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Hyperactivity and depression-like traits in Bax KO mice

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Abstract

The Bax gene is a member of the Bcl-2 gene family and its pro-apoptotic Bcl-associated X (Bax) protein is believed to be crucial in regulating apoptosis during neuronal development as well as following injury. With the advent of mouse genomics, mice lacking the pro-apoptotic Bax gene (Bax KO) have been extensively used to study how cell death helps to determine synaptic circuitry formation during neurodevelopment and disease. Surprisingly, in spite of its wide use and the association of programmed neuronal death with motor dysfunctions and depression, the effects of Bax deletion on mice spontaneous locomotor activity and depression-like traits are unknown. Here we examine the behavioral characteristics of Bax KO male mice using classical paradigms to evaluate spontaneous locomotor activity and depressive-like responses. In the open field, Bax KO animals exhibited greater locomotor activity than their control littermates. In the forced swimming test, Bax KO mice displayed greater immobility times, a behavior despair state, when compared to controls. Collectively, our findings corroborate the notion that a fine balance between cell survival and death early during development is critical for normal brain function later in life. Furthermore, it points out the importance of considering depressive-like and hyperactivity behavioral phenotypes when conducting neurodevelopmental and other studies using the Bax KO strain.

Keywords

Neuronal cell death; Bcl-2 protein family; apoptosis; behavior despair; locomotor hyperactivity; depressive-like behavior; behavioral phenotyping

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Conflict of interest statement

The authors declare that they have no competing interests.

1. Introduction

The underlying mechanisms and functional significance of programmed cell death during neuronal development, adult life or in response to injury have been topics of intense interest for decades (Lindsten et al., 2005; Martin, 2001). More recently, great progress in this field has been achieved using transgenic and knockout mice (Vogel, 2002). In particular, Bax knockout (Bax KO) mice have been used in a number of works to investigate the role of neuronal death in cell migration (Kim et al., 2007), traumatic brain injury (Tehrani et al., 2008), and ischemia (Hochhauser et al., 2003), to name a few. The Bax gene, a potent regulator of cell death (Karbowski et al., 2006), belongs to the Bcl-2 family of genes and its expression has been associated with Parkinson's disease (Tatton et al., 2003), Alzheimer's disease (Su et al., 1997), and Schizophrenia (Jarskog et al., 2004). Translocation of Bax protein to the mitochondria triggers the release of cytochrome c and subsequent caspase-mediated cell death (Cregan et al., 1999; Oltvai et al., 1993). Neuronal expression of Bax is seen in both central and peripheral nervous systems of humans and rodents (Hara et al., 1996; Krajewski et al., 1995; Menshanov et al., 2006; Oltvai et al., 1993) including neurons in the cerebral cortex, basal nuclei, hippocampus, brain stem, cerebellum, choroid plexus, and spinal cord anterior horn. In spite of such widespread expression, ablation of the Bax gene does not lead to a hypertrophied mouse brain. This is probably due in large part to the fact that Bax regulation of cell death occurs after cell proliferation and does not influence the programmed cell death of non-neuronal cells (White et al., 1998). Nonetheless, compared to wild type mice, Bax KO animals display a 62% increase in the number of neurons in the dentate gyrus (Sun et al., 2004), a 51% increase in the number of sympathetic and motor neurons (Deckwerth et al., 1996), a 30% increase in the number of cerebellar Purkinje cells (Fan et al., 2001), a 35% increase in the number of axons in the optic nerves, and a 24–35% increase in the number of motor and sensory nerves (White et al., 1998).

Despite the wide use of Bax KO mice in anatomical, physiological and pathological studies, few articles focused their attention on its behavioral phenotypes. Behavior analysis of transgenic and knockout mice is an important tool to determine the functional roles of genes in the central nervous system (Crawley, 2008). Moreover, specifically for the Bax KO mouse, such observations are extremely important to evaluate how disruption in the balance between neurogenesis and cell death affects particular behavioral traits. While previous studies have demonstrated that Bax KO mice have learning deficits (Lee et al., 2009), altered socio-sexual behaviors (Jyotika et al., 2007), increased anxiety (Luedke et al., 2013) as well as disrupted defensive behavior (Luedke et al., 2013), significant questions still remain as to the Bax role in spontaneous locomotor activity and depression-like behaviors.

Programmed neuronal loss is thought to play a vital role in motor activity and mood disorders. Increased rates of apoptosis in the adult brain have been recently associated to depression (Berk et al., 2014; Garcia-Fuster et al., 2014), whereas enhanced apoptosis caused by alcohol, anesthetics or hypoxia-ischemia during development seem to be linked to hyperactivity later in life both in humans and animal models (Anand and Scalzo, 2000; Dell'Anna et al., 1991; Fredriksson and Archer, 2003; Gramatte and Schmidt, 1986; Ieraci and Herrera, 2006; Van et al., 2007). Of particular interest, Bax function has been associated to neuropathological conditions such as depression-induced behavior (Wang et al., 2011),

bipolar disorder (Kim et al., 2007), fetal alcohol syndrome (Ikonomidou et al., 2000; Olney et al., 2002; Olney, 2003), and ischemic stroke (Gibson et al., 2001; Krajewski et al., 1995). It is thus conceivable to hypothesize that Bax KO mice would display altered locomotor activity and depression-like behaviors relative to control littermates. To investigate these possibilities, we examined the behavior of Bax null mice in the open field and in the forced swimming tests, classical assays used to evaluate spontaneous locomotor activity (Crawley et al., 1997; Walsh and Cummins, 1976) and depression-like phenotypes (Castagne et al., 2011).

2. Results

2.1 Open field

Bax KO mice were significantly more active than their control littermates. As shown in Figures 1A and B, despite the fact that spontaneous locomotor activity in the open field decreases over time for both groups (Figure 1A; RM ANOVA: *time-interval*, $F = 11.46$, $df = 2.1/38.4$, $p < 0.001$), Bax KO mice displayed significantly higher locomotion values than control animals (Figure 1B; RM ANOVA: *genotype*, $F = 13.84$, $df = 1.0/18.0$, $p < 0.01$). Note that for the control group, spontaneous activity decreased rapidly after the first minute of the testing session and remained constant afterwards. No significant differences were observed in the percentage of locomotor activity in the center of the open field between Bax KO and control animals (Figure 1C-D).

2.2 Forced swimming test

While there was a marked increase in immobility along the testing session for both groups (Figure 2A; RM ANOVA: *time-interval*, $F = 15.90$, $df = 2.9/63.8$, $p < 0.001$), immobility times of Bax KO were significantly higher than controls (Figure 2A-B; RM ANOVA: *genotype*, $F = 4.82$, $df = 1.0/22.0$, $p < 0.05$). Interestingly, Bax KO mice presented a peculiar immobility pattern characterized by a “freezing state” in which animals would suddenly stop swimming and stay floating motionless with their bodies crooked and their tails and limbs twisted (Figure 2C).

To determine whether the immobility in the forced swimming test was related to the magnitude of locomotor activity in the open field, we measured the correlation between immobility times and the total distance traveled in the open field for both Bax KO and control animals (Figure 2D-E). Interestingly, while no correlation between these two variables was found in control animals (Figure 2D; $df = 18$, $R = 0.09$, $p = 0.7$), Bax KO mice displayed a significant positive correlation between immobility times and the locomotor activity observed in the open field (Figure 2E; $df = 14$, $R = 0.72$, $p < 0.01$). In other words, Bax animals that showed high immobility time values also exhibited high locomotor activity.

Regarding the animals' turning activity, consistent with our previous studies (Krahe et al., 2002; Schmidt et al., 1999), the number of 30° turns declined within the testing session (Figure 3A; RM ANOVA: *time-interval*, $F = 47.9$, $df = 2.6/56.2$, $p < 0.001$). Contrary to what was observed in the open field test, significant differences between Bax KO and control animals were observed only in the first minute of the testing session, where controls

displayed on average more 30° turns than Bax KO mice (Figure 3A-B; ANOVA: $F = 5.6$, $df = 1/23$, $p < 0.05$).

Similarly to what was observed for immobility, the number of total 30° turns was correlated to the total distance traveled in the open field only for Bax KO mice (Figure 3C-D). However, Bax KO mice displayed a significant negative correlation between 30° turns and locomotor activity: animals that swam less exhibited high open field locomotion (Figure 3D; $df = 14$, $R = 0.64$, $p < 0.01$). This correlation was somewhat expected since there was a strong correlation between immobility values and 30° turns ($df = 14$, $R = 0.86$, $p < 0.0001$).

3. Discussion

Here we evaluated the behavioral performance of the Bax KO mouse strain in the open field and forced swimming tests. These tests were chosen to gain insights to the spontaneous locomotor activity and depressive-like behavior of mice. We observed that deletion of the pro apoptotic gene Bax significantly affected these behavioral traits.

Apoptosis plays a vital role in the morphogenesis and functional organization of the nervous system by controlling the number of neurons in different areas of the developing brain (Vanderhaeghen and Cheng, 2010; Williams and Herrup, 1988). Disruption of this complex and highly conserved process by endogenous or environmental factors can lead to a wide range of deficits (Creeley and Olney, 2013; McCann and Soriano, 2012). For instance, abnormally high rates of cell death are observed in Down syndrome, bipolar disorder, and mood disorders such as depression (Berk et al., 2014; Busciglio and Yankner, 1995; Garcia-Fuster et al., 2014; Uribe and Wix, 2012; Wisniewski and Kida, 1994). Moreover, reduction in brain size, a telltale sign of fetal alcohol syndrome, is associated with ethanol-induced apoptosis (Archibald et al., 2001; Ikonomidou et al., 2001; Lebel et al., 2008; Nardelli et al., 2011). On the other hand, our results draw attention to the fact that a decrease in programmed cell death can also lead to abnormal adult behaviors. In humans, perturbations in early neuronal death leading to an excess of neurons have been associated with neuro-developmental psychiatric disorders (Mirzaa and Poduri, 2014). For example, children with autistic syndrome may present hyperplasia in the cerebellum (Saitoh and Courchesne, 1998), while disruption of developmental cell death may play a role in schizophrenia and depression (Garcia-Fuster et al., 2014). Similarly, mice lacking specific apoptotic genes show abnormal brain development, which is believed to result in anxiety (Einat et al., 2005; Luedke et al., 2013), depression, and other emotional deficits (Chen et al., 2011; Ruan et al., 2015) as well as learning and memory problems (Rondi-Reig and Mariani, 2002). Moreover, the *fmri* KO mouse, a model for fragile X syndrome has decreased apoptosis due to an increased expression of the antiapoptotic protein, Bcl-xL (Cheng et al., 2013). Thus, a fine balance between cell survival and death early during development is critical for normal brain function later in life.

Locomotor hyperactivity in the open field is commonly associated to dysfunction of mesencephalic dopaminergic neurons and their connections with the prefrontal and cingulate cortices (Sagvolden et al., 2005). Given the observed increase in spontaneous locomotor activity of Bax KO mice in the open field compared to controls, it is reasonable to suppose

that such behavioral change could be a consequence of a disrupted mesocorticolimbic dopamine system carrying an excessive number of neurons and connections. However, it was previously reported that adult Bax KO and wild type mice have similar striatal and cortical neuronal density (Gavalda et al., 2008). This finding suggests that other Bcl-2 family members might compensate for the Bax deficiency and thus allow developmental programmed cell death in the rodent mesocorticolimbic dopamine system to take place.

Similar compensatory mechanisms do not seem to occur in the hippocampus and cerebellum where significant tissue hyperplasia was observed in Bax KO animals (Fan et al., 2001; Sun et al., 2004; White et al., 1998). Moreover, this scenario is worse in the hippocampus since neurogenesis extends to adult life leading not only to a continuous increase in neuronal numbers but also to aberrant migratory patterns as well as altered maturation of adult-generated neurons (Sun et al., 2004). Interestingly, a vast body of research demonstrates that both the hippocampus and cerebellum are implicated in novelty detection, spatial-contextual processing and exploratory behavior (Ammassari-Teule and Passino, 1997; Anderson, 1994; Pierce and Courchesne, 2001; Restuccia et al., 2007; Rochefort et al., 2011; Saab et al., 2009). Particularly, several animal models with hippocampal or cerebellar dysfunctions show increased locomotor activity in the open field test, which has been commonly associated to an excess of exploratory activity when exposed to a novel environment (Clark et al., 1997; Lo et al., 2015; Maksimovic et al., 2014). Thus, impaired hippocampal and cerebellar function may underlie the observed Bax KO hyperactivity phenotype as well as the slower habituation to the open field arena (note that the decline in spontaneous locomotor activity in Bax KO mice was much more gradual than that of controls, Figure 1A).

The fact that Bax mice had significantly greater immobility times than controls in the forced swimming test and that their immobility values were positively correlated to their locomotor activity in the open field reinforces the association between environment factors and the appearance of abnormal behaviors. Rats or mice forced to swim in a water container will initially make vigorous attempts to escape that diminish over time giving way to periods of immobility when animals float at the water surface (Krahe et al., 2002). This immobile state is interpreted as a passive stress-coping strategy or evidence of behavioral despair - the loss of hope of escaping from an unpleasant environment. The latter is further supported as a sign of depression-like behavior since immobility is reduced by the administration of antidepressant medications as well as by other antidepressant treatments such as REM sleep deprivation (Porsolt et al., 1978) and electroconvulsive therapy (Porsolt et al., 1977). In this sense, the higher immobility times displayed by Bax KO animals are in accordance with the idea that disruption of programmed cell death is associated to abnormal emotional states (Kim et al., 2007; Wang et al., 2011). Nonetheless, regardless of whether immobility is a stress-coping strategy or behavior despair, it seems safe to assume that immobility is a consequence of the exposure to a novel and stressful environment, which in turn could trigger the “freezing state” displayed by Bax KO mice during the testing session. In this context, one could further conceive that such “freezing posture” reflects motor abnormality. In fact, Bax KO mice show a significant increase in both motoneuron and motor axon numbers (Deckwerth et al., 1996; White et al., 1998). However, previous studies demonstrated that Bax KO animals have normal motor behavior on tests of balance and

coordination and an increased performance on behavioral tests used to assess muscle strength (Buss et al., 2006; Gould et al., 2006; Kim et al., 2009). Similarly, despite the “freezing posture”, we could not grossly distinguish the motor performance of Bax mice from control animals in both the open field and forced swimming tests.

As mentioned above with respect to locomotor hyperactivity, several studies also point to the role of the hippocampus and cerebellum in eliciting immobile behavior in the forced swimming test (Frye and Walf, 2002; Jang et al., 2009; Luo et al., 2015; Yamamoto et al., 2015). Thus, deficits in programmed cell death and/or neuronal migration in these regions of the Bax mouse may be related to both the hyperactivity activity in the open field as well as to the higher immobility times in the forced swimming test.

The lack of significant differences in locomotor activity in the center of the open field between Bax KO and control animals (Figure 1C-D) suggests that, contrary to previous findings, Bax KO mice did not display signs of reduced anxiety (Luedke et al., 2013). The reason for the apparent discrepancy may reside in the fact that in the previous work animals were tested in the elevated plus maze. Several studies in rodents point out to conflicting results between open field and elevated plus maze regarding anxiety states (Carola et al., 2002; File, 1991; Ramos et al., 1997; Trullas and Skolnick, 1993). Nonetheless, it is important to mention that similar to our findings, Luedke and collaborators (2013) reported that Bax KO mice exhibited increased locomotor activity in the elevated plus maze, which supports our interpretation of an augmented exploratory behavior phenotype in these animals.

Overall, our results show that Bax deficient male mice display clear and significant behavior alterations in the open field and forced swimming tests, highlighting the importance of performing behavioral analyses of transgenic and knockout mice.

4. Experimental procedure

4.1 Animals

All procedures were performed in compliance with the institutional animal care and use committee at (IACUC) Virginia Commonwealth University. Animals were maintained on a 12:12 h light/dark cycle (lights on: 6:00 h, lights off: 18:00 h) at controlled temperatures (22 ± 2 °C). Access to food and water was unrestricted. Bax mutant mice (B6.129X1-Bax^{tm1Sjk/J}) were maintained on a C57BL/6J background and were purchased from the Jackson Laboratory (Bar Harbor, ME). Mating of heterozygotes yields animals with the wild-type (+/+), heterozygous (+/-), and homozygous recessive (-/-) genotypes for the Bax allele in expected Mendelian ratios. At weaning (21st postnatal day) animals from the same litter were separated by sex and, as previously described (White et al., 1998), were genotyped by PCR amplification of tail DNA using a set of three primers (Figure 4). Male homozygous Bax^{-/-} (Bax KO, n = 17) and Bax^{+/+} (n = 13) as well as heterozygous Bax^{+/-} (n = 12) were used for this study (mean age = 79.1 ± 4.7 days, number of litters = 15). Females were not used in this study due to hormone-regulated, sexually dimorphic cell death observed in the developing rodent brain (Forger, 2006) and because previous results showed that both spontaneous locomotor activity and depressive-like responses in females are

markedly influenced by estrous cycle hormonal fluctuations (Morgan et al., 2004). All behavioral testing was performed during the light period of the light-dark cycle and were carried out on a single day. Animals were tested on the open field first (between 09:00 and 10:30 h) and then in the forced swimming test (between 14:00 and 15:30 h). To minimize nonspecific stress responses, all mice were handled daily by the experimenter for at least 1 week prior to the testing sessions and were also habituated to the testing room for 20 min before the behavioral procedures.

4.2 Open field

The open field consisted of a plastic container (diameter = 55.0 cm) surrounded by 18 cm high walls. The floor was divided in two concentric circles of equal area (inner circle diameter = 19.4 cm), and it was colored white to ensure good contrast between the animal and the background. Animals were placed in the center of the arena and the total distance ambulated was tracked and quantified for 5 min by an automated device (Videomot2, TSE Systems, Midland, MI). At the end of the testing session, the animal was returned to its home cage, and the open field's arena was thoroughly cleaned before testing another animal. The total distance traveled (in cm) was used to quantify spontaneous locomotor activity in the periphery and in the central portion of the open field. Activity data were lost for 6 control animals due to problems with the open field tracking system.

4.3 Forced Swimming test

Each animal was placed in the center of a plastic container (diameter = 26 cm, height = 32 cm) filled with water (depth = 27 cm) at about 25°C. The animal's behavior was continuously recorded for 5 min by an overhead video camera and the container was cleaned and the water exchanged across testing sessions. Later, the time the animal remained floating with all limbs and tail motionless (immobility time) as well as the animal's swimming displacements, expressed by the number of 30° turns (Krahe et al., 2002; Schmidt et al., 1999) were measured by an observer blind to the animal's genotype. Briefly, a transparent overlay with 30° axes was matched with the image of the circular container on the screen of the video monitor (Schmidt et al., 1999). For each animal, the number of consecutive 30° turns was counted for the total testing session (5 min). Measurements were interrupted whenever the animal presented a shift in direction, ceased to move, or floated passively. The video file of 1 knockout animal was lost because of a hard-disk failure.

4.4 Statistical analysis

For all behaviors evaluated, the total time of each test was divided in five equal 1 min intervals. Comparisons were carried out using repeated measures analyses of variance (RM ANOVA). The time interval was considered the within-subjects factor. When a significant effect of time interval or interaction between time-interval and genotype were detected in RM ANOVAs, appropriate lower-order ANOVAs were performed for each time interval. To minimize litter effects and avoid over-sampling, for all variables, data from animals of the same litter were averaged (Wainwright, 1998). For all measurements no differences were observed between Bax $+/+$ and Bax $+/-$ and data were pooled together (control group; Supplementary Table 1).

For simplicity, we will report results based only on the averaged univariate F tests. The univariate approach is considered more powerful than the multivariate criteria (Huynh and Feldt, 1976). However, each univariate test requires that the variances of all transformed variables for an effect to be equal and their covariances to be zero (Huynh and Feldt, 1976). Therefore, the extent to which the covariance matrices deviate from sphericity was estimated by Mauchly's test. Whenever the sphericity assumption was violated, we used the Greenhouse-Geisser correction, which adjusts the degrees of freedom, in order to avoid Type I errors. All analyses were carried out with IBM SPSS Statistics v.20 (IBM Corp, Chicago, IL) and all data are shown as mean and $\pm$ SEM. Significance was assumed at the level of $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Increased locomotor activity in Bax KO mice. **A and B**, Bax KO mice show increased overall locomotor activity in the open field compared to control animals, both across (A) and in the total testing period (B). **C and D**, mean percent activity in the inner area of the open field for Bax KO and control animals across 1 min time-intervals (C) and the total testing period (D). Bax KO, n = 17 animals from 14 litters; Controls, n = 19 animals from 7 litters. Symbols and Bars are means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 2.

Bax KO mice show greater immobility time than controls, but similar turning activity. **A and B**, Bax KO mice are more immobile than control animals in the forced swimming test, both across (A) and in the total testing period (B). Bax KO, $n = 16$ animals from 13 litters; Controls, $n = 25$ animals from 9 litters. Symbols and Bars are means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$. **C**, frame grabbed video images depicting the immobile behavior of 3 distinct Bax KO animals. Arrows highlight hind limb positions. **D and E**, plots showing the correlation between immobility time and overall locomotor activity in the open field for control (left)

and Bax KO animals (right). Each dot represents a single mouse. The solid line represents the linear fit (**D**, $p = 0.7$; **E**, $p < 0.01$).

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Figure 3.

Swimming activity in Bax KO mice. **A and B**, number of total 30° turns (turns to the left and right) during (A) and in the whole testing session (B) for Bax KO and control mice. Bax KO, $n = 16$ animals from 13 litters; Controls, $n = 25$ animals from 9 litters. Symbols and Bars are means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$. **C and D**, plots showing the correlation between total 30° turns and overall locomotor activity in the open field for control (left) and Bax KO animals (right). Each dot represents a single mouse. Solid lines represent the linear fit (C, $p = 0.5$; D, $p < 0.01$).

Figure 4.

Identification of Bax KO mice. Representative cases of PCR-based genotyping on tail DNA of Bax-deficient ($-/-$) and control ($+/+$ and $+/-$) mice. GAPDH was used as a loading control. $n = 3$ animals (samples in duplicate).