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Research report

Altered anxiety and defensive behaviors in *Bax* knockout mice

Angela C. Luedke[a], Pierre O. Boucher[a], Lee Niel[b], Melissa M. Holmes[a,c,*]

[a] Department of Psychology, University of Toronto Mississauga, Mississauga, ON, Canada L5L 1C6
[b] Department of Population Medicine, Ontario Veterinary College, University of Guelph, Guelph, ON, Canada N1G 2W1
[c] Department of Cell and Systems Biology, University of Toronto, Toronto, ON, Canada M5S 3G5

HIGHLIGHTS

- *Bax* deletion results in increased neuron number in many phenotypic populations.
- *Bax*−/− mice show reduced anxiety based on elevated-plus maze performance.
- *Bax*−/− mice employ a different defensive profile in response to aversive odors.
- Supernumerary neurons in *Bax*−/− mice appear to influence fear and anxiety.

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ABSTRACT

Developmental neuronal cell death is critically regulated by the pro-death protein Bax. *Bax*−/− mice exhibit increased neuron number, the elimination of several neural sex differences, and altered socio-sexual behaviors. Here we examined the effects of *Bax* gene deletion on anxiety and defensive behaviors by comparing the responses of male and female wildtype and *Bax*−/− mice to two different tests. On the elevated plus maze, *Bax*−/− mice of both sexes made more entries into and spent more time in the outer portion of open arms, indicating decreased anxiety compared to wildtype animals. Next, we exposed mice to two odors: trimethylthiazoline (TMT), an olfactory component of fox feces that rodents find aversive, and butyric acid (BA), an aversive odor without ecological significance. Each odor was presented individually and all animals were tested with both odors in a counterbalanced design. TMT was consistently more aversive than BA across a variety of behaviors (e.g., mice spent less time close to the odor source). Overall, *Bax* −/− mice showed fewer stretch approaches to both TMT and BA than wildtypes, but they avoided the odor source more (e.g., fewer contacts and less time spent in proximity). Finally, no effect of genotype was seen in baseline olfactory behavior; all mice were able to locate a buried food item, demonstrating that *Bax*−/− mice do not have impaired olfaction per se. Collectively, these data suggest a change in strategy with anxiety and defensive behaviors in *Bax*−/− mice, indicating that alterations in cell number affect more general mechanisms of fear and anxiety in addition to behaviors directly related to reproduction.

1. Introduction

Bax, a member of the Bcl-2 protein family, controls neuronal cell death during the postnatal period [1,2] and *Bax* knockout (*Bax*−/−) mice fail to show neural sex differences resulting from differential cell death in males and females. For example, male mice have more neurons than females in the principal nucleus of the bed nucleus of the stria terminalis (BNST) [3,4], likely the result of increased perinatal cell death in females [5,6], and the adult sex difference in neuron number is eliminated in *Bax*−/− mice [3,4]. A similar pattern is seen in brain regions where the sex difference in neuron number favors females. While females have more cells than males in the anteroventral periventricular nucleus (AVPV), this sex difference is also eliminated in adult *Bax*−/− mice [3]. Importantly, mechanisms controlling phenotypic differentiation are not necessarily altered by *Bax* deletion. In the BNST, sex differences in androgen receptor immunoreactive cells are absent in *Bax*−/− mice and knockouts have more of these cells overall [4]. In contrast, the sex difference in the number of BNST cells expressing vasopressin, a neuropeptide important in the control of social behaviors, is still present in *Bax*−/− mice, though again, both sexes have increased

Abbreviations: AVPV, anteroventral periventricular nucleus; BA, butyric acid; BNST, bed nucleus of the stria terminalis; EB, estradiol benzoate; TMT, 2,4,5-trimethylthiazoline.

* Corresponding author at: Department of Psychology, University of Toronto Mississauga, 3359 Mississauga Road North, Mississauga, ON, Canada L5L 1C6.
Tel.: +1 905 828 3874; fax: +1 905 569 4326.
E-mail address: melissa.holmes@utoronto.ca (M.M. Holmes).

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vasopressinergic cell numbers [7]. Similarly, in the AVPV, *Bax* deletion fails to eliminate the sex difference in tyrosine hydroxylase- and kisspeptin-immunoreactive populations though, in this case, there is no net increase in number of these particular cell types [3,8]. Thus, the *Bax*−/− mouse provides a unique opportunity to investigate how changes in specific neural populations affect behavior, both in terms of alterations (or lack thereof) in sexually differentiated populations but also a net gain in neuron number.

The overall motor and exploratory behavior of young *Bax*−/− mice appears grossly normal [e.g., 9,10] though, interestingly, specific social behaviors are altered. For example, *Bax* deletion decreases both male and female sexual behavior [11] and *Bax*−/− mice fail to show sex-typical olfactory preferences for conspecifics [12]. However, *Bax* knockouts appear more social overall; they spend more time chemoinvestigating conspecifics and in proximity to a novel stimulus animal, though no effects of gene deletion were seen with aggression [12]. These behavioral effects are likely due to altered signaling involving the BNSTp and medial amygdala, given their pivotal role in the processing of socially relevant olfactory cues [13,14]. However, the role of the BNST goes beyond the processing of social information. Indeed, androgen receptor and vasopressinergic populations within the BNST are important for regulating stress, anxiety, and fear responses. For example, individual differences in defensive burying are associated with variation in the number of androgen receptor immunoreactive neurons and vasopressin expression [15]. Furthermore, hippocampal function is also significantly altered in *Bax*−/− mice due to supernumerary adult-generated neurons [16]. Specifically, *Bax*−/− mice show deficits in contextual but not cued fear memory [10,16]. Thus, we hypothesized that non-social behaviors mediated by the BNST and hippocampus (e.g., fear and anxiety) would also be altered in *Bax*−/− mice. To test this we compared wildtype and *Bax*−/− mice of both sexes on the elevated plus maze and in response to aversive odors to assess anxiety and defensive behavior, respectively.

2. Materials and methods

2.1. Animals

Wildtype (*Bax*+/+) and *Bax*−/− mice were bred locally by pairing mice heterozygous for *Bax* gene deletion on a C57Bl/6J background; genotype was confirmed with PCR amplification of ear punch DNA using established primer sequences [2]. A total of 40 mice were used (8 male *Bax*−/−, 14 male *Bax*+/+, 7 female *Bax*−/− and 11 female *Bax*+/+). Animals were single-housed between 7 and 9 weeks of age in cages containing corn cob bedding and maintained in a 12:12 light/dark cycle with ad libitum access to water and laboratory chow (Lab Diet #5012). Behavior testing began at 12 weeks of age and one test per week was conducted for a total of three weeks, in the same order for all animals: olfactory test, elevated plus maze, and defensive behavior. Testing was done during the light phase of the light/dark cycle. All procedures adhered to institutional and federal guidelines for the care and use of animals in research. All behavior testing and scoring was performed by experimenters blind to the genotype of the animals.

2.2. Olfactory test

Olfactory tests were conducted in the animals' home cages in the colony room. Mice were exposed to a single Froot Loop (Kellogg's cereal) one week prior to testing to eliminate novelty effects. On test day, mice were transferred into a clean holding cage for approximately 30 s while a single Froot Loop was buried approximately 1 cm below the bedding surface in one of the four corners of the home cage. Mice were then placed back into the center of the home cage and time to discover the Froot Loop was recorded up to a maximum of 15 min.

2.3. Elevated plus maze

Testing for the elevated plus maze occurred in a separate testing room. Mice were transported to the testing room 1 h prior to testing to allow for habituation to the new environment. Mice were then placed in the center of the maze and behavior was recorded for a period of 5 min. The arms of the maze were 10 cm wide, 51 cm long, and 53 cm above the floor. The maze was wiped down with 70% ethanol between animals. The number of entries into open and closed arms, as well as duration spent in each arm, was scored using Observer 10 software (Noldus, Inc.). Bax−/− mice made significantly more arm entries compared to wildtypes ($F_{1,36} = 8.34$, $p = 0.007$) and females tended to make more entries than males ($F_{1,36} = 3.9$, $p = 0.06$), indicating group differences in overall activity. Therefore, percentage scores were calculated for the number of entries into open arms and duration of time spent in open arms. For example, the number of entries into open arms was divided by the total number of entries into any arm. Because the arms were visually divided in half based on distance from the center platform, we were also able to score the number of entries into and duration of time spent in the outer portion of open and closed arms. These data are also presented as percentage scores.

Table 1
Time (s) required to complete olfactory test.

Group	Mean (±SEM)
Bax+/+ males	192.73 (56.22)
Bax+/+ females	146.96 (65.57)
Bax−/− males	80.88 (17.60)
Bax−/− females	128.11 (24.32)

No significant effects were detected.

2.4. Defensive behavior

We compared defensive behavior following exposure to either 2,4,5-trimethylthiazoline (TMT; PheroTech) or butyric acid (BA; Sigma). TMT is a component to fox feces and is commonly used as an ecologically relevant stressor for laboratory rodents, demonstrated to induce fear beyond its aversive odorant properties, while BA is a non-ecologically relevant aversive odor [17]. By comparing the two, we aimed to determine whether *Bax* deletion might affect behavioral responses to aversive odors in general or whether any effects were specific to fear-inducing stimuli.

Testing was conducted over a 5-day period, 10 min each day, in a separate testing room. Testing chambers were 10 L glass aquaria containing approximately 1–2 in. of corn cob bedding, which were placed under a fume hood to minimize dispersal of the strong test odors. A glass scintillation stimulus vial containing a Kim wipe was placed in one corner of each aquarium prior to the introduction of a mouse; vial placement was counterbalanced across animals but kept consistent for each animal across days. Tape on the side of each aquarium divided it into 3 equal sections to allow calculation of the amount of time spent in proximity to the stimulus vial. Days one through three were habituation sessions: animals were placed into the test chamber but the stimulus vial did not contain any odor. On days four and five, the Kim wipe was soaked with 20 μl of either TMT or BA in a counter balanced design. Thus 20 animals received TMT on day 4 and BA on day 5 and 20 animals received BA on day 4 and TMT on day 5. All sessions were video-recorded for later analysis.

Videos were scored for avoidance and defensive behaviors using Observer 10 software (Noldus, Inc.). Specifically, we examined the number of stretch approaches (planting of hind feet and elongation of body and neck toward the stimulus vial), rears (both front limbs off of bedding), jumps (all limbs off bedding), and contacts with the vial (including with limbs or nose), and the duration of freezing (crouched posture with no movement), defensive burying (forward pushing of bedding toward stimulus vial using limbs and nose), digging (backward pushing of bedding using limbs not in relation to vial), and percent time spent in proximity to the vial (the one-third portion of the chamber containing the vial) were also recorded.

2.5. Statistical analyses

Data for the olfactory test and elevated plus maze were analyzed using 2-way (sex by genotype) ANOVAs. Defensive behaviors following exposure to BA and TMT were analyzed using repeated measures 2-way ANOVAs with sex and genotype as the independent variables and stimulus type as the repeated measure. All analyses were performed with Statview software, and statistical significance was set at $p < 0.05$.

3. Results

3.1. Olfactory test

For the olfactory test, no significant effects of sex ($F_{1,36} = 0.00$; $p = 0.99$) or genotype ($F_{1,36} = 1.31$; $p = 0.26$) were detected, nor was the sex × genotype interaction statistically significant ($F_{1,36} = 0.66$; $p = 0.42$) (Table 1).

3.2. Elevated plus maze

Bax−/− mice tended to make more entries ($F_{1,36} = 3.22$; $p = 0.08$; Fig. 1A) and spend more time ($F_{1,36} = 3.42$; $p = 0.07$; Fig. 1B) in open

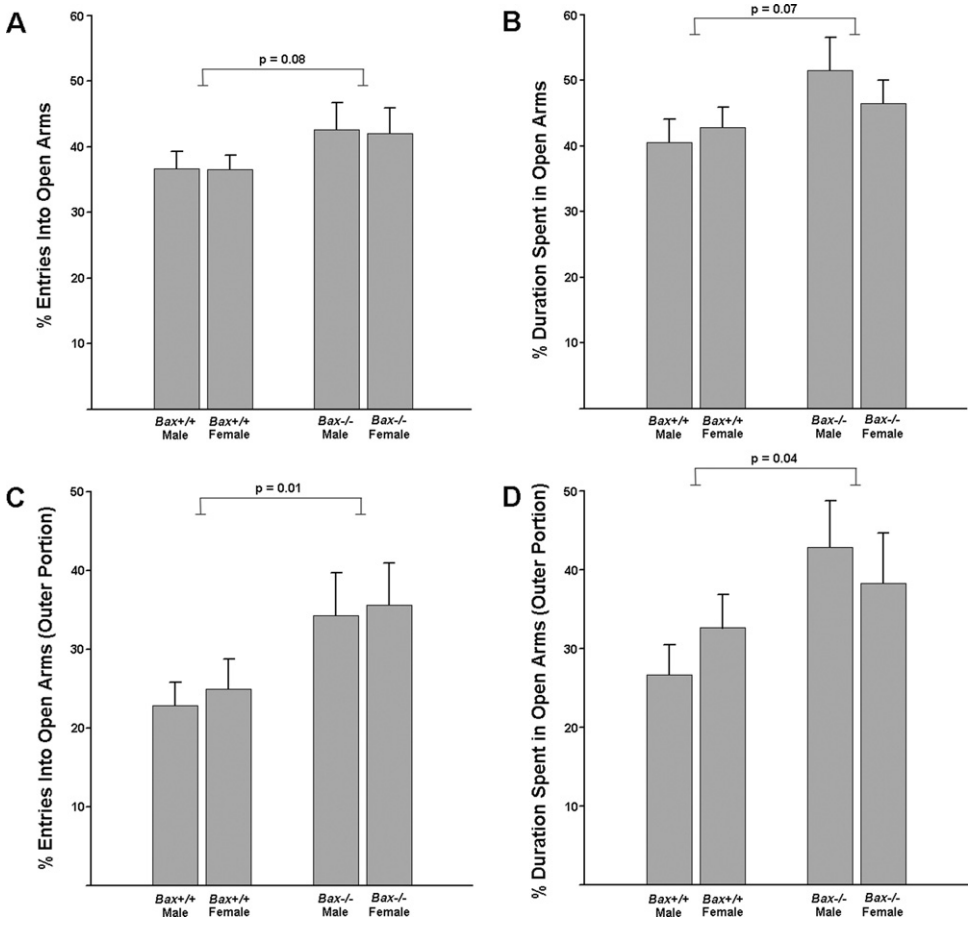

Fig. 1. Mean (+SEM) number of entries into (A) and (C) and duration of time spent in (B) and (D) the open arms of the elevated plus maze; (C) and (D) are the outer portion of the arms only. All data are presented as the percentage relative to total entries (both open and closed arms combined).

arms. This effect of genotype reached statistical significance when looking at the outer portion of the open arms (percent entries into outer open arms: $F_{1,36} = 6.81$; $p = 0.01$; percent duration in outer open arms: $F_{1,36} = 4.82$; $p = 0.04$; Fig. 1C and D).

No significant sex effects were detected when looking at percentage scores for open arms (entries: $F_{1,36} = 0.008$; $p = 0.93$; duration: $F_{1,36} = 0.11$; $p = 0.74$) or outer portion of open arms (entries: $F_{1,36} = 0.16$; $p = 0.69$; duration: $F_{1,36} = 0.02$; $p = 0.88$). The sex × genotype interactions also failed to reach significance for both open arms (entries: $F_{1,36} = 0.005$; $p = 0.94$; duration: $F_{1,36} = 0.84$; $p = 0.37$) and outer open arms (entries: $F_{1,36} = 0.01$; $p = 0.92$; duration: $F_{1,36} = 1.11$; $p = 0.30$). In fact, the only significant sex effect was seen in the raw number of entries into closed arms ($F_{1,36} = 6.09$; $p = 0.02$) where females made more entries into closed arms than males (female average = 45.95; male average = 40.27). However, even in this case, the sex × genotype interaction was not significant ($F_{1,36} = 0.46$; $p = 0.50$).

3.3. Defensive behavior

A significant effect of stimulus was seen for number of stretch approaches ($F_{1,36} = 7.87$; $p = 0.008$; Fig. 2A), number of jumps ($F_{1,36} = 7.61$; $p = 0.009$; Fig. 2B), number of contacts ($F_{1,36} = 26.28$; $p < 0.001$; Fig. 2C), and percent time spent in proximity to the vial ($F_{1,36} = 16.35$; $p < 0.001$; Fig. 2D) where animals clearly found TMT more aversive than BA (i.e., they performed more stretch approaches and jumps, contacted the vial less, and spent less time in proximity to the vial).

Significant effects of genotype were seen on several behaviors. Wildtypes performed more stretch approaches than *Bax*−/− mice, regardless of stimulus ($F_{1,36} = 4.55$; $p = 0.04$; Fig. 2A). There were significant genotype × stimulus interactions for number of jumps ($F_{1,36} = 8.45$; $p = 0.006$), number of contacts with the vial ($F_{1,36} = 7.85$; $p = 0.008$), and percent time spent in proximity to the vial ($F_{1,36} = 7.23$; $p = 0.01$) where *Bax*−/− mice jumped more, contacted the vial less, and spent less time in proximity with the vial when exposed to TMT (Fig. 2B and D). There was also a trend for a genotype × stimulus interaction ($F_{1,36} = 4.06$; $p = 0.051$) on defensive burying where wildtype mice showed increased burying in response to TMT relative to BA and *Bax*−/− mice did not (Table 2).

No significant main effects of sex were seen on any assessed defensive behavior (all $p < 0.16$). One significant sex × stimulus interaction was seen for percent time in proximity to the vial ($F_{1,36} = 5.00$; $p = 0.03$) where females spent less time in proximity to the vial than males when exposed to TMT (Fig. 2D). There was also a significant sex × genotype effect on the number of jumps ($F_{1,36} = 5.53$; $p = 0.02$) where male *Bax*−/− mice jumped more than other groups (Fig. 2B). No significant effects of genotype, sex, or stimulus were seen on duration of digging or number of rears (Table 2).

4. Discussion

Deletion of the *Bax* gene has striking effects on the brain, reducing or eliminating neural cell death during development [2] and adulthood [18]. As such, mice lacking the *Bax* gene show markedly different organization in several neural populations; those best

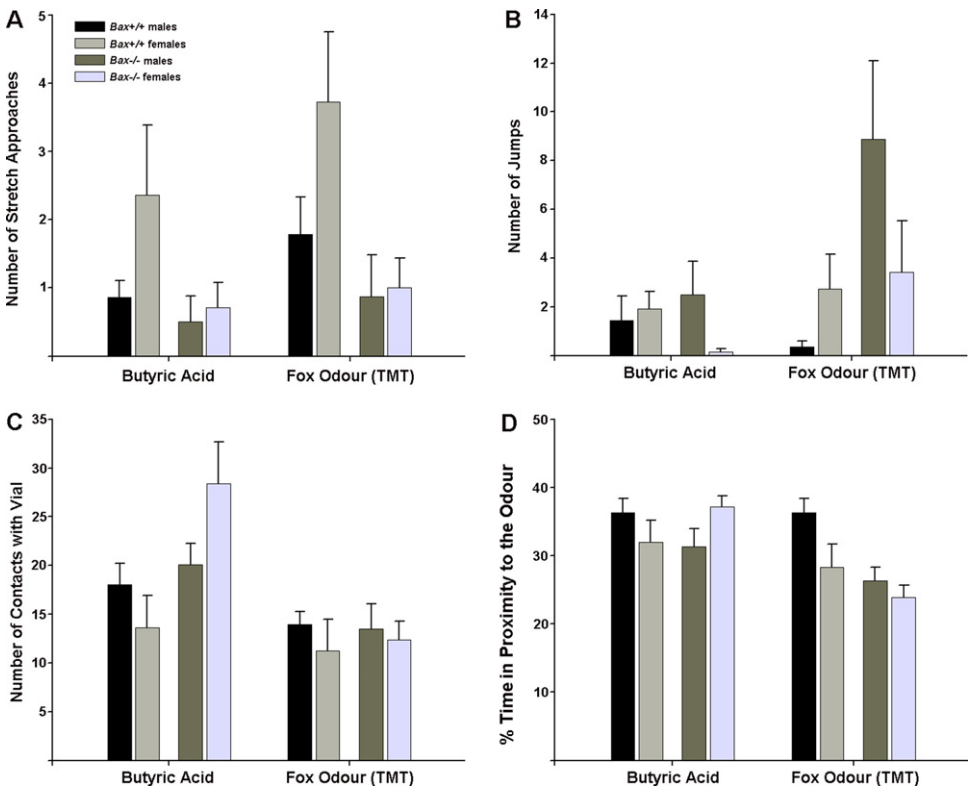

Fig. 2. Mean (+SEM) number of stretch approaches (A), jumps (B), and contacts with the vial (C) and the percent time spent in proximity to the odor source (D) following exposure to either butyric acid or trimethylthiazoline (TMT), an olfactory component of fox feces.

characterized to date are the hippocampus, BNSTp and AVPV. A growing body of work is revealing how these altered neural circuits influence behavior, including motor, sexual, social, and cognitive function. The present data suggest that *Bax*−/− mice are less anxious than wildtypes; they made more entries and spent more time in the outer portion of the open arms on the elevated-plus maze. We have also demonstrated alterations in the defensive response whereby *Bax*−/− mice exhibited fewer 'approach' defensive behaviors (e.g., stretch approach, defensive burying, and contact with odor source) but more 'avoidance' behaviors (e.g., they spend more time away from the stimulus). Importantly, these knockout animals can clearly perform both approach and defensive behaviors, further demonstrating that motor and sensory functions appear to be intact (e.g., olfactory test reported here; [9,19]). Finally, regardless of sex or genotype, we saw virtually no freezing behavior, likely due to the large size of our testing chambers with only a single odor source, which allowed animals to more readily avoid or escape the odor source [20]. Together, these data demonstrate that the neural effects resulting from Bax deletion cause alterations in non-social behaviors.

Stimulus intensity might contribute to the magnitude of behavioral responses we observed. It is important to acknowledge that in the defensive behavior paradigm, we used the same amount of fully concentrated liquid for both odor stimuli (20 μl each). We report stimulus differences where TMT was more aversive than BA, which might be due to the ecological relevance and fear-inducing properties of TMT. However, TMT induces avoidance at a lower concentration than BA [17]; therefore, our increased response to TMT relative to BA might be due to its increased stimulus intensity. Regardless, while this issue may temper the interpretation that *Bax* deletion has specific effects on fear processing, it does not negate the clear effects of *Bax* deletion on defensive behavioral responses to more general aversive stimuli.

Sex differences in anxiety and defensive behavior have been reported for laboratory rodents, though these effects appear to be more consistent for rats [reviewed in 21,22] than mice. In DBA/2 and T1 mice, there are subtle sex differences in elevated plus maze performance where males make more arm entries overall [23]. These authors also report that males make more entries into closed arms, however, this is strain dependent [23]. In their gonadally intact control C57Bl/6J mice, similar to the mice used here, Adamec et al. [24] did not see sex differences in entries into or time spent in open arms (corrected for total entries and total time spent in arms). Similarly, sex differences in defensive behavior are inconsistent and may be stimulus dependent. Female rats show greater avoidance of a potential cat threat and also make more stretch approaches

Table 2
Mean (±SEM) duration of defensive burying and digging and number of rears in response to either butyric acid (BA) or trimethylthiazoline (TMT) exposure.

Group	Burying (s)		Digging (s)		Rears (number)	
	BA	TMT	BA	TMT	BA	TMT
Bax+/+ males	0.15 (0.1)	1.03 (0.6)	14.54 (3.2)	17.81 (5.7)	85.43 (7.8)	82.0 (7.5)
Bax+/+ females	2.34 (1.3)	5.17 (3.1)	23.93 (5.1)	19.46 (3.0)	77.91 (10.7)	67.55 (5.6)
Bax−/− males	6.07 (4.6)	4.08 (4.0)	24.20 (6.8)	20.1 (4.8)	72.25 (7.2)	57.88 (7.9)
Bax−/− females	8.27 (3.2)	5.76 (2.8)	23.46 (4.0)	36.55 (13.7)	68.0 (5.8)	65.0 (8.2)

No significant effects were detected. A trend for a genotype × stimulus interaction was seen for defensive burying ($p = 0.051$).

[25]. Though no sex differences in defensive behavior were seen in rats following TMT exposure [26], female mice avoid TMT more than males [27]; Fig. 2D). We did not see robust sex differences in either elevated plus maze performance or in response to aversive odors. When we did see a significant effect of sex (e.g., females made more entries into closed arms, data not shown; females spent less time in proximity to odor source, Fig. 2D), *Bax* deletion had no effect, indicating that Bax-mediated sex differences in neuron number are not responsible for controlling putative sex differences in anxiety or defensive behavior. However, our animals were quite young and there is an age-related increase in anxiety in female mice [28]. Perez et al. [10] report an effect of *Bax* deletion in the open field test in 15–17-month-old female mice but not 2–3-month-old female mice, which is comparable to the young mice we used. It would thus be of interest to examine whether *Bax* deletion alters age-related changes in sex differences in anxiety and/or fearfulness.

Given that sex differences in these behaviors exist, it is not surprising that gonadal steroids can influence behavioral expression. Importantly, our animals were gonadally intact and we did not track ovarian cycling in the females. Cycle phase in gonadally intact females has been shown to influence elevated plus maze performance in mice on a C57BL/6 background [29]. Specifically, female mice show more open arm entries and spend more time in the open arms during proestrus when levels of estrogen and progesterone are elevated. This effect is likely the result of 5-α-reductase activity because variation across the estrous cycle is not seen in 5-α-reductase type 1 knockout mice [30]. Similarly, testosterone treatment in adulthood reduces anxiety in some paradigms (e.g., novel object), though it fails to affect behavior on the elevated plus maze [31]. Defensive behavior also appears to be affected by cycle phase in mice, as TMT approach distance has been shown to decrease during proestrus [27]. In addition, EB heightens fear responses with a non-predator stimulus in mice [32]. In rats, estradiol and progesterone, either systemically or directly in the amygdala, mediate the anti-nociceptive effects of TMT [33] and there are significant effects of estradiol and androgen manipulation on TMT-induced stretch approach, but not defensive burying [26,34]. Collectively, these data indicate that gonadal steroids might influence performance on the tasks we employed. That being said, there is no evidence for differences in organizational or activational effects of androgens in *Bax* knockouts: testosterone levels and seminal vesicle weights do not differ in *Bax*−/− mice of either sex [3] and anogenital distance is also unchanged by genotype [3]. Furthermore, at least for older females (15–17 months), *Bax* deletion does not change circulating estradiol [10], though female sexual behavior is impaired in young adults [11]. Thus, it remains to be determined whether the effects of genotype that we report are mediated by circulating gonadal steroids.

We propose that the alterations in anxiety and defensive behaviors we report are due to the supernumerary neurons, likely in the BNST and/or hippocampus, that result from *Bax* deletion, and not due to alterations in sexually differentiated populations per se. The BNST is involved in fear and anxiety behavior [35,36] and is critically involved in the behavioral response to TMT. Both rats and mice show increased c-fos activity in this region following TMT exposure [37,38] and inactivation of the BNST, but not the amygdala, decreases TMT-induced freezing in rats [39]. While several sex differences in cell number in the BNST are eliminated by *Bax* deletion, including neuronal nuclei (NeuN) and androgen receptor-expressing populations [4], *Bax*−/− mice of both sexes have more neurons overall in this brain region [4,7]. Furthermore, *Bax*−/− mice show deficits in contextual but not cued fear memory [10,16], likely the result of impaired hippocampal function resulting from adult-generated supernumerary neurons [16]. It is interesting to note that depletion of adult hippocampal neurogenesis in mice increases anxiety behavior on the elevated plus maze [40]. Though pure speculation at this point, perhaps the reduction in anxiety we report is the result of increased cell number in the hippocampus resulting from *Bax* deletion.

An additional consideration that is warranted stems from Bax protein levels in the mitochondria of adult neurons, independent of changes in neuron number. While Bax translocation to mitochondria is key for triggering apoptosis, it is the ratio between the pro-apoptotic Bax and the anti-apoptotic members (e.g., Bcl-xl) of the Bcl-2 family that controls cell fate [reviewed in 41]. Increased resilience to behavioral stress is associated with an increased Bcl-xl/Bax ratio in the adult hippocampus [42]. Given that mitochondrial dysfunction has been linked to psychiatric conditions including anxiety and depression [43], it would be of interest to determine if alterations in the ratios between pro-and anti-apoptotic Bcl-2 family proteins in the *Bax* knockout brain are involved in the altered defensive and anxiety behaviors that we report.

Bax deletion causes increased neuron number in the adult brain, as well as the elimination of several sex differences in cell number. It is not necessarily surprising, therefore, that these mice show alterations in various socio-sexual and cognitive behaviors [10–12,16]. We further demonstrate here a reduction in anxiety and alteration in defensive behaviors, likely the result of a change in active vs. passive strategy, in mice lacking the *Bax* gene. However, in general, these mice seem to function quite normally as there are no gross alterations in motor or sensory function [9,11,16,19]. Ultimately, there appears to be striking compensation for the supernumerary neurons and reduced sexual dimorphism that result from *Bax* deletion.

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