Increased anxiety-like behaviors and mitochondrial dysfunction in mice with targeted mutation of the Bcl-2 gene: Further support for the involvement of mitochondrial function in anxiety disorders

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Abstract

There is growing evidence that anxiety disorders are associated with impairments of cellular plasticity and resilience. Paralleling these advances in our understanding of the neurobiologic underpinnings of anxiety disorders is the growing appreciation of the diverse functions that mitochondria play in regulating integrated CNS function. The emerging data suggest that mitochondrial Ca2+ sequestration has a key role in modulating the tone of synaptic plasticity in a variety of neuroanatomical regions, including those implicated in the pathophysiology of anxiety disorders. Furthermore, activation of peripheral mitochondrial benzodiazepine receptors resulted in reduced anxiety in rats. One of the major modulators of mitochondrial function is Bcl-2 protein imbedded in the inner mitochondrial membrane. Bcl-2 overexpression increases mitochondria Ca2+ uptake capacity and resistance to Ca2+ inhibition of respiration and upregulation of Bcl-2 increases maximal uptake capacity of mitochondria. We have, therefore, explored the significance of Bcl-2 in the association between mitochondrial function and affective disorders testing Bcl-2 heterozygote mice in models of affective and anxiety disorders.

Mutant mice have reduced mitochondrial Bcl-2 levels, and although they have no gross behavioral abnormalities, they demonstrate a significant increase of anxiety-like behaviors. Bcl-2 heterozygote mice spent less time in the center of an open field, spent less time outside an enclosure in the "emergence test", were less likely to explore the transparent part of a black/white box or the open arms of an elevated plus maze compared with WT controls. Mutant mice did not differ from WT in measures of locomotion or in the forced swim test for depression-like behavior suggesting a specific effect on anxiety-like behaviors.

Our study, therefore demonstrates that Bcl-2 may be a key factor in anxiety disorders and that its effects may possibly originate from its role in the mitochondria.

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In mood disorders by utilizing a variety of biochemical and neuroendocrine strategies. While such investigations have been heuristic over the years, they have been of limited value in elucidating the unique biology of anxiety disorders. The recognition of the clear need for better treatments and the lack of significant advances in our ability to develop novel, improved therapeutics for these devastating illnesses has led to the investigation of the putative roles of intracellular signaling cascades and non-aminergic systems in the pathophysiology and treatment of anxiety disorders. Consequently, recent evidence demonstrating that impairments of cellular plasticity and resilience may underlie the pathophysiology of anxiety disorders, and that effective treatments (notably antidepressants) exert major effects on signaling pathways which regulate neuronal circuitry, the regulation of intracellular calcium (Ca^{2+}), and critical underpinnings of these disorders. Paralleling these advances in our understanding of the neurobiologic underpinnings of anxiety disorders is the growing appreciation of the diverse functions that mitochondria play in regulating integrated CNS function. Thus, mitochondria are intracellular organelles best known for their critical roles in regulating energy production via oxidative phosphorylation, the regulation of intracellular calcium (Ca^{2+}), and critical mediators of apoptosis; however, increasing evidence suggests mitochondria may be integrally involved in the general processes of synaptic plasticity. In a detailed investigation of the relative roles of mitochondrial and ER Ca^{2+} buffering, it was found that the dendra mitochondrial quickly accumulates Ca^{2+}, while the endoplasmic reticulum displays a more delayed increase in Ca^{2+} during high frequency stimulation [49]. Furthermore, increased synaptic activity has been shown to induce the expression of mitochondrial-encoded genes, suggesting that the regulation of metabolism is an important component in the long-term regulation of synaptic strength [66]. This regulation occurred even with stimulation that were under the threshold for long-term potentiation induction, suggesting that a sort of ‘metabolic priming’ might take place. All in all, these findings suggest that mitochondrial Ca^{2+} sequestration has a key role in modulating the tone of synaptic plasticity in a variety of neuroanatomical regions, including those implicated in the pathophysiology of anxiety disorders. In total, these observations suggest that regulation of mitochondrial function is likely to play important roles in regulating synaptic strength neuronal circuitry mediating complex behaviors [56]. For further discussion of recent research into the mitochondrion’s role in synaptic plasticity, the reader is referred to Matson & Liu’s recent review [39]. For the purposes of the present discussion, it is noteworthy that there has already been indirect evidence accumulating suggesting that mitochondrial function may be related to the pathophysiology and treatment of behavioral disorders. Thus, monoamine oxidase inhibitors (MAO-I) which are arguably amongst the most potent anxiolytics and antidepressants, improve mitochondrial efficiency [21,38,62] and protect mitochondrial function against a variety of insults [1,7,10,38,48,58]. Moreover, in vivo animal studies demonstrated that activation of peripheral mitochondrial benzodiazepine receptors resulted in reduced stress and anxiety in rats [4,51]. Additionally, mitochondria have specific binding sites for neurosteroids that are major modulators of Ca^{2+} efflux [24] and were shown to protect mitochondria from a variety of insults (e.g. [27,44]. Neurosteroids are well known for their anxiolytic properties in animal models (e.g. [2,52]) and it is, therefore conceivable that their properties as anxiolytics may be related to their actions in the mitochondria. These studies suggest that targeting mitochondrial function may represent a novel avenue for the development of therapeutics for the treatment of anxiety disorders. In this context, one of the major modulators of mitochondrial function is the protein Bcl-2 (originally identified from b cell leukemias). Bcl-2 is the first gene shown to be involved in apoptosis. The Bcl-2 family consists of both pro- and anti-apoptotic proteins. The expression of bcl-2 was localized to a large population of neurons and some glial cells in CNS and PNS [19,32]. It is present in the outer mitochondrial membrane, endoplasmic reticulum and nuclear membrane interacting with other Bcl-2 family members (such as Bax and Bad). A number of mitochondrial related mechanisms have been proposed for the action of Bcl-2, including increasing mitochondrial calcium buffering capacity and protecting mitochondrial membrane integrity, so preventing the release of cytochrome c and the formation of the apoptosome and activation of caspases. It can prevent mitochondrial dysfunction such as membrane potential loss and the permeability transition (PT) precedes cell death. In studies of isolated mitochondria, Murphy et al have shown that Bcl-2 overexpression increases mitochondrial Ca^{2+} uptake capacity, increasing the resistance of mitochondria to Ca^{2+}-inhibition of respiration [45]. Thus, Bcl-2 overexpressing cells appear to be particularly resistant to the destructive influence of elevated intracellular Ca^{2+}. Bcl-2 likely exerts significant effects on cellular Ca^{2+} buffering not only under the extreme conditions of Ca^{2+}-induced apoptosis, but potentially even during normal synaptic activity. Thus, Murphy et al. [45] demonstrated that upregulation of Bcl-2 increases the maximal uptake capacity of mitochondria, effects which likely maintain mitochondrial activity during very high frequency stimulation. Recent studies suggest that Bcl-2 may have a more general role in regulating mitochondrial metabolism and function [34], and that its protective effect may not be limited to an antiapoptotic role. Its overexpression reduces the rate of mitochondrial ATP consumption under conditions in which ATP hydrolysis is stimulated [25]. Overexpression of bcl-2 in the desmin null heart results in correction of mitochondrial defects [65]. Interestingly, in the context of anxiety disorders, it was demonstrated that mice with overexpression of the Bcl-2 gene exhibited reduced anxiety-like (fear) behavior [54]. Inactivation of bcl-2 results in progressive degeneration of the nervous system.
motoneurons, sympathetic and sensory neurons during early postnatal development, and enhanced oxidative stress and susceptibility to oxidants as well as altered levels of anti-oxidant enzymes have been observed in brains of Bcl-2-deficient mice [22]. Most of the studies that have contributed to elucidation of the roles of Bcl-2 have been performed using overexpression or deficient rodent systems. Nevertheless, it is conceivable that the study of animal behaviour when Bcl-2 is down-regulated may contribute further to the understanding of its functions. In many neurological disease models, altered Bcl-2 family member mRNA and protein expression has been observed. These findings provide hope that development of targeted pharmacological agents that enhance anti-apoptotic Bcl-2 family function or inhibit pro-apoptotic Bcl-2 family function will prove useful in the treatment of human neuropathological conditions.

To further explore the involvement of mitochondrial function in affective disorders and particularly in anxiety disorders, and the importance of Bcl-2 in this context, the present study was designed to explore the behavioral outcome of deletion of the Bcl-2 gene in mice in behavioral models of psychiatric disorders in mice with targeted mutation of the Bcl-2 gene (heterozygote mice). We found that these mutant mice – which have reduced mitochondrial Bcl-2 levels – do not show gross behavioral abnormalities but demonstrate significant increase in a number of animal models of anxiety-like behavior.

2. Methods

2.1. Animals

Male Bcl-2 heterozygote mice and colony inlernates Wild Type controls (Strain Name: 129S1/SvImJ-Bcl2tm1Mpin/J) originally developed by Dr. S.J. Korsmeyer [64] were purchased from Jackson Laboratories (Jackson Laboratories, ME). We chose not to use null mutants because they were reported to have retarded growth, a variety of peripheral disorders and they die at young age [64] and accordingly are not appropriate for behavioral studies whereas clear pathologies were not reported for the heterozygote animals [41,64]. Breeding was not performed in our lab and the source of all mice was Jackson Labs and all details are available at their web site at www.jax.org. Mice were transported to our laboratory and experimentation started no less than a one week later, to allow for appropriate acclimation time. At the beginning of experiments mice weighed 25–30 g and were housed, two per cage in an animal room with constant temperature (22 ± 1 °C) and 12h light/dark cycle (lights on/off at 6:00 a.m./p.m.), with free access to food and water. All experiments were performed in the early phase of the light cycle under standard room fluorescent lights. All experimental procedures were approved by the Animal Use Committee of the National Institute of Mental Health (Protocol number 1171) and were conducted according to NIH guidelines.

2.2. Sequence of experiments

The sequence of experiments was designed from less to more intrusive in order to minimize the effects of previous experiments on future behaviors [9]. Accordingly, mice were tested in the following sequence: emergence test; open field test; black/white box test; elevated plus-maze test; forced swim test. Mice had at least 3 days separating between consecutive tests. Approximately, a week after the end of all behavioral testing, mice were decapitated, their brains extracted and used for the mitochondria assay.

2.3. Equipment and procedures

2.3.1. Large open field

A 120 cm × 120 cm transparent Plexiglas platform with 35 cm high transparent walls, elevated 35 cm above the floor served to study locomotor and exploratory behavior. Such large open fields have previously been demonstrated to be much more effective in the study of behavior compared with the more commonly used, smaller activity monitors [11]. As done previously [16] the center square of the open field, measuring 40 X 40 cm was defined as the “Center” area of the field. A video camera was placed 2.3 m above the center of the open field and interfaced with a computer and a VCR. Data were collected by the Noldus Ethovision system, a video tracking system designed to study spatial behavior and simultaneously videotaped for further analysis. The Ethovision system follows the center of the body of the mouse and records its position according to the location of this point. Each mouse was placed individually in the center of the open field and its behavior tracked for a 45 min session. At the end of the session, mice were returned to their home cages. Locomotor activity measures as well as time and frequency measures for center visits were collected. The open field was wiped clean between trials with a 10% alcohol solution.

2.3.2. Elevated plus-maze

A Plexiglas + shaped maze with two dark, enclosed arms and two open and lit arms, without walls, elevated 50 cm above ground served to examine anxiety-like behavior. The size of the arms was 30 cm × 5 cm with a 5 cm × 5 cm center area and the walls of the closed arms were 40 cm high. A video camera tracking system (Ethovision, Noldus VA) was used to track mouse activity in the maze as described above. Each mouse was placed in the center of the maze for a 5 min session and then placed back in its home cage. Time and frequency of visits to the different zones of the maze, as well as locomotion measures were collected for later analysis. The plus-maze was wiped clean between trials with a 10% alcohol solution.

2.3.3. Emergence test

A 65 cm × 45 cm gray metal table served as the platform for the emergence test designed to examine anxiety-like behaviors. A white (opaque) cup, 6 cm in diameter and 10 cm deep, was attached to the corner of the platform facing the center of the table. At the beginning of the test session each mouse was placed inside the cup and its behavior videotaped for 5 min. The latency to exit the cup, number of exits from the cup to the open arena and time spent outside the cup were manually scored from videotapes for later analysis. At the end of the session mice were returned to their home cages and the cup and area wiped clean with a 10% alcohol solution.

2.3.4. Black/white box

The black white box test is another model for anxiety-like behavior. A Plexiglas box (50 cm × 25 cm with 30 cm walls) was divided into two compartments: black (one-third of the box) and transparent (two-thirds of the box) with a separating door that can be open to

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2.4.3. Western blotting

and the rate was calculated on the graph automatically. Oxygen consumption was recorded for 5 min, using Clark type oxygen electrodes (Hansatech Instruments, UK). Fifty microliters of fresh homogenate sample was added to the oxygraph chamber which containing 300 μl respiration medium (same as isolation buffer). It has previously been shown that this procedure yields pure mitochondrial preparations which are practically free from cytosolic contamination. Protein concentration was measured using Bio-Rad Protein Assay Reagent. The forebrain region was chosen because it provides enough tissue to isolate enough high-quality mitochondria fraction. We assume there is an overall reduction in Bcl-2 levels in all brain regions because this transgenic KO is not conditional or region-specific.

2.4.4. Measurement of oxygen consumption

Measurement of oxygen consumption was carried out at 28°C using Clark type oxygen electrodes (Hansatech Instruments, UK).

2.4.5. Western blotting

5x loading buffer (0.3 M Tris, pH 6.8, 50% glycerol, 10% sodium dodecyl sulfate, and 12.5% 2-mercaptoethanol), was added to the sample aliquot, and heated at 95°C for 5 min. One microgram of sample was loaded onto 4–20% SDS-polyacrylamide gels and resolved by standard electrophoresis. Gels were blotted electrophoretically to nitrocellulose membrane in a transfer tank using a constant current of 350 mA for 3 h. The membrane blots were blocked for 1 h at room temperature in 5% dry milk dissolved in Tris-buffered saline (TBS-T; 50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20). Then blots were incubated with an anti-Bcl-2 (Santa Cruz Biotechnology, Inc., diluted 1:500) antibody in blocking buffer. The blots were washed four times with TBS-T for 10 min each wash and then incubated with an HRP-conjugated anti-rabbit secondary antibody (Amersham Biosciences, diluted 1:3000). After four more washes, the blots were developed with Enhanced Chemiluminescence (Amersham Biosciences, Arlington Heights, IL) and visualized with Kodak Biomax MR imaging film. Densitometric analysis of immunoreactivity was conducted with a Kodak Image Station 440CF.

2.5. Data analysis

Data for all tests were analyzed using a Student’s t-test. Significance level was set at p < 0.05. Since some of the behavioral experiments were run in two batches, results for these experiments were first tested for homogeneity of variance across trials. Since variance was not significantly different (Levene’s test) data from the two replications were pooled.

3. Results

3.1. Bcl-2 heterozygote mice have reduced mitochondrial Bcl-2 levels

Mice heterozygote for the Bcl-2 gene had less than 40% Bel-2 mitochondrial protein compared with wild type controls (Fig. 1; N = 4/group, p < 0.005).

3.2. Bcl-2 heterozygote mice spend less time in the center of the open field

Bcl-2 heterozygote mice did not differ from their colony WT controls in measures of locomotor activity across the ses-

![Graph](image-url)

Fig. 1. Bcl-2 mitochondrial levels. Heterozygote mice had a significantly reduced Bcl-2 mitochondrial protein level than WT controls. Measures reflect densitometric analysis of immunoreactivity. *N = 4/group, (*) denotes p < 0.05, N = 10/group.
Table 1

<table>
<thead>
<tr>
<th>Test</th>
<th>Behavior</th>
<th>Results (mean ± S.E.)</th>
<th>N</th>
<th>Statistics</th>
</tr>
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<tbody>
<tr>
<td>Black/white box</td>
<td>Frequency of visits to white</td>
<td></td>
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<td></td>
<td>WT: 24.7 ± 2.2</td>
<td>6</td>
<td></td>
<td>t(10)=1.2, p=0.25</td>
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<tr>
<td></td>
<td>Het: 21.0 ± 2.2</td>
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<tr>
<td>Forced swim test</td>
<td>Immobility time (s)</td>
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<tr>
<td></td>
<td>WT: 141 ± 14.9</td>
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<td></td>
<td>t(14)=0.8, p=0.87</td>
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<tr>
<td></td>
<td>Het: 137 ± 19.1</td>
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<td></td>
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<tr>
<td>Elevated plus maze</td>
<td>Entries to closed arms</td>
<td></td>
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<tr>
<td></td>
<td>WT: 20.1 ± 1.3</td>
<td>10</td>
<td></td>
<td>t(18)=0.05, p=0.96</td>
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<tr>
<td></td>
<td>Het: 20.2 ± 1.7</td>
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<td>Time in closed arms (s)</td>
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<tr>
<td></td>
<td>WT: 172.6 ± 12.0</td>
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<td>t(18)=0.96, p=0.35</td>
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<tr>
<td></td>
<td>Het: 190.4 ± 14.3</td>
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<td>Distance (m)</td>
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<tr>
<td></td>
<td>WT: 15.0 ± 0.7</td>
<td>10</td>
<td></td>
<td>t(18)=1.6, p=0.13</td>
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<tr>
<td></td>
<td>Het: 13.5 ± 0.7</td>
<td>10</td>
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sion (Table 1), however, the mutant mice spent significantly less time in the center of the open field (Fig. 2, t(18)=2.26, p<0.04), a measure reflecting increased anxiety-like behavior.

3.3. Bcl-2 heterozygous mice spend less time in the sheltered area in the emergence test

Bcl-2 heterozygote mice spent less time outside the "sheltered" area compared with the WT controls (Fig. 3; t(18)=2.48, p<0.03). No differences between the groups were observed in the latency for the first exit out of the shelter or the distance traveled outside of the shelter (Table 1). The Emergence test data, therefore, supports an anxiety-like behavior in the mutant mice.

3.4. Bcl-2 heterozygous mice spend less time in the transparent part of the box in the black-white box

As in the previous tests, Bcl-2 heterozygote mice demonstrated anxiety-like behavior spending less time in the transparent part of the box compared with the WT controls (Fig. 4; t(10)=2.56, p<0.03) but there was no effect on the number of transitions between the parts of the box (Table 1). Inter-

Fig. 2. Behavior in the open field: heterozygote mice spent significantly less time in the center of a large novel open field (t(18)=2.26, p<0.04; A), without changes in total activity levels (t(18)=0.7, p=0.49; B), behavior that is representative of increased anxiety levels. (*) denotes p<0.05, N=10/group.

Fig. 3. Behavior in the emergence test: the time spent outside of an enclosed shelter, and on an unfamiliar open arena was significantly shorter for heterozygote Bcl-2 mice compared with their wild type counterparts (A t(18)=2.48, p<0.03) but there was no effect on the amount of locomotion; (B t(18)=0.3, p=0.97) (* denotes p<0.05, N=10/group.
Fig. 4. Black/white box: time spent in the transparent part of the black/white box was significantly shorter for the heterozygote Bcl-2 mice compared with wild type controls. (*) denotes \( p < 0.05; \ N = 10/\text{group}.\)

Fig. 5. Elevated plus-maze: number of entries into the open (threatening) arms of the elevated plus maze was significantly lower in the heterozygote Bcl-2 mice compared with wild type controls. (\(*\) denotes \( p < 0.05; \ N = 10/\text{group}.\))
well being, the previous reports as well as the specificity of changes reported here do suggest that the increased anxiety-like behavior is indeed directly related to the genetic mutation.

Notably, mitochondrial function has been recently associated with the pathophysiology and treatment of affective and anxiety disorders. Thus, decreases in mitochondrial production rate (MAPR) and enzyme ratios were found in patients with major depression compared with healthy controls with a correlation between MAPR and anxiety (Karolinska Scale of Personality) [17] and polymorphisms in genes coding for mitochondrial DNA were found to be a risk factor in bipolar disorder [28]. Furthermore, MAO-I’s, known for their anti-anxiety (as well as antidepressant) properties, improve mitochondrial function [65], activation of mitochondrial benzodiazepine receptors reduced stress and anxiety in rats [45] and neurosteroids that had been extensively documented as having anti-anxiety properties [2,52] have specific binding sites on mitochondria and were demonstrated to modulate mitochondrial 

Expression of Bcl-2 in rat hippocampus [33] and a variety of psychiatric disorders. For example, the Bax/Bcl-2 ratio varies and can be regulated at the transcriptional level [57,69], although it is not markedly upregulated by activation events. It has been proposed that transcription may be negatively regulated, at least in part, by the tumor suppressor p53 [42,43]. The half-life of bcl-2 mRNA is approximately 2.5 h [57], whereas the Bcl-2 protein demonstrates considerable stability, with a half-life of 10 h or better [40,53]. Bcl-2 is a key modulator of mitochondrial function. Bcl-2 overexpression was demonstrated to increase the resistance of mitochondria to the destructive influence of elevated intracellular Ca²⁺ and enhance the maximal uptake capacity of mitochondria [45]. Bcl-2 had been implicated in a number of psychiatric disorders. For example, the Bax:Bcl-2 ratio was found to be higher in the temporal lobe of schizophrenic patients [26], repeated stress was shown to decrease brain expression of Bcl-2 in rat hippocampus [33] and a variety of psychiatric drugs including atypical antipsychotics [3], mood stabilizers [6,37] and antidepressants [67] were found to increase Bcl-2 expression and levels. Tissue culture studies showed that neurons from Bcl-2 deficient mice are more susceptible to insults compared to neurons from WT animals [18,60] offering additional support to the possible involvement of the gene in neurodegenerative (including psychiatric) disorders although no data is available at this time regarding neurodegeneration in the brain of the heterozygote mice used in the present experiments. Furthermore, in the context of anxiety it was recently reported that Bcl-2 transgenic mice with overexpression of Bcl-2 demonstrate less fear-like behavior and neophobia in the elevated plus-maze and the emergence test models than their WT controls [54]. Our present study further demonstrates that Bcl-2 may be a factor in anxiety disorders and that its effects in that context originate from its role in the mitochondria. It is still possible that the effects of Bcl-2 manipulations on anxiety levels may be related to the activity of the molecule outside the mitochondria as Bcl-2 is also expressed in other intracellular areas, however, the crucial role of Bcl-2 in the mitochondria (e.g. [12,30]) and the increasing support for the relationship between mitochondrial function and affective disorders (e.g. [17,28]) substantiate the possibility that the currently demonstrated increased anxiety in mice with reduced Bcl-2 levels (as probably the decreased anxiety in mice overexpressing Bcl-2 [54]) are related to changes in the mitochondria.

Attention to potential drugs that can regulate the activity of Bcl-2 is already given in relation to other disorders. For example, the neuroprotective activity of propargylamines-based drugs developed in the context of dementia-related disorders has been attributed to the ability of propargylamines-inducing the antiapoptotic family proteins Bcl-2 and Bcl-xl, while decreasing Bad and Bax and preventing opening of mitochondrial permeability transition pore [68]. Yet, the future development of treatments that more directly target molecules involved in critical CNS cell survival may be a promising avenue for the development of improved long-term treatments for anxiety and affective disorders [36].

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Acknowledgments

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