ELSEVIER

Physiology & Behavior 81 (2004) 545–555

PHYSIOLOGY & BEHAVIOR

Changes in anxiety-related behaviors and hypothalamic–pituitary–adrenal activity in mice lacking the 5-HT-3A receptor

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Received 31 July 2003; received in revised form 12 January 2004; accepted 21 January 2004

Abstract

The serotonin-3 (5-HT-3A) receptor has been localized in limbic and brainstem structures that regulate anxiety-related behavior and hypothalamic–pituitary–adrenal (HPA) activity, but its role in regulating anxiety-related behaviors is equivocal, and evidence for its role in regulating HPA activity is limited. Therefore, we used 5-HT-3A receptor knockout (KO) mice to further study these issues. Behavior in the elevated plus maze, open field, light–dark box and after Pavlovian fear conditioning was examined in addition to HPA activity under basal and acute stress conditions. Compared to age-matched adult male wild-type (WT) controls, adult male KO mice exhibited increased distance traveled in the open arms of the elevated plus maze, consistent with decreased measures of anxiety. There were no differences between the two genotypes in exploratory behavior in the open field or light–dark test. KO mice displayed enhanced fear conditioning indexed by fear-induced freezing behavior. KO mice displayed lower adrenocorticotropin (ACTH) responses to restraint or lipopolysaccharide (LPS). In addition, lower vasopressin mRNA in the paraventricular nucleus of the hypothalamus (PVN) and higher corticotropin-releasing hormone (CRH) mRNA in the central amygdala were observed in KO compared to WT mice. Therefore, deletion of the 5-HT-3A receptor revealed an important role for this receptor in regulating HPA responses to acute stress and a potential interaction between the 5-HT-3A receptor and CRH in the amygdala. Together, these data suggest that the 5-HT-3A receptor does not have a unitary role in the regulation of anxiety- and fear-related behaviors but has a potentially substantial role in the regulation of HPA activity.

Keywords: Serotonin-3 receptor; Anxiety; Stress; CRH; Vasopressin; Amygdala

1. Introduction

The serotonin-3 (5-HT) receptor is a serotonin-gated ion channel [1] that mediates fast excitatory responses [1,2]. Two subunits (5-HT-3A and -3B receptors) are believed to comprise native 5-HT-3 receptor. Only 5-HT-3A receptor can form homomeric channels on its own [1], and this subunit is essential for the expression of functional 5-HT-3 receptor complexes [3]. The localization of the 5-HT-3A receptor in limbic regions, such as cortex, amygdala, bed nucleus of the stria terminalis, and hippocampal formation [4–9], suggests its involvement in anxiety, cognition, and hypothalamic–pituitary–adrenal (HPA) function. However, the exact role of the 5-HT-3A receptor in anxiety-related behaviors has been difficult to determine, and the role of this receptor in regulation of HPA activity is largely unexplored.

The role of 5-HT-3 receptors in mediating anxiety-related behaviors has been studied in a wide variety of paradigms [10]. Peripheral administration of 5-HT-3 antagonists, such as ondansetron and tropisetron, decrease measures of anxiety consistently in the (black–white box) [11–14]. However, such decreases in other tests of anxiety-related behaviors, such as the elevated plus maze or open field, are not always observed ([15–21]; for review see Refs. [10,22]). The discrepant results in these studies with respect to behavioral effects of 5-HT-3 antagonists may be due to

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doi:10.1016/j.physbeh.2004.01.018

differences in type of behavioral test used, dose or type of antagonist tested, species used, or housing and testing conditions. These discrepancies underline the need to better define the role of 5-HT-3 receptors in anxiety-related behaviors.

Some evidence suggests a potential involvement of the 5-HT-3 receptor in the regulation of function within the HPA axis. Exposure to stress stimulates the activity of the HPA axis. Stress-activated inputs to the parvocellular division of the paraventricular nucleus of the hypothalamus (pPVN) release corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) [23]. CRH and AVP act at the anterior pituitary to release adrenocorticotropin (ACTH) [23,24], which in turn stimulates the release of corticosterone from the adrenal cortex. There is no evidence for the presence of 5-HT-3 receptors in the PVN, but these receptors are expressed in brain regions known to regulate PVN activity, such as the amygdala, bed nucleus of the stria terminalis, and hippocampus [6,7]. Since as 5-HT-3 receptors have also been identified in the pituitary gland, it is not clear whether any effects of 5-HT-3 antagonists on HPA activity would be centrally or peripherally mediated [25].

We used a 5-HT-3A-receptor-subunit-deficient mouse [5-HT-3A knockout (KO) mouse] in an attempt to clarify the role of the 5-HT-3A receptor in anxiety-related behaviors and the regulation of HPA activity. This genetic approach allows us to examine the role of the 5-HT-3A receptor in these functions under consistent housing and testing conditions. We examined measures of anxiety in WT and KO mice in the elevated plus maze, open field, light–dark box, and after Pavlovian fear conditioning. To characterize HPA function in WT and KO mice, we measured basal and stress-induced ACTH and corticosterone levels following exposure to two distinct stress stimuli, restraint and lipopolysaccharide (LPS). We also examined pituitary sensitivity to CRH. Finally, we examined density of CRH and AVP mRNA levels in brain regions important for anxiety and/or HPA activity in WT and KO mice.

2. Materials and methods

The generation of 5-HT-3A KO mice is described by Zeitz et al. [26]. Briefly, F1 hybrid C57Bl/6J × 129 heterozygous progeny were backcrossed with C57Bl/6J mice for 5–7 generations to produce the homozygous null mutant (KO) and wild-type (WT) mice used in these studies. Deletion of the 5-HT-3A receptor has been previously confirmed by autoradiography and Southern and Northern blotting (see Ref. [26]).

2.1. Animals

Adult male mice (ranging from 7 to 20 weeks of age) were used. WT and KO mice born within 1 week of one another were used in any given experiment. All mice were housed on a 12:12-h light–dark schedule (lights on at 0700 h) with food and water available ad libitum. For collection of plasma ACTH and corticosterone at 60 min after 30-min restraint, we used C57Bl6 males instead of WT mice. All experiments were conducted between 1000 and 1400 h and were approved by the Animal Care Committee at UCSF and the University Committee on Use and Care of Animals at the University of Michigan.

2.2. Behavioral testing in the elevated plus maze

Anxiety levels were assessed using an elevated plus-shaped maze consisting of two open arms and two closed arms equipped with rows of infrared photocells interfaced with a computer (Hamilton-Kinder, Poway, CA). Rodents avoid the open arms of the plus maze so that decreases in time spent in and entries into the open arms are thought to reflect enhanced measures of anxiety [27]. Mice were placed individually in the center of the maze and allowed free access for 10 min. They could spend their time either in a closed safe area (closed arms), in an open area (open arms), or in the middle, intermediate zone. Recorded beam breaks were used to calculate the time spent and the distance moved in the open and closed arms and the number of times the mice extended over the edges of the open and closed arms. After behavioral testing, the equipment was cleaned with 1 mM acetic acid to remove odors. There were six KO mice and seven WT mice used in this experiment.

2.3. Behavior in the open field

Mice were taken from their home cages into an adjacent testing room and placed in the middle of a brightly lit, white open field. A four-unit open field (50 × 50 × 38 cm) was used, allowing all mice from a single cage (three to four mice per cage) to be tested concurrently. For data analysis, each open field was divided into concentric rectangles with an outer zone (8.3 cm in from the walls), a middle zone (8.3 cm from the outer zone), and an inner zone (8.3 cm from the middle zone). Behavior was monitored using a PolyTrack system (San Diego Instruments) for a total of 30 min. Distances traveled in, time spent in, and entries into the outer, middle, and inner zones were recorded and summed into 10-min bins and were also summed into one 30-min value. Increases in these variables in the inner zone are thought to reflect decreased measures of anxiety [27]. After behavioral testing, the enclosures were cleaned with 1 mM acetic acid to eliminate odors. There were 10 WT and 9 KO mice used in this experiment.

2.4. Behavior in the light–dark test

The apparatus consisted of a Plexiglas rectangular box (45 cm long × 27 cm wide × 27 cm high) divided into a dark region (15 cm long) and a larger light region (30 cm long) as in Ref. [28]. The light and dark regions were

separated by an opening (7.5 × 7.5 cm) that allowed the animals to move between the two compartments. The dark region was made of black Plexiglas and covered with a black lid. The light portion was made of white Plexiglas, and a 60-W light was positioned directly over it. On the day of testing, each mouse was transported individually from the housing room to the testing room. The mouse was placed in the light compartment (as in Refs. [28–31]) and was allowed to move freely between the two compartments. Behavior was video-recorded for a total of 10 min, and the videotapes were scored for latency to return to the light compartment, the number of transitions between the light an dark compartments as well as total time in the light or dark compartments by an investigator blind to the groups assignments of the animals. In this experiment, because of lack of sufficient WT mice, we used age-matched C57 male mice instead and compared them to our male KO mice. There were eight mice per group in this experiment.

2.5. Pavlovian fear conditioning

Testing was conducted using a standard fear-conditioning paradigm, as previously described [32]. Briefly, fear conditioning took place in four identical observation chambers (30 × 24 × 21 cm; MED-Associates, Burlington, VT). The chambers were constructed from aluminum (side walls) and Plexiglas (rear wall, ceiling, and hinged front door) and were situated in sound-attenuating cabinets located in a brightly lit and isolated room. The floor of each chamber consisted of 36 stainless steel rods (3 mm in diameter), spaced 8 mm apart (center to center). Rods were wired to a shock source and solid-state grid scrambler (MED-Associates) for the delivery of footshock USs. A speaker mounted outside a grating in one wall of the chamber was used for the delivery of acoustic CSs. A 15-W house light was mounted on the opposite wall. The chambers were cleaned with 1% ammonium hydroxide solution, and stainless steel pans containing a thin film of the same solution were placed underneath the grid floors to provide a distinct odor before the mice were placed inside. Ventilation fans in each cabinet supplied background noise (65 dB; A scale).

Mice were conditioned and then tested for fear to the conditioning context and to the tone CS on the two subsequent days. For fear conditioning, mice were transported in squads of four (counterbalanced for genotype; $n = 8$ per group) and placed in the conditioning chambers; chamber position was counterbalanced for each squad. The mice received five tone (10 s, 2 kHz, 85 dB)–footshock (1.0 s, 0.5 mA) trials (74-s intertrial interval) beginning 192 s after placement in the chambers. Sixty-four seconds after the final shock, the mice were returned to their home cages. Twenty-four hours after the conditioning session, the mice were returned to the conditioning chambers and tested for fear to the context in which they had received the tone–footshock trials. This test for fear to the context lasted 512 s, after which the mice were returned to their home cages. Twenty-four hours after the context test, the mice were tested for fear to the tone CS in "Context B." This context consisted of the same chambers used for "Context A;" however, the room lights and chamber houselights were turned off (three 40-W red lights provided illumination). In addition, the doors on the sound-attenuating cabinets were closed, the ventilation fans were turned off, and the chambers were cleaned with 1% acetic acid solution. To provide a distinct odor, stainless steel pans containing a thin film of acetic acid were placed underneath the grid floors before the mice were placed inside. In this context, the mice received a single continuous tone (512 s, 5 kHz, 85 dB) beginning 128 s after being placed in the chambers.

Fear to the context and to the tone CS was assessed by measuring freezing behavior. The output from video cameras mounted above each chamber was fed into a video processor (Robot, San Diego, CA), and the mice were videotaped throughout each of the sessions. Freezing behavior, defined as the absence of all movement except for that necessitated by breathing, was scored using a time-sampling procedure by an experimenter who was blind to the ages and genotypes of the mice. Measurements were made every 8 s for each mouse, yielding 64 observations during the 512-s test. Freezing was quantified by computing the percentage of observations in which the mouse had been scored as freezing during the test.

2.6. Blood sampling in response to restraint or LPS

Mice previously group-housed were transferred into individual plastic tub cages and allowed at least 1 week to acclimate to their housing conditions before these experiments were conducted. Previous data indicate that within a week following transfer to individual housing conditions, feeding patterns have stabilized [33]. Mice were killed by decapitation to collect sufficient blood to perform both the ACTH and corticosterone assays. Therefore, separate groups of mice were decapitated immediately following removal from the home cage (the 0-min time point) and at the end of a 30-min period of restraint in a plastic cylindrical tube [34]. For plasma ACTH, the number of mice used were the following: $n = 11$ for WT and $n = 13$ for KO under basal conditions, and $n = 14$ for WT and $n = 14$ for KO at 30-min restraint. For plasma corticosterone, $n = 9$ for WT and $n = 8$ for KO under basal conditions, and $n = 8$ for WT and $n = 8$ for KO at 30-min restraint. In a later experiment, we examined plasma corticosterone and ACTH at 60 min following onset of 30-min restraint. Due to lack of sufficient numbers of WT mice, we used C57Bl6 age-matched male mice and compared them to KO mice. C57 and KO mice were restrained for 30 min, returned to their home cage, and were decapitated 30 min later. This 60-min measure provided an indication of recovery from acute restraint. Plasma ACTH and corticosterone were determined at 60 min for five KO and six C57 mice.

A different cohort of singly housed mice was injected intraperitoneally with either 1 μg or 5 μg of LPS (Sigma, St. Louis, MO) or saline vehicle 2 h prior to decapitation. These doses of LPS and time intervals between injection and blood collection have been used previously in mice to study HPA function [35–40]. For plasma ACTH, the number of mice used were the following: $n=7$ for WT and $n=6$ for KO after vehicle injection; $n=8$ for WT and $n=7$ for KO after 1.0 μg injection; and $n=6$ for WT and $n=7$ for KO after 5.0 μg injection. For plasma corticosterone, the figures were as follows: $n=8$ for WT and $n=7$ for KO after vehicle injection; $n=9$ for WT and $n=7$ for KO after the 1.0-μg injection; and $n=8$ for WT and $n=8$ for KO after the 5.0-μg injection.

2.7. Pituitary sensitivity to CRH

Mice were singly housed as described above and received either 10 μg/kg CRH (Peninsula Laboratories, Belmont, CA) ip at 40 min, 100 μg/kg CRH ip at 60 min, or 0.2 ml saline vehicle ip at either 40 or 60 min prior to sampling by decapitation. Samples were collected at different time points for the two doses of CRH to assess peak ACTH levels in plasma. These doses of CRH have been previously used in mice to test pituitary sensitivity to CRH [41]. There were 7–8 mice in all groups at the end of the study.

2.8. ACTH and corticosterone radioimmunoassays

Mice were decapitated, and the blood was collected in tubes containing sodium EDTA. The blood was centrifuged, and the plasma was frozen at −20 °C until assayed. Plasma ACTH was measured by radioimmunoassay using a specific antiserum generously donated by Dr. William Engeland (University of Minnesota) at a final dilution of 1:120,000 and [I^{125}] ACTH as tracer (Diasorin, Stillwater, MN). The ACTH antiserum cross-reacts 1% with $ACTH_{1-39}$, $ACTH_{1-18}$, and $ACTH_{1-24}$ but not with $ACTH_{1-16}$, B-endorphin, alpha-MSH, or B-lipotropin (<0.1%). Plasma was incubated for 48 h at 4 °C with antiserum and tracer, then precipitation serum (Peninsula Laboratories) was added and incubated for 2 h. Bound peptide was obtained by centrifugation at 5000 × *g* for 45 min. The minimum level of detection of the assay was 10 pg/ml. Plasma corticosterone was measured using a kit from ICN Biomedicals (Orangeburg, NY), and the minimum level of detection was 0.6 μg/dl.

2.9. In situ hybridization for CRH and AVP

Naive mice that had been singly housed for at least 1 week were decapitated in the morning, and brains were collected in OTC compound in brain molds and stored at −80 °C. Brains were sliced at 10 μm and stored until in situ hybridization analysis. Hybridization localization of CRH and AVP mRNAs using ^{33}P-labeled antisense cRNA probes was performed as previously described ([42]; CRH cDNA was obtained from Dr. K. Mayo, Northwestern University, and AVP cDNA was obtained from Dr. D. Richter, University of Hamburg). Material was analyzed by semiquantitative dark-field densitometric analysis of the relative levels of the mRNA of interest. Optical density measures of the sampled area were generated against a standard curve that best fit the relationship between the optical density of brain paste standards and the amount of radioactivity per unit area of standard using Macintosh-driven NIH Image software. The area of interest was first defined by Nissl-stained material and then redirected and aligned over the dark-field image. Optical density readings were taken at regularly spaced intervals through the largest extent of the PVN or through the rostral (−1.88 to −2.40 mm from bregma) part of the central nucleus of the amygdala, as defined by Nissl staining. Density readings were corrected for background and averaged for each animal. Background estimates were produced by optical density measurements over non-positively hybridized regions. There were eight WT and seven KO mice for analysis of CRH in the PVN, six WT and six KO mice for analysis of AVP in the PVN, and six WT and five KO mice for analysis of CRH in the central amygdala.

2.10. Statistical analyses

Data were expressed as mean ± S.E.M.. Significant differences were determined by analysis of variance (ANOVA) tests and were followed by Fisher's post hoc comparisons when appropriate. One-way ANOVAs were used for all experiments, except the pituitary sensitivity experiment and the LPS experiment in which two-way ANOVAs [Dose of drug × Genotype (WT or KO)] were used. For the fear-conditioning experiment, two-way ANOVAs were also used (Genotype × Time block) on each measure (training or conditioning, context test, and tone test). The level of significance was set at $P<.05$.

3. Results

3.1. General observations

The KO mice did not exhibit any overt physical or behavioral abnormalities based on visual examination and testing on the rotorod apparatus for balance. Furthermore, WT and KO mice did not exhibit any differences in body weight, food or water intake during the light and dark periods, adrenal or thymus weight, or weights of white (perirenal, subcutaneous, and mesenteric) or brown (intrascapular and perirenal) adipose tissue depots (data not shown).

3.2. Behavior in the elevated plus maze

Measures of anxiety in naïve WT and KO mice were assessed under basal conditions in the elevated plus maze

for 10 min (Table 1). KO animals traveled significantly more distance in the open arms [$F(1,12) = 9.09$; $P \leq .01$] and spent more time in the open arm [$F(1,12) = 8.89$; $P \leq .01$] compared to WT mice. Accordingly, both time and distance traveled in the open arm as a percentage of total time and total distance were significantly higher in KO mice than WT mice [$F(1,12) = 10.84$; $P \leq .01$ and $F(1,12) = 6.31$; $P \leq .03$, respectively]. Entries into the open arm and percent of entries into the open arm as a function of total entries were not significantly different between groups. Time spent in the closed arm and percent of total time in the closed arm was significantly higher in WT mice than in KO mice [$F(1,12) = 7.72$; $P \leq .02$ and $F(1,12) = 18.59$; $P \leq .01$, respectively]. Distance traveled or entries into the closed arm or distance and entries in the closed arm as a percent of the total were not significantly different between WT and KO mice. Total (closed and open arms) activity was not different between WT and KO mice (WT: 1127 ± 53 cm; KO: 1287 ± 56 cm), indicating that potential differences in overall activity level did not contribute to the differences found in the open arms. In summary, KO mice exhibited greater distance traveled and time spent in the open arm (both absolute and as a percentage of total) as well as decreased time spent in the closed arm (absolute and percent of total) but no difference in total distance traveled. Therefore, KO mice exhibited indices of decreased anxiety compared to WT mice in the elevated plus maze.

3.3. Behavior in the open field

Naïve WT and KO mice were placed in the open field for 30 min, and their locomotor activity was recorded (Table 1). No differences were found between WT and KO mice in total distance traveled, or in distance in, time spent in, or entries into the outer, middle, or inner zones of the open field under these conditions. Furthermore, there were no differences between WT and KO mice in any of these variables in the first, second, or third 10-min bin. Therefore, WT and KO mice did not differ in their behavior in the open field.

Table 1
Behavior in the elevated plus maze and open field

	WT	KO
Elevated plus maze		
Distance traveled in open arm (cm)	82.1 ± 8.4	138.3 ± 18 *
Total distance traveled in open arm (%)	7.2 ± 0.6	10.8 ± 1.4 *
Time in open arm (s)	64 ± 6	113.7 ± 16.6 *
Total time in open arm (%)	10.1 ± 0.9	18.9 ± 2.8 *
Number of entries into open arm	5.7 ± 0.9	7.8 ± 1.3
Total entries into open arm (%)	11.9 ± 1.6	14.8 ± 2.1
Distance traveled in closed arm (cm)	848 ± 40	933 ± 49
Total distance traveled in closed arm (%)	75.4 ± 1.7	72.4 ± 1.8
Time in closed arm (s)	452.0 ± 13.5	395.1 ± 15.5 *
Total time in closed arm (%)	77.3 ± 1.3	65.8 ± 2.6 *
Number of entries into closed arm	18 ± 0.8	19 ± 1.8
Total entries into closed arm (%)	39 ± 1.7	36 ± 2.1
Open field		
Distance traveled in outer zone (cm)	7317 ± 1496	6335 ± 445
Distance traveled in middle zone (cm)	2033 ± 153	2273 ± 230
Distance traveled in inner zone (cm)	857 ± 70	904 ± 105

Male WT and 5-HT-3A receptor KO mice were tested in the elevated plus maze for 10 min and in the open field for 30 min.

* Denotes significant difference between groups, $P < 0.05$.

Table 2
Behavior in the light–dark test of anxiety was examined under basal conditions in male C57 and 5-HT-3A receptor KO mice

	C57	KO
Latency to enter light compartment (s)	26.7 ± 4.5	44.6 ± 13.9
Number of transitions	22.5 ± 4.3	22.4 ± 2.7
Total time in light compartment (s)	143.1 ± 19.1	186.6 ± 25.9
Total time in dark compartment (s)	456.9 ± 19.1	413.4 ± 26

Mice were placed in the light–dark box for a total of 10 min. There were eight mice per group in this experiment.

3.4. Behavior in the light–dark test

We found no significant differences between KO and C57 mice in the latency to enter the light compartment and the number of transitions from the dark to the light compartment (Table 2). Furthermore, no significant differences were observed between KO and C57 mice in the total amount of time spent in either the dark or the light compartment.

3.5. Pavlovian fear conditioning

There was a significant enhancement in conditional freezing in the KO mice. On the conditioning day, KO mice froze significantly more than WT mice after footshock delivery [Fig. 1a; main effect of genotype; $F(1, 13) = 5.7$, $P < .05$]. There was a tendency for enhanced freezing by KO mice to the context (Fig. 1b), but this was not statistically significant. Significant enhanced conditional freezing was also evident during the tone extinction tests conducted 48 h after conditioning [Fig. 1c; Genotype × Test minute interaction; $F(7, 91) = 4.1$, $P < .001$] and was manifested as a pronounced enhancement of conditional freezing during the first minute of the tone. Enhanced conditional freezing in the KO mice was not likely due to altered activity levels. As described above, there were no differences between KO and WT mice in open field behavior. Moreover, there were no differences between these groups in activity levels prior to footshock on the conditioning day [cage crossovers (mean ± S.E.M.): WT, 9.4 ± 0.8; KO, 11.0 ± 0.7]. Collectively, these data suggest that KO mice exhibited enhanced fear conditioning, rather than a nonspecific enhancement in freezing behavior brought about by lower locomotor activity levels, for example.

3.6. HPA responses to restraint and LPS

We examined HPA responses to two distinct acute stressors: a 30 min period of restraint or injection with

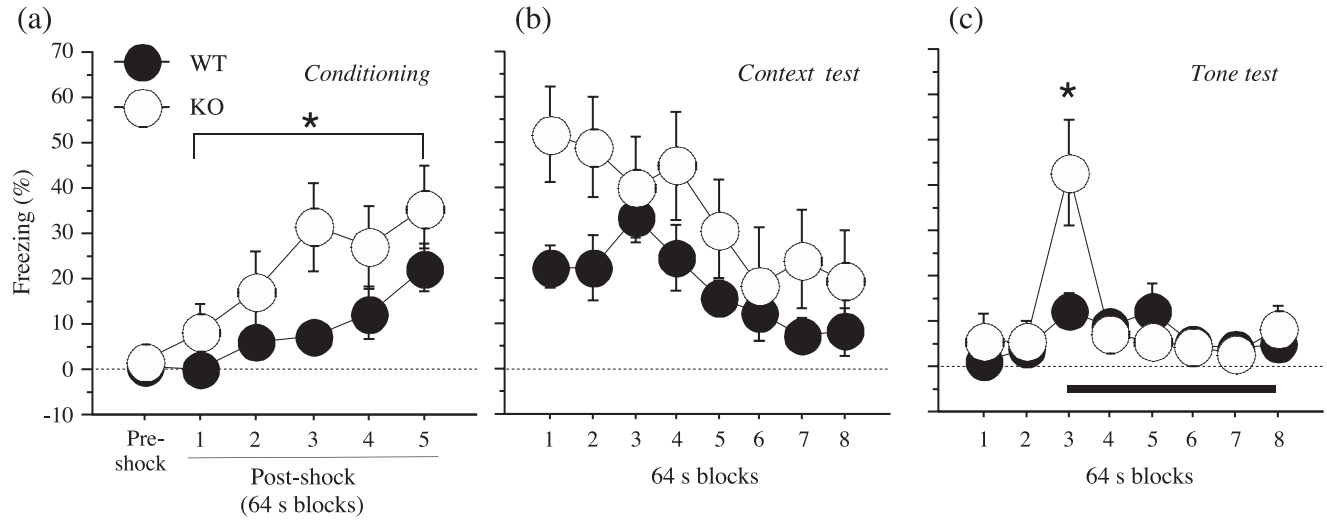

Fig. 1. Mean (± S.E.M.) percentage of conditional freezing behavior in WT and KO mice. (a) Freezing on the conditioning day during the preshock period (an average of three 64-s blocks) and after each of the conditioning (tone–footshock) trials (five 64-s blocks). (b) Freezing during the context extinction test 24 h after fear conditioning; freezing was assessed in the conditioning context. (c) Freezing during the tone extinction test 48 h after fear conditioning; freezing to the tone conditional stimulus (CS) was assessed in a novel context. Onset of the tone CS occurred 128 s after the rats were placed in the novel chambers; the duration of the tone CS is indicated by the black bar. KO mice exhibited a significant enhancement in conditional freezing on the conditioning day (a) and during the tone extinction test (c). $*P \leq .05$.

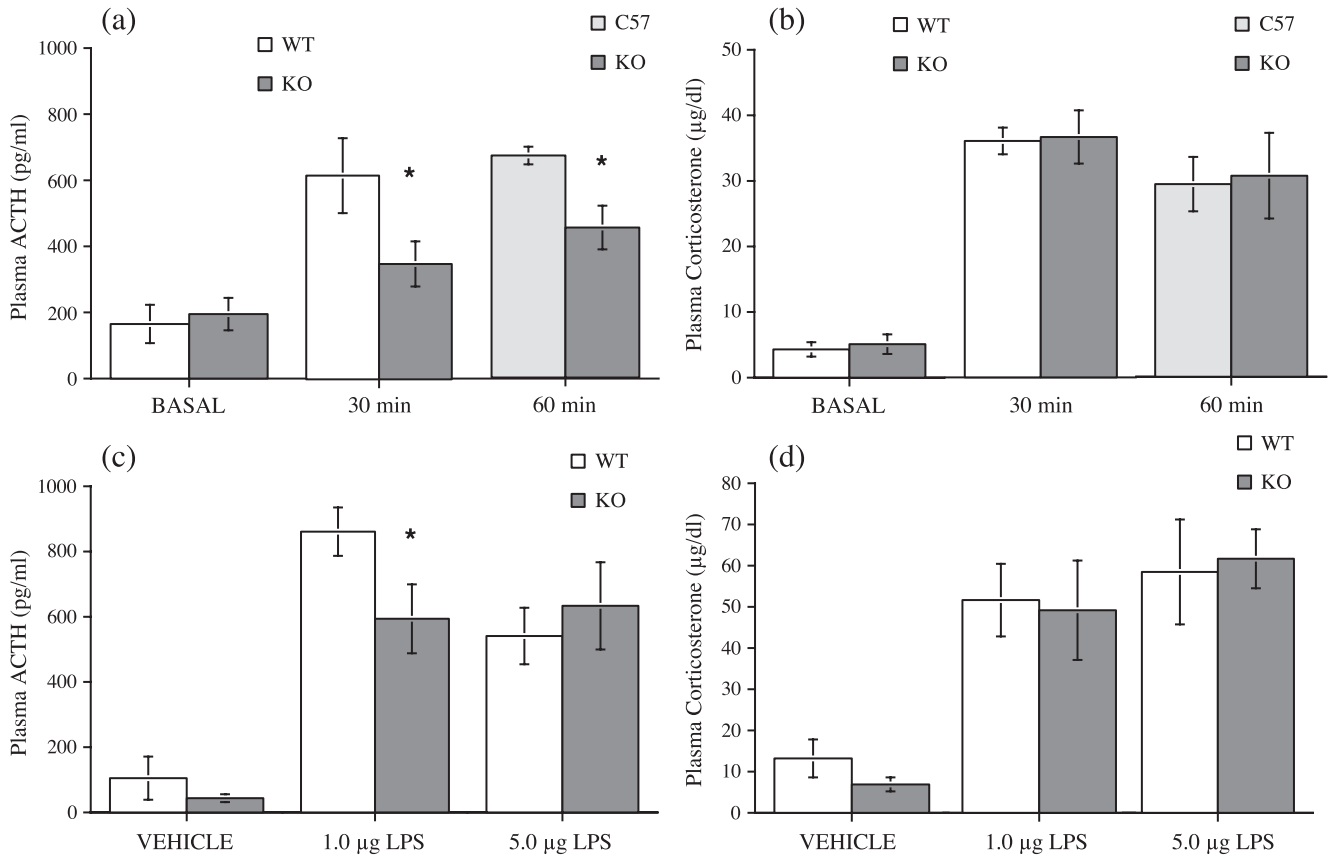

Fig. 2. Plasma ACTH (a) and corticosterone levels (b) are shown in WT and 5-HT-3A receptor KO mice under basal conditions and at the end of a 30-min period of restraint. Plasma ACTH (a) and corticosterone (b) at recovery from restraint (60 min following onset of 30-min restraint) are also shown for 5-HT-3A KO mice and control C57mice. Plasma ACTH (c) and corticosterone (d) in WT and 5-HT-3A mice following injection of vehicle, 1.0 µg, or 5.0 µg LPS ip 2h prior to sampling. KO mice exhibited lower ACTH responses to both restraint and 1.0 µg ip LPS. $*P \leq .05$.

Table 3
ACTH and corticosterone responses to intraperitoneal injection of vehicle, 10 μg/kg CRH, or 100 μg/kg of CRH

	Vehicle	10 μg/kg	100 μg/kg
ACTH (pg/ml)			
WT	327 ± 123	376 ± 116	707 ± 203
KO	227 ± 71	362 ± 107	604 ± 174
Corticosterone (μg/dl)			
WT	4.0 ± 1.5	28.6 ± 3.3	36.7 ± 3.7
KO	4.4 ± 1.5	23.7 ± 3.5	31.7 ± 1.7

Blood was sampled at 40 min following injection of the 10-μg/kg dose and at 60 min following injection of the 100-μg/kg dose. Vehicle injected animals were sampled at either 40 or 60 min postinjection, and data from these time points were pooled for analysis. No significant differences between WT and KO mice were observed.

LPS. There was no difference in basal (0 min) ACTH (Fig. 2a) or corticosterone (Fig. 2b) levels between the groups prior to onset of restraint. ACTH responses to restraint were significantly lower in KO mice than in WT mice at the end of 30-min restraint [$F(1,27) = 4.09$; $P \leq .05$; Fig. 2a] and at recovery from restraint (60 min following onset of 30-min restraint) [$F(1,21) = 10.59$; $P \leq .01$; Fig. 2a]. However, there were no significant group differences in plasma corticosterone responses at either 30 min or 60 min following onset of restraint (Fig. 2b).

There were significant Dose [$F(1,34) = 36.8$; $P \leq .01$] and Dose × Genotype [$F(1,34) = 3.6$; $P \leq .03$] effects for plasma ACTH following injection of LPS. Post hoc tests indicated that both the 1- and 5-μg doses of LPS elevated ACTH above that found following vehicle injection. Furthermore, KO mice displayed significantly lower ACTH than did WT mice following injection of the 1-μg dose of LPS.

There was also a significant Dose effect [$F(1,34) = 36.8$; $P \leq .01$] with plasma corticosterone (Fig. 2d). Post hoc tests indicated that both the 1- and 5-μg doses of LPS elevated corticosterone compared to vehicle injection. There were no significant Genotype or Interaction effects with plasma corticosterone, indicating that WT and KO mice did not differ in their corticosterone response to LPS, similar to the findings with corticosterone in response to restraint.

3.7. Pituitary sensitivity to CRH

The 5-HT-3 receptor has been identified in the anterior pituitary [10]. The absence of the 5-HT-3 receptor in the pituitary of KO mice may alter HPA responses to stress and contribute to the dampened ACTH responses to restraint and LPS by decreasing sensitivity of pituitary corticotrophs to the stimulatory effects of CRH. Therefore, we examined pituitary sensitivity to peripherally administered CRH (10 μg/kg CRH or 100 μg/kg CRH ip) in KO and WT mice (Table 3). For ACTH, we observed significant Drug effects [$F(2,40) = 3.95$, $P \leq .03$] with the Vehicle group and the 10-μg/kg group being significantly lower than the 100-μg/kg injected group. Similarly, for corticosterone, we also observed a significant Drug effect [$F(2,42) = 64.3$, $P \leq .01$]. Post hoc tests indicated both WT and KO mice injected with either the 10-μg/kg or 100-μg/kg dose of CRH exhibited higher corticosterone levels than their vehicle-injected counterparts. However, there were no significant Genotype or Genotype × Drug effects, indicating that WT and KO mice did not differ from one another in terms of ACTH or corticosterone responses to CRH injection. Therefore, pituitary sensitivity to CRH was not different between WT and KO mice.

3.8. CRH and AVP mRNA

We determined the expression of the ACTH secretagogues, CRH and AVP, in the PVN because of the differences in ACTH responses to acute stress between WT and KO mice. In addition, we examined CRH mRNA in the central nucleus of the amygdala, often associated with changes in anxiety-related behaviors [37,45]. While basal CRH mRNA in the PVN was not different between KO and WT mice (Fig. 3a), AVP mRNA in the PVN was significantly lower in KO mice compared to WT mice [$F(1,11) = 13.5$, $P \leq .004$; Fig. 3b]. Furthermore, CRH mRNA was significantly increased in the central nucleus of the amyg-

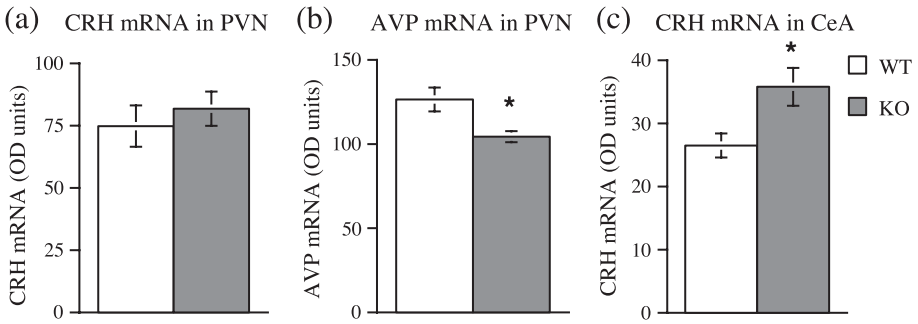

Fig. 3. In situ hybridization for CRH (a) and AVP (b) mRNA in the PVN of WT and 5-HT-3A KO mice. CRH mRNA (c) in the central nucleus of the amygdala of WT and KO mice. * $P \leq .05$; KO mice significantly different from WT mice.

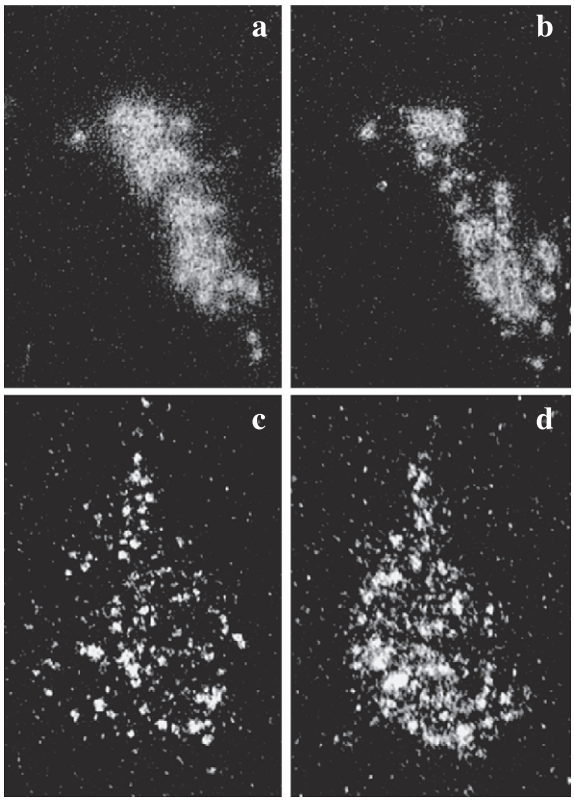

Fig. 4. Representative photomicrographs showing lower AVP mRNA in the PVN of KO (b) mice compared to WT (a) mice and increased CRH mRNA in the central nucleus of the amygdala in KO (d) compared to WT (c) mice.

dala in KO mice compared to WT mice [$F(1,10) = 8.5$; $P \leq .01$; Fig. 3c]. Representative photomicrographs are shown in Fig. 4.

4. Discussion

We found that 5-HT-3A KO mice exhibited increased distance traveled and time spent in the open arm of the elevated plus maze compared to WT mice (both in absolute terms as well as a percentage of total time and distance traveled). Such increases in exploration in the open arm of the plus maze are generally interpreted as reflecting decreased measures of anxiety [27]. This finding is consistent with previous investigations that have found increased exploration of the open arm in the elevated plus maze following peripheral administration of 5-HT-3 antagonists such as ondansetron or tropisetron [12,43,44]. In contrast to the elevated plus maze, we found no differences between WT and KO mice in their behavior in either the open field or light–dark box test. Such seemingly discrepant results in different tests of anxiety following manipulations of the 5-HT-3 receptor system, such as those found here in the open field, light–dark test, and elevated plus under basal conditions, have been previously reported (see Introduction). Inasmuch as our examination of the role of the 5-HT-3A receptor in tests of anxiety used a single species and did not require injection of antagonists, we suggest that discrepancies in the present behavioral results are not due to type or dose of antagonist used or the species studied.

One interpretation of the present results is that the 5-HT-3A receptor does not have a major role in mediating anxiety-related behavior. Although it is preferable to test anxiety using several different tests [45], it is difficult to interpret discrepancies in behavior in different tests. We chose the elevated plus maze, open field, and light–dark tests because such tests are based on the natural neophobia of rodents [46]. However, the elevated plus test is considered a more sensitive test of anxiety [27], and behavior in one test does not always predict behavior in the other [47]. A recent study using the 5-HT-3A KO mice that we used [48] confirmed our findings of anxiolytic behavior in the plus maze but also reported anxiolytic behavior in the light–dark box (we did not). This discrepancy in the light–dark box test is likely due to important methodological differences since as Kelley et al. [48] did not state their housing conditions (we singly housed mice), acclimated the mice to the testing room for 60 min prior to testing (we did not acclimate), and used a different test duration. Although the possibility that the 5-HT-3A receptor plays different roles in different tests of anxiety still remains, on the whole, our results suggest that the 5-HT-3A receptor does not play a major role in the regulation of anxiety-related behaviors.

We observed increased CRH mRNA in the central nucleus of the amygdala in KO mice compared to WT mice. These data suggest that, normally, activation of the 5-HT-3A receptor inhibits expression of CRH in the central nucleus of the amygdala. Elevated levels of CRH mRNA in the central nucleus of the amygdala tend to be correlated or associated with increased anxiety in the elevated plus maze [49,50]. However, the increased CRH mRNA we observed in the central amygdala of KO mice was not associated with increased measures of anxiety in the plus maze, open field, or light–dark tests. One explanation for these findings is that CRH produced in cell bodies of the central amygdala does not play an important role in mediating anxiety-related behavior or that the increased mRNA does not produce elevated levels of the peptide. Alternatively, the 5-HT-3A receptor interaction with CRH in the central amygdala may play an indirect role in regulating anxiety-related behaviors. Since as there are no reports of 5-HT-3 receptor localization in the central nucleus of the amygdala (see Refs. [5–8]), the elevated CRH population in the central nucleus of the amygdala in mice lacking the 5-HT-3A receptor is likely secondary to alterations in some other structure and/or peptide such as cholecystokinin, a peptide known to be involved in anxiety [51,52].

In contrast to the discrepant data with the open field and light–dark tests' comparison to the elevated plus maze, KO mice exhibited a reliable behavioral phenotype in Pavlovian fear conditioning. KO mice exhibited a significant enhancement of conditional freezing on the conditioning day, during

the postshock periods and during the tone extinction tests conducted 48 h after conditioning. There was also a trend for enhanced conditional freezing to the conditioning context. Enhanced conditional freezing could not be accounted for by a decrease in locomotor activity in the KO mice and is not likely due to increased sensitivity to shock since as Zeitz et al. [26] have shown that 5-HT-3A KO mice do not differ from WT mice in responsivity to a variety of acute, physiologically relevant nociceptive stimuli. Therefore, the enhancement of fear conditioning in the KOs suggests that deletion of the 5HT-3A receptor enhances fear memory formation, possibly by affecting CS–US association formation in the lateral amygdala. One mechanism that could account for this pattern of results is the removal of serotonergic inhibition of lateral amygdala neurons that normally blunts glutamatergic transmission in the amygdala [53]. In support of this possibility, 5-HT-3 receptors have been reported in GABA-ergic neurons in the lateral amygdala [54]. Alternatively, the upregulated CRH mRNA in the central nucleus could mediate the enhanced fear conditioning since as the central nucleus has a critical role in fear conditioning [55]. Lang et al. [56], for example, have suggested that the central amygdala has a selective role in fear conditioning and a lesser role in mediating anxiety-related behavior but that the bed nucleus of the stria terminalis has a greater role in mediating anxiety- vs. fear-related behavior. Whether this particular neuroanatomical distinction could explain the dissociation between the anxiolytic behavior of KO mice in the elevated plus maze but enhanced fearful behavior in the Pavlovian fear-conditioning task remains to be determined. In addition, this dissociation may also reflect a differential role for the 5-HT-3A receptor in mediating conditioned vs. unconditioned behaviors.

Absence of the 5-HT-3A receptor produced marked effects on stress-induced, but not basal, HPA activity. 5-HT-3A receptor KO mice exhibited lower ACTH responses in response to acute restraint as well as recovery from restraint (the 60-min time point). This decreased stress responsivity was not limited to restraint since as lower ACTH responses to the low dose of LPS were also observed in KO compared to WT mice (Fig. 2). No differences were observed with the high dose of LPS. It is possible that the high dose of LPS produced a higher HPA response than the lower dose at earlier times than we examined, and this could be one explanation for why the higher dose did not produce elevated HPA responses at 2 h compared to the low dose. Alternatively, the higher dose may have produced a smaller response than the lower dose but for a longer period of time (beyond the time points we studied). Unlike ACTH, corticosterone levels following acute restraint, recovery from restraint, or either dose of LPS were not different between KO and WT mice. One explanation for the different effects of 5-HT-3A receptor deletion on ACTH and corticosterone is based on the fact that the adrenal integrates the ACTH signal over time to release corticosterone [57]. Therefore, examination of corticosterone levels at later time points could demonstrate lower corticosterone responses to restraint or LPS in KO mice, in accordance with the ACTH data. However, this explanation is unlikely since as we found lower ACTH but not corticosterone responses at 60 min following onset of restraint in KO compared to WT mice. An alternative explanation for the discrepancy between the ACTH and corticosterone data in KO mice is that the adrenal glands of KO mice are more sensitive to ACTH than those of WT mice. Further work will be required to determine if the 5-HT-3A receptor regulates adrenal sensitivity to ACTH.

A previous study had found that intracerebroventricular administration of a 5-HT-3A receptor antagonist blocked corticosterone responses to acoustic stress but not to footshock or restraint [58]. However, this study was conducted in rats, ACTH was not examined, and we found a similar lack of an effect of 5-HT-3A KO on plasma corticosterone responses to restraint. Therefore, the present findings of dampened ACTH responses to acute stressors as diverse as restraint and LPS in KO mice suggest that the 5-HT-3A receptor normally has a more general stimulatory effect on acute stress-induced HPA activity than previously indicated.

We examined potential mechanisms by which the 5-HT-3A receptor might regulate HPA responses to acute stress. Inasmuch as this receptor has been localized in the pituitary, it is possible that the lower ACTH responses to acute stress in KO mice were due to lack of the 5-HT-3A receptor in the pituitary. Therefore, we studied pituitary sensitivity to CRH in WT vs. KO mice by injecting three doses of CRH intraperitoneally (0, 10, and 100 μg/kg body weight). ACTH levels were higher after the 100-μg/kg dose compared to the 0- and 10-μg/kg doses, but this increase was not significant. It is likely that ACTH peaked earlier in response to 10- and 100-μg/kg doses of CRH, and we did not observe these elevations because we sampled at 40 and 60 min, respectively. Consistent with this likely earlier elevation of ACTH, we found that corticosterone secretion was significantly higher in both WT and KO animals after 10 or 100 μg/kg CRH compared to vehicle injections and corticosterone responses to the 100-μg/kg dose were higher than responses to the 10-μg/kg dose. Importantly, there were no differences between WT and KO mice at any dose, indicating that pituitary sensitivity to CRH was similar in WT and KO mice. These data suggest that the lower ACTH responses to restraint or LPS in KO mice are not due to differences in pituitary sensitivity to CRH but are instead centrally mediated.

We observed lower AVP, but not CRH, mRNA in the PVN of KO mice compared to WT mice. AVP potentiates CRH effects on ACTH release and is the driving force behind acute stress-induced ACTH release [24]. Therefore, it is likely that the diminished ACTH response to acute restraint or LPS in KO mice is related to decreased AVP mRNA in the PVN. However, the possibility remains that this decreased AVP mRNA in the PVN is due to changes in

magnocellular and not parvocellular AVP or that it does not result in decreased synthesis and availability of the peptide itself. The mechanism by which AVP mRNA in the PVN is decreased by the absence of the 5-HT-3A receptor is not clear. Since as there are no reports of the 5-HT-3A receptor being localized in the PVN, AVP mRNA in the PVN may be regulated by afferents to the PVN that contain the 5-HT-3A receptor. The PVN receives strong afferent input from brainstem regions such as the parabrachial nuclei and nucleus tractus solitarius, in which the 5-HT-3 receptor has been localized [6,8]. Whether CRH in the central amygdala can regulate AVP in the PVN is not known. In sum, these data indicate that activation of the 5-HT-3A receptor normally stimulates acute stress-induced HPA activity, possibly through AVP in the PVN.

Together, the present results suggest that the 5-HT-3A receptor has a more substantial role in regulation of acute stress-induced HPA activity than previously envisioned and that it regulates CRH mRNA in a site-specific manner (in the central amygdala but not PVN). Finally, the ability of the 5-HT-3A receptor to differentially regulate anxiety- and fear-related behaviors may reflect its distinct role in mediation of conditioned vs. unconditioned behaviors and/or may depend on its interactions with CRH.

Acknowledgements

We are indebted to Alan Chu and Liza Soriano for the in situ hybridization analyses and Ki Goosens and Jennifer A. Hobin for assistance with the fear-conditioning procedure. We are grateful to Dr. Audrey Seasholtz for help in genotyping some of the mice used in these studies. This research was funded by NIDDK 28172 to MFD, NIMH to DJ, and NARSAD Young Investigator Awards to SB.

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