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

INTRODUCTION

The etiology of depression and other mood-related disorders is still poorly understood, yet it is increasingly evident that interactions between genetic and environmental factors, like exposure to stress, have 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 that these transporters might have a physiologic role in the response to stress. Here, we report that 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 (UCMS) 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 humans 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.

MATERIALS AND METHODS

Animals

OCT2−/− mice were generated previously by homologous recombination. Heterozygous animals with 10 backcross generations into C56BL/6J 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 08:00 hours) and 8–16-week-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−1) paraformaldehyde/phosphate-buffered saline. Brains were removed, postfixed by immersion overnight in 4% paraformaldehyde/ phosphate-buffered saline 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−1) Triton and 4% (wt vol−1) 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 (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 kg−1; Sigma-Aldrich) dissolved in 0.45% (wt vol−1) 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−1 ip.; Sigma-Aldrich) 2 h before saline or cosyntropin injection (50 μg kg−1 ip.; 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 hours) 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 radioimmuno assay kits (MP Biomedicals, Illkirch, France) and expressed as ng ml−1 of plasma. For the dexamethasone suppression test, mice were injected with dexamethasone (100 μg kg−1 ip.; Sigma-Aldrich) 30 min before swim 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 a small wire mesh cage (10 × 6.5 cm) located at an extremity of the home cage field and the time spent exploring displaced objects was measured over a 5-min session by video tracking. For nest building, a square cotton nestlet (5 × 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’); and (5) the cotton nestlet was gathered into a hollow sphere with one opening for entry. For the sucrose preference test, single-housed mice were first habituated for 48 h to drink water from two bottles. On the following 3 days, the mice could choose between 1% water bottle and 1% (wt vol−1) 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 multibarreled glass micropipettes as described previously.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 (HT, 25 μm in 0.2 μM NaCl pH 4) or norepinephrine (NE, 1 μM−HEC, 25 μm in 0.2 NaCl pH 4) and quisqualate (1.5 μM in 0.2 μM 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 and 50–100 MΩ, respectively. The five-barreled glass micropipettes were positioned (in mm) relative from bregma: A/P, −2.5 to −2.3; M/L, +2.3 to 2.8; and 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−1, 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; Milltex, 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 (15–50 μg) were loaded in NuPage LDS sample buffer (Invitrogen, Carlsbad, CA, USA) were separated by Bis-Tris sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred with 10% (wt vol−1) sucrose membranes (Invitrogen). Transfer efficiency 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 (1/200), 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 700DQ and IRDye 800CW, 1/5000; Rockland, Gilbertsville, 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−1 ip.) 3.5 h before the mice were killed.
The functional activity of 5-HT receptors was assessed as described previously with the following modifications. Cytoswells were pre-incubated 30min at 20°C in HEPESS buffer (50mM HEPESS, pH 7.4, 100mM NaCl, 3mM MgCl₂, 0.2mM EGTA, 0.2mM DTT and 2mM GDP) for 5-HT1A receptors and in Tris buffer (50mM Tris-HCl, pH 7.4, 100mM NaCl, 3mM MgCl₂, 0.2mM EGTA, 0.2mM DTT and 2mM GDP) for 5-HT2A receptors, and then incubated with 25nM [³⁵S]GTPγS in the same buffers for 2h in the presence of selective agonists: 8-OHDPAT (10μM) and DOI (5μM) were used to stimulate [³²P]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 (1μM) for 5-HT1A receptors and ketanserin (10μM) for 5-HT2A receptors. Basal binding was determined in the absence of agonists. The sections were washed in cold HEPESS buffer for 2min, 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 the MCID software (Imaging Research, St. Catherine, ON, Canada).

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. 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 described previously (Figure 1b), in neurons of the polymorph layer of the DG and diffusely in the lacunomus molecular layer of CA1–3 (Figure 1b). Notable OCT2 levels were found in ventral subiculum (Figures 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 PVN of the thalamus (Figure 1g). Finally, OCT2 immunolabeling was detected in the median eminence (Figure 1h) and in the pituitary (Figures 1i and j). In this last structure, OCT2 was expressed in a non-corticotrope sub-population of the anterior pituitary, as shown with colabeling with ACTH antibodies (Figure 1i) and uniformly distributed in the posterior pituitary, apposed to pituicytes labeled with glial fibillary acidic protein (Figure 1j). No labeling was detected in OCT2−/− mice, 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 perPVN regions, the activation of the HPA axis, which culminates in corticosterone secretion. We thus investigated in OCT2−/− mice the neuroendocrine response to acute stress. The circulating corticosterone levels under basal conditions and following a 15min swim stress were evaluated in wild-type and knockout mice. Basal plasma corticosterone levels were increased by 87% in OCT2−/− mice compared with wild-type mice (P = 0.0058; Figure 2a). More importantly, swim stress-induced corticosterone release was also strongly enhanced in OCT2−/− mice compared with 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. In particular, OCT2 labeling was found in the adrenal cortex and in some of the noradrenergic transporter-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 the pituitary with little or no penetration in the brain, and routinely used to evaluate adrenal sensitivity. 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 supraptitory 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. 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, deteriorated progressively throughout the UCMS procedure in both genotypes. However, this deterioration occurred significantly more intensely (P < 0.05 and < 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 the hippocampus often impaired in stress-related disorders, was assessed by the measure of performance in the object location test. 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 with 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), whereas 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 with wild-type mice ($P = 0.0248$ and $P = 0.0457$, respectively), whereas at later stages the 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, 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, that is, 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, as 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).

**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 and 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 sub-population of the anterior lobe of pituitary (AL), as shown by colabeling with ACTH antibodies (i) and uniformly distributed in the posterior lobe (PL, j). No labeling was detected in OCT2$^{-/-}$ mouse brain, shown for cingulate and motor cortices (Cx, k) and the 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 = 100 μm (a–c, f, h, k and l) and 20 μm (d, e, g, i and j).
Exogenous corticosterone can modulate 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- and low-affinity transporters in vivo. Four-day corticosterone oral administration raised circulating concentrations to 115.9 ± 14 and 112.8 ± 18 ng ml⁻¹ for wild-type and OCT2⁻/⁻ mice, respectively. The activity of postsynaptic neurons in the hippocampus and 112.8 ± 18 ng ml⁻¹ for NE (F 1,35 = 82.900; P < 0.0001), time (F 7,106 = 49.81; P < 0.0001) and genotype × 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 with saline injection (dashed line). Two-way ANOVA (n = 4–6) reveals no main significant effects of genotype (F 1.137 = 3.8; P = 0.0603). Results are given as mean ± s.e.m.

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, t₂ = 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 × 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 with saline injection (dashed line). Two-way ANOVA (n = 4–6) reveals no main significant effects of genotype (F 1.137 = 3.8; P = 0.0603). Results are given as mean ± s.e.m.
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Figure 3. OCT2−/− mice show increased vulnerability to the effects of unpredictable chronic mild stress (UCMS). (a) OCT2−/− mice show enhanced deterioration of coat state during the UCMS procedure. Two-way analysis of variance (ANOVA) (n = 6–15) reveals significant effects of time (F8,211 = 17.82; P < 0.0001), genotype (F1,211 = 77.25; P < 0.0001) and genotype × time interaction (F8,211 = 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 increased vulnerability to the effects of unpredictable chronic mild stress (UCMS). Two-way ANOVA (n = 10) reveals significant main effects of object (F1,114 = 52.13; P < 0.0001) and object × UCMS interaction (F3,114 = 5.813; P = 0.0010). Fisher’s post hoc test reveals significant differences in the exploration time of the displaced object compared with 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), whereas 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 (F1,90 = 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, t21 = 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 (F2,30 = 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 (F1,36 = 4.589; P = 0.039) but not of genotype (F1,36 = 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 (F1,26 = 24.57; P < 0.0001) and genotype × treatment interaction (F1,26 = 5.166; P = 0.0309). Fisher’s post hoc test reveals significant differences between OCT2−/− and wild-type mice before (t6 = 0.0127) but not after UCMS, and significant effects of UCMS for 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

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phosphorylation at Akt ($P = 0.0268$), but not the total amount of these proteins (not shown), were increased in OCT2−/− mice hippocampus compared with wild-type mice 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−/− mice before but not after venlafaxine injection. Three-way analysis of variance (ANOVA) of RT50 values ($n = 4–6$) reveals significant main effects of genotype (5-HT: $F_{1,35} = 5.880, P = 0.0206$; 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 $\times$ 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.

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. As 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.32,35 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 the hippocampus CA3.38 These experiments indicated that the 5-HT1A receptor was overactivated in the 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 before swim stress and the functional state of the Akt/GSK3β pathway explored in the hippocampus. WAY100635 restored in OCT2−/− mice a ratio of phosphorylated to total GSK3β and Akt comparable to wild-type mice, with an effect in the 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 postsynaptic 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.
Figure 5. OCT2−/− mice show profound alterations of Akt/GSK3β signaling at basal state and during acute stress. (a and 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 the hippocampus (HPC) and paraventricular hypothalamic nucleus (PVN) of OCT2−/− mice compared with 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, \( t_{13} = 3.122, **P = 0.0037, n = 17–18 \); PVN, \( t_{13} = 2.196, *P = 0.038, n = 13 \); Akt: HPC, \( t_{13} = 5.288, ***P < 0.0001, n = 17–18 \); PVN, \( t_{13} = 2.905, *P = 0.0194, n = 13 \)). (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 analysis of variance (ANOVA) of phosphorylated protein over non-phosphorylated protein ratios (\( n = 5–18 \)) reveals a significant effect of swim stress for GSK3β (F(1,25) = 116.2; \( P < 0.0001 \)) and TrkB (F(1,25) = 14.94; \( P = 0.0005 \)), and of genotype for GSK3β (F(1,25) = 11.52; \( P = 0.0013 \)) and Akt (F(1,25) = 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 with 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(1,25) = 129.8; \( P < 0.0001 \)), Akt (F(1,25) = 14.57; \( P = 0.0003 \)) and TrkB (F(1,25) = 20.99; \( P < 0.0001 \), and of genotype for GSK3β (F(1,25) = 8.761; \( P = 0.0045 \)) and Akt (F(1,25) = 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(1,25) = 9.522; \( P = 0.0033 \), Akt (F(1,25) = 94.97; \( P < 0.0001 \)) and TrkB (F(1,25) = 17.60; \( P = 0.0002 \)), and of genotype × UCMS interaction for GSK3β (F(1,25) = 4.839; \( P = 0.0324 \)) and Akt (F(1,25) = 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).
DISCUSSION

Stress and HPA axis dysregulation constitute important risk factors for psychiatric disorders such as depression and post-traumatic stress disorder.\(^{39,40}\) Chronic stress in rodents can mimic these noxious effects, inducing gradual neurobiologic and behavioral anomalies resembling symptoms of human depression.\(^{19,27}\) In addition, HPA axis activity has been suggested to have a role in ‘passive’ resilience, that is, resilience mediated by the absence of key neurochemical, molecular or endocrinal responses in certain individuals.\(^{41,42}\) The major key neurochemical, molecular or endocrinal responses in certain social withdrawal symptoms that occur in MDD patients.\(^{44,45}\) Negative feedback was observed in OCT2 which remain to be determined. Moreover, a normal state of HPA-axis activity profoundly modulates the hormonal response to acute stress in normal mice,\(^{47}\) our investigation of adrenal sensitivity to ACTH 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 even 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, PVN of the thalamus and pituitary, as well as in adrenals.\(^{2,5,25}\) 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 adenral sensitivity to ACTH allowed us to exclude periphery-based mechanisms involving sympathetic innervation, circulating catecholamines and/or paracrine control within adrenal glands.\(^{54,23}\) 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 postmitotic neurons but inhibited by phosphorylation in response to various upstream pathways implicating neurotrophins\(^{57}\) 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 with 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 concluded 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, whereas the inhibition of this kinase was able to correct the mood-related behavioral anomalies of these depleted mice.\(^{53}\) Reciprocally, elevation of 5-HT level was shown to inhibit considerably GSK3β by Ser-9 phosphorylation in several brain regions including the hippocampus, through the activation of 5-HT1A receptors\(^{55}\) and Akt signaling.\(^{57}\) In agreement with this mechanism, our results suggest that increased 5-HT tonus leading to the overactivation of 5-HT1A receptor could at least in part the anomalies in Akt/ GSK3β signaling in OCT2/−/− mice (Figure 6c). Interestingly, postsynaptic 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,\(^{61}\) as well as IGFII-dependent memory enhancement,\(^{62}\) whereas its inhibition was linked to impaired N-methyl-D-aspartate receptor-dependent LTD,\(^{63}\) a process required for spatial memory consolidation.\(^{64,65}\) Other studies suggest that forebrain GSK3β has a role in sociability.\(^{66}\) Interestingly, Akt/GSK3β signaling in the brain was also implicated in susceptibility to social defeat\(^{67}\) and to post-traumatic stress disorder-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, whereas 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 micro- iontophoretic electrophysiology. Such an interaction has been demonstrated in vivo\(^{13,14}\) 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.\(^{10}\) OCT2 blockade by corticosterone could thus contribute to the neurochemical and molecular adaptive 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 feedforward mechanism to modulate this pathway. This possibility is for
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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 anti-viral compounds,69,70 could have important consequences on the vulnerability to environmental stress.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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