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Behavioral effects of Bcl-2 deficiency: implications for affective disorders

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Abstract:
New hypotheses regarding affective disorders suggest a critical role for cellular resilience and plasticity. Bcl-2 is a central protein in these processes and is elevated by mood stabilizers and antidepressants. In previous studies, mice with targeted mutations of Bcl-2 showed anxiety-related behavioral changes. The present study further explored the relationship between Bcl-2 and behavior using mice with a targeted mutation but with a different background strain than previously tested.
Bcl-2 heterozygous mice (B6;129S2-Bcl-2<tm1Sjk>/J) were tested in models of depression, mania and anxiety. Compared to Wild Type (WT) controls, mutant mice showed behaviors modeling two facets of mania: increased reward seeking and amphetamine sensitization. Moreover, the sensitization was attenuated by chronic pretreatment with lithium. In contrast to previous data, the mutation did not affect measures of anxiety. Although data are still minimal, it supports additional studies of the role of Bcl-2 in affective and anxiety disorders. The importance of background strain in behavioral phenotypes of mutant mice is known and the current lack of effect on anxiety measures may be related to high baseline anxiety of WT animals. More precise studies of Bcl-2 in affective and anxiety disorders will be possible when specific pharmacological modulators of Bcl-2 become available.

Key words:
animal models, bipolar disorder, mania, depression, Bcl proteins

Introduction

In recent years, much evidence was presented implicating cellular resilience and plasticity in mood and anxiety disorders and their treatment [3, 8, 13, 29]. Bcl-2 is the first gene shown to be involved in apoptosis and the Bcl family of both pro- and anti-apoptotic proteins is closely involved in cellular processes related to resilience, plasticity and death. The expression of Bcl-2 is localized to a large population of neurons and some glial cells in the central nervous system (CNS) and peripheral nervous system (PNS) [22, 28].

Multiple studies demonstrated that treatment with the mood stabilizers lithium and valproate [4, 32], the antidepressants amitriptyline, desipramine and venlafaxine [26, 49] and the atypical antipsychotic drugs olanzapine and clozapine [2, 30] all increased Bcl-2 levels in the brain. These findings link affective disorders and mood regulation with cellular resilience and the ability to withstand a variety of insults [31, 43]. A few behavioral studies in mice suggested that Bcl-2 may also be related to anxiety. Specifically, mice with a Bcl-2 transgene (and hence, elevated Bcl-2 levels) showed less anxiety-like behaviors [41, 42], whereas

mice heterozygous for the Bcl-2 gene demonstrated increased anxiety-like behaviors [17].

Since psychiatric disorders are expressed mostly with behavioral features, molecular hypotheses related to the pathophysiology and treatment of these disorders must be evaluated at the behavioral level with appropriate animal models [45]. However, the existing data related to Bcl-2 involvement in affective and anxiety disorders is still limited. Moreover, to the best of our knowledge, specific Bcl-2 pharmacological modulators that can be used *in vivo* are not commercially available, and the data acquired using mice with targeted mutations may carry significant limitations [5, 6], including behavioral effects related to the background strain of the mutants [7]. Given that mice with a targeted mutation in the Bcl-2 gene were developed from more than one background strain, the present study further explored the effects of Bcl-2 gene deletion on behavior using Bcl-2 heterozygous mice from the B6;129S2-Bcl-2<tm1Sjk>/J strain. This strain is different from the 129S1/SVIMJ BCL2<TM1MPIN>/J strain evaluated in an earlier study [17].

Materials and Methods

Animals

Male Bcl-2 heterozygous mice and colony littermate Wild Type (WT) controls (N = 53 in total, 25 WT and 28 heterozygous mice; Strain Name: B6;129S2-Bcl-2<tm1Sjk>/J), 11–12 weeks old at the beginning of experiments, were bred for the current project from a cryopreserved strain by 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 early death and, therefore, are not appropriate for behavioral studies. Such clear pathologies were not reported for heterozygous animals [46]. Breeding was performed at Jackson Laboratories 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 one week later, to allow for appropriate acclimatization time. Mice were transported in two batches, the first one including 10 WT and 8 heterozygous mice and the second, approximately a month later, included 15 WT and 20 heterozygous. Mice were weighed at the beginning of the experiment. Weights of mice from the second batch were also followed every week thereafter. Animals were singly housed in an animal room with constant temperature (22 ± 1°C) and 12 h light/dark cycle (lights on/off at 7:30 a.m./p.m.), with free access to food and water. All experiments were performed in the early/mid phase of the light cycle under standard room fluorescent lights. All experimental procedures were approved by the University of Minnesota IACUC (Protocol number 0504A68808) and were conducted according to NIH guidelines.

Sequence of experiments

Considering the difficulty in obtaining mice with targeted mutations, it was not possible to use each mouse for just one behavioral test. Mice from the first batch (N = 18) were tested for locomotor activity, saccharin preference, black white box and response to acute amphetamine administration (see below for detailed descriptions of tests). Mice from the second batch (N = 35) were tested for locomotor activity, saccharin preference, elevated plus maze, resident-intruder, forced swim test and amphetamine/lithium interaction (see below for detailed descriptions of tests). Order of tests was planned from less to more intrusive to minimize the effects of previous experiments on future behaviors [6].

Drugs

Amphetamine sulfate (Sigma, St. Lewis, MI) was dissolved in saline and injected *ip* at a 1 or 2 mg/kg dose (for the acute experiment with the first batch of mice) or in a 1 mg/kg dose (for the amphetamine/lithium interactions experiment). Regardless of dose, amphetamine was diluted in saline to 10 ml/kg volume. These doses were chosen based on studies demonstrating their behavioral effects [44]. This 1 mg/kg dose is relatively low [23] and was selected based on the results of the preliminary experiment and to minimize the possible effects of amphetamine to induce locally oriented (stereotypical) movements in high doses.

Lithium chloride (Sigma, St. Lewis, MI; 100 mg/kg) was diluted in saline and injected *ip* once daily (as detailed below) at a 10 ml/kg volume. This dose was chosen as it was demonstrated to ameliorate the behavioral effects of amphetamine in many strains of mice [21, 24]. In addition to tap water, a bottle of sa-

line was available for mice receiving lithium to minimize any electrochemical imbalance that may occur because of the diuretic properties of the drug [16].

Equipment and procedures

Spontaneous activity in a large open field

An 80 × 80 cm white painted wooden box with walls 35 cm high served to study locomotor and exploratory behavior [9]. A 30 × 30 cm central square of the open field was defined as the "center" of the field. A video camera was placed above the center of the open field to digitally record each session to be later analyzed by the Ethovision videotracking system (Noldus, VA), a video tracking system designed to study spatial behavior. Each mouse was placed individually in the center of the open field and its behavior was tracked for a 60 min session. Locomotor activity (distance traveled), time spent in "center", and frequency of "center" visits were collected. The open field was wiped clean between trials with a 10% alcohol solution.

Black/white box test

The black/white box test is a frequently used model for anxiety-like behavior that assesses the balance between nocturnal rodents' fear of open, well lit areas versus their exploratory drive [17]. A painted wooden box (60 × 40 cm with 45 cm high walls) was divided into a black, covered, compartment comprising one-third of the box and a white, well lit, compartment comprising the remaining two-thirds. The compartments were separated by a door that remained open during sessions to allow free transitions between the compartments. Each mouse was placed in the white part of the box and allowed to freely move for a 5 min session. The sessions were digitally recorded and behavior was later manually scored from digital files for time spent and frequency of visits in the white part of the box. At the end of the session mice were returned to their home cages and the box was wiped clean with a 10% alcohol solution.

Forced swim test (FST)

The FST is a frequently used test for antidepressant activity [9, 12, 38]. For the present experiment, each mouse was placed in a transparent cylindrical container (25 cm tall and 18 cm diameter) filled 3/4 with water at 22–23°C so that a mouse cannot touch the ground or climb out of the container. Behavior was digitally recorded from the side for a 6 min session. The last 4 min of each session were then manually scored from digital files for active (swimming and struggling) versus passive (floating with only minimal movements) behaviors. Water in the container was changed after each session.

Saccharin preference

A sweet solution preference test evaluates hedonistic-like or reward seeking behavior [47]. The sweet solution preference test is commonly utilized to evaluate anhedonia after induction of depression-like state in rodents and to evaluate the effects of antidepressants [35, 36]. Similarly, it can be used to explore increased reward seeking, a facet of mania [10, 11]. Mice were supplied with a bottle of 1% saccharin solution (Sigma, St. Lewis, MI) in addition to their regular supply of water and food. Bottles were present for 2 days. Weights of saccharin solution and water bottles were taken at the beginning of the experiment and every 24 h thereafter. Daily saccharin preference (consumption of saccharin solution as a percentage of total liquid consumption) was computed. This computation is needed to overcome differences that may be related to the different weights or different drinking patterns of mice.

Elevated plus-maze (EPM) test

The EPM test is an additional frequently used model testing anxiety-like behaviors [25] and risk evaluation [1] in rodents. A sealed natural wooden + shaped maze with two dark, closed arms and two open, lit arms without walls, elevated 60 cm above ground served to examine anxiety-like behavior. The size of the arms was 30 × 5 cm with a 5 × 5 cm central area and the walls of the closed arms were 24 cm high. Each mouse was placed in the central area of the maze and behavior was digitally recorded with an overhead camera for a 5 min session. Recordings were used for manual scoring of time and frequency of visits in the open and the closed arms of the maze. The plus-maze was wiped clean between trials with a 10% alcohol solution.

Resident-intruder test

The resident intruder test is commonly used for aggression research [34] and was recently validated as a possible model for the aggression facet of mania [11]. For the present experiment, resident mice were transferred in their home cages to an experimental room and the cage covers were removed. After a 2 min adaptation period, an intruder animal (naive C57bl/6 mice, approximately the same age and size, housed 4/cage) was introduced into the cage and behavior was digitally recorded for a 10 min session. At the end of the session the intruder was removed and placed back in its home cage, the resident's cage was covered and both mice were moved back to the colony room.

As repeatedly demonstrated [11, 34], resident mice attacked the intruders during the session. When such attacks happened and became vicious, the experimenter used a probe to separate the animals and, therefore, avoid serious harm and injury and minimize pain and distress. In these events, the experimenter kept the animals separated for a few seconds and then withdrew the probe so the experiment could continue. Because of this design, measures of total time spent in aggressive interactions could not have been scored. This simplified version of the test was recently used to detect the effects of mood stabilizers to reduce aggressive behavior in mice [11]. Recordings were used to score the number of aggressive interactions during the session. Incidents of non-aggressive interactions were also scored. Aggressive interactions were defined as attempts to bite, actual bite, boxing postures and wrestling postures. Non-aggressive interactions were defined as other types of body contact, including sniffing, allogrooming and body contact. Behaviors performed by the mice when not interacting with each other were not scored.

Acute amphetamine response

Sensitivity to psychostimulant drugs is one of the domains of bipolar disorder and the acute response to amphetamine is a frequently used model to screen for mood stabilizing agents [15, 18, 20]. For the present study, mice were divided to three groups treated with acute 1 mg/kg, 2 mg/kg amphetamine or with vehicle. Immediately after injection, each mouse was placed individually in the center of a large open field and behavior was videotracked for a one hour session (as described above for the spontaneous activity test).

Amphetamine sensitization

Sensitized response to psychostimulants is an accepted model for manic-like behavior in rodents [39] and has been reported to be ameliorated by the prototypic mood stabilizers lithium and valproate [19, 40]. For the present study, mice were divided into two groups receiving daily lithium/vehicle (saline) injections for 10 days after which amphetamine treatment and testing began. Lithium administration continued throughout the entire experiment. On test days, 30 min after the last lithium injection, mice received an *ip* injection of amphetamine and were each placed in an activity monitor (35 × 28 cm with 25 cm high walls, transparent plastic cages; OptoM-3, Columbus Inst. Columbus, OH) for a 60 min session. Activity levels (number of crossings of infrared beams) were collected in 10 min intervals for the entire session. The experiment included 4 test sessions spread 2 days apart so each mouse was exposed to amphetamine and tested 4 times.

Statistical analysis

Student's *t*-test, repeated measures ANOVA and one-way ANOVA were used to analyze the results of the different experiments as was appropriate for each experimental design (specific statistical method for each experiment is shown in the results section). Level of statistical significance was set at $p < 0.05$. Data for the open field experiment and the saccharin preference experiment were pooled for the two batches of mice after no statistical differences were found between the two groups.

Results

As previously demonstrated with Bcl-2 deficient mice from the 129S1/SVIMJ BCL2<TM1MPIN>/J strain [17], heterozygous B6;129S2-Bcl-2<tm1Sjk>/J mice were not different from their wild type littermate in initial weight and weight gain, spontaneous activity levels in the large open field, behavior in the FST and response to acute amphetamine (Tab. 1). Regarding

Tab. 1. Tests where no differences were found between Bcl-2 heterozygous (+/–) and wild type control (+/+) mice

Test	Measure	+/+ Mean (SE)	+/– Mean (SE)	Statistics
Weight (g)	Initial	21.6 (0.7)	21.2 (0.4)	t(33) = 0.54, NS
	Gain in 3 weeks	2.6 (0.5)	2.3 (0.3)	t(33) = 0.53, NS
Open field	Spontaneous activity (cm)	5713 (774)	5265 (615)	t(49) = 0.40, NS
	"Center" time (s)	26.4 (4.8)	34.7 (8.2)	t(49) = 0.80, NS
Black/white box	Entries to white (number)	3.7 (0.6)	3.5 (0.6)	t(49) = 0.20, NS
	Time in white (s)	19.5 (4.7)	17.5 (3.7)	t(49) = 0.40, NS
FST	Immobility time (s)	184 (8.4)	169 (10.3)	t(33) = 1.00, NS
Elevated plus maze	Entries to open/ total arms entries (ratio)	0.16 (0.07)	0.19 (0.05)	t(33) = 0.34
	Time in open/total arms time (ratio)	0.11 (0.04)	0.16 (0.09)	t(33) = 0.42, NS
Resident-intruder	Number of attacks	15.8 (5.0)	11.5 (4.5)	t(33) = 0.70, NS
	Social interactions	28.3 (3.2)	26.8 (3.0)	t(33) = 0.40, NS
Acute amphetamine	Vehicle (distance, cm)	4341 (2107)	2761 (788)	ANOVA by strain by dose; strain: $F(1,12) = 0.3$, NS; amphetamine: $F(2,12) = 5.3$, $p < 0.03$; interaction: $F(2,12) = 0.3$, NS. *Post-hoc* for amphetamine: group 1 mg/kg different than other groups
	Amphetamine 1 mg/kg (distance, cm)	14575 (9459)	19046 (9953)	
	Amphetamine 2 mg/kg (distance, cm)	3834 (1408)	6479 (2038)	

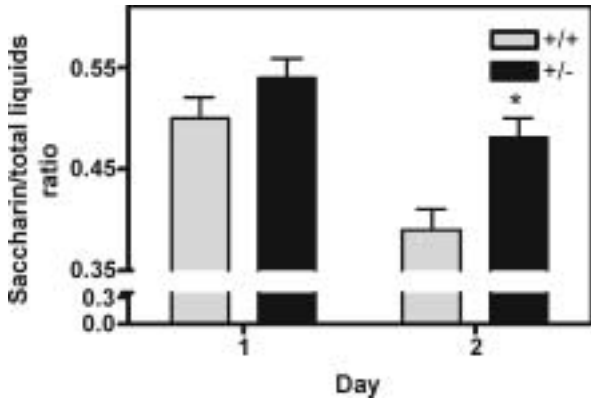

Fig. 1. Bcl-2 deficient mice (+/–) demonstrated increased saccharin preference compared with WT littermate mice (+/+). The values represent the ratio between saccharin solution and total liquid (saccharin solution + water) consumption for each 24 h period. * signifies difference between groups ($p < 0.05$)

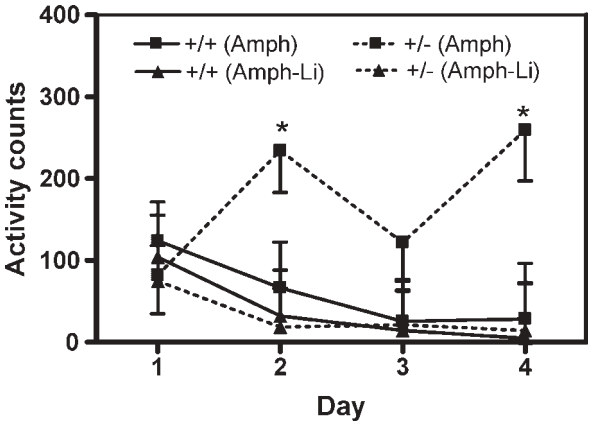

Fig. 2. Bcl-2 deficient mice (+/–) showed a sensitized response to amphetamine (Amph) treatment compared with WT controls (+/+). This response was attenuated by lithium (Li) pretreatment. Measures represent activity counts (crossings of infrared beams) in a 60 min session. * signifies difference from all other groups ($p < 0.05$)

the effects of acute amphetamine, both WT and heterozygous mice showed hyperactivity when administered 1 mg/kg dose but not in response to a 2 mg/kg dose.

Nevertheless, Bcl-2 deficient mice were different in two tests: the saccharin preference test and the amphetamine sensitization model. For saccharin preference (the proportion of sweet solution/total liquid drinking), the heterozygous mice were higher than the wild type mice [Fig. 1; Repeated measures ANOVA across days: Strain: $F(1, 52) = 7.36$, $p < 0.01$; Day: $F(1, 52) = 31.5$, $p < 0.001$; Strain x Day: NS]. For the amphetamine sensitization test, Bcl-2 deficient mice were not different from wild type mice in their acute response to amphetamine, but, whereas the wild type animals did not show sensitized response at this dose and schedule of administration, the Bcl-2 deficient animals demonstrated sensitization as shown by a strain x day statistically significant interaction (see below). Moreover, lithium pretreatment attenuated sensitization in amphetamine-treated Bcl-2 deficient mice [Fig. 2; Repeated measures ANOVA across days: Group: $F(3, 19) = 3.2$, $p = 0.047$; Day: $F(3, 19) = 1.66$, NS; Day x Group: $F(3, 57) = 2.37$, $p = 0.024$]. *Post-hoc* LSD test revealed a significant difference between the amphetamine-treated Bcl-2 deficient group and the other groups on days 2 and 4.

Bcl-2 deficient mice were not different from WT mice in the resident intruder test (Tab. 1). Moreover, in contrast to the results of an earlier study [17], these mice did not show increased anxiety-like behavior in the open field, the black/white box or the elevated plus-maze (Tab. 1).

Discussion

The present study demonstrates that mice heterozygous for the Bcl-2 gene are different from WT controls in two behaviors that were previously suggested to model domains of mania, increased reward seeking and increased sensitivity to psychostimulants expressed as a sensitized response to a low dose of amphetamine [15].

Interestingly, the difference in saccharin preference between the heterozygous and the WT mice was not because of a very high preference by the mutants but because of a low preference of the WT mice. As discussed below, the use of these specific WT mice was inevitable as they are the only appropriate controls. Regardless of the source of the difference, whether it is high saccharin preference of the mutants or a low preference of the WT mice, the results show that the mutation increased saccharin preference in this specific strain.

Based on the response to acute amphetamine, the 1 mg/kg dose was selected for the sensitization study as the higher dose did not induce hyperactivity. The sensitized response of the mutant mice to amphetamine was modified by the prototypic mood stabilizer lithium suggesting that these changes are relevant to bipolar disorder and its treatment. Since lithium was administered before amphetamine, it is not possible to determine whether the effects of lithium were to attenuate the development or the expression of the sensitized response. These are two separate processes that might be related to different mechanisms [27], but previous data suggest that the effects of lithium may be related more to attenuating the development rather than the expression of sensitization [37]. It is noteworthy that the WT mice did not show sensitization in this dose and schedule of administration. Perhaps this unexpected lack of effect was related to the relatively low dose used in the experiment [23]. More knowledge could have been obtained if it was possible to test a range of amphetamine doses and compare a dose response curve for both acute and sensitized responses of the WT and mutant mice, but due to the limited number of available animals that was not possible as the mice used in the current study were specifically bred for the project in a limited number. However, the present results do suggest that the heterozygous animals were sensitive to an amphetamine dose that had no sensitization effect in the WT mice and that lithium treatment attenuated this difference in behavior.

The demonstrated behavioral changes in the mutant mice are clearly not enough to suggest that reduced Bcl-2 levels result in a manic-like phenotype. However, such a general claim is nearly impossible under any circumstance because of the limitations of appropriate animal models for mania. Because there is no comprehensive, well-validated model for mania available, the approach that was used in the current study, was to test animals in a battery of models that represent several domains of manic-like behavior, including activity levels, reward seeking, aggression and response to acute or subchronic stimulants [10, 11, 14,

15]. Bcl-2 deficient mice demonstrated altered behavior in some tests as detailed above. However, a broader manic-like phenotype could have also resulted in increased spontaneous activity, increased aggression and possibly increased response to acute amphetamine. Although these effects were not demonstrated, the results of the study offer some additional support to a possible relationship between the observed behavioral changes and mania because chronic treatment of the mutant mice with the prototypic mood stabilizer lithium ameliorated the increased sensitivity to amphetamine.

The present study demonstrates differences between Bcl-2 heterozygous mice and WT controls that may be related to mania but shows no effect of Bcl-2 deficiency in behavioral tests related to depression or anxiety. As such, the results stand in partial contrast with a previous study that demonstrated an anxious-like phenotype of Bcl-2 heterozygotes [17]. Beyond some technical and methodological differences between these studies, the main dissimilarity is the background strain of mutant mice. Specifically, although both studies tested mice in some anxiety and mood-related models as well as for spontaneous activity, 129S1/SVIMJ BCL2<TM1MPIN>/J mice were used in the previous study, while B6;129S2-Bcl-2<tm1Sjk>/J mice were used in the current experiment. The results of both studies are in agreement regarding spontaneous and depression-like behavior (no effects of mutation) but are significantly different regarding the anxiety models. The 129S1/SVIMJ BCL2<TM1MPIN>/J heterozygous mice used in Einat et al. [17], showed increased anxiety-like behavior in the open field, the black white box, the emergence test and the elevated plus-maze compared to their WT controls, whereas in the current experiment B6;129S2-Bcl-2<tm1Sjk>/J heterozygous mice were not different from their WT controls in 3 of these 4 anxiety tests (and not tested in the emergence test). It is, therefore, conceivable that the background strain had an influence on the behavioral phenotype related to anxiety-related pathology expressed as a result of the mutation. One way to explain this disparity may be that the baseline level of anxiety of WT mice in the present experiment was very high and, therefore, any possible effect of the mutation in these measures was overshadowed by a ceiling effect. Indeed, the behavior of WT mice in the previous experiment was very different compared with the current study. For example, in the black/white box test, WT mice from the previous study spent an average of 183 s in the white portion of the box, whereas WT mice in the present study spent only 12.5 s on average in that area. WT mice from the previous study had an average of 10.9 exits to the open arms of the elevated plus-maze, whereas the current WT mice averaged only 2.8 exits per session. A formal comparison of results from the two studies is not possible as they were performed in different laboratories, at a different time, different housing conditions and somewhat different protocols. Yet, the large differences in anxiety-related measures of WT mice between the studies may suggest that the lack of effects of mutation on anxiety measures in the current study may be the consequence of baseline differences in WT anxiety levels between strains. The use of these specific WT mice in the present study was inevitable as they are the WT littermate of the Bcl-2 mice and are the ones that share the same genetic background with them. Studies of mice with targeted mutations must use the WT littermates as controls because of the significant heterogeneity in genotype and phenotype between different mouse strains [5].

These differences in responses that might be related to the background strain are a good example and reminder to the complicated issues related to working with mice with targeted mutations and the importance of the background strain in the interpretations of results from such studies [6].

However, beyond the problems that arise from the use of mutant mice in general and ones from different background strains in particular, the accumulating data regarding the behavioral effects of Bcl-2 deficiency support the notion that Bcl-2 may be involved in the pathology and/or treatment of affective and anxiety disorders [33, 42, 43]. While no strong conclusions can be made based on existing behavioral data, it is suggested that the behavioral changes that were previously demonstrated with 129S1/SVIMJ BCL2<TM1MPIN>/J heterozygous mice [17] and the additional effects demonstrated in the present study support the need for additional research.

A more direct approach to explore these relationships will be possible when specific Bcl-2 modulators, which can be administered *in vivo* and penetrate into the brain become available. Such specific pharmacological agents are being developed [48] and would be an important tool to further explore the significance of Bcl-2 in the context of psychiatric disorders.

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