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DISTINCT BEHAVIORAL PHENOTYPES IN MALE MICE LACKING THE THYROID HORMONE RECEPTOR $\alpha 1$ OR β ISOFORMS

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Abstract

Thyroid hormones influence both neuronal development and anxiety via the thyroid hormone receptors (TRs). The TRs are encoded by two different genes, TR α and TR β . The loss of TR $\alpha 1$ is implicated in increased anxiety in males, possibly via a hippocampal increase in GABAergic activity. We compared both social behaviors and two underlying and related non-social behaviors, state anxiety and responses to acoustic and tactile startle in the gonadally intact TR $\alpha 1$ knockout ($\alpha 1$ KO) and TR β (β KO) male mice to their wild-type counterparts. For the first time, we show an opposing effect of the two TR isoforms, TR $\alpha 1$ and TR β , in the regulation of state anxiety, with $\alpha 1$ knockout animals ($\alpha 1$ KO) showing higher levels of anxiety and β KO males showing less anxiety compared to respective wild-type mice. At odds with the increased anxiety in non-social environments, $\alpha 1$ KO males also show lower levels of responsiveness to acoustic and tactile startle stimuli. Consistent with the data that T4 is inhibitory to lordosis in female mice, we show subtly increased sex behavior in $\alpha 1$ KO male mice. These behaviors support the idea that TR $\alpha 1$ could be inhibitory to ER α driven transcription that ultimately impacts ER α driven behaviors such as lordosis. The behavioral phenotypes point to novel roles for the TRs, particularly in non-social behaviors such as state anxiety and startle.

Keywords

thyroid hormone receptor; sexual behavior; arousal; anxiety

Introduction

Thyroid hormone receptors (TRs) are nuclear receptors that play an important role in neuronal development (Bernal et al., 2003; Nunez et al., 2008) and lipid metabolism (Davis) using both ligand-independent and ligand-dependent signaling (Bernal et al., 2003; Yen,

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2001). For genes whose mRNA is increased by tri-iodothyronine (T3), the TRs bind to thyroid hormone response elements (TREs) and repress basal transcription while unliganded; on binding ligand, they transactivate from the TREs (Chin, 1991). Two different genes, α and β , give rise to differentially spliced mRNAs that, in turn, yield four isoforms, the $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$ in mammals (Lazar, 1993). Despite considerable similarities in ligand and DNA binding, the differences in tissue distribution (Keijzer et al., 2007) predict differences in biological functions e.g., the TR $\beta 1$ isoform in ear hair cells is critical for auditory function (Rusch et al., 1998) (Abel et al., 1999), the TR $\beta 2$ in the anterior hypothalamus is needed for the negative regulation of thyrotropin stimulating hormone (TSH) by T3 (Forrest et al., 1996) (Abel et al., 1999) and the TR $\alpha 1$ in the myocardium (Stoykov et al., 2006) maintains basal heart rate and regulates increased heart rate on T3 stimulation (Wikstrom et al., 1998). TR knockout mice (TRKO), which lack either $\alpha 1$ ($\alpha 1$ KO) or β (β KO) isoforms, have, therefore, been used as genetic tools to investigate the comparative contribution of isoforms to the various physiological functions attributed to thyroid hormone (TH) (Cheng et al.; Forrest and Vennstrom, 2000; O'Shea and Williams, 2002).

Previously, molecular data have implicated the TR, specifically TR $\alpha 1$, in the inhibition of estrogen-dependent gene expression (Vasudevan et al., 2002). TH administration inhibited lordosis behavior in estrogen-primed female mice (Morgan et al., 2000) and rats (Dellovade et al., 1996). Surprisingly, while the $\alpha 1$ KO females showed poorer lordosis than wildtype ($\alpha 1$ WT) females and β KO females showed greater lordosis than wildtype females (β WT) (Dellovade et al., 2000), the effect of TR deletion on social behaviors in the male mouse is unknown. Moreover, the contribution of these isoforms to related underlying states such as generalized arousal or trait anxiety that are important for goal-directed social behaviors (Churchland and Winkielman, 2012) has not been examined. Several lines of evidence, including the distribution of both TR $\alpha 1$ (Wallis et al., 2010) and TR β (Carlson et al., 1994; Ercan-Fang et al., 1996) in the amygdala, the BNST, and hippocampus and the behavior of isoform specific knockout and transgenic mice, point to a role of TRs in the regulation of anxiety. In mice that lack TR $\alpha 1$, an increase in anxiety was seen in the open field test (Guadano-Ferraz et al., 2003). In a transgenic male mouse that possesses a dominant negative TR $\alpha 1$ with a ten fold lower affinity for T3 and hence is presumably unliganded, extreme anxiety was seen in the open field, the elevated plus maze (Venero et al., 2005), and in the light dark transition tests (Pilhatsch et al., 2010). These findings suggest that a balance of unliganded TR and liganded TR signaling is required to maintain state anxiety. Administration of T3 to these transgenic animals during adulthood alleviated the anxiety in all these tests (Pilhatsch et al., 2010; Venero et al., 2005), implying that ongoing T3 action via TR $\alpha 1$ during testing is critical in mediating anxiolytic behavior and that these behaviors are not developmentally organized by T3. In this study, we compared adult gonadally intact $\alpha 1$ KO and β KO males to their wild-type counterparts in tests that measure socially motivated behaviors and arousal (Experiment I) and on measures that denote anxiety (Experiment II). We found subtle increases in sex behavior in the $\alpha 1$ KO, but not β KO, males, although the $\alpha 1$ KO males showed decreased acoustic and tactile startle responses and hence decreased arousal. In addition, the $\alpha 1$ KO males were more anxious while the β KO males were less anxious compared to the corresponding wild-type mice. Hence, the decrease seen in arousal by the deletion of the TR $\alpha 1$ isoform may be independent of the increase seen in socially motivated behaviors such as male sex behavior.

Materials and Methods

Animals

Gonadally intact male TR $\alpha 1$ ($\alpha 1$ KO) and TR β (β KO) knockout mice and their corresponding wild-type ($\alpha 1$ WT and β WT) control mice (12–18 weeks of age) were used.

They were obtained from the breeding colony at the Rockefeller University maintained by mating of heterozygous mice for each gene. Details of the $\alpha 1$ KO mice (background of the 129Hsd/B6CBA/Balb/C) (Wikstrom et al., 1998) and β KO mice (with mixed background of the 129/Sv and C57BL/6J mouse strains) have been described previously (Forrest et al., 1996). Animals were individually housed in plastic cages (30 cm $\times$ 20 cm $\times$ 13 cm) throughout the studies starting 7–14 days prior to the first behavioral test. They were given food and water *ad libitum* and maintained on a reversed 12hr light: 12hr dark cycle (lights off at 11 am) with constant temperature (22°C).

Experimental Designs and General Procedure

Two different sets of $\alpha 1$ KO and β KO animals were compared to their $\alpha 1$ WT and β WT counterparts in two different series of behavioral tests, designated as Experiment I and II. All testing except the step down passive avoidance tests (see below) took place in a sound attenuated room adjacent to the colony room, starting 2–3 hr after lights off, under red light. All animals were acclimatized to the behavioral testing room for 2 hours before testing. Testing order was counterbalanced between genotypes on all tests and animals were returned to the colony room before the next animal was tested. All acquisition, handling, and other animal procedures were done the NIH and Rockefeller University IACUC guidelines.

Experiment I—Experiment I was designed to evaluate locomotor activity levels, social behaviors such as aggression and sexual behaviors, as well as the startle response to acoustic and tactile stimuli. A total of 54 mice ($\alpha 1$ WT $n=12$; $\alpha 1$ KO $n=11$; β WT $n=13$; β KO $n=18$) were tested in the order shown in Figure 1A: open field tests on three consecutive days, followed a week later by three consecutive days of aggression tests. A week after the last aggression test, mice were tested for sexual behavior three times, each of which was a week apart. A week after the last sexual behavior test, baseline, acoustic and tactile startle responses were examined. Ten days after the last behavioral test, mice were sacrificed, perfused by cardiac puncture and blood samples were collected for the determination of serum T4 levels.

Experiment II—A set of behavioral tests was performed to evaluate anxiety levels and passive avoidance learning. We used a total of 43 mice ($\alpha 1$ WT $n=8$; $\alpha 1$ KO $n=15$; β WT $n=10$; β KO $n=10$). All animals were tested, as shown in Figure 1B, once for open field activity, once for elevated plus maze (EPM) activity, and twice for light-dark transitions (LDT) in two consecutive days. Four days after the last light-dark transition test, a step down passive avoidance (SDA) test took place in three trials: a training trial on the first day and test trials 24 hours and 7 days after training.

Open field behavior tests

Open field behavior was tested for 5 min in a transparent acrylic chamber (40.5 $\times$ 40.5, 30 cm high) with infrared beams for the automatic recording of horizontal activity. The total moving distance (total distance), moving distance in the center area (center distance), and time spent in the center area (center time) were recorded by the Digiscan Analyzer and Digiscan software (Accuscan Instruments, Columbus, OH) for each mouse. The center area was defined as the area one inch away from the walls of the chamber. In Experiment I, mice were tested on three consecutive days and the mouse was placed gently in the left corner of the chamber with his head facing the corner. In experiment II, the mice were tested only once and the mouse was placed in the center of the chamber. The paradigms chosen in Experiment I and II were different because we aimed to investigate general locomotor activity and desensitization in Experiment I versus generalized anxiety in a novel environment in Experiment II. The data in Experiment I were analyzed using a two way ANOVA for repeated measures with genotype as the between subjects factor and the days of

testing (day 1, 2 and 3) as the within subject variable (SPSS, IBM Corp, NY). The data obtained in a single day in Experiment II were analyzed using t-tests for genotype differences (Graph Pad Prism, CA).

Aggressive behavior tests

Aggressive behavior was assessed on three consecutive days using a resident-intruder paradigm as described previously (Ogawa et al., 1996). Briefly, the male resident mice were tested in their home cage against weight-matched, group-housed (4–5 mice/cage), olfactory bulbectomized male intruders (C57BL/6 mice heterozygous for the ER β locus) for 15 min. The resident mouse was tested against different intruder mice on each of the three tests. All tests were videotaped and male typical aggressive behavior acts (Ogawa et al., 1996) scored by observers blind to the genotype. Data obtained were analyzed using a two way ANOVA for repeated measures with genotype as the between subject factor and the days of testing (Days 1, 2 and 3) as the within subject variable (SPSS, IBM Corporation).

Sexual behavior tests

Male mice were tested for sexual behavior for 30 min on each of three tests a week apart. Each male was tested on all three tests with a different ovariectomized female (C57BL/6 mice heterozygous for the ER β locus) in the male's home cage. All females were primed with two s.c. injections of 10 μ g estradiol benzoate in sesame oil given 24 hours apart, starting 48 hours before testing, and one s.c. injection of 500 μ g of progesterone in sesame oil 5–6 hours before testing, to ensure high sexual receptivity. All tests were videotaped for further analysis by observers blind to the genotype. For each male, the numbers and latencies of mounts, intromissions and ejaculations were noted. The latency was recorded as 1800 seconds when the male mouse did not show the behavior. The data were analyzed using a two way ANOVA for repeated measures with genotype as the between subjects factor and the days of testing (day 1, 2 and 3) as the within subject variable (SPSS, IBM Corp, NY).

Acoustic and tactile startle response tests

Mice were tested for responses to acoustic and tactile stimuli in one of two soundproof chambers (San Diego Instruments) amidst background white noise. Chamber assignment was counter balanced between the genotypes. At the center of each chamber, a transparent acrylic cylinder (4 cm diameter) was placed on a frame equipped with a motion sensor that detects and transduces the mouse's movement to voltage. Response amplitude (mV), recorded automatically using the SR-LAB software, was defined as the maximum voltage changes occurring during the 100 ms after the presentation of acoustic or tactile stimuli. These tests were carried out over a period of five days; the first day was used to record baseline data; on the second day we measured responses to acoustic stimuli while on the fifth day, 3 days after the acoustic startle test, we measured responses to tactile stimuli (See Fig 1A for timeline). Both chambers were calibrated for equivalent stimulus intensities and response detection sensitivities prior to each test. At the beginning of each test on each of the three testing days, mice were gently placed in the cylinder and acclimatized for 4 min. On the first day, baseline response amplitude in the absence of any stimuli (i.e., random body movement inside the cylinder) was determined for each mouse by measuring response amplitude in 60 trials with 15 sec inter-trial intervals. The next day, mice were tested for startle responses to three varying levels of acoustic stimuli for a total of 60 trials (20 trials of each of the three different decibel levels, presented in a pseudo-random order) with a 15-second inter-trial interval. In each trial, a 0, 95 or 115-decibel white burst noise of 40 ms duration was presented. On the third test, startle responses to tactile stimuli were measured by presenting 20 air puffs of 4 psi (40 ms duration) from a compressed air cylinder toward the neck of the animal with a 15-second intertrial interval. The data from the acoustic stimuli

tests were analyzed by a two way ANOVA for repeated measures with genotype as the between subjects factor and the two stimulus levels (95 and 115db) as the within subject variable (SPSS, IBM Corp, NY). The data from the tactile startle response tests were averaged across the twenty trials for each mouse and analyzed using t-tests for genotype differences (Graph Pad Prism, CA).

Elevated plus maze tests (EPM)

Mice were tested for their anxiety behaviors in a plus maze, that was elevated 40 cm above the floor. The apparatus consisted of a 5×5 cm center platform from which extended four acrylic arms (30cm long $\times$ 5cm wide) in a cross formation, with two opposing arms enclosed by opaque acrylic side walls (15cm high) and two arms left open. Testing took place under a red light (50-Watt) suspended 180 cm above the maze. The mouse was gently placed in the center of the maze with its head oriented towards an open arm. The number of entries into and time spent in both open and closed arms were recorded during 5 min tests. The data were analyzed using t-tests for genotype differences (Graph Pad Prism, CA).

Light-dark chamber transition (LDT) tests

Testing took place in the same apparatus as that used for open field tests, except that an enclosed black opaque acrylic box ($40 \times 20.5 \times 20.5$ cm) was inserted into the right half of the chamber. The inserted box had an opening (13×5 cm) allowing mice to move freely between the two compartments. The open compartment was illuminated by a white light (450 lumens) placed 40 cm above the floor of the compartment and directed such that a crisp dark-light line existed exactly at the doorway between the two compartments. Animals were gently inserted into the dark compartment, and data collection commenced immediately for 5 min. Latency to emerge into the light compartment, the number of transitions between the two compartments (with a transition defined as all four feet crossing the line between the two compartments), and activity and time in the light compartment were recorded. The data were analyzed using a two way ANOVA for repeated measures with genotype as the between subjects factor and the days of testing (day 1 and 2) as the within subject variable (Graph Pad Prism, CA).

Step-down passive avoidance (SDA) learning tests

Mice were tested on a passive avoidance task in a transparent acrylic test chamber ($29 \times 26 \times 28$ cm) illuminated by a 40 W red light placed 40 cm above the floor of the compartment. On the first day (training trial), mice were placed on a small plastic platform (14×10 cm) located in one corner of the chamber at a height of 1.5cm, with their heads facing the corner of the chamber. Once they stepped off the platform and all four paws touched a grid floor (model E10-18 MF; Coulbourn Instruments, Lehigh Valley, PA), an electrical shock (0.25mA, 1sec) was delivered to the grid floor via an animal shocker (SanDiego Instruments Programmable Animal Shocker, CA). Mice were then tested twice for memory retention at twenty-four hours (short-term retention) and a week later (long-term retention), after the training trial. In the retention trials, the mice were tested in the same way as in the training trials except that no electrical shock was presented. All trials were limited to 360sec and latency to step off the platform was recorded. Latency differences between genotypes were compared by the non-parametric Mann-Whitney Test, with trials as the within subject variable (SPSS, IBM Corp, NY).

Radioimmunoassay for total T4

Blood samples were collected from each mouse via cardiac puncture under deep anesthesia with Nembutal (i.p.). Serum levels of total T4 were assayed in duplicate using the DPC kit

for total T4 (Diagnostics products Corporation, Los Angeles, CA). The data was analyzed using t-tests for genotype differences (Graph Pad Prism, CA).

Statistics

All data was analyzed, as detailed above for the individual experiments, in Graph Pad Prism software (CA). Before being subject to t-tests or ANOVA's, data was analyzed for homogeneity of variance using Bartlett's test and outliers (2 standard deviations from the mean) were removed. Data was plotted in Prism as mean $\pm$ SEM. A $p < 0.05$ was considered significant.

Results

Experiment I

The experimental paradigm outlined in Fig. 1A was designed to test locomotor/anxiety (open field test), social motivational behaviors (sexual behavior and aggression), and arousal (startle tests).

Open field behavior in $\alpha 1$ TR and β TR knockout mice—The distance traveled or the time spent in the center as well as the total distance traveled in the open field were not significantly different on any of the three test days between the $\alpha 1$ WT ($n=10$) and $\alpha 1$ KO ($n=12$) mice. The β KO ($n=18$) also showed no difference when compared to the β WT ($n=12$) in either center distance, center time, or total distance on any of the three test days.

Sex and aggressive behavior of $\alpha 1$ TR and β TR knockout mice—Despite the fact that the $\alpha 1$ KO showed a significantly shorter latency to the first mount (Fig. 2A) and to the first intromission (Fig. 2B) on all three tests, the $\alpha 1$ KO did not differ from the α WT in the number of mounts and intromissions over the three tests (data not shown). The β KO showed no difference in the number of mounts and intromissions or the latency to any sexual act studied in any of the three tests, when compared to β WT (data not shown). Preference for a particular female was not a factor in these tests since on every test day, a different female was used. Neither $\alpha 1$ WT nor β WT mice showed any aggressive behavior and therefore, aggressive behavior analysis was not included in this study.

Effect of TR deletion on startle responses—The startle tests were used to investigate arousal state in mice deleted for either of the TR isoforms. By initially investigating the response of the mice to trials where no stimulus was presented, we obtained a baseline arousal measure to the chamber itself. The $\alpha 1$ KO showed a larger baseline response than the $\alpha 1$ WT (Fig. 3A). The $\alpha 1$ KO showed significantly lower acoustic startle response (ASR) at 115 decibels, but not at 95 decibels, than the $\alpha 1$ WT (Fig. 3B). Both $\alpha 1$ KO and $\alpha 1$ WT animals showed an increase response at 115 decibels compared to 95 decibels (Fig. 3B). The β KO showed no differences compared to the β WT in either baseline arousal (Fig. 3C) or at 95 and 115 decibels (Fig. 3D), though there was a significantly higher response at 115 decibels than 95 decibels in both genotypes. The tactile response of the $\alpha 1$ KO male mice was lower than the $\alpha 1$ WT mice (Fig. 3E;) and this lowered response was not confined to any particular set of trials (data not shown). The β KO and the β WT did not differ significantly in tactile startle across 20 trials (Fig. 3F).

Experiment II

The behavioral tests outlined in Fig. 1B (Experiment II) were conducted to investigate differences between $\alpha 1$ WT and $\alpha 1$ KO and β WT and β KO on anxiety related tasks. In addition, the last task in this series of experiments was done to examine learned fear responses using an inhibitory avoidance paradigm.

Effect of TR deletion on the open field behavior in male mice—In Experiment II, $\alpha 1$ KO animals spent a greater proportion of their time in the center of the open field than did $\alpha 1$ WT (Fig. 4A). The total distance traveled by the animals was not significantly different by genotype (data not shown). Since all animals were initially placed in the center, it is possible that center time may not be a measure of a true anxiolytic phenotype particularly if longer center time was due to immobility at the beginning of the test. However, $\alpha 1$ KO mice also traveled a greater distance (Fig. 4B) in the center than the $\alpha 1$ WT animals. In accordance with the results from Experiment I, there were no significant differences between β WT and β KO animals on open field measures, including time in the center of the field, distance traveled in the center of the field and overall total activity (data not shown).

Effect of TR deletion on the performance of mice on the elevated plus maze (EPM) test— $\alpha 1$ KO animals spent a lesser percent of total time in the open arms than did $\alpha 1$ WT (Fig. 5A). The percent of total entries that were entries into the open arms was just shy of significant (Fig. 5B). The difference in total arm entries was not significant (Fig. 5C), indicating that $\alpha 1$ KO animals were not less active overall, just more anxious in the EPM. Though β KO animals spent an equivalent amount of time in the open arms as the β WT (Fig. 5D), β KO animals had a greater number of total arm entries (Fig. 5E) and made a greater percent of total arm entries into the open arms (Fig. 5F) than the β WT animals.

Effect of TR $\alpha 1$ or TR β gene deletion in the light dark transition (LDT) test—Data from Day 1 of the LDT test on $\alpha 1$ WT and $\alpha 1$ KO mice were lost due to instrument error. On the second LDT test, $\alpha 1$ KO animals did not differ from $\alpha 1$ WT animals in time spent in the light compartment (data not shown). β KO animals spent more time in the light compartment than β WT animals (Fig. 6A) on Day1. However, they were not more active than β WT in the dark compartment (data not shown), indicating that they were not more active, just less anxious.

Effect of TR $\alpha 1$ and TR β gene deletion in the Step-down Inhibitory Avoidance Test—Animals were measured 24 hours after the training trial (Test 1) and 7 days after the training trial (Test 2). The $\alpha 1$ KO and the β KO did not differ from their corresponding WT group in the latency differences between the training trial and either of the test trials. There was a significant difference between latencies to step off the platform between training and the test days in all groups, indicating that both $\alpha 1$ KO and β KO showed significant fear learning (data not shown).

Total T4 levels in the sera of the animals from Experiment I—Consistent with previously published reports (Forrest et al., 1996), the β KO animals were hyperthyroid with higher T4 levels compared to β WT animals (Table 1). Though previous work has indicated that the $\alpha 1$ KO is slightly hypothyroid (Wikstrom et al., 1998), there was no difference in T4 levels in the $\alpha 1$ KO as compared to the $\alpha 1$ WT in the radioimmunoassay (Table 1).

Discussion

The conservation of all four TR isoforms in evolution argues for a pivotal and perhaps unique role for each of the isoforms in various physiological processes. Though underexplored, some physiological and behavioral phenotypes seem to point to an opposing, or balancing function of the two different isoforms, TR α and TR β . For example, the loss of TR $\alpha 1$ led to slight hypothyroidism and a low baseline heart rate (Wikstrom et al., 1998), while the loss of TR β led to increased heart rate (Johansson et al., 1999). Behaviorally, lordosis behavior increased in β KO female mice while the $\alpha 1$ KO female mice exhibited

decreased lordosis as compared to their wildtype counterparts (Dellovade et al., 2000). KCDN3, a potassium channel in rat cardiomyocytes is downregulated by liganded TR β 1 but upregulated by liganded TR α 1, supporting the idea that TR α 1 may oppose TR β in some circumstances (Gassanov et al., 2009). Mutant TR α 1 animals are more impaired in adipogenesis than the TR β isoform mutated at the same site, possibly because of a higher affinity for the nuclear co-repressor, NCoR by the mutant TR α 1 (Zhu et al., 2011).

Effect of TR isoform deletion on sex behavior in gonadally intact male mice

Previous work from this laboratory has addressed the hypothesis that the liganded TR signals environmental changes and modulates the reproductive capacity controlled by estrogen (Zhu et al., 1997). Among others, preproenkephalin and oxytocin receptor are ER dependent gene products which facilitate lordosis behavior in female rodents (Nicot et al., 1997) (Quinones-Jenab et al., 1997) (Arletti et al., 1992; Arletti and Bertolini, 1985) (Carter, 1992). Cotransfection of TR α 1 into cell lines results in TR α 1 inhibition of ER α -mediated induction of both these gene promoters (Vasudevan et al., 2001a; Vasudevan et al., 2001b). Therefore, it was expected that loss of TR α 1 isoform would lead to an increase in estrogen-controlled lordosis behavior in female mice. Unexpectedly, the α 1KO females demonstrated lower lordosis while β KO females showed higher lordosis behavior (Dellovade et al., 2000). However, contrary to the results in females and consistent with the initial hypothesis, the α 1KO gonadally intact males showed a subtle increase in sex behavior by lower latencies to mounts and intromissions in the present study. This small effect might be a modulating TH influence on reproductive behavior in males that is strongly driven by gonadal steroids. There was no difference in sex behavior observed with the β KO male mice. In summary, neither social behavior, i.e. aggression or sex, appears to be robustly influenced by the TR isoforms in gonadally intact males; these behaviors are possibly more critically dependent on gonadal steroids in males than in females.

The effect of TR isoform deletion on startle responses in male mice

The acoustic and tactile startle responses are indices of sensory alertness, which underlies arousal (Frohlich et al., 1999) and affect-related defensive responding (Hubbard et al., 2011). Arousal can be measured by the acoustic and tactile startle paradigms (Frohlich et al., 1999). Previous findings that TRH administration to rats increased startle amplitude (Thompson and Rosen, 2000) and T4 administration to ovariectomized female mice increased acoustic startle responses (ASR) (Frohlich et al., 2002) support the idea that TH influences both arousal and startle responses. Rat pups that were made hypothyroid by the administration of 900 mg/kg/day thiourea to pregnant dams showed a reduction in the acoustic startle response (ASR), when tested on postnatal day 10 (Schneider and Golden, 1987) while adult Sprague-Dawley rats made hypothyroid in adulthood also had lower reactivity to stimuli (Rial et al., 1987). Adult male Wistar rats treated with the thyroxine synthesis inhibitor, propylthiouracil (PTU), showed decreased ASR (Tikhonova et al., 2005). Hence, decreased ASR might be expected in the TR knockout animals. However, since β KO animals have a developmental delay in the expression of a potassium channel in inner hair cells, their auditory brainstem response (ABR) thresholds range from elevation by just a few decibels to complete deafness (Abel et al., 1999) and we expected a lack of ASR in β KO compared to β WT animals. Contrary to expectation, the β KO mice showed no differences in the ASR from β WT mice. This could be due to the unknown individual ABR thresholds of these β KO animals and the fact that we presented animals with white noise, which encompassed a range of frequencies, possibly accounting for the lack of difference that we saw with β WT animals. Consistent with the lack of differences in the acoustic startle test, the β KO showed no differences in the tactile startle test when compared to β WT. The α 1KO male mice, however, did show a lower response to an acoustic stimulus of 115 decibels despite a higher baseline arousal state. Strengthening the idea that the ASR and airpuff startle responses are linked (Plappert

and Pilz, 2005; Plappert et al., 2005), reduction in the ASR in the $\alpha 1$ KO males was accompanied by a reduction in the tactile startle response. Despite being slightly hypothyroid, a motor deficit is unlikely since the $\alpha 1$ KO did not show any locomotor deficit over three days of testing in the open field. The lower ASR at higher decibels was not seen in a male mouse that is heterozygous for a mutant TR $\alpha 1$ that shows 10 fold lower affinity for T3 and acts in effect, as an unliganded receptor (Pilhatsch et al., 2010). The decreased response to acoustic and tactile stimuli parallels the response seen in hypothyroidism and argues that the defect in the hypothyroid model is primarily due to an inability of liganded TR $\alpha 1$ to regulate target genes. In the β KO animal, the loss of both TR β isoforms appears to be compensated at least to a large extent by the presence of the ligand-binding TR $\alpha 1$ isoform.

Effect of TR isoform deletion on anxiety-related behaviors and learned fear in gonadally intact male mice

A separate set of mice was used to test behaviors that connote anxiety. In an open field lit only by red light, $\alpha 1$ KO placed in the center showed a tendency to remain in the center. This was not due to freezing in the center or impairment of general locomotor activity since they moved more in the center than their wildtype counterparts. Though there is a trend on Day 1 of open field testing in Experiment I for the $\alpha 1$ KO to spend greater time in the center, this is not statistically significant. The subsequent light dark transitions and elevated plus tests are widely used as tests of inherent, unconditioned anxiety and fear (Crawley and Goodwin, 1980) (Rodgers and Dalvi, 1997) whereas inhibitory or passive avoidance is primarily a measure of learned or conditioned fear (Kim et al., 1997). The $\alpha 1$ KO showed fewer entries and less time in the open arms in the EPM, suggesting that they are more anxious. This is consistent with data from a transgenic mouse that carries a TR $\alpha 1$ receptor that has 10 fold less affinity for T3 (Venero et al., 2005). The β KO, on the other hand, appears to be more active with greater number of entries into arms in the EPM; however, compared to the β WT, a greater percentage of open arm entries were made. This anxiolytic behavior is also seen in Day 1 in the LDT as the β KO spends a greater amount of time in the light. Both groups of wildtype ($\alpha 1$ WT and β WT) and knockout ($\alpha 1$ KO and β KO) mice learned the step down inhibitory avoidance paradigm, as could be seen by the increased latency to step down onto the grid relative to their own performances on Day 1 both on the first (Test 1) and seventh (Test 2) day after training. However, in contextual but not cued fear conditioning, a related learning paradigm for learned fear, $\alpha 1$ KO males show greater retention of the fear memory (Guadano-Ferraz et al., 2003) than the $\alpha 1$ WT. Therefore, two different paradigms suggest that the loss of TR $\alpha 1$ or TR β does not affect learning of fear but that the loss of TR $\alpha 1$ influences memory retention.

The effect of TR deletion on both startle and anxiety states

The ASR has been linked to anxiety; for example, in human volunteers given alprazolam, an anxiolytic benzodiazepine, the amplitude of the acoustic startle reflex was reduced (Rodriguez-Fronells et al., 1999). Conversely, patients with panic disorders showed an increase in startle responses (Grillon et al., 1994). Higher ASR was also correlated with higher anxiety in rat with testicular feminization (Tfm) (Zuloaga et al., 2011). Since the $\alpha 1$ KO male mice had lower responses to startle stimuli, we initially hypothesized that this might be due to decreased anxiety; this provided impetus to perform Experiment II. However, contrary to prediction, the $\alpha 1$ KO animals were more anxious in the elevated plus maze but did not show anxiety in tests that utilize an open/bright field (the OF and the LDT). In a previous study (Guadano-Ferraz et al., 2003), the $\alpha 1$ KO showed higher anxiety than the $\alpha 1$ WT in the open field. However, the animals were tested in the light phase under the light; this could be more anxiety provoking than in our study where animals were tested in the dark phase under a dim red light, to monitor locomotor activity. Our data in the EPM

are consistent with the data obtained using a TR α 1 mutant with 10 fold less affinity for thyroid hormone (Venero et al., 2005). This TR α 1 mutant also showed lower time in the light compartment; unfortunately, these data were lost in our study due to instrument error. The EPM is currently the most sensitive and popularly used test for anxiety (Ramos, 2008); the anxiolytic behavior of the α 1KO matches data from other studies (Guadano-Ferraz et al., 2003; Venero et al., 2005). Though anxiety and startle are presumably linked by common substrates, the disconnect between the ASR and anxiety data in the α 1KO suggests that TR α 1 may decrease non-social anxiety but increase ASR via independent mechanisms. Despite the fact that the β KO mice do not show significantly lower ASR than the β WT mice, they showed less anxiety on both elevated plus and the dark-light transition tasks. The decreased anxiety in the β KO mice might be a result of increased exploration. In humans, high T4 levels correlate with “thrill seeking” attributes (Arque et al., 1987). In the EPM, the β KO had a higher number of total arm entries but a lower number of closed arm entries, suggesting that they preferred the open arm. The hyperactivity of the β KO manifested by a greater total number of entries into EPM arms could be due to higher T4 levels acting via TR α 1.

The HPA axis may play a role in both the ASR and anxiety. Propylthiouracil-induced hypothyroidism caused a reduction in CRH mRNA in the PVN and circulating corticosterone (Shi et al., 1994) while T3 coadministration blocked this reduction, showing that circulating T4 has a role in maintaining an intact hypothalamic-pituitary-adrenal axis (Shi et al., 1994). An i.c.v injection of TRH decreased anxiety (Gutierrez-Mariscal et al., 2008), suggesting that this is centrally mediated. Intraventricular administration of corticotropin releasing hormone CRH/CRF (Davis, 1998) enhanced the ASR in male rats (Liang et al., 1992) (Servatius et al., 2005) while CRF receptor 1 (CRF-R1) conditional knockouts in the limbic system were hypersensitive to stress (Muller et al., 2003). In future studies, HPA axis function in the α 1KO and β KO should be compared to their wildtype counterparts.

The genetic background of the wildtype TR mice

The wildtype mice for the TR α 1 and TR β are from different genetic backgrounds and behave differently on the range of tasks studied. For example, the β WT mice showed low percentage of open arm entries (about 23%) in the EPM compared to α 1WT (about 47%), possibly due to the fact that the genetic background of β WT i.e. the 129 strain is more anxious than the C57BL/6 strain (Rodgers et al., 2002) in both elevated plus and light dark transition tests. In addition, they spend less time in the center in the open field test than other strains (Holmes et al., 2002). The higher baseline anxiety of the 129 strain makes detection of anxiogenic effects difficult and biases towards the detection of anxiolytic effects; for e.g. – in serotonin transporter null mice, an anxiogenic effect was readily seen with a B6 background but not with a 129 background (Holmes et al., 2003). In a social behavior test, the β WT females performed lower on lordosis tests than the α 1WT females (Dellovade et al., 2000). Also, the α 1WT mice have a lower baseline level of serum cholesterol, a blunted response to T3 and a lower increase of cholesterol when deprived of T3 than β WT male mice, showing strain differences (Gullberg et al., 2000). Therefore, it must be emphasized that the knockouts are compared only to their respective age-matched wildtype littermates; the TR α 1 and TR β knockout mice are not compared to each other. It should also be emphasized that these are global knockout mice and hence peripheral effects of thyroid hormone action may subtly influence behavior. For e.g. the β KO is deficient in cholesterol 7 hydroxylase (CYP7A) in the liver, exhibits mild hyperthyroidism and does not develop hypercholesterolemia in response to dietary cholesterol (Gullberg et al., 2000). Hypercholesterolemia has been associated with generalized anxiety disorder in humans (Jakovljevic et al., 2007). The mild hypothyroidism and bradycardia exhibited by the α 1KO

could also influence locomotion and exploratory behavior via decreases in TRH and TSH secretion (Knight et al., 2006), suggesting that metabolic control by the TR's could be important in the regulation of behavior in the CNS.

Conclusion

This study demonstrates that many of the behavioral phenotypes of the ligand-binding TR isoforms are distinct and the loss of a particular TR isoform cannot be compensated by the other TR isoforms. We propose that the TR α 1 isoform is essential for the maintenance of arousal and that the balance of TR α 1 and TR β 1 is important for the maintenance of state anxiety. Furthermore, the TR α 1 isoform subtly decreases the sex behavior of gonadally intact male mice. This lends support to the molecular data that demonstrate that TR α 1 can decrease the transcriptional activation mediated by the ER isoforms; this interaction may modulate sex behavior, albeit solely in males.

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Highlights

- a)** We have studied social and nonsocial behaviors in TR α 1 and TR β knockout male mice.
- b)** The deletion of TR α 1 increases anxiety while TR β deletion decreases anxiety.
- c)** The deletion of TR α 1 decreases acoustic and tactile startle.
- d)** The deletion of TR α 1 subtly increases male sex behavior.

Figure 1A and 1B.

Behavioral timeline for Experiment I (Panel A:Top) and II (Panel B: Bottom). **Panel A:** In the first set of behavioral tests, animals were tested on open field for three days (Days 7, 8 and 9), followed by three consecutive days of aggression testing (Days 14–16), followed by sex testing on Day 21, 28 and 35. After testing for socially motivated behaviors, animals were tested for responses to both acoustic startle (Day 41) and tactile startle (Day 44) after recording baseline startle responses on Day 40. Sacrifice of animals by cardiac perfusion takes place on Day 48 after isolation. **Panel B:** In the second series of behavioral tests, a new set of animals were tested for anxiety-related behaviors on open field for a single day (Day 14), followed by elevated plus maze (EPM) on Day 18, the light dark transition (LDT) tests on Days 21 and 24 and the step down avoidance (SDA) training on Day 28. The SDA tests are on Day 29 and Day 35, followed by sacrifice of the animals on Day 38.

Figure 2.

A–B: Effect of loss of the TR α 1 isoform on sex behavior in gonadally intact male mice. Three 30-minute sexual behavior tests were performed on $\alpha 1$ TR (KO n=11; WT n=12) male mice, with each of the three test days separated by a week. Each test was conducted under red light and every male analyzed was tested on all three tests with a different sexually receptive female as detailed in methods. Repeated measures (RM) ANOVA compares across days and between genotypes. $\alpha 1$ KO and $\alpha 1$ WT differed significantly ($p < 0.05$) on a number of parameters such as the latency to the first mount, (Panel A) and latency to intromit (Panel B). Data represent mean $\pm$ SEM.

Figure 3.

A–F: The baseline startle (Panel 3A) and acoustic startle response (Panel 3B) in mice lacking either TR α 1 isoform ($\alpha 1$ WT n=11; $\alpha 1$ KO n=10) or TR β isoform (Panels C and D; β WT n=13 for baseline startle and β WT n=11 for acoustic startle response; β KO n=14). Panel A and C: The knockout animals were compared to the wildtype animals over 60 null trials with no stimulus to investigate the baseline startle. The mean of the maximum voltage change (Vmax) across the 60 trials for the WT and the KO was compared statistically using the t-test. Panel B and D: Startle responses to 20 presentations each of two levels of acoustic stimuli (95 dB and 115 dB), randomly counterbalanced with 20 trials with no sound (0 decibel trials) were compared between WT and KO animals using RM ANOVA with genotype as the between subject variable and the two decibel levels as the within subject variable. $\alpha 1$ KO mice were more aroused than the $\alpha 1$ WT (Panel A) and responded to a lesser extent than $\alpha 1$ WT at 115 dB but not at 95 decibels (Panel B). β KO showed no difference compared to β WT in baseline arousal (Panel C) or at either decibel level (Panel D). The effect of air-puff tactile startle on mice ($\alpha 1$ WT n=10; $\alpha 1$ KO n=11; β WT n=7; β KO n=10) deleted for TR α 1 (Panel E) and TR β isoforms (Panel F) over 20 trials of an air puff delivered to the back of the neck. The $\alpha 1$ KO mice showed a decreased response to air puff stimulus averaged across 20 trials, as analyzed by t-test (Panel E). There was no difference in the response to air puffs between β WT and β KO, as analyzed by t-test (Panel F). Data represent mean $\pm$ SEM.

Figure 4.

A–B: Activity of the TR $\alpha 1$ ($\alpha 1$ WT n=8; $\alpha 1$ KO = 15) null mice in a single 5 minute open field test (Experiment II). A second set of mice were run on a series of tests as outlined in Fig. 1B. The $\alpha 1$ KO spent significantly more time (Panel A) and traveled a greater distance (Panel B) in the center area of the open field. Data, analyzed by t-test, represent mean $\pm$ SEM.

Figure 5.

A–F: The impact of genotype ($\alpha 1$ WT $n=8$; $\alpha 1$ KO $n=15$; β WT $n=10$; β KO $n=10$) on activity in the elevated plus maze during a 5-min test under red light. The $\alpha 1$ KO spent lesser time in the open arms (Panel A) and made fewer entries into the open arms (Panel B) than the $\alpha 1$ WT. The number of total entries into arms, however, did not differ between $\alpha 1$ WT and $\alpha 1$ KO (Panel C). Though the β KO did not spend more time in the open arms (Panel D), they made more entries in the open arms (Panel E). They had a greater number of arm entries (Panel F) than the β WT. Data, analyzed by t-test, represent mean $\pm$ SEM.

Figure 6A.

The effect of the TR β deletion on behavior in the light dark compartment. Animals (β WT n=10; β KO n=10) were initially placed in the dark compartment, and time spent in each compartment was measured during 5min tests, administered for two consecutive days. The β KO animals spent more time in the light compartment than β WT animals on Day 1. Data was analyzed by RM ANOVA. Data represent mean $\pm$ SEM.

Table 1

T4 levels in sera of TR knockout and wildtype adult males.

Genotype	Number of animals	T4 ($\mu\text{g/dl}$) in sera
$\alpha 1\text{WT}$	10	3.30 ± 0.23
$\alpha 1\text{KO}$	10	3.16 ± 0.16
βWT	5	3.46 ± 0.12
βKO	8	$4.97 \pm 0.43^*$