal cortex, hippocampus and amygdala were identified as OCT2-expressing sites.[11] We further investigated by fluorescent immunohistochemistry the distribution of this transporter in the adult mouse brain and found notable expression in several regions implicated in the response to stress. In the telencephalon, OCT2 was prominently expressed in the prelimbic, infralimbic and anterior cingulate cortices, as shown for layers II-III to VI of prelimbic and infralimbic cortices (Figure 1a). In the hippocampal formation, OCT2 was expressed in pyramidal cells of CA1, CA2 and CA3 as previously described[11] (Figure 1b), in neurons of the polymorph layer of the DG and diffusely in the lacunosum molecular layer of CA1–3 (Figure 1b). Notable OCT2 levels were found in ventral subiculum (Figure 1c and d), amygdalohippocampal area (Figure 1c) and medial amygdaloid nucleus (Figure 1e). OCT2 was also expressed in several nuclei of the diencephalon, with intense labeling in dorsomedial, ventromedial and arcuate nuclei of the hypothalamus (Figure

1f) and in paraventricular nucleus of the thalamus (Figure 1g). Finally, OCT2 immunolabeling was detected in the median eminence (Figure 1h) and in pituitary (Figure 1i and j). In this last structure, OCT2 was expressed in a non-corticotrope subpopulation of the anterior pituitary, as shown with co-labeling with ACTH antibodies (Figure 1i) and uniformly distributed in the posterior pituitary, apposed to pituicytes labeled with GFAP (Figure 1j). No labeling was detected in $OCT2^{-/-}$ mice brain, as shown for cingulate and motor cortices (Figure 1k) and hippocampus CA1 (Figure 1l).

OCT2 in the brain controls the hormonal response to acute stress

A number of the above-mentioned circuits expressing OCT2 have been shown to trigger, through direct and indirect connections with the PVN and periPVN regions, the activation of the HPA axis, which culminates in corticosteroid secretion.[23] We thus investigated in $OCT2^{-/-}$ mice the neuroendocrine response to acute stress. The circulating corticosterone levels under basal conditions and following a 15-min swim stress were evaluated in wild-type and knockout mice. Basal plasma corticosterone levels were increased by 87% in $OCT2^{-/-}$ mice compared to wild-type mice ($P = 0.0058$, Figure 2a). More importantly, swim stress-induced corticosterone release was also strongly enhanced in $OCT2^{-/-}$ mice compared to wild-type mice, with an increase of 56% of peak levels after stress ($P < 0.001$, Figure 2a). Besides central circuits, peripheral processes involving circulating catecholamines or paracrine control within adrenal glands may also modulate locally corticosterone response during acute stress.[24, 25] In particular, OCT2 labeling was found in the adrenal cortex and in some of the norepinephrine transporter (NET)-positive secretory cells of the adrenal medulla, as shown by immunohistochemical labeling (Supplementary Figure 1a). In agreement, significant OCT2-specific uptake was detected *ex vivo* in adrenal cellular extracts (Supplementary Figure 1b). Therefore, to tease apart the respective contributions of central and peripheral processes in

corticosterone oversecretion, we performed an ACTH stimulation test after dexamethasone suppression. Dexamethasone at low doses is known to inhibit the HPA axis at the level of pituitary with little or no penetration in the brain and routinely used to evaluate adrenal sensitivity.[26] We found a comparable sensitivity of OCT2$^{-/-}$ and wild-type mice adrenal glands to physiological doses of ACTH, precluding a local effect of OCT2 (Figure 2b). These results demonstrate that the enhanced hormonal response to stress of OCT2$^{-/-}$ mice is a direct consequence of the absence of OCT2 in the brain, at pituitary or suprapituitary sites.

OCT2 is implicated in vulnerability to UCMS

We made the hypothesis that their increased hormonal response to acute stress could confer to OCT2$^{-/-}$ mice vulnerability to repeated stressful conditions. To challenge this possibility, we used UCMS, a depression model with high translational value for the investigation of interactions between genetic factors and environmental stress.[19, 27] Mice of both genotypes were subjected to UCMS for 8 weeks and their behavioral assessment was carried out throughout the procedure. OCT2$^{-/-}$ mice showed increased vulnerability to UCMS for the development of several anomalies reflecting distinctive symptoms of depression, involving grooming, spatial memory, social interaction and stress-sensitive spontaneous behavior. Coat state, an indicator of self-grooming behavior extremely sensitive to chronic stress,[19, 28] deteriorated progressively throughout the UCMS procedure in both genotypes. This deterioration occured however significantly more intensely ($P < 0.05$ and $P < 0.001$) in OCT2$^{-/-}$ mice than in wild-type mice (Figure 3a). The effect of UCMS on short-term spatial memory, a cognitive function implicating hippocampus[29] often impaired in stress-related disorders, was assessed by measure of performance in the object location test (OLT). We found that UCMS induced notable spatial memory deficits much earlier in OCT2$^{-/-}$ mice than in wild-type mice (Figure 3b). Significant differences in the exploration time of the displaced

object compared to the non-displaced object at basal state were found before UCMS for both genotypes (wild-type, $P < 0.0001$; OCT2$^{-/-}$, $P = 0.0003$). UCMS affected spatial memory performance of both genotypes, albeit in a different manner. Strikingly, after 3 and 5 weeks of UCMS, only wild-type mice maintained the ability to discriminate the relocated object ($P = 0.0039$ and $P = 0.0453$, respectively) while OCT2$^{-/-}$ mice had lost this ability (Figure 3b), indicating increased vulnerability in the early stages of the stress procedure. Finally, at later stages during the procedure, both genotypes lost the capacity to distinguish between the displaced and non-displaced objects. Next, spontaneous social interaction, an indicator of diminished appeal for social interaction, another anomaly found in MDD, was assessed before and after UCMS (Figure 3c). At basal state, both genotypes showed a comparable time of interaction with target. At 3 and 4 weeks after the start of UCMS, OCT2$^{-/-}$ mice showed a significant decrease in interaction time with target compared to wild-type mice ($P = 0.0248$ and $P = 0.0457$, respectively), while at later stages time of interaction with target became undistinguishable between the two genotypes (Figure 3c). These results reveal the increased vulnerability of OCT2$^{-/-}$ mice as regards social behavior, again at early stages of the UCMS procedure. Finally, decreased performance in nest building, a hippocampus-dependent task,[30] was detected in OCT2$^{-/-}$ mice after 8-week UCMS ($P = 0.0198$), indicating a stress-driven inhibition of spontaneous behavior (Figure 3d). In contrast, sucrose preference was significantly decreased in both OCT2$^{-/-}$ and control mice by the UCMS procedure ($P < 0.0001$), without leading to a full anhedonic state i.e., loss of preference at the end of the procedure, but no significant differences were detected between genotypes (Figure 3e). To determine the state of the negative feedback of the HPA axis in OCT2 mutants, the mice were submitted to a dexamethasone suppression test under swim stress conditions, before and after the complete UCMS procedure (Figure 3f). This evaluation showed a significant suppression (63–70%) of corticosterone secretion by dexamethasone for both genotypes, indicative of

normal HPA negative feedback control in the mutants. HPA feedback in either genotype was poorly sensitive to UCMS, since no significant modifications in dexamethasone suppression were found after completion of the procedure (Figure 3f). Interestingly, after UCMS, corticosterone secretion induced by swim stress was decreased in OCT2$^{-/-}$ mice, leading to levels comparable to those of wild-type mice (Figure 3g). Finally, genotype differences in immobility time in the forced-swim test, an acute behavioral despair paradigm, also leveled off after UCMS (Supplementary Figure 2).

Exogenous corticosterone can modify both high-affinity transporter-mediated and OCT2-mediated 5-HT and NE clearance in hippocampus *in vivo*

Previous studies raised the possibility that during the stress response corticosterone might impair clearance of 5-HT or other monoamines through inhibition of OCTs *in vivo*. To explore this possibility, we investigated the effect of subchronic corticosterone treatment on 5-HT and NE clearance mediated by the high-affinity and low-affinity transporters *in vivo*. 4-day corticosterone oral administration raised circulating concentrations to 115.9 ± 14 ng/ml and 112.8 ± 18 ng/ml for wild-type and OCT2$^{-/-}$ mice, respectively. The activity of postsynaptic neurons in hippocampus CA3, a region of high OCT2 density, was investigated *in vivo* by a combined microiontophoretic and electrophysiological approach, before and after subchronic corticosterone treatment. Injection of the dual inhibitor venlafaxine was used to inhibit the 5-HT and NE transporters and reveal OCT-mediated clearance. As previously reported,[11, 31] microiontophoretic applications of 5-HT (Figure 4a, left) or NE (Figure 4b, left) resulted in the suppression of the firing activity of CA3 pyramidal neurons. In pyramidal neurons of both control and corticosterone-treated hippocampus, the firing gradually recovered upon the cessation of the application of monoamines (Figure 4a–b, left). The recovery time for pyramidal neuron firing, with or without administration of corticosterone,

and before and after acute systemic administration of venlafaxine, was expressed as RT_{50} values (Figure 4a–b, right). In wild-type mice, RT_{50} values before treatment with venlafaxine were significantly higher for corticosterone-treated mice than for vehicle-treated controls (5-HT, $P = 0.0205$; NE, $P = 0.0394$), indicating a notable decrease of monoamine clearance after subchronic corticosterone when the high-affinity 5-HT and NE transporters are fully active. Venlafaxine at 16 mg/kg had a main significant effect on RT_{50} for 5-HT ($F_{1,35} = 14.052$; $P = 0.0006$, Figure 4a) and for NE ($F_{1,22} = 82.900$; $P < 0.0001$, Figure 4b) reflecting partial blockade of the high-affinity transporters, and the differences between control and corticosterone-treated mice were maintained after its administration (5-HT, $P = 0.0339$; NE, $P = 0.0342$). In contrast, $OCT2^{-/-}$ mice showed a similar increase of RT_{50} values after corticosterone treatment in absence of venlafaxine (5-HT, $P = 0.0021$; NE, $P < 0.0001$), but this effect of corticosterone was no more detectable after venlafaxine treatment (Figure 4a-b, right). This last observation indicates that subchronic corticosterone treatment inhibits 5-HT and NE clearance *in vivo* when the NET and SERT are partially pharmacologically inactivated in wild-type mice, but not in $OCT2^{-/-}$ mice. Taken altogether these experiments suggest that subchronic corticosterone administration at physiological concentrations may influence both high-affinity transporter-mediated and OCT2-mediated uptake.

$OCT2^{-/-}$ mice show dysregulation of Akt/GSK3β signaling in the hippocampus

Glycogen synthase kinase-3β (GSK3β) is a serine/threonine kinase with multiple cellular functions.[32] It was recently proposed to be a downstream intracellular mediator of the mood-related actions of 5-HT,[33, 34] an *in vivo* substrate of OCT2.[11] In particular, stimulation of 5-HT release in various brain regions including hippocampus was shown to inhibit considerably GSK3β by Ser-9 phosphorylation through the activation of 5-HT1A receptors,[35] an effect mediated by the principal GSK3β regulator, Akt.[36] We thus searched for anomalies of GSK3β

signaling in OCT2$^{-/-}$ mice, by assessing the functional state of this pathway in stress-related brain regions amenable to quantitative Western blot analysis (Figure 5a). We found that the phosphorylation of GSK3β at Ser-9, a phosphorylation site controlling negatively its activity,[36] was significantly increased in two regions of OCT2$^{-/-}$ mice brain compared to wild-type, dorsal hippocampus (P = 0.0037) and PVN (P = 0.038), while total levels of GSK3β were unchanged (not shown), indicating decreased activity selectively in these brain regions. This increase in GSK3β phosphorylation was associated with increased activity of its modulator Akt, which showed increased Threo-308 phosphorylation (P < 0.0001 and P = 0.0194, respectively for hippocampus and PVN). This phosphorylation increase occurred independently of TrkB signaling, the canonical GSK3β-activating pathway activated by neurotrophins[37] as shown by unchanged phosphorylated to total TrkB ratios (Figure 5a) and total TrkB levels (not shown).

To further study GSK3β dysregulation, we focused on a single region, hippocampus, and explored the functional state of this kinase after acute and chronic stress. Ser-9 phosphorylation of GSK3β was found highly increased after acute swim stress in hippocampus of both wild-type and OCT2$^{-/-}$ mice (P < 0.0001) while total amounts of GSK3β were unchanged (not shown). This was associated with increases in total amount of phosphorylated TrkB in wild-type mice (P = 0.0006) and of both phosphorylated Akt (P = 0.0007) and phosphorylated TrkB (P = 0.0101) in OCT2$^{-/-}$ mice (Figure 5b, bottom, pAkt or pTrkB/β-actin ratio) while phosphorylated over non-phosphorylated ratios of these proteins were unchanged or decreased by swim stress (Figure 5b, top), suggesting the implication of neurotrophin/TrkB signaling in acute stress-induced GSK3β inhibition. Interestingly, after swim stress, Ser-9 phosphorylation at GSK3β (P = 0.0021) and Threo-308 phosphorylation at Akt (P = 0.0268) but not total amount of these proteins (not shown) were increased in OCT2$^{-/-}$ mice hippocampus compared to wild-type with, as at basal state, no genotype-specific

differences in TrkB relative activity (Figure 5b) or level (not shown). Thus, the absence of OCT2 potentiates through Akt but independently of TrkB the effects on GSK3β inhibition occurring during acute stress. UCMS in contrast had limited effects on the GSK3β intracellular pathway. This procedure leveled out the differences between genotypes in Ser-9 phosphorylation, which was decreased in OCT2$^{-/-}$ (P = 0.0005) but not wild-type mice, while decreasing effects on Akt and TrkB activity were detected for both genotypes (Figure 5b).

Link between Akt/GSK3β signaling in OCT2$^{-/-}$ hippocampus during acute stress and 5-HT1A receptor activity

As shown above, the levels of phospho-Ser-9-GSK3β and phospho-Threo-308-Akt were significantly increased in certain regions (hippocampus, PVN) of OCT2$^{-/-}$ mice, revealing a notable perturbation of this intracellular pathway at basal state. Since TrkB was not involved in this dysregulation of Akt/GSK3β signaling, we tested whether it could be linked to altered serotonergic tonus and in particular 5-HT receptor activation.[33, 35] To further identify the mechanisms leading to GSK3β inhibition in OCT2$^{-/-}$ mice brain, we tested by stimulation of [35S]GTPγS binding on brain sections the functional state of 5-HT1A and 5-HT2A receptors, both highly expressed in hippocampus CA3.[38] These experiments indicated that the 5-HT1A receptor was overactivated in hippocampus of OCT2$^{-/-}$ mice compared to wild-type mice (P = 0.0163), while no significant modification of the activity of 5-HT2A receptors was detected (Figure 6a). To determine whether this increase in 5-HT1A receptor activity in the mutants could participate in modifications of Akt/GSK3β signaling during acute stress, the specific 5-HT1A receptor antagonist WAY100635 was injected prior to swim stress to the mice, and the functional state of the Akt/GSK3β pathway explored in hippocampus. WAY100635 restored in OCT2$^{-/-}$ mice a ratio of phosphorylated to total GSK3β and Akt comparable to wild-type mice, with an effect in hippocampus of OCT2$^{-/-}$ mice (GSK3β, P = 0.0058; Akt, P = 0.0036)

but not wild-type mice (Figure 6b). These experiments suggest that increased 5-HT tonus and in particular post-synaptic 5-HT1A receptor activation could be responsible at least in part for Akt/GSK3β signaling dysregulation in the mutants during acute stress in the hippocampus.

Discussion

Stress and HPA axis dysregulation constitute important risk factors for psychiatric disorders such as depression and PTSD.[39, 40] Chronic stress in rodents can mimic these noxious effects, inducing gradual neurobiological and behavioral anomalies resembling symptoms of human depression.[19, 27] In addition, HPA axis activity has been suggested to play a role in "passive" resilience i.e., resilience mediated by the absence of key neurochemical, molecular or endocrinal responses in certain individuals.[41, 42] The major finding of this study was that OCT2 activity profoundly modulates the hormonal response to acute stress and vulnerability to repeated stress. $OCT2^{-/-}$ mice showed an aggravation of several alterations induced by UCMS (Figure 6c), some of which evocative of the working memory deficits[43] and social withdrawal symptoms that occur in MDD patients.[44, 45] Intriguingly, these genotype-specific differences in social interaction and spatial memory appeared transiently in the first weeks of the UCMS procedure, suggesting a role of OCT2 in the acquisition of this phenotype in the early phases. Contrasting with these behaviors, no significant differential effect of UCMS on anhedonia, evaluated by sucrose preference, was detected in the OCT2 mutants. Anhedonia, a cardinal symptom of MDD, is only present in half of the patients[46] and also prominent in other mental disorders. Since OCT2 has a similar localization in human and rodent brain,[47] our findings suggest that its activity could be a determinant of individual variability in vulnerability to MDD and contribute to the clinical heterogeneity of this disorder.

It is commonly admitted that circulating corticosteroids mediate for a large part the deleterious effects of repeated stress on the brain. Enhanced corticosterone release is thus probably implicated in the differential effects of UCMS in OCT2$^{-/-}$ mice. Several lines of data argue in favor of this possibility. First, in agreement with our observations, stress and corticosterone have been shown to disrupt hippocampal-mediated learning[5, 48-50] and spatial memory retrieval.[51] Second, in keeping with a transitory action of UCMS, swim stress-induced corticosterone secretion was completely blunted in OCT2$^{-/-}$ mice at the end of the full UCMS procedure. Finally, chronic administration of corticosterone, another long-term depression paradigm which very rapidly enhances and evens out the levels of circulating corticosterone, was shown to induce in contrast to UCMS a comparable deterioration of the coat state of both genotypes.[11] These observations support a causal relationship between increased corticosterone release in OCT2$^{-/-}$ mice and some of the anomalies found in these mice during the first weeks of the chronic stress procedure.

OCT2 was detected in several brain regions implicated in the response to stress, such as prelimbic and infralimbic cortices, hippocampus, amygdala, dorsomedial and arcuate nuclei of hypothalamus, paraventricular nucleus of the thalamus and pituitary, as well as in adrenals.[7, 23, 52] It is however difficult at this stage to attribute HPA activation in the mutants to the absence of OCT2 within specific brain regions. Investigation of adrenal sensitivity to ACTH allowed us to exclude periphery-based mechanisms involving sympathetic innervation, circulating catecholamines and/or paracrine control within adrenal glands.[24, 25] Hence, enhanced hormonal response to acute stress in OCT2$^{-/-}$ mice appears to occur through mechanisms based in circuits controlling HPA activation upstream in the brain, at pituitary or suprapituitary sites, which remain to be determined. Moreover, a normal state of HPA negative feedback was observed in OCT2$^{-/-}$ mice, consistent with a role for OCT2 in the initial steps of HPA axis activation, rather than in indirect modulation of this response

through negative feedback.

Along with increased HPA axis activation during acute stress, OCT2$^{-/-}$ mice show important dysregulation of brain GSK3β signaling, a pathway previously implicated in the regulation of mood.[53-55] GSK3β is constitutively active in post-mitotic neurons but inhibited by phosphorylation in response to various upstream pathways implicating neurotrophins[37] and GPCRs.[56] To investigate the GSK3β modulation during stress, we focused on dorsal hippocampus and found that a profound inhibition of this kinase occurs during acute stress in normal mice, associated with an activation of the neurotrophin receptor TrkB. Remarkably, in OCT2$^{-/-}$ mice, GSK3β activity was significantly inhibited both at basal state and during acute stress compared to wild-type mice, accompanied by the activation of its main regulator Akt. As consequence of this modulation of hippocampal signaling, the inhibitory action of acute stress on GSK3β was notably aggravated in mutant mice (Figure 6c).

We excluded the canonical neurotrophin/TrkB pathway as a main mechanism for OCT2-mediated GSK3β regulation but found in mutant mice hippocampus a differential coupling with 5-HT receptor activation, another major GSK3β regulatory pathway in neural cells.[56, 57] 5-HT deficiency in Tph2 knockout mice was previously shown to activate GSK3β, while the inhibition of this kinase was able to correct the mood-related behavioral anomalies of these depleted mice.[33] Reciprocally, elevation of 5-HT level was shown to inhibit considerably GSK3β by Ser-9 phosphorylation in several brain regions including hippocampus, through the activation of 5-HT1A receptors[35] and Akt signaling.[57] In agreement with this mechanism, our results suggest that increased 5-HT tonus leading to overactivation of 5-HT1A receptor could drive at least in part the anomalies in Akt/GSK3 signaling in OCT2$^{-/-}$ mice (Figure 6c). Interestingly, post-synaptic 5-HT1A receptor activation was also shown to be involved in corticosterone secretion,[58, 59] suggesting a potential mechanistic link between 5-HT tonus, corticosterone secretion and GSK3β inactivation.

We do not at this point know whether differential GSK3β inhibition plays a role in the exacerbated vulnerability of OCT2$^{-/-}$ mice to chronic stress. Nevertheless, several observations support this possibility. GSK3β activation was shown to be important for memory retrieval[60] and reconsolidation[61] and IGFII-dependent memory enhancement[62], while its inhibition was linked to impaired NMDA receptor-dependent LTD,[63] a process required for spatial memory consolidation.[64, 65] Other studies suggest that forebrain GSK3β play a role in sociability.[66] Interestingly, Akt/GSK3 β signaling in the brain was also implicated in susceptibility to social defeat [67] and to PTSD-like symptoms following inescapable stress.[68] In agreement with a transitory action of GSK3β during UCMS, our study demonstrates that this kinase was strongly inhibited during acute stress in normal mice hippocampus, while its activation state was unchanged after completion of the UCMS procedure. In this perspective, the potentiation of GSK3β inhibition in OCT2$^{-/-}$ mice could contribute to their vulnerability during the first weeks of UCMS and constitute at these early stages a marker of vulnerability to chronic stress.

Finally, while these results clearly demonstrate that OCT2 deletion has major consequences on corticosterone secretion, a reciprocal action of basal or peak corticosterone on OCT-mediated transport cannot be excluded, as demonstrated here by microiontophoretic electrophysiology. Such an interaction has been demonstrated *in vitro*[13, 14] and suggested to occur *in vivo* by recent studies showing that repeated swim stress could reduce 5-HT clearance rate in mice hippocampus through corticosterone release.[16] OCT2 blockade by corticosterone could thus contribute to the neurochemical and molecular adaptative modifications taking place during short- or long-term stress. In this case, corticosterone blockade of OCT2 during the response to stress would be expected to reinforce 5-HT signaling and GSK3β inhibition, providing an additional feed-forward mechanism to modulate this pathway. This possibility is for the time being highly speculative, as this

interaction could occur during acute stress in only certain brain regions or neurons, generating radically different consequences than full-blown OCT2 invalidation.

In conclusion, the present study describes a novel mechanism at the core of the genetic-environment interactions that underlie individual variability in the response to stress. We identify OCT2 as a genetic factor that directly impacts on the molecular and hormonal events taking place during acute stress, accentuating the noxious effects of repeated stress on the development of stress-related phenotypes. This suggests that genetic polymorphisms modifying OCT2 transport activity or long-term administration of drugs inhibiting OCT2, such as certain antidiabetics, cytostatics or antiviral compounds,[69, 70] could have important consequences on the vulnerability to environmental stress.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We thank M. Nosten-Bertrand for advice concerning behavior and statistics and F. Machulka for expert assistance in animal care. T.C. A.B. and L.B. were recipients of fellowships from the French Ministry for Research and the Société Française de Pharmacologie et Thérapeutique. Financial support was provided by the Institut National pour la Santé et la Recherche Médicale (INSERM), the Fondation de France and the Agence nationale de la recherche (ANR-13-SAMENTA-0003-01).

Supplementary Information accompanies the paper on the Molecular Psychiatry website.

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Legends to figures

Figure 1 OCT2 is expressed in stress-related circuits in the brain. (**a**–**l**) Immunofluorescent histochemistry of coronal mice brain sections. OCT2 is notably enriched in layers II–III, V and VI of the prelimbic (PrL) and infralimbic (IL) cortices (**a**). In the hippocampal formation, high OCT2 expression is found in the pyramidal cell layer (pyr) of CA1, CA2 and CA3, in the polymorph (po) layer of the DG and diffuse labeling is detected in the lacunosum molecular (LMol) layer of CA1-3 (**b**). OCT2 positive cells are detected in the ventral subiculum (vSub, **c**, **d**) and in the amygdalohippocampal area (AHi, **c**). OCT2 is also detected in the medial amygdaloid nucleus (MeA, **e**), dorsomedial (DMH), ventromedial (VMH) and arcuate (Arc) nuclei of the hypothalamus (**f**), paraventricular nucleus of the thalamus (PVA, **g**) and median eminence (ME, **h**). OCT2 is found in a non-corticotrope subpopulation of the anterior lobe of pituitary (AL), as shown by co-labeling with ACTH antibodies (**i**) and uniformly distributed in the posterior lobe (PL, **j**). No labeling was detected in $OCT2^{-/-}$ mice brain, shown for cingulate and motor cortices (Cx, **k**) and hippocampus (**l**). Cortical layers are identified by roman numerals. Mol, molecular layer; ec, external capsule; gr, granular layer; Pe, periventricular nucleus of the hypothalamus. Scale bars represent 100 μm (**a**–**c**, **f**, **h**, **k**, **l**) and 20 μm (**d**, **e**, **g**, **i**, **j**).

Figure 2 OCT2 in the brain controls the hormonal response to acute stress. (**a**) Plasma corticosterone levels in basal conditions (**left**) and after a 15-min swim stress (**right**) are considerably increased in $OCT2^{-/-}$ mice. Student's *t*-test, unpaired, two-tailed, $t_{29} = 3.0$, $^{**}P = 0.0058$, n = 15–16 for basal conditions. Two-way analysis of variance (ANOVA) reveals main significant effects of genotype ($F_{1,106} = 58.18$; $P < 0.0001$), time ($F_{7,106} = 49.81$; $P < 0.0001$) and genotype x time interaction ($F_{7,106} = 2.727$; $P = 0.0121$). Fisher's post-hoc test, $^{**}P < 0.01$, $^{***}P < 0.001$, $OCT2^{-/-}$ mice versus wild-type mice (n = 4–12) for swim stress.

(**b**) OCT2$^{-/-}$ mice adrenal glands respond normally to ACTH stimulation. Plasma corticosterone secretion was induced by a single ACTH injection (solid line) after dexamethasone (Dex) pretreatment in wild-type and OCT2$^{-/-}$ mice, compared to saline injection (dashed line). Two-way ANOVA (n = 4–6) reveals no main significant effects of genotype ($F_{1,37} = 3.8$; $P = 0.0603$). Results are given as mean ± s.e.m.

Figure 3 OCT2$^{-/-}$ mice show increased vulnerability to the effects of UCMS. (**a**) OCT2$^{-/-}$ mice show enhanced deterioration of coat state during the UCMS procedure. Two-way ANOVA (n = 6–15) reveals significant effects of time ($F_{8,211} = 17.82$; $P < 0.0001$), genotype ($F_{1,211} = 77.25$; $P < 0.0001$) and genotype x time interaction ($F_{8,211} = 3.975$; $P = 0.0002$) and Fisher's post-hoc test show differences between wild-type and mice from week 2 to the end of the procedure (*$P < 0.05$, ***$P < 0.001$). (**b**) OCT2$^{-/-}$ mice show spatial memory deficits during the UCMS procedure. Two-way ANOVA (n = 10) reveals significant main effects of object ($F_{1,114} = 52.13$; $P < 0.0001$) and object x UCMS interaction ($F_{3,114} = 5.813$; $P = 0.0010$). Fisher's post-hoc test reveals significant differences in the exploration time of the displaced object compared to the non-displaced object at basal state before UCMS for both genotypes (wild-type, ***$P < 0.0001$; OCT2$^{-/-}$, ***$P = 0.0003$). At 3 and 5 weeks of UCMS, only wild-type mice retain a significant discrimination of the relocated object (Fisher's post-hoc test, $P = 0.0039$ and $P = 0.0453$), while OCT2$^{-/-}$ mice have lost this ability. (**c**) OCT2$^{-/-}$ mice show enhanced social interaction deficits during the UCMS procedure. Two-way ANOVA (n = 10) reveals significant main effects of genotype ($F_{1,86} = 5.087$; $P = 0.0266$). Fisher's post-hoc test indicate differences in interaction time at 3 weeks (*$P = 0.0248$) and 4 weeks (*$P = 0.0457$). (**d**) Decreased performance in nest building in OCT2$^{-/-}$ mice after 5-week UCMS (Student's *t*-test, unpaired, two-tailed, $t_{27} = 2.478$, $P = 0.0198$, n = 14–15). (**e**) UCMS alters sucrose preference of both OCT2$^{-/-}$ and wild-type mice. Two-way ANOVA (n =

10) show significant main effects of UCMS ($F_{2,30}$ = 16.89; P < 0.0001) but not genotype. Results are given as mean ± s.e.m. (**f**) HPA feedback is comparable between OCT2$^{-/-}$ and wild-type mice before and after UCMS. Two-way ANOVA (n = 10) show significant effect of UCMS ($F_{1,36}$ = 4.589; P = 0.039) but not of genotype ($F_{1,36}$ = 1.684; P = 0.2026). (**g**) Swim stress induced corticosterone secretion differences between OCT2$^{-/-}$ and wild-type mice are blunted after UCMS. Two-way ANOVA (n = 6–10) show significant effect of UCMS ($F_{1,28}$ = 24.57; P < 0.0001) and genotype x treatment interaction ($F_{1,28}$ = 5.166; P = 0.0309). Fisher's post-hoc test reveals significant differences between OCT2$^{-/-}$ and wild-type mice before ($^{\#}P$ = 0.0127) but not after UCMS, and significant effects of UCMS for OCT2$^{-/-}$ mice (***P < 0.0001).

Figure 4 *In vivo* OCT2-mediated clearance in hippocampus is sensitive to physiological levels of corticosterone. Recovery of pyramidal neuron firing activity *in vivo* after 5-HT (**a**) or NE (**b**) application in wild-type mice after subchronic corticosterone treatment. (**a**, **b**, **left**) Representative integrated firing histograms show the effects of microiontophoretically applied 5-HT or NE on the spontaneous activity of CA3 pyramidal neurons, before and following systemic administration of venlafaxine (16 mg/kg). White boxes indicate the duration of iontophoretic ejection of 5-HT or NE, respectively. The arrow indicates the time at which the injection of venlafaxine was completed. (**a**, **b**, **right**) Recovery time (RT50) values for pyramidal neuronal activity following microiontophoretic applications of 5-HT (**a**) or NE (**b**) are increased by corticosterone treatment in OCT2$^{-/-}$ mice before but not after venlafaxine injection. Three-way ANOVA of RT50 values (n = 4–6) reveals significant main effects of genotype (5-HT: $F_{1,35}$ = 5.880; P = 0.206; NE: $F_{1,22}$ = 42.925; P < 0.0001), corticosterone treatment (5-HT: $F_{1,35}$ = 14.220; P = 0.0006; NE: $F_{1,22}$ = 104.305; P < 0.0001), venlafaxine treatment (5-HT: $F_{1,35}$ = 14.052; P = 0.0006; NE: $F_{1,22}$ = 82.900; P < 0.0001) and genotype x

treatment interaction (5-HT: $F_{1,35} = 5.773$; $P = 0.0217$; NE: $F_{1,22} = 44.197$; $P < 0.0001$) for corticosterone. Fisher's post-hoc tests reveal significant increases after corticosterone before venlafaxine in both in wild-type mice (5-HT: $*P = 0.0205$; NE: $*P = 0.0394$) and $OCT2^{-/-}$ mice (5-HT: $**P = 0.0021$; NE: $***P < 0.0001$), as well as after venlafaxine treatment in wild-type (5-HT: $*P = 0.0339$; NE: $*P = 0.0342$) but not $OCT2^{-/-}$ mice.

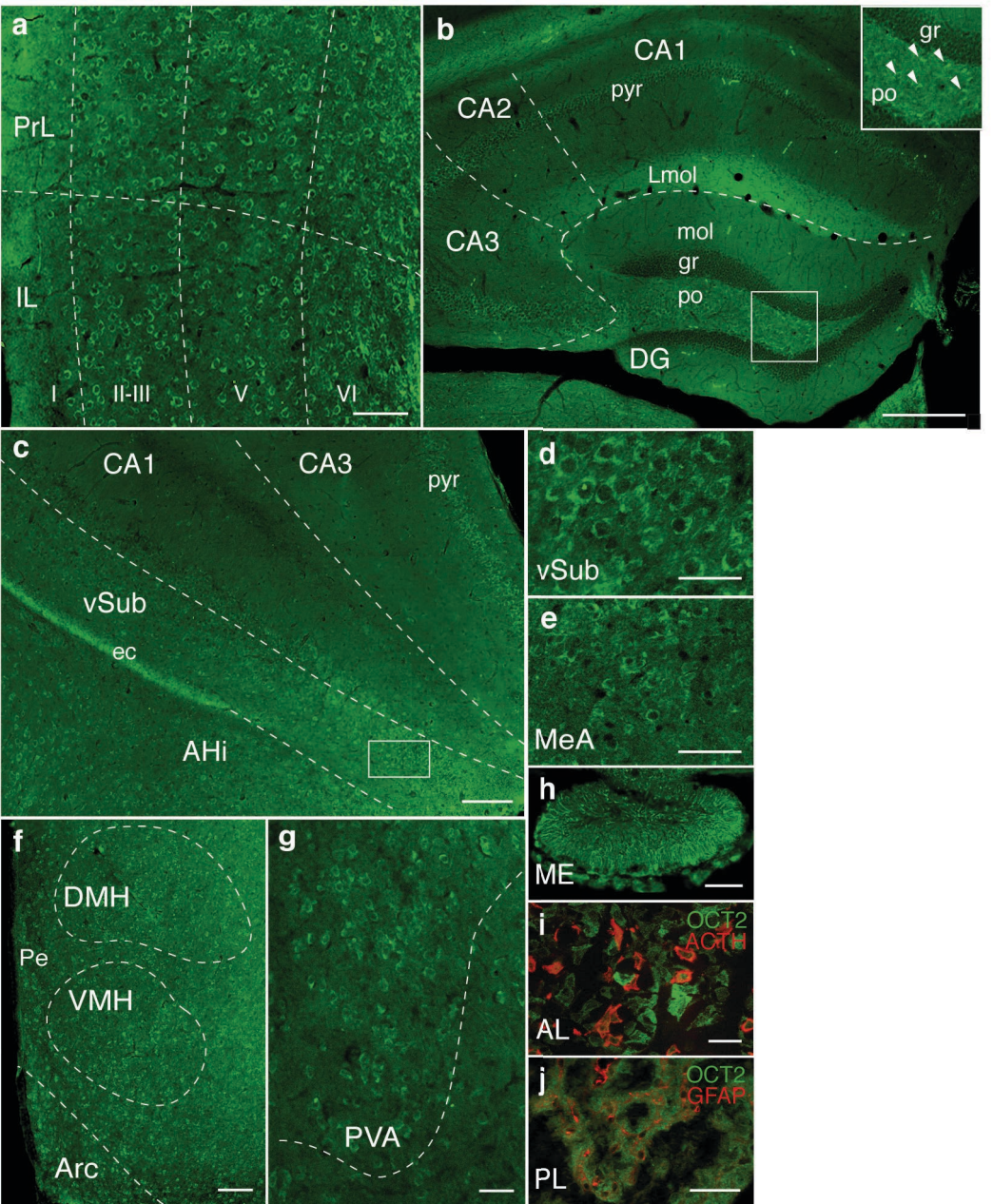
Figure 5 $OCT2^{-/-}$ mice show profound alterations of Akt/GSK3β signaling at basal state and during acute stress. (**a**, **b**) Increased inhibitory phosphorylation of GSK3β and activatory phosphorylation of Akt in the brain at basal state (**a**) and during swim stress and UCMS (**b)**. (**a**) Quantitative Western blot analysis show alterations of phosphorylation of GSK3β (pSer9), and Akt (pThr308) independently of phosphorylation of TrkB (pTyr516) in hippocampus (HPC) and paraventricular hypothalamic nucleus (PVN) of $OCT2^{-/-}$ mice compared to wild-type mice, but not in prefrontal cortex (PFC) and ventral subiculum (vSub). Results are given as mean ± s.e.m of phosphorylated over non phosphorylated protein ratio (Student's t-test, unpaired, two-tailed, GSK3β: HPC, $t_{33} = 3.122$, $**P = 0.0037$, n = 17–18, PVN, $t_{24} = 2.196$, $*P = 0.038$, n = 13 ; Akt: HPC, $t_{33} = 5.288$, $***P < 0.0001$, n = 17–18, PVN, $t_{24} = 2.505$, $*P = 0.0194$, n = 13). (**b)** Quantitative Western blot analysis show alterations of phosphorylation of GSK3β (pSer9), Akt (pThr308) and TrkB (pTyr516) in the hippocampus of wild-type and $OCT2^{-/-}$ mice exposed to acute swim stress or UCMS. Results are given as mean of phosphorylated over non phosphorylated protein ratio ± s.e.m (**top**) and mean of phosphorylated protein over β-actin ratio ± s.e.m (**bottom**). Phospho-TrkB and TrkB are indicated by an arrow. Two-way ANOVA of phosphorylated over non phosphorylated protein ratios (n = 5–18) reveals a significant effect of swim stress for GSK3β ($F_{1,55} = 116.2$; $P < 0.0001$) and TrkB ($F_{1,35} = 14.94$; $P = 0.0005$) and of genotype for GSK3β ($F_{1,55} = 11.52$; $P = 0.0013$) and Akt ($F_{1,55} = 28.55$; $P < 0.0001$). Fisher's post-hoc test reveals significant increase

of phosphorylated over non phosphorylated GSK3β ratios after swim stress in wild-type and OCT2$^{-/-}$ mice (***P < 0.0001), and of GSK3β ($^{\#\#}P$ = 0.0021) and Akt ($^{\#}P$ = 0.0268) phosphorylated over non phosphorylated ratio in OCT2$^{-/-}$ mice compared to wild-type mice after swim stress. Two-way ANOVA of phosphorylated protein over β-actin ratios reveals a significant effect of swim stress for GSK3β ($F_{1,55}$ = 129.8; P < 0.0001), Akt ($F_{1,55}$ = 14.57; P = 0.0003) and TrkB ($F_{1,35}$ = 20.99; P < 0.0001) and of genotype for GSK3β ($F_{1,55}$ = 8.761; P = 0.0045) and Akt ($F_{1,55}$ = 26.70; P < 0.0001). Fisher's post-hoc test reveals a significant increase of phosphorylated GSK3β over β-actin ratios after swim stress in wild-type and OCT2$^{-/-}$ mice (***P < 0.0001). This increase was associated with significant increases in phosphorylated Akt and TrkB over β-actin ratios (wild-type, TrkB: ***P = 0.0006; OCT2$^{-/-}$, Akt: ***P = 0.0007, TrkB: *P = 0.0101). Two-way ANOVA of phosphorylated protein over non phosphorylated protein ratios (n = 4–18) reveals a significant effect of UCMS for GSK3β ($F_{1,51}$ = 9.522; P = 0.0033), Akt ($F_{1,51}$ = 94.97; P < 0.0001) and TrkB ($F_{1,35}$ = 17.60; P = 0.0002) and of genotype x UCMS interaction for GSK3β ($F_{1,51}$ = 4.839; P = 0.0324) and Akt ($F_{1,51}$ = 15.24; P = 0.0003). Fisher's post-hoc test reveals a significant decrease of GSK3β phosphorylation after UCMS in OCT2$^{-/-}$ (***P = 0.0005) but not wild-type mice, and of Akt and TrkB phosphorylation after UCMS in both genotypes (wild-type, Akt: ***P = 0.0001; TrkB: *P = 0.0125; OCT2$^{-/-}$, Akt: ***P < 0.0001; TrkB: **P = 0.0017).

Figure 6 Enhanced 5-HT1A receptor activity restores Akt/GSK3β signaling in OCT2$^{-/-}$ mice during swim stress. (**a**) 5-HT1A receptor activity is selectively increased in OCT2$^{-/-}$ mice hippocampus. Stimulated [35S]GTPγS binding for the 5-HT1A and 5-HT2A receptors are expressed as mean ± s.e.m. of percentage values of basal level (Student's t-test, unpaired, two-tailed, t_9 = 2.947, *P = 0.0163, n = 5–6) and representative sections of specific ligands binding are shown below. (**b)** Quantitative Western blot analysis of phosphorylation of

GSK3β (pSer9) and Akt (pThr308) following swim stress shows that administration of the 5-HT1A receptor antagonist WAY100635 blunts the Akt/GSK3β signaling alterations in OCT2$^{-/-}$ mice hippocampus (n = 7–18). Two-way ANOVA reveals an effect of WAY100635 treatment ($F_{1,45} = 6.573$; $P = 0.0138$) and genotype ($F_{1,45} = 4.846$; $P = 0.0329$) for GSK3β and of genotype x treatment interaction for Akt ($F_{1,45} = 3.081$; $P = 0.0121$). Fisher's post-hoc test reveals significant increases in GSK3β ($^{\#\#}P = 0.0010$) and Akt ($^{\#\#\#}P = 0.0001$) phosphorylation in OCT2$^{-/-}$ mice compared to wild-type mice before but not after WAY100635 treatment, and significant decreases in GSK3β (**$P = 0.0058$) and Akt (**$P = 0.0036$) phosphorylation in WAY100635 treated OCT2$^{-/-}$ mice compared to wild-type mice. (**c**) Schematic representation of the role of OCT2 during stress. Acute stress activates the brain TrkB and HPA axis, leading to corticosterone release in circulation (1). OCT2 transport activity decreases extracellular 5-HT concentrations, modulating Akt/GSK3ß signaling in the brain (2) and inhibiting corticosterone release (3). Repeated HPA activation and corticosterone secretion in early stages of chronic stress exert persisting effects on the brain leading to depression-like behaviors (4). Absence of OCT2 thus exacerbates corticosterone secretion, GSK3 inhibition during acute stress and depression-like behaviors provoked by early repeated stress, leading to vulnerability in the mutant mice. Corticosterone could also inhibit OCT2, reinforcing 5-HT tonus and GSK3ß inhibition. Activation is shown by arrows and inhibition is shown by T-bars. HPA axis is represented in orange. Potential inhibition by corticosterone is indicated by dotted lines.

Figure 1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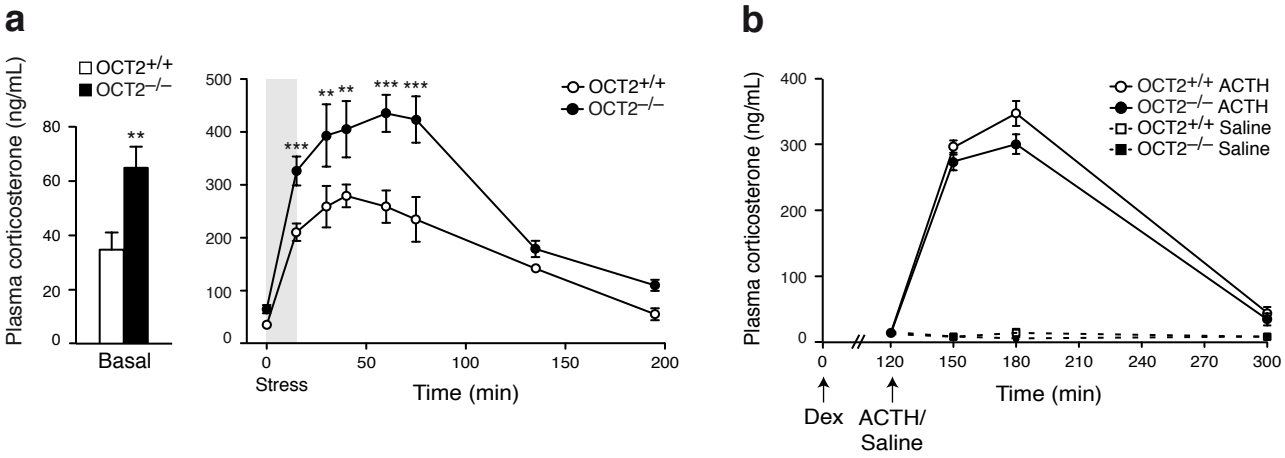
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OCT2+/+
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Basal
Stress
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b
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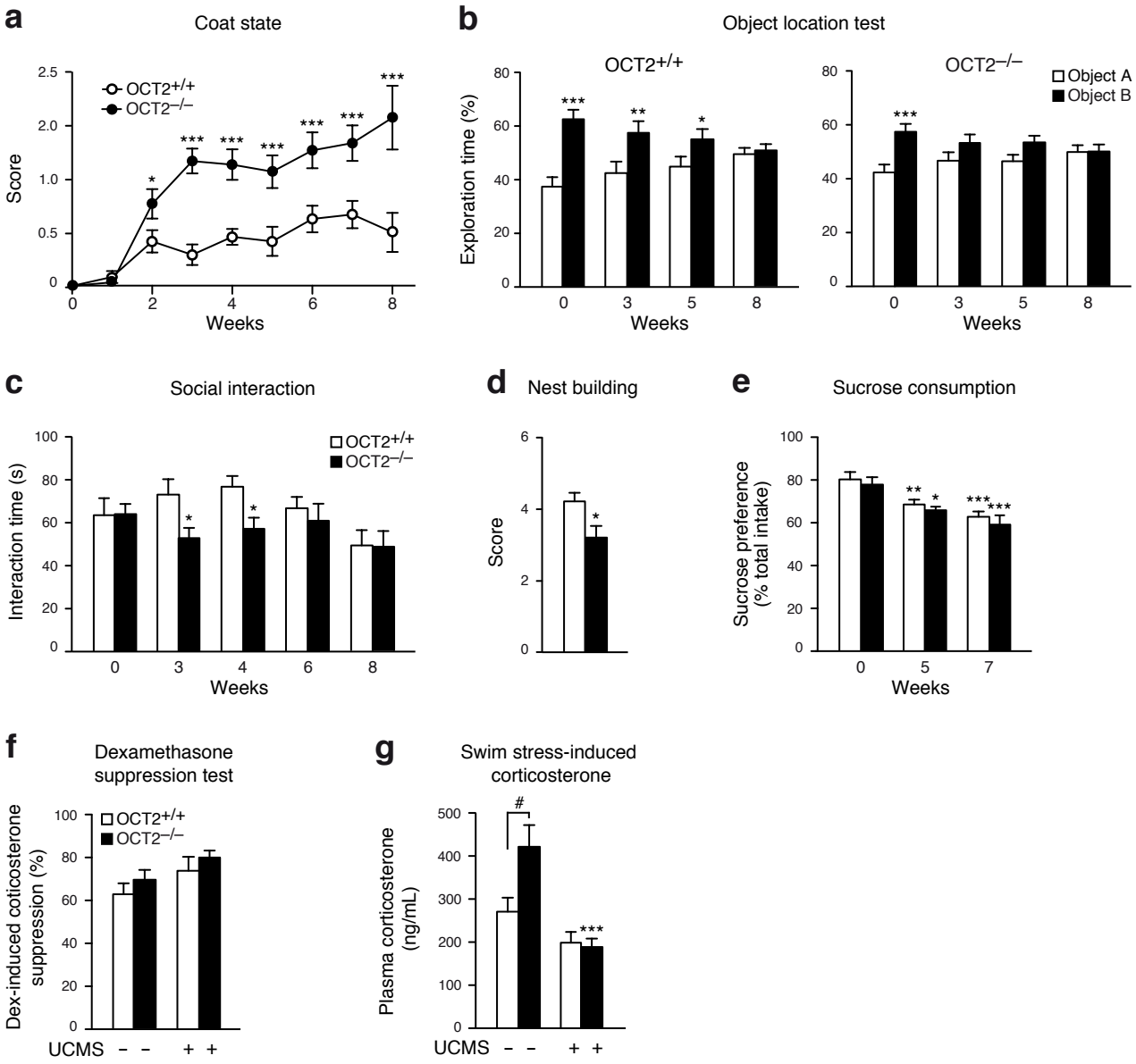
Dex
ACTH/
Saline
Time (min)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Coat state
Score
Weeks
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OCT2−/−
b
Object location test
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OCT2−/−
Exploration time (%)
Object A
Object B
Weeks
c
Social interaction
Interaction time (s)
OCT2+/+
OCT2−/−
Weeks
d
Nest building
Score
e
Sucrose consumption
Sucrose preference (% total intake)
Weeks
f
Dexamethasone suppression test
Dex-induced coticosterone suppression (%)
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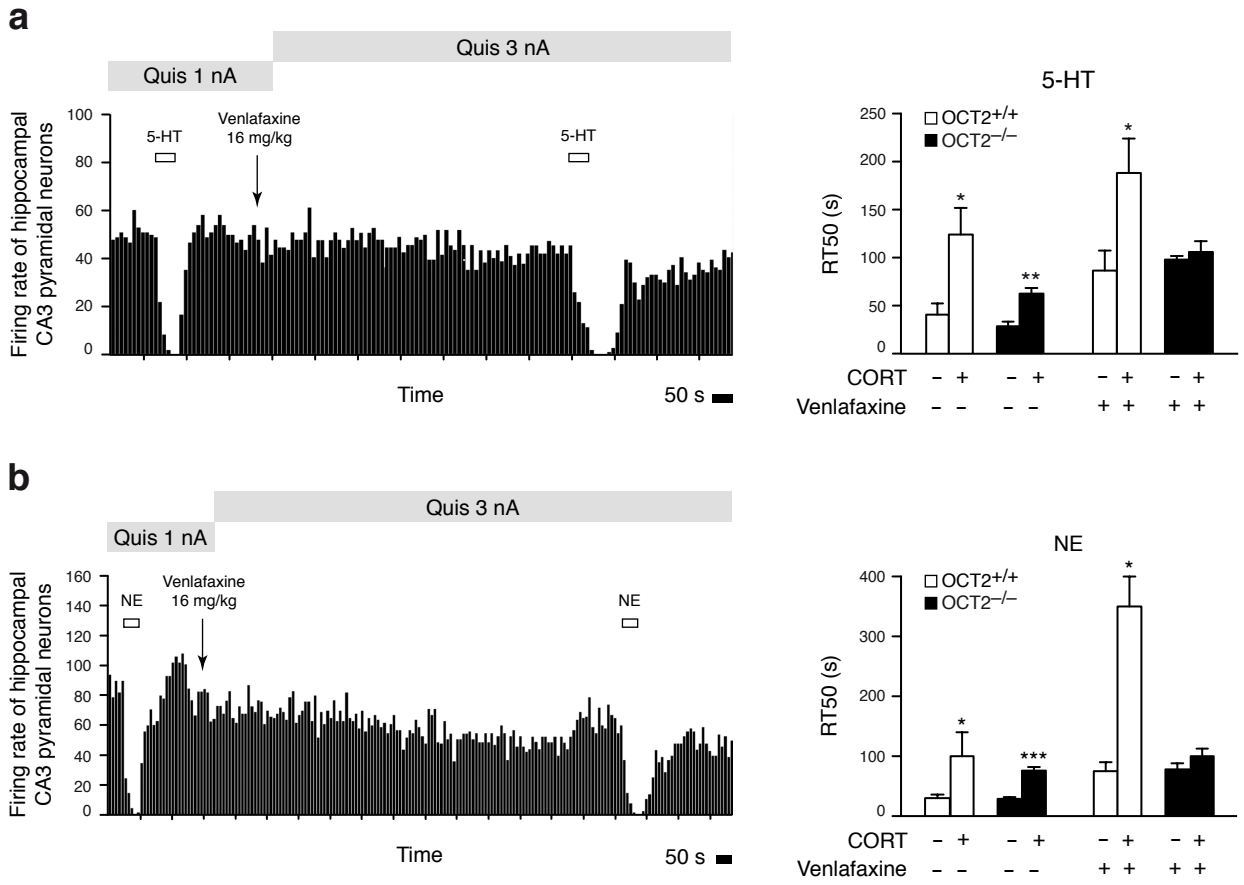
OCT2−/−
UCMS − − + +
g
Swim stress-induced corticosterone
Plasma corticosterone (ng/mL)
UCMS − − + +


a
Quis 3 nA
Quis 1 nA
Firing rate of hippocampal CA3 pyramidal neurons
5-HT
Venlafaxine 16 mg/kg
5-HT
Time
50 s
5-HT
OCT2+/+
OCT2−/−
RT50 (s)
CORT
Venlafaxine
b
Quis 3 nA
Quis 1 nA
Firing rate of hippocampal CA3 pyramidal neurons
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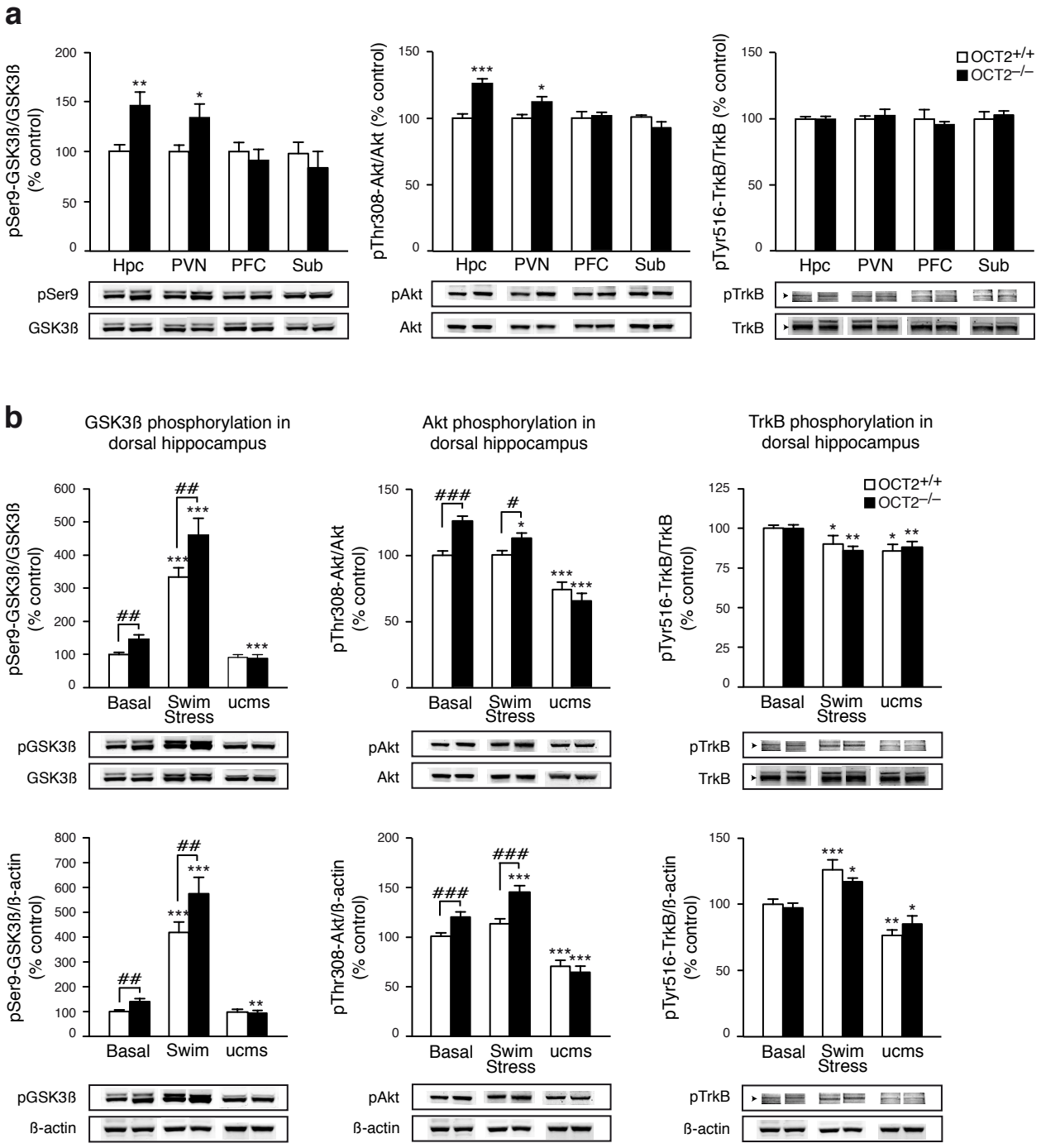
CORT
Venlafaxine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pSer9-GSK3ß/GSK3ß (% control)
pThr308-Akt/Akt (% control)
pTyr516-TrkB/TrkB (% control)
OCT2+/+
OCT2−/−
Hpc
PVN
PFC
Sub
pSer9
GSK3ß
pAkt
Akt
pTrkB
TrkB
b
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Akt phosphorylation in dorsal hippocampus
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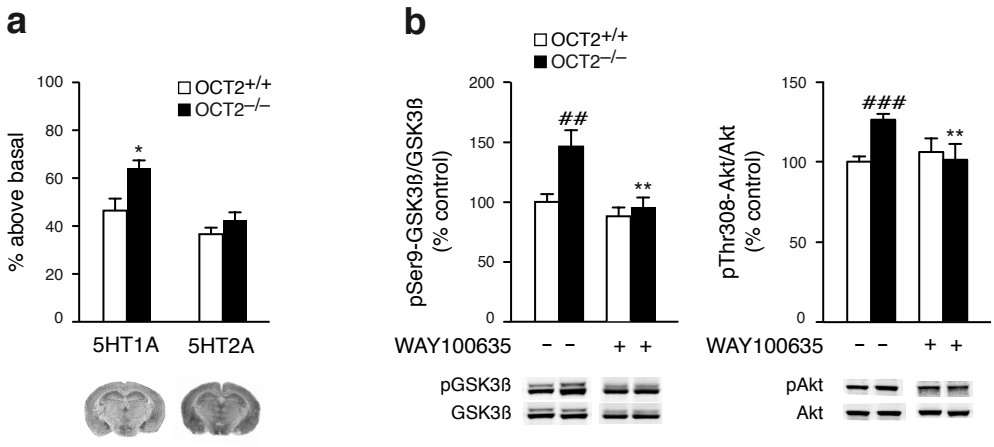
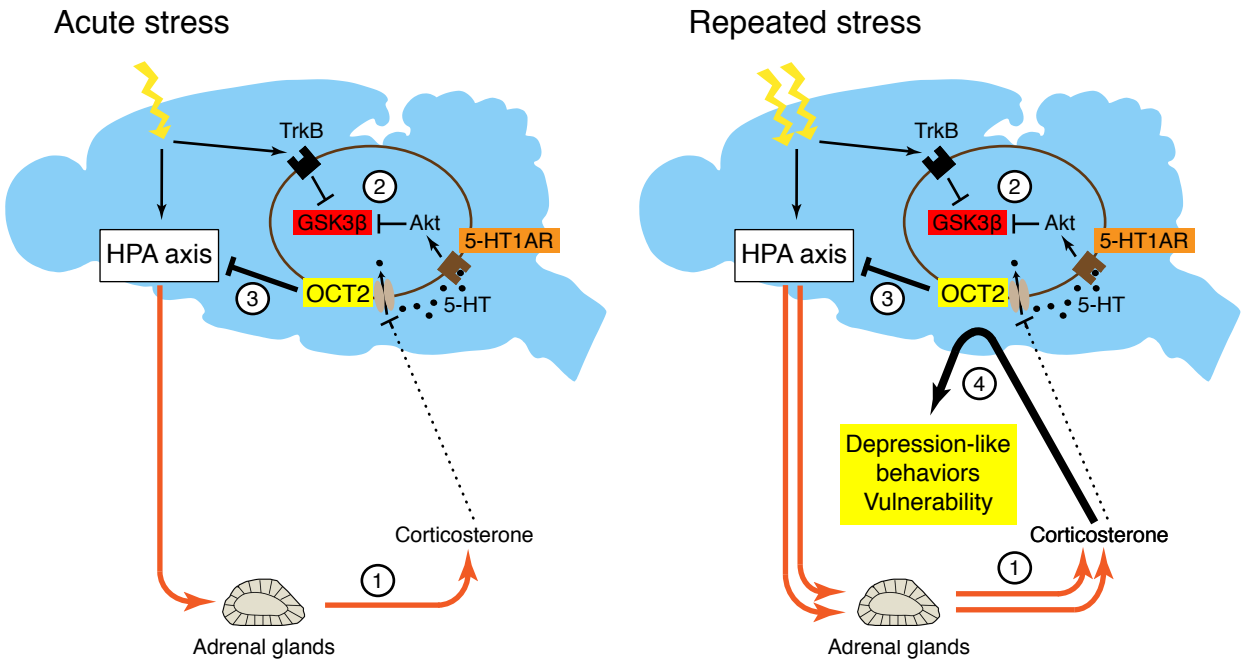
Basal
Swim Stress
Swim
ucms
pGSK3ß
ß-actin


a
b
OCT2+/+
OCT2−/−
% above basal
100
80
60
40
20
0
*
5HT1A
5HT2A
pSer9-GSK3β/GSK3β
(% control)
200
150
100
50
0
##
**
WAY100635
− − + +
pGSK3β
GSK3β
pThr308-Akt/Akt
(% control)
150
100
50
0
###
**
WAY100635
− − + +
pAkt
Akt

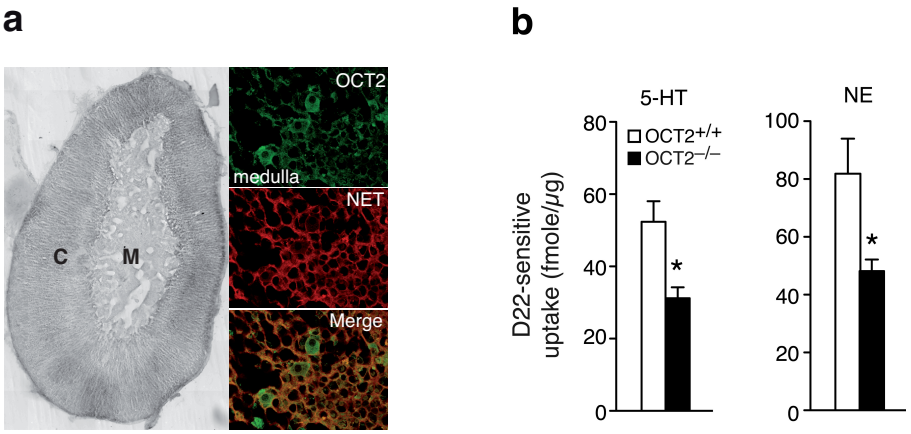
c


Acute stress
Repeated stress
TrkB
GSK3β
Akt
5-HT1AR
HPA axis
OCT2
5-HT
Corticosterone
Adrenal glands
Depression-like
behaviors
Vulnerability
1
2
3
4

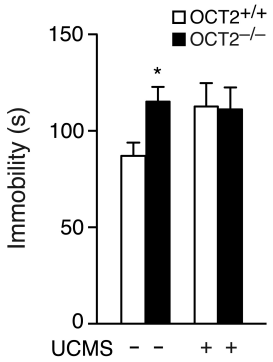
BRAIN ORGANIC CATION TRANSPORTER 2 CONTROLS RESPONSE AND VULNERABILITY TO STRESS AND GSK3β SIGNALING

Thomas Couroussé, Alexandre Bacq, Catherine Belzung, Bruno Guiard, Laure Balasse, Franck Louis, Anne-Marie Le Guisquet, Alain M Gardier, Alfred H Schinkel, Bruno Giros and Sophie Gautron

Supplementary Figures 1-2



Supplementary Figure 1 OCT2 is expressed in the adrenal glands and participates locally in 5-HT and NE uptake. (**a**) Immunochemical and immunofluorescent OCT2 labeling in adrenal cortex (C) and in a fraction of the NET-positive secretory cells of the adrenal medulla (M). (**b**) *Ex vivo* NE and 5-HT uptake is decreased in OCT2$^{-/-}$ mice adrenal glands. Decynium 22 (D22)-sensitive [^{3}H] 5-HT and NE uptake in cell extracts from wild-type and OCT2$^{-/-}$ adrenal glands is expressed as mean ± s.e.m. (n = 4). Student's t-test, unpaired, two-tailed, 5-HT: $t_6 = 3.3$, $^{*}P = 0.0169$; NE: $t_6 = 2.6$, $^{*}P = 0.039$



Supplementary Figure 2 UCMS blunts the genotype-specific differences in the forced-swim test, a behavioral despair paradigm. As previously described,[1] $OCT2^{-/-}$ mice show increased immobility in the forced-swim test (FST) compared to wild-type mice before (Student's t-test, unpaired, two-tailed, $t_{14} = 2.724$, $*P = 0.0165$, n = 7–9) but not after UCMS. Results are given as mean ± s.e.m.

Reference

1. Bacq A, Balasse L, Biala G, Guiard B, Gardier AM, Schinkel A *et al.* Organic cation transporter 2 controls brain norepinephrine and serotonin clearance and antidepressant response. *Mol Psychiatry* 2012; **17**(9)**:** 926-939.