Modulation of OCT3 expression by stress, and antidepressant-like activity of decynium-22 in an animal model of depression

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A B S T R A C T

The organic cation transporter-3 (OCT3) is a glucocorticoid-sensitive uptake mechanism that has been shown to regulate the bioavailability of monoamines in brain regions that are implicated in the pathophysiology of depression. In the present study, the relative impacts of acute stress alone and acute stress with a history of repeated stress (chronic + acute) were evaluated in two strains of rats: the stress-vulnerable Wistar–Kyoto (WKY) strain and the somewhat more stress-resilient Long–Evans (LE) strain. OCT3 mRNA was significantly upregulated in the hippocampus of LE rats 2 h after exposure to acute restraint stress, but not in acutely-restrained rats with a history of repeated social defeat stress. WKY rats exhibited a very different pattern. OCT3 mRNA was unaffected by acute restraint stress alone but was robustly upregulated after repeated + acute stress. There was also a corresponding increase in cytosolic OCT3 protein following repeated + acute stress in WKY rats 3 h after presentation of the acute stressor. These results are consistent with the hypothesis that altered expression of the OCT3 may play a role in stress coping, and strain differences in regulation of this expression may contribute to differences in physiological and behavioral responses to stress. Furthermore, the OCT3 inhibitor, decynium 22 (1 and 10 μg/kg, i.p.) reduced immobility of WKY rats, but not that of LE rats, in the forced swim test, suggesting that blockade of the OCT3 has antidepressant-like effects. Since WKY rats also appear to be resistant to the behavioral effects of traditional antidepressants, this also suggests that OCT3 antagonism may be an alternative therapeutic strategy for the treatment of depression in individuals who do not respond to conventional antidepressants.

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1. Introduction

Major depressive disorder has been described as a disorder of stress regulation that can impact monoaminergic systems in the brain, producing profound alterations in mood and emotional behavior (for a review see Hamon and Blier, 2013). Indeed, elevated activity of the hypothalamic–pituitary–adrenocortical (HPA) axis is implicated in the pathophysiology of depression, and many of the antidepressant drugs that specifically modify serotonergic and/or adrenergic tone concurrently restore normal HPA axis functioning (Schüle, 2007).

It is widely recognized that some individuals are more susceptible to depression than others. The biochemical basis of these individual differences is not fully characterized, but evidence is accruing that reciprocal interactions between stress responses and monoaminergic neurotransmission play key roles in the pathogenesis of depression. In agreement with this perspective, there is evidence that polymorphisms in genes that regulate the bioavailability or post-synaptic effects of serotonin may contribute to individual differences in vulnerability. For instance, carriers of the s allele of the SLC6A4 gene that encodes the serotonin transporter (SERT) are at increased risk for depression (Caspi et al., 2003). These individuals have dysregulated HPA axis function, resulting in elevated waking cortisol and adrenocorticotropic hormone (ACTH) levels (Chen et al., 2009; Wüst et al., 2009).

Alterations in noradrenergic neurotransmission also appear to contribute to the pathophysiology of depression. Norepinephrine (NE) is released by terminals of neurons that originate in the locus coeruleus, a nucleus that is thought to be sensitized in major depression, and normalized by antidepressant treatment (Chandley et al., 2014). Acute treatment with various antidepressant medications each reduce spontaneous and sensory-evoked firing of LC neurons (Grant and Weiss, 2001; West et al., 2009), and chronic treatment with these drugs restores extra-neuronal concentrations of NE in cortical regions that are innervated by LC projections (Linner et al., 1999; Matteo et al., 2001). Thus, antidepressant drugs may potentially reverse the pathology of depression (at least in part) through noradrenergic re-regulation (Grant and Weiss, 2001).

The organic cation transporter-3 (OCT3) represents a potential link between monoaminergic function and stress neurobiology that may help modulate vulnerability for, and recovery from major depression. This transporter belongs to a family of polyspecific solute carriers that
increase the elimination of cationic species (Pritchard and Miller, 1993; Ullrich, 1994), including monoamines from the extracellular space (Gründemann et al., 1998, 1999; Schömig et al., 2006). Whereas the OCT1 and OCT2 subtypes are predominantly expressed in visceral organs, OCT3 mRNA is abundantly expressed within the brain, especially in cerebellum, hippocampus, pontine nuclei and cortex (Kekuda et al., 1998; Wu et al., 1998). In these regions, it is distributed along monoaminergic pathways, where it mediates a low-affinity, high-capacity, glucocorticoid-sensitive system for removing extracellular monoamines (Amphoux et al., 2006; Gasser et al., 2006, 2009).

Overall, it appears that the low-affinity, high-capacity function of the OCT3 transport system may be especially salient when the classical high-affinity monoamine transporters are saturated or in low abundance. This was demonstrated in mice with a homozygous deletion of the serotonin transporter (SERT). These interesting knockout experiments exhibit an abnormal phenotype that includes elevated anxiety-related behavior and exaggerated stress responses (Holmes et al., 2003). However, these mice maintain hippocampal 5HT levels at relatively normal concentrations due to upregulation of the OCT3 in the hippocampus (Schmitt et al., 2003; Baganz et al., 2008). Since corticosteroids and stress provoke rapid increases in extracellular monoamine concentrations (Lowry et al., 2001, 2003), we propose that stressors may provide the necessary conditions for the OCT3 to exert a physiologically meaningful effect on monoaminergic neurotransmission.

The first goal of this study was to examine OCT3 expression after stress in Wistar–Kyoto (WKY) and Long–Evans (LE) rat strains, focusing primarily on the hippocampus, medial prefrontal cortex, and striatum, where monoaminergic and stress–response systems converge. The WKY rat exhibits relatively greater behavioral and neuroendocrine responses to stress, which may indicate a heightened sensitivity to the effects of corticosterone (Paré and Redei, 1993; Tejani-Butt et al., 1994; Bielajew et al., 2002; Rittenhouse et al., 2002). Furthermore, brain 5HT systems appear to be dysregulated in WKY rats after stress compared to responses in other strains (Paré and Tejani-Butt, 1996; De La Garza and Mahoney, 2004). A recent study suggests that stress, corticosterone sensitivity, and OCT3 expression levels are positively correlated (Baganz et al., 2010). Accordingly, we hypothesized that OCT3 expression would be upregulated more in the stress-sensitive WKY rat than in the LE rat. We also examined hippocampal expression of the glucocorticoid receptor (GR) and SERT, both of which exhibit plasticity in animal models of stress and depression (Paré and Tejani-Butt, 1996; Marini et al., 2006; Wu et al., 2011).

In a second experiment, we assessed the effects of decynium 22 (D22; a cyanine dye derivative that inhibits OCT3 transport) on WKY and LE rats in the forced swim test. Previous studies revealed that D22 reduced immobility in the tail suspension test (an antidepressant-like effect) in SERT knockout mice while failing to alter the behavior of wild type conspecifics (Baganz et al., 2008). Furthermore, microdialysis studies have shown that intracranial administration of D22 into the dorsomedial hypothalamus (a region implicated in endocrine, autonomic, and behavioral responses to stress) elicits robust increases in extracellular 5HT concentrations (Feng et al., 2005), providing a potential neurochemical mechanism that could contribute to an antidepressant-like effect. In accordance with these observations, we hypothesized that D22 would exert an antidepressant-like effect selectively in the stress-sensitive WKY strain.

2. Materials and methods

2.1. Experiment 1 — effect of stress on OCT3 gene and protein expression

All experimental protocols were pre-approved by the University of Florida Institutional Animal Care and Use Committee (IACUC) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2003).

2.1.1. Animals and housing

Male LE and WKY rats (250–300 g; n = 48 per strain) were pair-housed in standard polycarbonate cages (43 × 21·5 × 25·5 cm) in a climate controlled vivarium with a 12-h light/dark cycle (lights on at 7 am) and were given 7 days to adapt to the AAALAC-approved housing facility before the experiments began. The rats were allowed ad libitum access to standard laboratory chow (Lab Diet 5001) and tap water. These rats were randomly assigned to control, acute restraint stress, or repeated social defeat (i.e., intruder rats) plus acute restraint stress groups (n = 8 per group). An additional 8 male LE rats (i.e., resident rats; 600–650 g) were singly-housed in standard polycarbonate cages and were trained to exhibit dominant home-cage behaviors in the social defeat paradigm. An additional 4 male LE rats (i.e., training rats; 250–300 g) were used to train the dominant residents.

2.1.2. Dominant resident training

During each daily dominance–training session, a training rat was placed inside the resident’s home cage for up to 5 min. If the training rat was defeated 3 times (i.e., maintained a supine posture for a minimum of 2 s), the resident was classified as dominant for that session. Once dominance was established, the test session was terminated. This procedure was repeated daily (up to 30 days) until there were 3 consecutive days in which the resident defeated the training rat 3 times per session. Resident rats were used in social defeat sessions only after they passed this training.

2.1.3. Experimental design

Each of the rats in the acute stress groups was handled once daily on each of seven consecutive days, exposed to a single 30 min session of restraint stress on day 8, and was killed 120 min (n = 24 rats per strain) or 180 min (n = 24 rats per strain) after the beginning of the restraint session. Each of the intruder rats in the chronic + acute stress groups was exposed to one daily social defeat session on each of 7 consecutive days. On day 8, these rats were exposed to 30 min of restraint stress, and were killed 120 min (n = 24 rats per strain) or 180 min (n = 24 rats per strain) after the beginning of the restraint session. The control rats were handled once daily but were otherwise undisturbed, and were killed at the same time as the other rats on day 8 (n = 8 LE and 8 WKY rats in each of the control, acute stress, and chronic + acute stress conditions).

2.1.4. Social defeat

The social defeat procedure is based on previous methods described in detail inMarcinkiewcz et al. (2009). Briefly, this procedure consisted of two stages. In the first stage, the intruder LE or WKY rat was placed into a 10 × 10 × 15 cm (inner dimensions) double-walled wire mesh protective cage. The rat was then placed, inside the protective cage, into the home cage of the resident rat for 45 min. The cage served to separate the rats, avoiding physical contact, but maintaining stressful sensory stimuli. During the second stage, the intruder rat was removed from the wire mesh cage and placed directly inside the resident’s home cage. Dominance/submission behavior was scored as described for the training sessions. This direct interaction between resident and intruder rats continued for a maximum of 5 min, or until 3 defeats occurred. Every intruder rat was exposed to a different resident rat in each of the 7 defeat sessions. After each exposure to a resident rat, the intruder rat was removed and returned to its home cage.

2.1.5. Restraint stress

Restraint stress was performed according to the methods that we have previously described (Devine et al., 2003; Simpkins and Devine, 2003; King et al., 2007). Briefly, each rat was individually removed from its home cage and placed into a restraining tube for 30 min. The restraint tube was composed of a flexible sheet of plastic (11” × 7 3/4”) mounted to a rigid Plexiglas cradle (8 1/2” × 3” × 3”), with a vertical slot to allow comfortable placement of the tail, and ventilation holes to allow unrestricted breathing. The plastic sheet was gently rolled
around the animal and held securely in place with two Velcro straps. At the end of the 30 min restraint stress, each rat was returned to its home cage.

2.1.6. Post-mortem tissue processing
All the rats were killed by rapid decapitation 2 or 3 h after the beginning of the last stressor for mRNA and protein quantification, respectively. The time at which OCT3 mRNA was assayed was chosen on the basis that a previous report described increases in cortical corticotropin releasing hormone (CRH) mRNA following acute restraint (Greetfeld et al., 2009). The 3 h time point for protein assays was chosen to allow sufficient time following mRNA synthesis for de novo protein synthesis. Each rat brain was rapidly dissected on a glass plate over ice to remove the medial prefrontal cortex (mPFC), hippocampus and striatum according to a modified version of the technique first described by Iversen and Glowinski (1966). Briefly, the brain was placed on an ice-cold Petri dish with the ventral surface facing up to reveal the hypothalamus, which was carefully teased apart from the surrounding tissue with forceps. With the dorsal surface facing up, the cortices were parted with forceps. With the dorsal surface facing up, the cortices were parted.

2.1.7. Real-time PCR
RNA isolation was carried out according to a standard protocol (Molecular Research Center, Cincinnati OH). Briefly, tissues were homogenized in 1 ml TRI Reagent RT per 50 μg of tissue using a Branson 450 sonifier. The use of TRI Reagent RT combined with phase separation in the cold (4 °C) produces an RNA sample that is free of DNA contamination as indicated by 35-cycle PCR, thus eliminating the need for DNase treatment. The absence of any amplification in the reverse transcriptase-negative control reaction also indicated that our samples were free of DNA contamination. Following homogenization, 50 μl 4-bromoisobutylmethanesulfonate (BAN) per 1 ml of TRI Reagent RT was added to each sample. Each sample was shaken vigorously for 15 s and then centrifuged at 12,000 × g for 15 min at 4 °C. The RNA-containing aqueous phase (0.5 ml) was then carefully transferred to a new tube for subsequent wash and precipitation steps. RNA was precipitated from the aqueous phase with the addition of 0.5 ml isopropanol, incubation for 10 min at room temperature, and centrifugation at 12,000 × g for 5 min at 4 °C. The resulting RNA pellet was then washed twice with 100 μl 70% ethanol and centrifuged again for 5 min at 12,000 × g at 4 °C. The ethanol wash was finally removed and the pellet reconstituted in nuclease-free water. The final RNA concentration was quantified using a NanoDrop spectrophotometer. The cDNA was generated from total RNA using a High Capacity RNA-to-cDNA kit according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA). Briefly, a master mix of the 2× RT buffer and 20× RT enzyme mix containing reverse transcriptase and both random and poly-T primers was prepared and 11 μl of this mixture was aliquoted to each reaction tube. An appropriate amount of nuclease-free water was added to achieve a final reaction volume of 20 μl together with 2 μg of RNA. Each tube was gently mixed, briefly centrifuged, and then incubated at 37 °C for 1 h, followed by 95 °C incubation for 5 min and a final holding step at 4 °C.

The real-time PCR was carried out using Taqman Gene Expression reagents with primer and probe sets from Applied Biosystems that are specific for the rat OCT3 (Assay ID—Rn00570264_m1), SERT (Assay ID—Rn00564737_m1), GR (Assay ID—Rn00561369_m1) or eukaryotic 18S ribosomal RNA (Assay ID—Hs99999901_s1). Information regarding primer and probe sequences is contained in Table 1. All samples were analyzed in duplicate on the Applied Biosystems 7500 Fast Real-time PCR system. The PCR reaction consisted of an initial holding stage at 50 °C for 20 s, a second holding stage at 95 °C for 10 min, and 40 cycles of PCR with a melting temperature of 95 °C for 15 s and an annealing temperature of 60 °C for 1 min. Cycle thresholds (Ct) were used to compute the fold change (2–ΔΔCt) in mRNA expression for each gene of interest. All experiments included in the analysis had Ct values < 35.

2.1.8. Protein quantification by Western blot
Tissues were processed for Western blot analysis using a Plasma Membrane Protein Extraction Kit (Biovision, Mountain View, CA) to obtain separate fractions of cell surface, organelle, and cytosolic proteins. OCT3 protein expression was quantified by Western blot using antibodies directed against the rat OCT3 (Abcam, Cambridge, MA). N-Cadherin (Abcam, Cambridge, MA) and β-actin (Cell Signaling Technology, Danvers, MA) were used as endogenous controls in plasma membrane and cytosol extracts, respectively.

Protein electrophoresis was performed on a Nu-PAGE 4–12% Tris-glycine gel (Invitrogen, Carlsbad, CA). Each sample was diluted in loading buffer (50 mM Tris–HCl (pH 6.8), 2% SDS (sodium dodecyl sulfate), 0.1% Bromophenol Blue, 10% glycerol, 50 mM DTT). Ten microgram of protein was loaded per lane, with molecular weight markers run in parallel. Electrophoresis was performed at 195 V for approximately 60 min. The gels were then equilibrated in a protein transfer buffer containing 25 mM Tris, 192 mM glycine, and 10% (v/v) methanol. The proteins were electrochemically transferred onto a polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer apparatus (Biorad, Hercules, CA). After the transfer, the membrane was rinsed with Tris–buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) with pH of 7.4 and blocked for 1 h with 5% non-fat dry milk in TBS-T. The membrane was then incubated with rabbit anti-rat OCT3 IgG (Abcam, Cambridge, MA) diluted 1:1000 in 5% non-fat dry milk in TBS-T overnight at 4 °C. After the overnight incubation they were washed three times in TBS-T and incubated for 1 h at room temperature in horseradish peroxidase (HRP)-conjugated goat anti-rabbit (1:2000 goat anti-rabbit peroxidase conjugate; Cell Signaling Technology, Beverly, MA) in 5% non-fat dry milk in TBS-T. Membranes were washed for 3 × 10 min in TBS-T at room temperature with rocking, then developed using a Phototope®-HRP Western Blot Detection System (Cell Signaling Technology) and exposed to Kodak Biomax Light chemiluminescence film. Chemiluminescent bands were quantified by optical densitometry using Image J software.

The PVDF membranes were stripped by immersion in a SDS stripping buffer (containing 62.5 mM Tris, 2% SDS and 150 mM 2-mercaptoethanol, pH 6.8) for 15 min. at 37 °C. Membranes were then washed for 3 × 10 min in TBS-T, reblocked in 5% non-fat dry milk in

Table 1
Sequence information for primers and probes used in real-time PCR assays.

<table>
<thead>
<tr>
<th>Assay ID</th>
<th>Interrogated sequence</th>
<th>Amplicon location*</th>
<th>Amplicon length</th>
<th>Probe location*</th>
<th>Exon boundary</th>
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</thead>
<tbody>
<tr>
<td>OCT3</td>
<td>Rn00570264_m1</td>
<td>NM_019230.1</td>
<td>1829–1951</td>
<td>61</td>
<td>1878–1902</td>
</tr>
<tr>
<td>GR</td>
<td>Rn00561369_m1</td>
<td>NM_012576.2</td>
<td>1180–1326</td>
<td>73</td>
<td>1241–1265</td>
</tr>
<tr>
<td>SERT</td>
<td>Rn00564737_m1</td>
<td>NM_013034.3</td>
<td>1228–1386</td>
<td>79</td>
<td>1295–1319</td>
</tr>
<tr>
<td>18S</td>
<td>Hs99999901_s1</td>
<td>X03205.1</td>
<td>417–791</td>
<td>187</td>
<td>592–616</td>
</tr>
</tbody>
</table>

Assay ID for inventoried assays, interrogated sequence, amplicon length and location, probe location, and exon boundary spanned by the probe. Asterisks (*) indicate that the location given is an approximation (information about the exact location of primers and probes is proprietary). Forward and reverse primers were approximately 25 bp and probes were 12 bp in length.
TBS-T, and re-probed with an antibody for N-cadherin (plasma membrane marker) or β-actin (cytosolic marker) to verify that an equivalent expression of this stable marker is present in blots from all the rats in each treatment group. Chemiluminescent bands were quantified by optical densitometry using a MCID camera system and Image J software. The optical density (OD) of bands corresponding to the OCT3, N-cad, and β-actin was measured using this program and used to compute the relative OD for each gene of interest.

2.1.9. Data analysis

All gene expression data is represented as a fold change \(2^{-\Delta\Delta Ct}\) over basal expression of the respective control group. Protein expression data is expressed as relative OD, which is the calculated as follows:

\[
\text{Adjusted OD} = \frac{\text{OD (gene of interest)}}{\text{OD (endogenous control)}}
\]

Relative OD = Adjusted OD (group mean) / adjusted OD (reference mean)

A one-way analysis of variance (ANOVA) was used to determine statistically significant differences in expression with stress condition as the independent variable. Bonferroni post-tests were used to make pair-wise comparisons between groups. All outcomes were treated as statistically reliable when the p-values were less than 0.05.

2.2. Experiment 2 — effect of OCT3 antagonism on immobility in the forced swim test

2.2.1. Animals, housing, and experimental design

Thirty-four male WKY rats (250–300 g) and 12 male LE rats (250–300 g) were pair-housed in standard polycarbonate cages as in Experiment 1. The rats were allowed 7 days acclimation to the housing conditions before the start of the experiment.

The WKY rats were randomly assigned to groups that received vehicle, 1 μg/kg or 10 μg/kg of D22. LE rats received vehicle or 1 μg/kg D22 only. All the rats underwent a 15-min exposure to the forced swim test (pre-test) 24 h before the experimental session began. On the test day, each rat received an intraperitoneal injection of decynium 22 (0, 1, or 10 μg/kg) 1 h before the 5-min forced swim test. This time point was selected based on a previous report by Baganz et al. (2008) showing that D22 administered by intraperitoneal injection is maximally effective in SERT knockout mice after 1 h.

2.2.2. Drugs and injections

D22 (Sigma Aldrich) was dissolved in 100% ethanol and then diluted with saline to a final concentration of 1% ethanol containing 0, 1, or 10 μg/ml D22. Each rat was injected with vehicle (1% ethanol in isotonic saline; i.p.) or D22 (1 μg/kg or 10 μg/kg; i.p.) 60 min prior to behavioral testing in the forced swim test. The lower dose was selected based on a previous report by Baganz et al. (2008) showing that 1 μg/kg D22 produced antidepressant effects in SERT knockout mice. We included a higher dose (10 μg/kg dose) in WKY rats to see if this would elicit a greater antidepressant response and found that it did not, so only the lowest effective dose was used in the LE rats.

2.2.3. Forced swim test

The procedure for the forced swim test was based on a modified version of that described by Porst et al. (1978). Briefly, rats were placed individually into cylindrical containers (45 cm height; 20 cm diameter) containing 30 cm of water at 23 °C, for 15 min during a “pre-test” session. Twenty-four hours later, a test session was conducted for 5 min and behavior was recorded with a video camera mounted over the test apparatus. At the end of the test session, each rat was dried with a clean towel, placed in a heated cage for 15 min, and then returned to its home cage. Fresh water was used for each rat and the cylinder was cleaned thoroughly with 4% bleach between sessions. A trained observer who was blind to the treatment conditions scored the total time spent immobile during the test session. A rat was considered immobile when floating and making only the necessary movements to keep its nostrils above the water surface.

2.2.4. Statistical analysis

A one-way ANOVA was used to analyze the effects of D22 on immobility, swimming, and climbing behaviors during the forced swim test with drug treatment as the independent variable. Pairwise comparisons between groups were made using Bonferroni post-tests, and all outcomes were treated as statistically reliable when the p-values were less than 0.05.

3. Results

3.1. Real-time PCR analysis

Hippocampal OCT3 expression was significantly altered by stress in LE rats (\(F(2,21) = 7.85, p < 0.01\)) and WKY rats (\(F(2,20) = 11.84, p < 0.001\)). Bonferroni post-tests indicated that OCT3 gene expression was upregulated after acute stress in LE rats (t = 3.71, p < 0.01) and chronic + acute stress in WKY rats (Fig. 1A and B). OCT3 mRNA was not significantly altered by stress in the mPFC (\(F(2,21) = 3.12, p > 0.05\)), mPFC (\(F(2,20) = 0.90, p > 0.05\)) or striatum (\(F(2,21) = 1.51, p > 0.05\)), or mPFC (\(F(2,20) = 0.04, p > 0.05\)) of LE rats or WKY rats, respectively (Fig. 1C-F).

A one-way ANOVA of hippocampal glucocorticoid receptor (GR) gene expression did not detect any significant effects of stress in LE rats (\(F(2,21) = 1.21, p > 0.05\)) or WKY rats (\(F(2,20) = 3.33, p > 0.05\)) (Fig. 2A and B). Hippocampal SERT gene expression also did not vary in a stress-dependent manner in LE rats (\(F(2,21) = 1.08, p > 0.05\)) or WKY rats (\(F(2,20) = 1.45, p > 0.05\)) (Fig. 2C and D).

In order to assess the reliability of 18S as a reference gene, we compared the mean Ct values of 18S between groups for all of the assays performed in this study. One-way ANOVAs with stress as the independent variable indicated that 18S Ct values did not vary in a stress-dependent manner for any of the assays performed (Table 2).

3.2. Western blot analysis

There was a significant reduction in plasma membrane expression of the OCT3 in the hippocampus of WKY rats following exposure to chronic + acute stress (\(F(2,21) = 11.77, p < 0.001\)) but no

### Table 2

Reference gene (18S) Ct values did not vary in a stress-dependent manner for any of the genes assayed.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>LE</th>
<th>WKY</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td>(F(2,21) = 2.01)</td>
<td>(F(2,20) = 1.76)</td>
</tr>
<tr>
<td>mPFC</td>
<td>(p = 0.16)</td>
<td>(p = 0.20)</td>
</tr>
<tr>
<td>Striatum</td>
<td>(p = 0.11)</td>
<td>(p = 0.68)</td>
</tr>
<tr>
<td>GR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td>(F(2,21) = 1.50)</td>
<td>(F(2,20) = 0.21)</td>
</tr>
<tr>
<td>Striatum</td>
<td>(p = 0.25)</td>
<td>(p = 0.01)</td>
</tr>
<tr>
<td>SERT</td>
<td>Hippocampus</td>
<td>Striatum</td>
</tr>
<tr>
<td>(F(2,21) = 1.77)</td>
<td>(F(2,21) = 1.77)</td>
<td></td>
</tr>
<tr>
<td>(p = 0.19)</td>
<td>(p = 0.19)</td>
<td></td>
</tr>
</tbody>
</table>

1-way ANOVAs of 18S Ct values for each of the gene expression assays in LE and WKY rats reveal no significant effects of stress. Reported here are the F statistics and p values for each of the ANOVAs.
corresponding stress-dependent effects in LE rats ($F(2,21) = 2.14, p > 0.05$) (Fig. 3A and B). However, OCT3 protein content in cytosolic fractions was significantly increased following chronic + acute stress in both LE rats ($F(2,21) = 4.43, p < 0.05$) and WKY rats ($F(2,21) = 18.78, p < 0.001$) (Fig. 3C and D).

3.3. Forced swim test

D22 reduced the time that WKY rats spent immobile on the forced swim test ($F(2,21) = 9.565, p < 0.001$), and post-tests revealed that both the 1 $\mu$g/kg ($t = 3.73, p < 0.01$) and 10 $\mu$g/kg D22 ($t = 3.47, p < 0.01$) significantly reduced immobility compared to vehicle. WKY rats did not exhibit climbing behavior in the forced swim test so all observed activity was due to swimming (Fig. 4B).

The LE rats were injected with 1 $\mu$g/kg D22 only since this was determined to be the lowest, maximally effective therapeutic dose in our studies. Overall, there were no significant effects of D22 on immobility ($t_{10} = 0.11, p > 0.05$), swimming ($t_{10} = 0.36, p > 0.05$), or climbing behavior ($t_{10} = 0.37, p > 0.05$) in the forced swim test in the LE rats (Fig. 4A). Interestingly, there was a profound strain difference in sensitivity to the FST as revealed by immobility time in vehicle-treated LE rats compared to vehicle-treated WKY rats ($t_{20} = 5.001, p < 0.001$). These data suggest that rats with low basal depressive-like behavior are not responsive to the antidepressant-like effects of D22, possibly due to floor effects.

4. Discussion

The LE and WKY strains exhibit substantial differences in the plasticity of OCT3 gene expression after acute and chronic + acute stress. Acute restraint stress induced a rapid and robust up-regulation of OCT3 mRNA in the hippocampus of LE rats. However, the effects of acute restraint were diminished in the LE rats with a history of repeated social defeat stress, indicating some degree of habituation. A different pattern was observed in WKY rats, in which hippocampal OCT3 mRNA was unaltered by acute restraint stress but substantially up-regulated.
after restraint in the rats with a history of repeated defeat. The delayed plasticity in OCT3 gene expression in the WKY rats' response to stress may provide further insight into the behavioral phenotype of these rats. WKY rats are slow to adapt to stress and manifest a helpless, depressive phenotype under stress conditions, whereas LE rats adapt more readily to stress after multiple exposures (Bielajew et al., 2002).

Fig. 2. GR and SERT gene expression in LE and WKY rats under basal and stressed conditions. GR (A, B) and SERT mRNA expression (C, D) in the hippocampus of LE (n = 24) and WKY rats (n = 23) under basal, acute stress, and chronic + acute stress conditions. Data represent fold change (mean ± SEM) over expression in the control group.

Fig. 3. OCT3 protein expression in the hippocampus of LE and WKY rats under basal and stressed conditions. Hippocampal OCT3 protein expression in plasma membrane (A, B) and cytosolic fractions (C, D) from LE (n = 24) and WKY rats (n = 24) under basal, acute stress, and chronic + acute stress conditions. Data represent relative optical density (mean ± SEM) relative to the control group. Asterisks (*), (**) or (***) denote p < 0.05, 0.01 and 0.001, respectively, for post-hoc comparisons with the control group.
Accordingly, we propose that habituation of the OCT3 mRNA response in LE rats may contribute to stress coping, whereas the pattern of delayed and augmented response in the WKY rats represents a failure to accommodate in the presence of repeated stress exposure. One limitation of this study is that we cannot ascertain whether the elevated OCT3 mRNA expression following chronic + acute stress in WKY rats is driven by chronic stress alone, or whether the addition of acute stress is required. For example, CRH mRNA returns to basal levels within about 4–24 h after acute restraint stress (Greetfeld et al., 2009). However, the time course of the OCT3 mRNA response has not been examined, and it is possible that repeated stress exposure could induce persistent and ongoing changes in OCT3 mRNA expression in the absence of any acute stress.

In contrast to the robust increase in hippocampal OCT3 mRNA, there was a small but statistically significant reduction in plasma membrane expression of OCT3 protein following chronic + acute stress in WKY rats (with no significant changes in LE rats). In order to resolve the apparent discrepancy between mRNA and membrane protein levels, we quantified OCT3 protein in cytosolic fractions, and found that OCT3 was significantly increased after chronic + acute stress in both LE and WKY rats. Taken all together, the data suggest that acute exposure to stress increased OCT3 mRNA expression in the LE rats, and this produced an enduring elevation in cytosolic (i.e., vesicular) OCT3 protein, without significant changes in plasma membrane trafficking. In WKY rats, the concomitant increase in OCT3 mRNA, decrease in membrane OCT3, and increase in cytosolic OCT3 following chronic + acute stress suggests that there were both de novo protein synthesis and internalization of OCT3 from the plasma membrane by the time of testing. It is worth noting that OCT3 protein was not significantly altered 3 h after acute stress in the cytosol or plasma membrane of either strain, even though OCT3 mRNA was upregulated following acute stress in LE rats. It is possible that the 3 h time point was too early to observe de novo protein synthesis. In future studies, it would be informative to determine whether restraint stress causes an upregulation of OCT3 protein 24 h after stress exposure.

Contrary to our expectations, there was no effect of stress on OCT3 mRNA expression in the mPFC or striatum of LE or WKY rats. These results suggest that OCT3 expression in these regions does not contribute to restraint stress-induced physiological and behavioral changes in either strain, or to the differences in stress-responding between WKY and LE rats.

In this study there was no significant effect of stress on GR mRNA expression. These results contrast with previous reports in which increases in hippocampal GR expression were elicited by acute social defeat stress (Marini et al., 2006), suggesting that restraint may be insufficient to elicit a robust GR mRNA response using the experimental protocols described herein. Likewise, SERT gene expression was not significantly altered by stress. The fact that GR and SERT gene expression were not substantially modified by the stress regimen used in this study, and yet OCT3 mRNA expression was robustly up-regulated in hippocampus, suggests that OCT3 expression levels were more highly responsive to our stress regimen than other known biomarkers of stress responsiveness. These data emphasize the potential importance of OCT3 plasticity in the impact of stress.

We further characterized the role of the OCT3 in the behavior of WKY and LE rats in the forced swim test, a widely used test of behavioral despair during inescapable stress. To our knowledge, this is the first study to report that D22, an inhibitor of the OCT3, has an antidepressant-like effect in the WKY rat. This effect does not appear to derive from general stimulant actions. Although we did not directly measure the effects of D22 on locomotor activity, previous studies have reported that a dose of D22 that exerts antidepressant-like actions (1 μg/kg) had no effect on locomotor activity in mice (Baganz et al., 2008). Rather, the antidepressant-like effect in the WKY rats, combined with the lack of an effect in LE rats, is interesting because WKY rats not only exhibit a depressive phenotype compared to other strains, but are typically resistant to the effects of SSRI antidepressants (Griebel et al., 1999; Lopez-Rubalcava and Lucki, 2000; Tejani-Butt et al., 2003). The dose of 1 μg/kg D22 used in this study was based on a previously published report indicating that this dose produced antidepressant-like effects in the tail suspension test in SERT KO mice (Baganz et al., 2008). Here we also included a 10 μg/kg dose to see if these antidepressant-like effects could be increased further. We found that both doses of D22 were equally potent in reducing immobility in WKY rats, suggesting that peak antidepressant effects were achieved with the lower (1 μg/kg) dose. For this reason, we used only the lower dose to test antidepressant actions in LE rats.

Overall, it appears that overexpression of hippocampal OCT3 may play an important role in the depressive phenotypes of WKY rats (although the dynamics of membrane trafficking are unclear), and in the antidepressant effects of D22. This contention is bolstered by evidence that genetic knockdown of the OCT3 has an antidepressant-like effect (Kitaichi et al., 2005) and OCT3 knockout mice have reduced anxiety-like behavior (Wultsch et al., 2009). We propose that overexpression of the OCT3 in repeatedly-stressed WKY rats is one potential mechanism of antidepressant resistance of this strain, since the OCT3 would be able to compensate for the loss of SERT-mediated 5HT clearance after SSRI treatment. It is possible that additional studies exploring the therapeutic potential of OCT3 antagonists could ultimately lead to new treatments for antidepressant resistance in major depression.

An additional possibility is that D22 could exert its antidepressant-like effects through inhibition of the plasma membrane monoamine transporter (PMAT; Engel and Wang, 2005), or through antagonism of α1 adrenoceptors (Russ et al., 1996). However, corticosterone, which...
inhibits OCT3 but has very low affinity for PMAT, has antidepressant-like effects in the murine forced swim test (Stone and Lin, 2008), and antagonism of α7 receptors should produce depressant actions (Stone et al., 2011), rather than the antidepressant-like effects that were observed. Taken together, these studies support the interpretation that the antidepressant-like effects of D22 are mediated by OCT3 inhibition, and not by α7 adrenoceptor antagonism, but the potential contribution of PMAT is unclear.

In summary, hippocampal expression of OCT3 mRNA exhibits stress-induced plasticity which is rapid yet transient in LE rats and delayed but perhaps more persistent in WKY rats. The functional significance of upregulated OCT3 expression remains somewhat unclear, but the large increases in cytosolic OCT3 protein in the WKY rats suggests that there is a large pool of protein that may participate in membrane trafficking. An acute blockade of the OCT3 with D22 reduced immobility in the forced swim test in the WKY rats, which is congruent with previous reports that OCT3 blockade by D22 or antisense oligonucleotides exerts antidepressant-like effects in mice (Kitachi et al., 2005; Baganz et al., 2008).

Overall, we propose that stress-induced OCT3 expression may play a role in individual differences in stress-responsive behavioral phenotypes, and our data concur with previous suggestions that OCT3 antagonists may be effective as antidepressants alone or in combination with other antidepressant drugs.

References
Lowry CA, Burke KA, Renner KJ, Moore FL, Orchick M. Rapid changes in monoamine levels following administration of corticotropin-releasing factor or corticoste- rone are localized in the dorsomedial hypothalamus. Horm Behav 2001;39:195–205.

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