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Altered Serotonergic Function may Partially Account for Behavioral Endophenotypes in Steroid Sulfatase-deficient Mice

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The X-linked gene *STS* encodes the steroid hormone-modulating enzyme steroid sulfatase. Loss-of-function of *STS*, and variation within the gene, have been associated with vulnerability to developing attention deficit hyperactivity disorder (ADHD), a neurodevelopmental condition characterized by inattention, severe impulsivity, hyperactivity, and motivational deficits. ADHD is commonly comorbid with a variety of disorders, including obsessive–compulsive disorder. The neurobiological role of steroid sulfatase, and therefore its potential role in ADHD and associated comorbidities, is currently poorly understood. The 39,X^{Y}*O mouse, which lacks the *Sts* gene, exhibits several behavioral abnormalities relevant to ADHD including inattention and hyperactivity. Here, we show that, unexpectedly, 39,X^{Y}*O mice achieve higher ratios than wild-type mice on a progressive ratio (PR) task thought to index motivation, but that there is no difference between the two groups on a behavioral task thought to index compulsivity (marble burying). High performance liquid chromatography analysis of monoamine levels in wild type and 39,X^{Y}*O brain tissue regions (the frontal cortex, striatum, thalamus, hippocampus, and cerebellum) revealed significantly higher levels of 5-hydroxytryptamine (5-HT) in the striatum and hippocampus of 39,X^{Y}*O mice. Significant correlations between hippocampal 5-HT levels and PR performance, and between striatal 5-HT levels and locomotor activity strongly implicate regionally-specific perturbations of the 5-HT system as a neurobiological candidate for behavioral differences between 40,XY and 39,X^{Y}*O mice. These data suggest that inactivating mutations and functional variants within *STS* might exert their influence on ADHD vulnerability, and disorder endophenotypes through modulation of the serotonergic system.
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INTRODUCTION

Steroid sulfatase, an enzyme encoded by the X-linked gene *STS* (Xp22.3) in humans, and by the pseudoautosomal *Sts* gene in mice, catalyzes the desulfation of steroid hormones (Reed *et al*, 2005). In the brain, sulfated and non-sulfated steroids (eg, dehydroepiandrosterone sulfate (DHEAS) and its non-sulfated form DHEA) act at a variety of neurotransmitter receptors (notably NMDA and $GABA_A$ receptors), and may exert distinct effects (Reddy, 2010).

Deletions encompassing *STS*, or inactivating mutations within it, are associated with an increased vulnerability to developing attention deficit hyperactivity disorder (ADHD) (Kent *et al*, 2008; Doherty *et al*, 2003; Lonardo *et al*, 2007; Tobias *et al*, 2001), a neurodevelopmental condition characterized by inattention, impulsivity, and hyperactivity (Wilens and Spencer, 2010). Where contiguous genes are deleted, ADHD may be present against a background of multiple physiological and behavioral abnormalities and may not necessarily result from steroid sulfatase dysfunction *per se*. However, in support of a specific role for *STS* in ADHD, polymorphisms within the gene are associated with disorder risk (Brookes *et al*, 2010) and with symptom severity/cognitive abnormalities (Brookes *et al*, 2008;

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Stergiakouli *et al*, 2011), whereas the gene is expressed in regions of the fetal brain associated with the later development of ADHD (Stergiakouli *et al*, 2011). The fact that steroid sulfatase-deficient individuals are vulnerable to developing the predominantly inattentive subtype of the disorder (Kent *et al*, 2008), together with the observation that *STS* polymorphisms are associated with inattentive symptomatology (Stergiakouli *et al*, 2011), imply that steroid sulfatase may have an important role in attentional processes.

39,X^{Y}*O mice, in which *Sts* is the only known deleted gene as a consequence of an end-to-end fusion of the X and Y chromosomes (Odorisio *et al*, 1998), exhibit a constellation of behavioral and hormonal abnormalities including inattention (Davies *et al*, 2009), hyperactivity, heightened emotional reactivity and aggression, increased water consumption, and lowered serum DHEA levels (Trent *et al*, 2011); thus, these mice show some phenotypic overlap with individuals diagnosed with ADHD. However, given that deletions encompassing *STS* have been observed in other psychiatric disorders (Kent *et al*, 2008; Milunsky *et al*, 1999; Thomas *et al*, 1999) it is important to appreciate that the 39,X^{Y}*O mouse may not necessarily be a model specific for, or limited to, ADHD (Gottesman and Gould, 2003).

Individuals with ADHD commonly show motivational deficits (Carlson *et al*, 2002). Experimentally, motivation (or perhaps more precisely the drive to obtain a reinforcer of a particular efficacy) can be assayed using a 'progressive ratio' (PR) task, in which individuals must complete progressively higher numbers of actions in order to obtain a fixed quantity of reinforcer; the maximum number of actions an individual is willing to perform to obtain the reinforcer is known as the 'break point', and a higher break point may reflect higher motivation to obtain the reinforcer (Roane, 2008). Unmedicated children with ADHD exhibit a lower break point than age and sex-matched healthy subjects on a PR task using a monetary reinforcer (Chelonis *et al*, 2011a, b); administration of methylphenidate in the ADHD group increased responding to levels seen in healthy children (Chelonis *et al*, 2011b). Here, we compared the performance of 39,X^{Y}*O mice with wild-type mice on a murine version of the PR task.

ADHD is commonly comorbid with other psychiatric conditions including obsessive–compulsive disorder (OCD) (Cormier, 2008; Geller, 2006). Given this, we compared 39,X^{Y}*O mice with wild-type mice on a marble-burying task, in which the extent to which mice are willing to bury non-aversive novel objects is thought to index compulsivity (Albelda and Joel, 2011); this particular behavior is sensitive to the administration of neurosteroids including DHEAS (Umathe *et al*, 2009) and selective serotonin reuptake inhibitors (SSRIs)(Shinomiya *et al*, 2005).

Currently, little is known about the brain functions of steroid sulfatase. In order to identify which neural systems steroid sulfatase may influence, and hence to identify neural correlates of behavioral differences between 39,X^{Y}*O and wild-type mice, we examined monoamine neurotransmitter levels in brain tissue from both groups. We focused on this system (dopamine (DA), 5-hydroxytryptamine (5-HT), noradrenaline, and metabolites) for two reasons: (i) because of its involvement in attention, activity, and anxiety phenotypes (Robbins and Arnsten, 2009; Webster, 2001) and (ii) because the most commonly used therapeutics for ADHD act to enhance levels of synaptic DA and/or noradrenaline (NA) (Rowles and Findling, 2010). We examined five brain regions selected for their high *Sts* expression (Compagnone *et al*, 1997), their sensitivity to steroid sulfatase axis modulation (Rhodes *et al*, 1997), and/or their altered structure/function in individuals with ADHD (Stergiakouli *et al*, 2011; Durston, 2008).

MATERIALS AND METHODS

Subjects

39,X^{Y}*O mutant and 40,XY wild-type mice were bred at the MRC National Institute for Medical Research, London. 39,X^{Y}*O mice were produced from three crosses: (i) 39,X^{Paf}O $\times$ 40,XY*, (ii) 39,X^{Paf}O $\times$ 39,X^{Y}*O, and (iii) 40,In(X)^{Paf}/X $\times$ 40,XY* (Trent *et al*, 2011). 39,X^{Y}*O males were identified though a combination of checking for gonadal type and the presence of patchy fur (caused by the *Paf* mutation) between postnatal days 7–10, and polymerase chain reaction for the *Sts* gene from tail biopsy at weaning (Davies *et al*, 2009). 40,XY mice were generated from 40,XY $\times$ 40,XX crosses. Care was taken to keep the genetic backgrounds of the crosses equivalent (ie, predominantly that of the MF1 albino strain but with two C3H strain-derived factors, which enable fertility of 39,X^{Y}*O males); also, the 40,XY males carry a Y chromosome derived from the LT strain from which the Y* chromosome originated. Two cohorts of mice were transferred to Cardiff University for the present experiments: the first was used for the behavioral experiments described in Trent *et al* (2011) (39,X^{Y}*O: $n = 19$, 40,XY: $n = 25$), subsets of which were used for the reinforcer preference/PR tests (39,X^{Y}*O: $n = 10$, 40,XY: $n = 15$), and for brain dissection/neurochemical analysis (39,X^{Y}*O: $n = 8$, 40,XY: $n = 8$); subjects from the second cohort were used for the marble-burying test only (39,X^{Y}*O: $n = 12$, 40,XY: $n = 12$).

Animal Husbandry

In Cardiff, 39,X^{Y}*O and 40,XY mice were treated with Baytril and Norodine-24 antibiotics for 1 month in a negative-pressure isolator to cure a *Pasteurella pneumotropica* infection before release onto the open racks. These mice were then housed in a holding room (1–4 mice of the same karyotype per cage) maintained at $21 \pm 2^{\circ}$C and $50 \pm 10\%$ humidity, with a 12 h light–dark cycle (lights on at 07:00 h). Animals were treated in accordance with the Animal (Scientific Procedures) Act (United Kingdom, 1986).

Reinforcer Preference

Before testing on the PR task, mice were subjected to a water restriction schedule, whereby they received 4 h of water per day for 4 days, and 2 h per day thereafter. Subjects were regularly checked and weighed to ensure that this restriction schedule had no adverse effects. Preference for a palatable reinforcer (10% condensed milk, Nestle, UK) relative to tap water was then tested in all subjects in a standard 5-day protocol (Humby *et al*, 1999).

PR

The task was conducted in nine-hole boxes (Campden Instruments, UK, Humby *et al*, 1999) configured such that only the central aperture of the response array (10 mm diameter, 10 mm from the chamber floor) was available. This aperture housed a small stimulus light and responses (nose pokes) could be recorded by the breaking of a vertically orientated infrared beam at its entrance. Reinforcer was delivered to a food magazine (20 × 20 mm) opposite the response aperture. The presence of reinforcer was signalled by illumination of a light within the food magazine and collection of the reinforcer was recorded via a microswitch on a clear Perspex door. Test chambers were enclosed in sound attenuated boxes, with a fan providing ventilation and background noise (60 dB). Stimuli presentations and subject responses were controlled/recorded by custom-written software programmes (Arachnid). For training, the mice were habituated to the test chambers for six 20 min sessions (one session per day) in which 20 μl of reinforcer was presented on a VI30 schedule, with the food magazine illuminated from the time of delivery until the mouse was recorded as collecting the reinforcer. The nose poke aperture was blocked for these initial training sessions. For the first three sessions, the door to the food magazine was wedged open so that the mice could gain access to the reinforcer easily, but for the remaining sessions mice were required to push the door open. On session 7, the response aperture was unblocked and mice were moved to a continuous reinforcement schedule (CRF), where they had to make a single nose poke to initiate reward delivery. Sessions were terminated after 20 min, 100 trials or a period of inactivity for 4 min. At the start of each trial, the response aperture was illuminated until the mouse made a nose poke response, which triggered reward delivery (20 μl). Collecting the reinforcer initiated the next trial. Following three sessions of CRF training, the PR schedule was introduced for a further three sessions. The basic session structure did not differ between CRF and PR schedules except that the number of nose pokes required to initiate reinforcer delivery was sequentially increased through a PR session (ie, 1, 2, 4, 6, 8, 10, etc), with four trials per ratio. At each ratio the stimulus light would remain illuminated until the correct number of nose pokes had been completed and reinforcer delivered. For the three PR sessions the following parameters were recorded: maximum ratio achieved ('break point'), total number of nose pokes, latency to first nose poke, intra-nose poke interval, latency to collect the reinforcer, and activity (indexed by infrared beam breaks). Mean values for each parameter over the three PR sessions were subjected to statistical analysis.

Marble-Burying Test

Mice between the ages of 6 and 8 months were tested under low illumination conditions (5 lux) using an identical procedure to that of Doe *et al* (2009). Subjects were individually placed in a plastic box (45 × 28 × 15 cm) filled with sawdust to a depth of 4–5 cm and covered with a clear plastic lid. Eight opaque red marbles (diameter 12 mm) were equally spaced in one half of the box on top of the sawdust. Animals were placed in the marble-free side of the box, and allowed to explore for 16 min. All sessions were recorded for subsequent data analysis from videotape; data were analyzed in four bins, each of 4 min duration. The main measures recorded at each timepoint were: (i) number of marbles totally buried (>90% covered by sawdust), partially buried (10–90% covered), or not buried (<10% covered); (ii) activity as indexed by number of transitions between the marble-free and marble-strewn halves of the box; and (iii) bouts of digging behavior.

Brain Dissections

39,X^{Y}*O and 40,XY mice (aged 13–15 months) were allowed *ad libitum* access to water and food for >14 days before being culled by cervical dislocation. Brains were removed immediately and dissected into five regions of interest on ice: the frontal cortex (comprising medial prefrontal, orbitofrontal, motor, frontal association, and agranular insular cortices), striatum (comprising caudate putamen and nucleus accumbens), thalamus, hippocampus, and cerebellum. Two additional 'negative control' regions exhibiting low-*Sts* expression and not implicated in ADHD pathology (the olfactory bulb and hypothalamus) were also dissected. Dissected regions (pooled from both hemispheres) were weighed to a high degree of accuracy using a microbalance, frozen on dry ice, and stored at −80°C.

High Performance Liquid Chromatography (HPLC) for Determination of Monoamine and Monoamine Metabolite Concentrations

HPLC was performed according to Cassano *et al* (2009). Briefly, tissue samples were ultrasonicated in 0.1M perchloric acid, centrifuged for 20 min at 15 000 *g* (4°C), and the supernatant used for HPLC assays. Samples from mutant and wild-type mice were run in parallel to eliminate the possibility of run effects. The endogenous levels of DA, DA metabolites (homovanillic acid and 3,4-dihydroxyphenylacetic acid), NA, and NA metabolite (4-hydroxy-3-methoxyphenylglycol (MOPEG)), 5-HT (serotonin) and 5-HT metabolite (5-hydroxyindolacetic acid (5-HIAA)) were assayed by microbore HPLC using a SphereClone 150 × 2-mm column (3-μm packing). Detection was accomplished with a Unijet cell (BAS) with a 6-mm-diameter glassy carbon electrode at +650 mV *vs* an Ag/AgCl reference electrode, connected to an electrochemical amperometric detector (INTRO, Antec Leyden, Netherlands). The chromatographic conditions were: (i) a mobile phase composed of 85 mM of sodium acetate, 0.34 mM EDTA, 15 mM sodium chloride, 0.81 mM of octanesulphonic acid sodium salt, 5% methanol (v/v), pH = 4.85; (ii) a rate flow of 220 μl/min; and (iii) a total runtime of 65 min. For each analysis, a set of standards containing various concentrations of each compound (monoamines and metabolites) was prepared in the acid solution and was injected before, and at the end, of any one run to account for changes in chromatographic conditions during the run. The calibration curves were calculated by linear regression using the mean of the standard values before and after the run. The retention times of standards were used to identify peaks, and peak areas were used to quantify

monoamine/metabolite levels. Results were normalized to wet tissue weight.

Statistics

Statistics were analyzed using SPSS16.0. (IBM Corporation, New York). Data were initially tested for normality with Shapiro–Wilks test. Normal data were analyzed by unpaired two-tailed *t*-test. Where sphericity assumptions were violated, Greenhouse–Geisser corrected degrees of freedom values are presented. Nonparametric data were analyzed by two-tailed Mann–Whitney *U*-test. Frequency data were analyzed by two-tailed χ^2-test. For the correlational analyzes, Pearson's test was used for normally distributed data, and Spearman's test for non-normally distributed data. *P*-values of $\leqslant 0.05$ were generally regarded as significant; however, for the neurochemical analysis, *P*-values of $\leqslant 0.0033$ (ie, 0.05/15) were regarded as significant to account for multiple testing across three different monoamine systems and five brain regions of interest. Given the interdependence of the brain regions and monoamine systems analyzed, this cutoff is likely to be conservative. Data are reported as mean values ± SE of the mean.

RESULTS

Reinforcer Preference

39,X^{Y}*O and 40,XY mice consumed equal volumes of fluid on the final day of the reinforcer preference task (2.4 ± 0.2 ml *vs* 2.7 ± 0.2 ml, respectively, $t(23) = 0.931$, $P = 0.361$), and showed an equally high preference for the 10% condensed milk reinforcer relative to tap water before commencing the PR task (81.7 ± 6.6% and 83.6 ± 2.7%, respectively, $U = 64.5$, $P = 0.56$).

PR Task

There was a significant difference in performance between 39,X^{Y}*O and 40,XY mice on the break point measure recorded in the PR task. Contrary to expectation, the former group achieved a significantly higher break point (18.1 ± 1.4 *vs* 13.5 ± 1.1, $t(23) = -2.544$, $P = 0.018$); they also performed significantly more nose pokes in total (357 ± 58 *vs* 218 ± 32, $t(23) = -2.290$, $P = 0.032$). 39,X^{Y}*O mice demonstrated a shorter latency to initiate nose poking (6.8 ± 0.8 *vs* 8.3 ± 0.9 s, $t(23) = 1.184$, $P = 0.249$), shorter intervals between nose pokes (2.6 ± 0.4 *vs* 6.9 ± 2.8 s, $t(23) = 1.216$, $P = 0.236$), and shorter latencies to collect the reinforcer (4.5 ± 1.8 *vs* 10.7 ± 5.5 s, $U = 51.0$, $P = 0.183$) than wild-type mice. Although 39,X^{Y}*O mice tended to be more active than 40,XY mice during the task (as indexed by the number of infrared beam breaks), this result was not significant (518 ± 54 *vs* 391 ± 47, $t[23] = -1.761$, $P = 0.091$).

Marble-Burying Test

All mice tested showed a tendency to bury the marbles to some extent. Although 39,X^{Y}*O mice were significantly more active than their 40,XY counterparts in this task (92.7 ± 6.2 *vs* 56.6 ± 3.4 transitions, $t(22) = -5.105$, $P < 0.001$), they did not engage in significantly more bouts of digging (26.8 ± 3.4 *vs* 21.3 ± 3.0, $t(22) = -1.19$, $P = 0.247$), and exhibited a similar marble-burying dynamic profile (Supplementary Figure 1); at the conclusion of the test, there was no significant difference in the pattern of marbles buried by the two groups ($\chi^2(2) = 5.44$, $P = 0.067$).

Monoamine Analysis

Of the 784 HPLC peaks examined in total, just two could not be analyzed reliably (DA in the hippocampus of one 40,XY mouse, 5-HIAA in the hypothalamus of one 39,X^{Y}*O mouse). Using our stringent cutoff of $P < 0.0033$, we identified three significant differences in monoamine levels between 40,XY and 39,X^{Y}*O mice: the latter group exhibited significantly elevated 5-HT levels in the striatum ($t(14) = -3.55$, $P = 0.003$) and in the hippocampus ($t(14) = -4.36$, $P = 0.001$), and significantly reduced MOPEG levels in the striatum ($t(14) = 4.08$, $P = 0.001$)(Table 1). Neither 5-HIAA levels in the striatum and hippocampus, nor-striatal NA levels differed between the two groups ($t(14) = -0.46$, $P = 0.66$, $t(14) = -0.75$, $P = 0.47$, and $t(14) = -0.43$, $P = 0.67$, respectively); hence, there was significantly reduced 5-HT turnover (ratio of 5-HIAA/5-HT) in the 39,X^{Y}*O striatum (0.48 ± 0.04 *vs* 0.71 ± 0.05, $t(14) = 3.75$, $P = 0.002$) and hippocampus (1.14 ± 0.09 *vs* 1.78 ± 0.16, $t(14) = 3.44$, $P = 0.004$), and reduced NA turnover (ratio of MOPEG/NA) in the 39,X^{Y}*O striatum (1.36 ± 0.24 *vs* 2.42 ± 0.49, $t(14) = 2.81$, $P = 0.014$). We found no strong evidence for group differences in the dopaminergic system in the brain regions examined. As expected, there were no significant differences in any of the neurochemical systems assayed in the two 'negative control' regions (the olfactory bulb and hypothalamus)(Supplementary Table 1).

Correlations Between Behavior and Monoamine Levels

Given that lesions of the dorsal or ventral hippocampus in mice result in increased break points in a PR task similar to the one employed here (Gourley *et al*, 2010), and genetic and pharmacological manipulations of the serotonergic system influence responding for food in a PR task (Sanders *et al*, 2007), we tested whether 5-HT levels in the hippocampus of our 40,XY and 39,X^{Y}*O were related to the break points achieved in the PR task; neurochemical and behavioral data were available for four 40,XY mice and five 39,X^{Y}*O mice. The data indicated a strong, and highly significant, positive correlation between 5-HT levels in the hippocampus and break point (Pearson's correlation (r) = 0.832, $P = 0.005$, Figure 1a). No significant correlation was observed between 5-HT levels in the striatum and break point ($r = 0.252$, $P = 0.513$), nor between MOPEG levels in the striatum and this measure ($r = -0.329$, $P = 0.387$), indicating a degree of regional and neurochemical specificity to the effect.

It has been previously shown that elevated levels of striatal 5-HT are associated with hyperactivity in mice (Avale *et al*, 2004). Hence, we also tested whether 5-HT levels in the striatum were correlated with an activity measure taken previously upon which 40,XY and 39,X^{Y}*O mice differed significantly (ie, aggregate value of distance travelled in homecages over a 24-h test period and total

Table 1 Concentrations of Monoamines and their Metabolites in the five Brain Regions of Interest in 40,XY and 39,X^{Y}*O Mice

Brain region of interest	Karyotype	Neurotransmitter/metabolite concentration (pg/mg wet brain weight)						
		Noradrenergic system		Dopaminergic system			Serotonergic system	
		NA	MOPEG	DA	HVA	DOPAC	5-HT	5-HIAA
Frontal cortex	40,XY ($n=8$)	231 ± 19	179 ± 14	21 ± 2	170 ± 12	94 ± 6	492 ± 46	472 ± 30
	39,X^{Y}*O ($n=8$)	231 ± 13	224 ± 16*	20 ± 3	196 ± 18	117 ± 6*	354 ± 44	482 ± 11
Striatum	40,XY ($n=8$)	243 ± 19	565 ± 55	3675 ± 437	2870 ± 105	3697 ± 218	983 ± 64	677 ± 25
	39,X^{Y}*O ($n=8$)	258 ± 29	307 ± 31***	6147 ± 853*	2567 ± 199	3245 ± 420	1492 ± 128***	694 ± 27
Thalamus	40,XY ($n=8$)	292 ± 32	192 ± 11	89 ± 14	274 ± 17	218 ± 18	512 ± 144	805 ± 37
	39,X^{Y}*O ($n=8$)	326 ± 23	133 ± 15**	93 ± 16	307 ± 24	179 ± 17	970 ± 172	764 ± 44
Hippocampus	40,XY ($n=8$)	325 ± 22	271 ± 25	38 ± 7	288 ± 166	85 ± 5	386 ± 26	663 ± 28
	39,X^{Y}*O ($n=8$)	396 ± 40	207 ± 15*	28 ± 2	152 ± 8	76 ± 4	633 ± 50***	695 ± 33
Cerebellum	40,XY ($n=8$)	179 ± 31	117 ± 27	12 ± 5	37 ± 8	128 ± 30	140 ± 28	263 ± 36
	39,X^{Y}*O ($n=8$)	116 ± 10	101 ± 36	7 ± 1	34 ± 5	67 ± 3	119 ± 15	215 ± 13

*$p<0.05$, **$p<0.01$, ***$p<0.0033$.

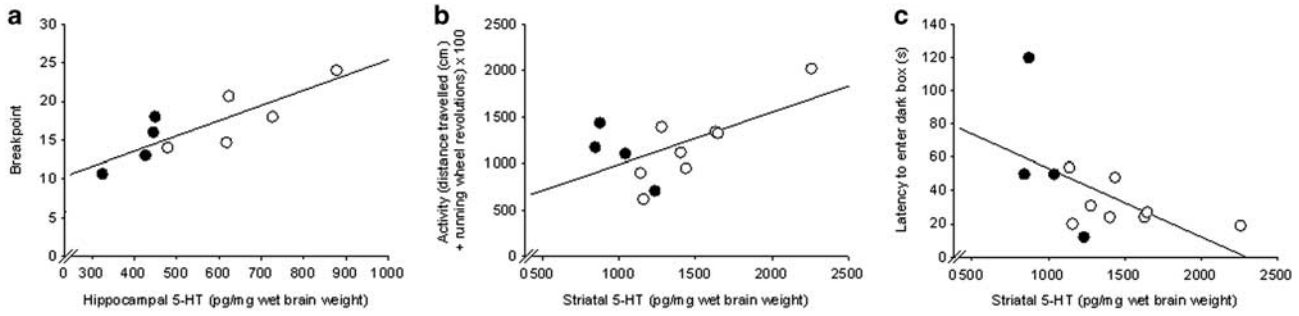

Figure 1 Correlations between behavioral endophenotypes and brain region 5-HT levels in 40,XY and 39,X^{Y}*O mice (black and white circles, respectively). There was a positive correlation between hippocampal 5-HT levels and the highest break point achieved in a PR task (a), a positive correlation between striatal 5-HT levels and a homecage activity measure (b), and a negative correlation between striatal 5-HT levels and latency to enter a relatively non-aversive environment from an aversive one in the light–dark box test (c).

number of revolutions in a running wheel over the same period, Trent *et al* (2011)); neurochemical and behavioral data were available for four 40,XY mice and eight 39,X^{Y}*O mice. There was a moderate, significant positive correlation between 5-HT levels in the striatum and this activity measure ($r=0.583$, $P=0.047$, Figure 1b), which was even more evident when the 39,X^{Y}*O group was considered in isolation ($r=0.884$, $P=0.004$). No significant correlation was observed between 5-HT levels in the hippocampus and this activity measure ($r=-0.035$, $P=0.914$), nor between striatal MOPEG levels and activity ($r=-0.123$, $P=0.704$), again indicating some degree of specificity to the effect.

There is some evidence for striatal, hippocampal, and serotonergic involvement in adult anxiety phenotypes in mice (Adhikari *et al*, 2011; Carola *et al*, 2008). We previously showed that 39,X^{Y}*O mice exhibit more 'anxiety-related' behaviors than 40,XY mice (notably shorter latencies to enter a less aversive (dark) environment from a more aversive (light) one, and an increased tendency towards thigmotaxis in an open field)(Trent *et al*, 2011). Therefore, we also correlated two previously obtained anxiety measures with 5-HT levels in the striatum and hippocampus; neurochemical and behavioral data were available for four 40,XY mice and eight 39,X^{Y}*O mice. We noted a significant negative correlation between striatal 5-HT levels and latency to enter the dark box in the light–dark box test (Spearman's correlation coefficient $=-0.618$, $P=0.032$, Figure 1c), but no significant correlation between hippocampal 5-HT levels (Spearman's correlation coefficient $=-0.333$, $P=0.290$), or striatal MOPEG levels (Spearman's correlation coefficient $=0.358$, $P=0.253$) on the same measure. Time spent in the periphery of the open field did not correlate with hippocampal 5-HT levels ($r=-0.231$, $P=0.471$), striatal 5-HT levels ($r=0.032$, $P=0.921$), or striatal MOPEG levels ($r=0.215$, $P=0.502$).

DISCUSSION

The purpose of this study was to further specify the behavioral functions underpinned by the novel ADHD candidate gene *Sts*, and to investigate, for the first time, how steroid sulfatase deficiency might impact upon brain

neurochemistry. We have shown, using a PR task, that 39,X^{Y}*O mice are more willing to work to obtain a palatable liquid reinforcer than wild-type mice. This increased willingness to undertake the sequence of actions that result in reinforcement could theoretically be due to an enhanced preference for the reinforcer in the 39,X^{Y}*O mice; however, we have shown here, and elsewhere (Davies *et al*, 2009), that 40,XY and 39,X^{Y}*O groups consume equal amounts of, and display an equal preference for, the reinforcer used in this study. The fact that 39,X^{Y}*O mice perform significantly more nose pokes than 40,XY mice, although exhibiting equivalent levels of general activity, suggests the possibility that rather than being due to general hyperactivity, the higher ratio achieved in 39,X^{Y}*O mice may reflect their increased tendency to perform repetitive, focused actions, potentially in a habitual (ie, reinforcement-independent) manner. Parametric behavioral studies examining the performance of the 40,XY and 39,X^{Y}*O mice in the PR task following prefeeding, with different reinforcers and in extinction will be necessary to specify the behavioral processes disrupted by steroid sulfatase deficiency more precisely. Future work may also investigate whether the present PR data are recapitulated in humans with steroid sulfatase dysfunction, and to see whether similar underlying behavioral processes are affected across the two species.

We did not observe any difference between the behavior of 40,XY and 39,X^{Y}*O mice on a marble-burying task thought to model aspects of compulsivity and known to be sensitive to neurosteroid manipulations in the form of acutely administered DHEAS, allopregnanolone, and progesterone (Umathe *et al*, 2009); this may be because 40,XY and 39,X^{Y}*O mice do not differ with respect to the levels of neurosteroids, which influence this behavior, or because the behavioral effects arising from acute neurosteroid administration differ from those arising from developmental alterations in neurosteroid levels. Future work might examine the extent to which steroid sulfatase-deficient individuals are affected by disorders of compulsivity such as OCD.

A third main finding of this study was that 39,X^{Y}*O mice show regionally-specific increases in levels of 5-HT (but not its metabolite 5-HIAA) in the striatum and hippocampus, as well as reduced levels of MOPEG in the striatum. Here, it should be acknowledged that for consistency of dissection, accuracy of weighing, and to ensure coverage of as many subregions of interest as possible, the striatum and hippocampus were not subdivided into more functionally dissociable regions. Therefore, we cannot exclude the possibility that these elevated levels are specific to one or more subregions. Moreover, the present analysis simply examined whole tissue 5-HT levels; whether these are reflected in the synaptic cleft remains to be determined, eg, through *in vivo* microdialysis. Potential explanations for elevated 5-HT levels in 39,X^{Y}*O striatum and hippocampus include increased 5-HT biosynthesis in neurons projecting from the dorsal and median Raphe nuclei, respectively, and/or increased numbers/size of serotonergic terminals. Acute administration of exogenous DHEA may both attenuate the activity of the rate-limiting enzyme in 5-HT biosynthesis, tryptophan hydroxylase, in rodent brain under certain conditions (Singh *et al*, 1994) and increase 5-HT turnover in the striatum (Perez-Neri *et al*, 2008). We have previously shown that 39,X^{Y}*O mice exhibit reduced systemic DHEA levels (Trent *et al*, 2011). Thus, we speculate that loss of steroid sulfatase in 39,X^{Y}*O mice results in reduced levels of systemic DHEA, which in turn (by some as-yet-undefined mechanism) results in an increase in tryptophan hydroxylase activity and therefore increased 5-HT levels (ie, reduced 5-HT turnover) in a regionally-selective manner. Parallel biochemical and histological analyzes across development will be necessary to understand the neurobiological mechanisms responsible for the group difference in neurochemistry. We found no evidence that the break point measure in the PR task, nor 5-HT levels in the striatum and hippocampus, were influenced by maternal genotype or housing condition (single *vs* group housing) in 39,X^{Y}*O mice (data not shown).

Here and elsewhere, we have identified numerous behavioral abnormalities in 39,X^{Y}*O mice. First, these mice exhibit deficits in response accuracy/stimulus detection and reduced premature responding in the 5-choice serial reaction time task (5-CSRTT) relative to wild-type mice, indicative of impaired attention and a greater degree of impulsive action control, respectively (Davies *et al*, 2009). Second, 39,X^{Y}*O mice are hyperactive relative to 40,XY mice, exhibit more 'anxiety-related' behaviors and appear more aggressive (Trent *et al*, 2011). Finally, they achieve a significantly higher ratio than wild-type mice on the PR task described here. We tested to see whether our neurochemical findings could account for some, or all, of these behavioral results. We found here that hippocampal (but not striatal) 5-HT levels were highly significantly correlated with PR performance, suggesting higher hippocampal 5-HT levels in 39,X^{Y}*O mice as an excellent candidate biological mechanism for this behavioral phenotype. We also found that striatal 5-HT levels were significantly correlated with both a homecage activity measure and the latency to enter a non-aversive environment from an aversive one; however, we found no correlation between striatal 5-HT levels and an independent measure of anxiety from the open field test. Hence, elevated striatal 5-HT levels may explain both the locomotor hyperactivity phenotype and the 'latency to enter the dark box' measure in 39,X^{Y}*O mice; the latter may primarily be indexing activity rather than anxiety *per se*. The fact that no correlation was observed between striatal 5-HT levels and break point in the PR task provides further evidence that the behavioral abnormality seen in 39,X^{Y}*O mice in the PR task is not activity dependent. It should be noted that the correlational analyzes presented herein used relatively small sample sizes, and as such may be sensitive to type I error generation and relatively underpowered to detect true effects. Nevertheless, our results are neurobiologically plausible and merit attempted replication in subsequent larger-scale studies.

Striatal 5-HT depletion, as a consequence of dorsal Raphe lesion, results in enhanced choice accuracy/stimulus detection and more premature responding in the 5-CSRTT (Harrison *et al*, 1997; Robbins, 2002; Eagle and Baunez, 2010). In the 39,X^{Y}*O mouse the opposite may be true, ie, elevated striatal 5-HT levels may result in the impaired choice accuracy and reduced premature responding phenotypes seen in these mice. 5-HT is a key modulator of aggressive behavior in rodents (Popova, 2006; Nelson and Trainor, 2007). Elevated 5-HT levels in the forebrain (the frontal cortex, striatum, and hippocampus) of monoamine

oxidase-deficient mice may explain their aggressive phenotype (Chen *et al*, 2007), and a similar mechanism may plausibly operate in 39,X^Y*O mice. The fact that the 39,X^Y*O mouse we previously identified as being particularly aggressive (Trent *et al*, 2011) had the highest striatal 5-HT level of all animals tested (but a median hippocampal 5-HT level) suggests that striatal 5-HT levels may be of particular significance to this behavioral phenotype. Our analyzes suggest that striatal MOPEG levels are unlikely to explain any of the main behavioral differences identified between 40,XY and 39,X^Y*O mice to date, but they may be pertinent to other, as yet, uncharacterized behavioral differences between the two groups.

Altered 5-HT levels may exert neurodevelopmental effects that perturb the expression and/or normal function of receptors for this neurotransmitter (Sodhi and Sanders-Bush, 2004). The marble-burying paradigm we employed here is sensitive to the effects of the SSRI paroxetine, but not to the effects of the 5-HT_{2c} receptor antagonist SB242084 (Doe *et al*, 2009); conversely, both PR performance and 5-CSRTT premature responding in rodents may be modulated by pharmacological manipulation of the 5-HT_{2c} receptor (Fletcher *et al*, 2010; Eagle and Baunez, 2010). Hence, we examined the expression of two distinct 5-HT_{2c} receptor splice variants giving rise to functionally different proteins in 39,X^Y*O and 40,XY adult hippocampus (Doe *et al*, 2009); both variants were more highly expressed in the former group. Moreover, there was some degree of specificity to this effect, in that the expression of a second hippocampally-expressed 5-HT receptor subunit, 5-HT_{1A}, did not differ between the two groups (Supplementary Methods and Supplementary Figure 2). Together, these data suggest that elevated 5-HT_{2c} receptor expression might underlie some of the behavioral endophenotypes seen in the 39,X^Y*O mouse, and that 40,XY and 39,X^Y*O mice might be differentially sensitive to the effects of pharmacological manipulations targeting these receptors.

Our present findings suggest: (i) that the apparent predisposition to ADHD (and possibly other psychiatric disorders) in individuals with steroid sulfatase deficiency (Kent *et al*, 2008) might be partially because of relatively large perturbations in the 5-HT system, and (ii) the more general possibility that functional polymorphisms within *STS* influence ADHD risk and disorder endophenotypes via more subtle effects on the same system. There is some limited evidence that certain ADHD endophenotypes may be underpinned by abnormal 5-HT function (Oades, 2007), but as yet, the precise role of 5-HT in ADHD vulnerability is unclear.

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DISCLOSURE

The authors declare no conflict of interest.

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