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WE tested the neuroprotective potential of the Bcl-2$^{20-34}$ peptide sequence in hippocampal slices. Treatment with Bcl-2 after fluid percussion trauma significantly improved recovery of CA1 antidromic PS to a mean of 92% ± 1 of initial amplitude, compared with only 16% ± 2 in unmedicated slices. The EC_{50} for trauma protection was 84 μM Bcl-2$^{20-34}$. Protection with Bcl-2$^{20-34}$ also extended to long-term potentiation. No protection was seen with the reverse sequence of Bcl-2$^{20-34}$. Treatment with Bcl-2$^{20-34}$ also protected against hypoxic damage, with treated slices recovering to 98% ± 2, while unmedicated slices recovered to 14% ± 2. Similar protection was seen against AMPA, NMDA and nitric oxide. These findings indicate that Bcl-2$^{20-34}$ provides specific neuroprotection against acute CA1 neuronal injury. *NeuroReport* 9: 4131–4136 © 1998 Lippincott Williams & Wilkins.

Key words: AMPA; Apoptosis; Bcl-2; Cerebral ischemia; Hypoxia; Nitric oxide; NMDA; Slice; Traumatic brain injury

Neuroprotection with Bcl-2$^{20-34}$ peptide against trauma

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Introduction

Members of the Bcl-2 proto-oncogene family have been identified as major modulators of programmed cell death, with Bcl-2 gene products either enhancing or diminishing the likelihood of neuronal survival. This regulation appears to be essential to normal brain development, where programmed cell death may eliminate up to 50% of neurons as a part of selective pruning during formation of neuronal networks.[1] In addition to this physiological role, the Bcl-2 family appears to be active in brain pathophysiology, by modulating neuronal survival after injury.[2] The Bcl-2 protein has been demonstrated to be neuroprotective, since the presence of Bcl-2 gene inhibits neuronal death from apoptosis.[3] In addition, the overexpression of the Bcl-2 gene is associated with enhanced neuronal survival after ischemia.[4]

Although the neuroprotective effects of the Bcl-2 protein may be substantial, the mechanism of this protection is not clearly understood. However, data suggest that the BH4 region near the N-terminus may be in involved, since deletion of N-terminal region in fibroblasts promotes rather than inhibits cell death.[5] Therefore, we examined neuroprotective effects of the peptide sequence Bcl-2$^{20-34}$, which is contained within the N-terminal region.

For our study, we utilized the hippocampal slice and monitored the electrophysiological function of CA1 pyramidal neurons subjected to fluid percussion trauma.[6] Electrophysiological function was utilized as a marker of neuroprotection, because the preservation of neuronal electrophysiological function is the ultimate goal of neuroprotective efforts. The response of hippocampal CA1 pyramidal neurons to Bcl-2$^{20-34}$ were monitored, because these neurons have been found not to express endogenous Bcl-2 in either a basal state or following hypoxia–ischemia injury.[7] In addition, CA1 pyramidal cells show great vulnerability to clinical head trauma[8] and hypoxia–ischemia.[9] Memory loss is the most frequent deficit seen after head trauma,[10] and the CA1 pyramidal neuron population has been found to play a critical role in memory formation.[11]

Materials and Methods

Male Sprague–Dawley rats (250–480 g) were briefly anesthetized with halothane and decapitated. As previously described,[12] 475 μm hippocampal slices were dissected and maintained in a submerged system recording chamber at 34.0 ±0.5°C, perfused with artificial cerebrospinal fluid (ACSF), composed of (in mM) NaCl, 126; KCl, 4.0; KH_2PO_4, 1.4; $MgSO_4$, 1.3; $CaCl_2$, 2.4; $NaHCO_3$, 26; and glucose, 4.0, and saturated with 95% O_2/5% CO_2 with pH adjusted to 7.4. Bcl-2 peptide was obtained from Oncogene Research Products, Inc. and contained equal amounts of bovine serum albumin (BSA). Bcl-2$^{20-34}$ was dissolved in 200 μl distilled water, and then diluted

with ACSF. Reverse sequence Bcl-2$^{20-34}$ was produced by Coast Scientific, Inc. All other reagents were purchased from Sigma, Inc.

To assess morphological evidence of CA1 injury, some hippocampal slices were analysed using standard electron microscopy techniques.[13] Hippocampal slices fixed in a mixture of 2% glutaraldehyde and 4% paraformaldehyde were stained with uranyl acetate/lead and embedded in osmium and araldite resin. Tissue sections of CA1 at a depth of 100 μm were sliced using an ultra microtome and placed on 200 μm mesh grids. Sections were coded to ensure absence of bias by the observer. Analysis involved visual inspection of the central region of CA1 using a Phillips Electron Microscope at a magnification of ×10 000–20 000. Hippocampal slices were collected for analysis at varying times following trauma. Representative electron micrographs of the CA1 region of sham hippocampal slices demonstrate normal nuclear morphology (Fig. 1A). In contrast, neuronal damage as evidenced by some cell loss within the CA1 subfield of hippocampal slices exposed to fluid percussive injury can be seen as early as 5 min after trauma (Fig. 1B). More extensive damage as demonstrated by the presence of distinct nuclear and cytoplasmic changes suggestive of necrosis are observed 60 min after trauma (Fig. 1C).

The orthodromic CA1 population spike (PS) amplitude of each slice was elicited every 30 s by Schaffer collateral stimulation. A bipolar electrode was used with square wave pulses of 40 μs duration, while responses in the CA1 pyramidal cell layer were recorded with a tungsten electrode. Stimulating current strengths were adjusted until a maximal PS amplitude was obtained, and were not changed thereafter. Only slices with an initial PS amplitude $\geqslant 3$ mV were utilized. To help exclude effects of possible synaptic depression, CA1 antidromic PS was also assessed by stimulation given over the alveus at the beginning and end of each experiment, while recordings were made from the CA1 pyramidal cell layer.

To induce trauma, slices were transferred to a 7 ml specialized chamber filled with ACSF and sealed with a rubber piston.[6] A 1 kg weight was then dropped upon the piston from a height of 61 cm, producing percussion of the fluid surrounding the slice. After trauma, each slice was returned to the recording chamber and electrodes were positioned

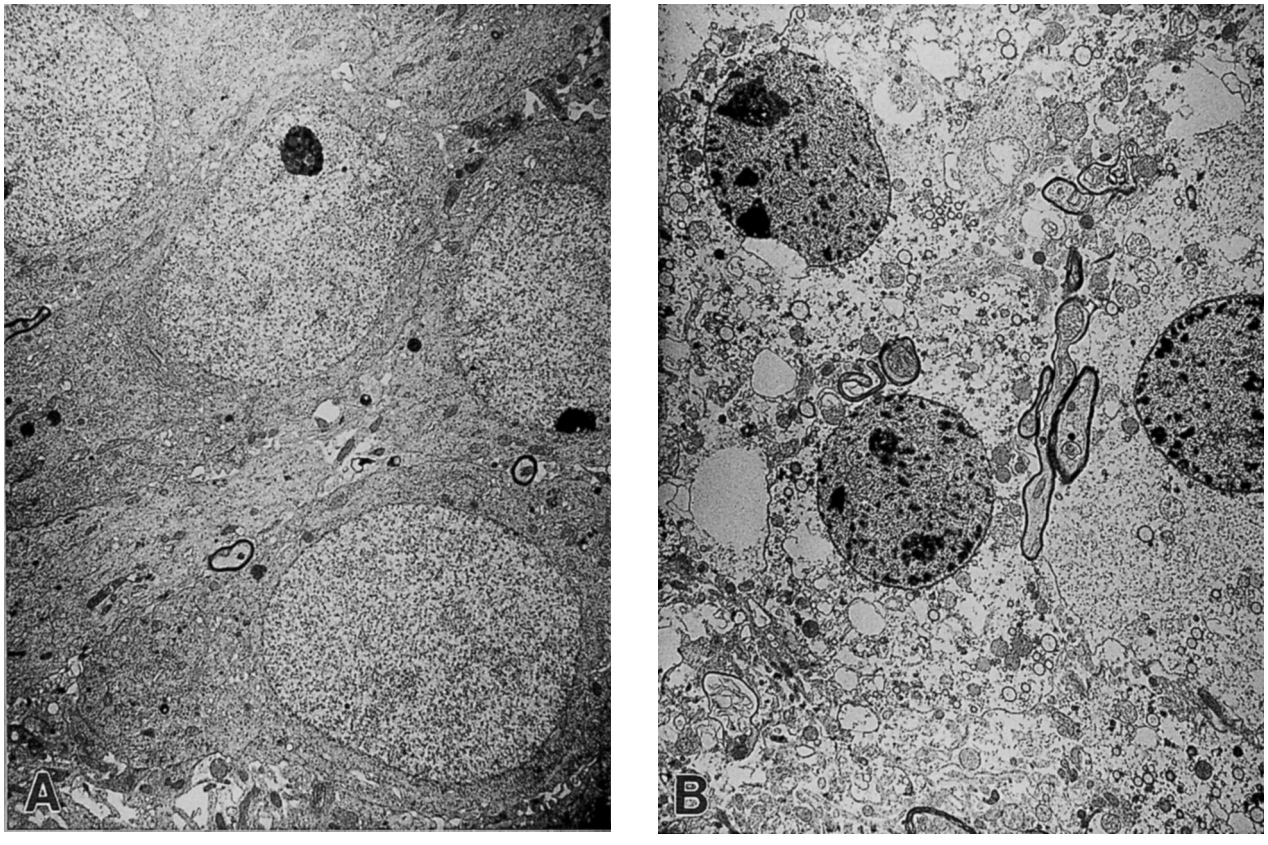

FIG. 1. Electron micrographs of CA1 cells in hippocampal slices. (**A**) Untraumatized slice demonstrating a large nucleus with finely dispersed chromatin and prominent nucleolus. Cystoplasmic components include rough endoplasmic reticulum and intact mitochondria (×6800). (**B**) Five minutes following fluid percussion injury, CA1 cells show darkened nuclei with chromatin aggregation, cytoplasmic vacuolization and cellular membrane rupture (×6800).

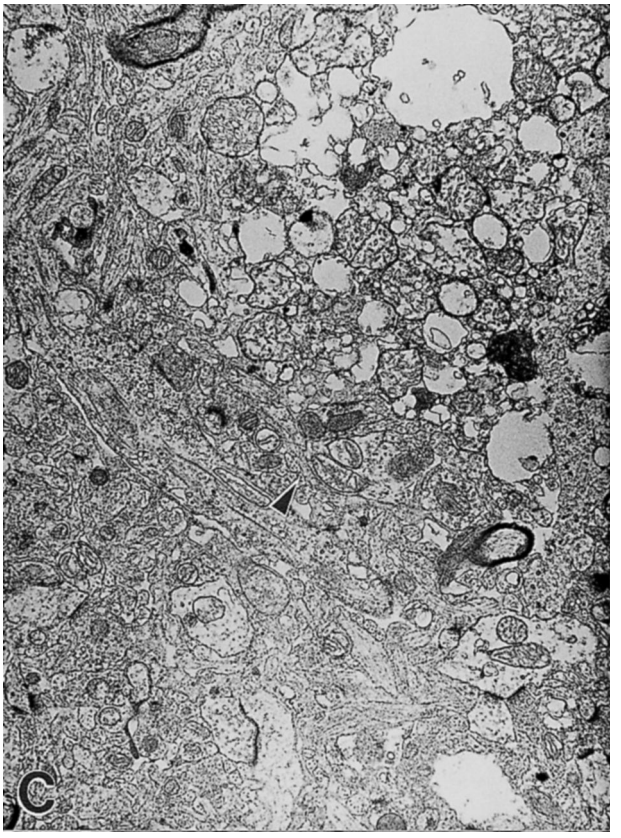

FIG. 1**C**. Electron micrographs of CA1 cells in hippocampal slices. Sixty minutes following fluid percussion injury, extensive cellular damage is observed. Arrowhead points to nuclear and plasma membrane disintegration and widespread vacuolization (×1 3600).

within one min. Treatment with Bcl-2^{20-34} was begun within 1 min following trauma and continued for 35 min. Final recovery was assessed 95 min after trauma. Following trauma and treatment with Bcl-2^{20-34}, four slice pairs were given extended monitoring for 8 h.

In hypoxic trials, paired slices taken from the same dissection were monitored with one slice receiving only hypoxia, while the other slice received hypoxia and Bcl-2^{20-34}. Hypoxic exposure was initiated by changing the perfusion fluid to ACSF saturated with 95% N_2/5% CO_2. Hypoxia was continued for 20 min. Treatment with Bcl-2^{20-34} was begun 15 min prior to hypoxia and continued through the first 15 min of reoxygenation. Final recovery was assessed after 60 min of reoxygenation.

Exposures to NMDA (20 μM) and AMPA (25 μM) were given for 8 min. Exposure to nitric oxide (150 μ)M lasted 10 min. As previously described, nitric oxide exposure was performed under hypoxic conditions with ACSF glucose increased to 10 mM to help prevent hypoxic injury.[14] Treatment with Bcl-2^{20-34} peptide was begun 15 min prior to exposure to NMDA, AMPA or nitric oxide and continued through the first 15 min of recovery. Final responses were assessed after 60 min recovery. Perfusion with ACSF saturated with helium for 2 min preceded nitric oxide exposure.

All experimental paradigms utilized five or more slices. Initial potentials of slice treatment groups did not differ significantly when assessed by one-way analysis of variance. Responses of paired experiments were compared using Student's correlated *t*-tests. Wilcoxon rank-sums test was used for other comparisons.

Results

The post-traumatic application of Bcl-2^{20-34} proved to be strongly protective against CA1 traumatic neuronal injury. When slices were treated with 150 μM Bcl-2^{20-34} for 35 min immediately after trauma, CA1 orthodromic PS amplitude recovered to a mean 92% ± 1 of initial amplitude. In contrast, unmedicated slices recovered to only 16% ± 3 of initial amplitude (Fig. 2A), which is similar to the degree of injury induced by trauma in prior studies using this preparation.[6] Severe neuronal cell body dysfunction from trauma was confirmed by loss of CA1 antidromic PS, which recovered in unmedicated slices to only a mean 16% ± 2. Treatment with Bcl-2^{20-34} again provided significant protection with CA1 antidromic PS recovery of 92% ± 1 ($p < 0.05$).

Neuroprotection against trauma with Bcl-2^{20-34} was concentration-dependent (Fig. 2B), with an EC_{50} of 84 μM for protection of CA1 orthodromic PS and 80 μM for protection of CA1 antidromic PS.

In extended electrophysiological monitoring over 8 h, protection by brief post-traumatic Bcl-2^{20-34} (150 μM) treatment was found to be long-lasting (Fig. 2C). In these slices CA1 orthodromic PS was stable, similar to that seen in non-traumatized slices. In contrast, traumatized slices showed only progressive dwindling of response over the same time course.

Protection with Bcl-2^{20-34} against traumatic injury also extended to the ability to induce long-term potentiation (LTP) after trauma (Fig. 3A). Tetanus (100 Hz for 1 s) given without change from supramaximal threshold current strengths to slices treated with 150 μM Bcl-2^{20-34} after trauma produced a mean increase in CA1 orthodromic PS amplitude of 131% ± 5 compared with pre-tetanic values. This was similar to the response of non-traumatized slices, where an amplitude increase of 132% ± 3 after tetanus was observed, while traumatized slices not given Bcl-2^{20-34} showed no increase with tetanus.

In contrast to the neuroprotection seen with forward sequence Bcl-2^{20-34} against trauma, reverse sequence Bcl-2^{20-34} provided no protection against trauma. While forward sequence Bcl-2^{20-34} treatment

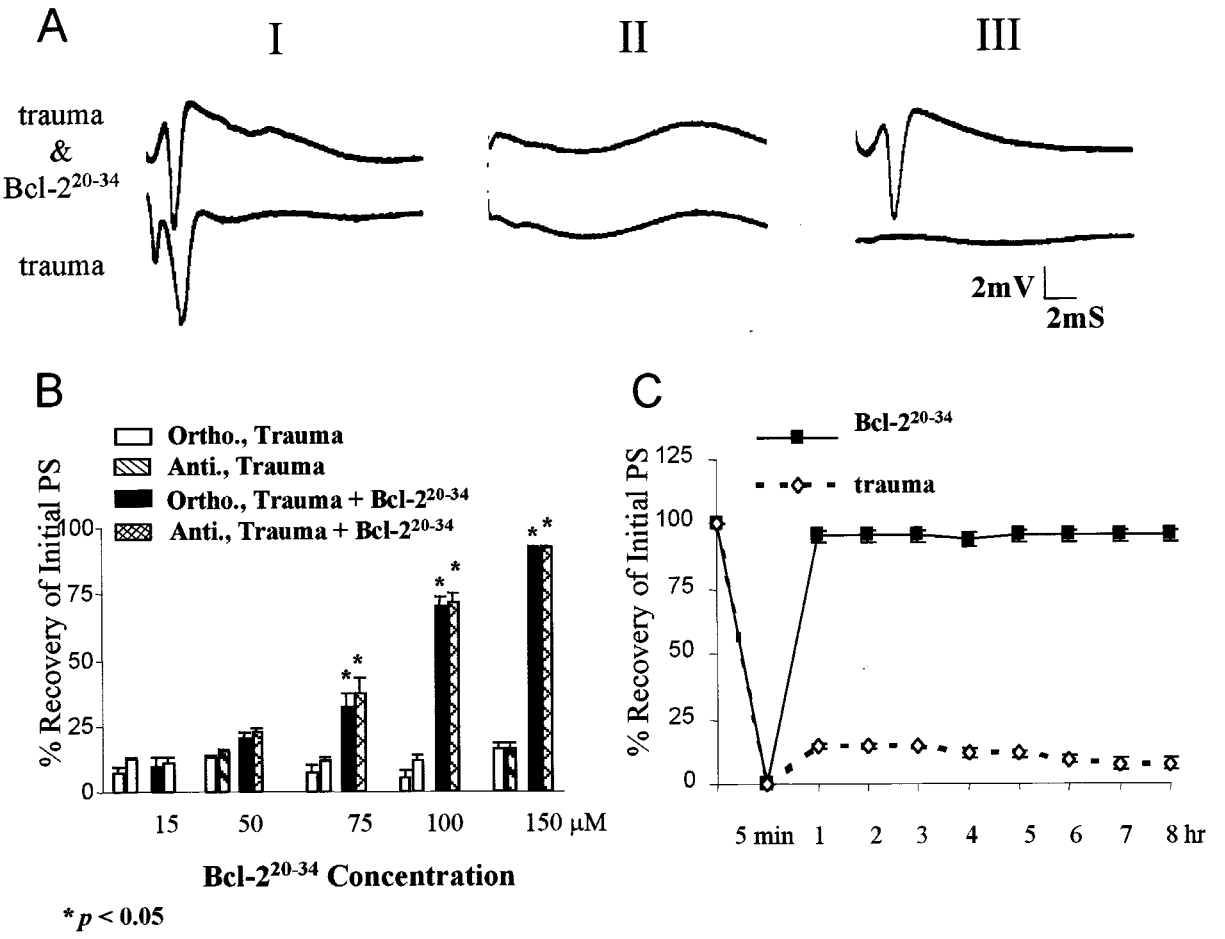

FIG. 2. (**A**) Neuroprotection of CA1 evoked response with Bcl-2^{20-34} peptide against fluid percussion trauma in the hippocampal slice. Tracings demonstrate CA1 orthodromic evoked response in paired hippocampal slices subjected to fluid percussion trauma. Upper tracings show evoked responses in slice treated with Bcl-2^{20-34}. Lower tracings show evoked response of the paired, unmedicated slice which received only artificial cerebral spinal fluid (ACSF). Initial tracings in paired hippocampal slices from the same dissection demonstrate similar CA1 population spike (PS) amplitude. One minute following trauma, CA1 PS is lost in both tracings. Treatment with 150 μM Bcl-2^{20-34} treatment was initiated at this time for upper tracing slice. Ninety-five minutes following trauma, upper tracing shows good recovery of CA1 PS in slice given Bcl-2^{20-34} treatment, while no recovery is seen in unmedicated slice. (**B**) Treatment with Bcl-2^{20-34} peptide protects against fluid percussion trauma. Neuroprotection with Bcl-2^{20-34} against traumatic loss of CA1 evoked response is concentration dependent. (**C**) Traumatic neuroprotection for CA1 neurons with Bcl-2^{20-34} treatment is long-lasting in extended monitoring. Responses of unmedicated traumatized slices differed significantly from those of non-traumatized, unmedicated slices at all points after trauma. All responses 1 h or more following trauma differed significantly when comparing Bcl-2^{20-34}-treated slices with unmedicated slices. $*p < 0.05$, Student's correlated *t*-test. Values are mean ± s.e.

after trauma yielded CA1 orthodromic and antidromic recoveries of 92% ± 1, reverse sequence Bcl-2 treatment gave recoveries of only 13 ± 1% and 14 ± 2%, similar to that seen with trauma alone (12% ± 1 and 14% ± 1). Increasing the concentration of reverse sequence Bcl-2^{20-34} by 10 times (1500 μM) did not improve recovery (Fig. 3B).

Neuroprotection with forward sequence Bcl-2^{20-34} also extended to CA1 hypoxic injury. Slices treated with Bcl-2^{20-34} recovered CA1 orthodromic and antidromic PS amplitudes of 97% ± 1 and 98% ± 2, compared with unmedicated slices which recovered to only 8% ± 2 and 14% ± 2 ($p < 0.05$). Similar protection with Bcl-2^{20-34} (150 μM) was seen against several forms of excitotoxicity including those produced by AMPA, NMDA and nitric oxide. Exposure to 25 μM AMPA for 8 min induced rapid neuronal injury, as evidenced by loss of evoked response and only minimal recovery of orthodromic and antidromic PS recoveries of 10% ± 1 and 12% ± 1, respectively. Treatment with Bcl-2^{20-34} provided significant protection against the injurious effects of AMPA exposure yielding both orthodromic and antidromic PS recoveries of 96% ± 2. Similarly, exposure to 20 μM NMDA for 8 min produced orthodromic and antidromic PS recoveries of only 9% ± 2 and 13% ± 2, while treatment with Bcl-2^{20-34} provided excellent protection of CA1 orthodromic and antidromic PS response with recoveries of 94% ± 2 and 93% ± 3. Lastly, treatment with Bcl-2^{20-34} improved CA1

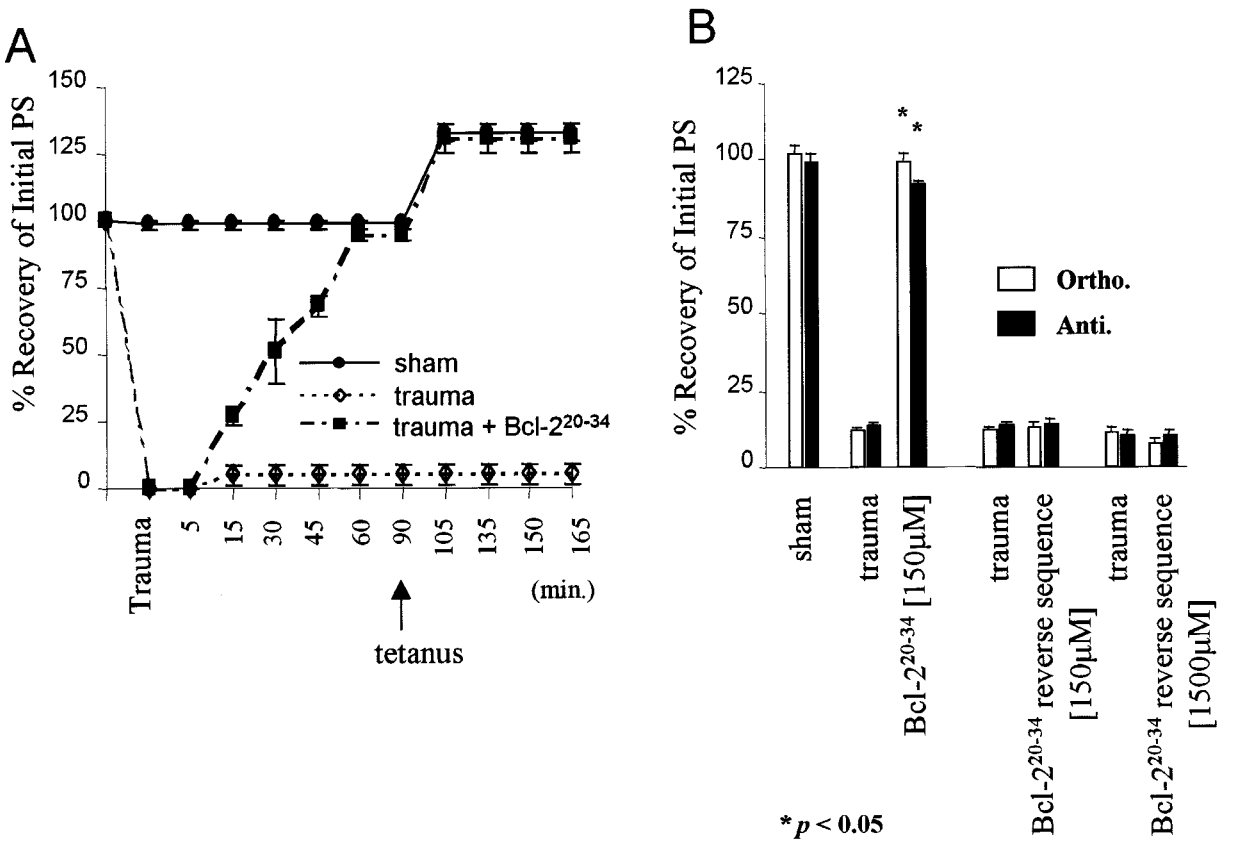

FIG. 3. Treatment with Bcl-2$^{20-34}$ peptide preserves LTP responses and shows specificity. (**A**) Protection with 150 μM Bcl-2$^{20-34}$ peptide from loss of LTP induction after trauma. (**B**) Protection with Bcl-2$^{20-34}$ (150 μM) against trauma is specific. No protection is seen with the reverse sequence Bcl-2$^{20-34}$ (150 μM and 1500 μM). Values are mean ± s.e.

orthodromic and antidromic PS recovery after exposure to 150 μM nitric oxide, from 8% ± 2 and 11% ± 1, to 95% ± 2 and 95% ± 1, respectively.

Discussion

The Bcl-2$^{20-34}$ peptide sequence provided robust neuroprotection of CA1 electrophysiological function in the hippocampal slice against the effects of fluid percussion trauma. Injury to CA1 neurons induced by trauma appeared to be severe as evidenced by electrophysiological correlates and ultrastructural analyses. Neuroprotection seen with Bcl-2$^{20-34}$ treatment also extended to the ability to induce LTP, which is an important electrophysiological correlate of memory and learning often lost with traumatic brain injury.[15] The protection seen with Bcl-2$^{20-34}$ was concentration dependent, and occurred over a rapid time course. This protection also showed specificity, and was not seen with reverse sequence Bcl-2$^{20-34}$.

The Bcl-2 protein has been found to be neuroprotective in several studies, however the amino acid sequences conferring this protection are not well delineated. One potential mechanism of protection is Bcl-2 heterodimerization with Bax, a pro-apoptotic member of the Bcl-2 family. The Bcl-2 peptide domains, BH1, BH2 and BH3 have been shown to be involved in heterodimerization with Bax.[16,17] However, different regions of the Bcl-2 protein may also provide neuroprotective effects through other mechanisms. In this regard, the BH4 domain has been shown not to be involved with dimerization with Bax, yet it is required for the anti-apoptotic action of the Bcl-2 protein.[18] The importance of the BH4 domain is also demonstrated by the fact that it is conserved in all anti-apoptotic members of the Bcl-2 family.

The Bcl-2$^{20-34}$ sequence used in these studies is largely contained within the BH4 domain (amino acids 10–30). The BH4 region is part of the NH-terminus of the Bcl-2 protein and is thought to lie free within the cytoplasm,[2] while the hydrophobic COOH terminus appears to serve as a membrane anchor for attachment to the outer mitochondrial, endoplasmic reticulum and the nuclear membranes.[19] The findings of our study suggest that the BH4 domain plays a critical role in neuroprotection against trauma to CA1 neurons, although it may have a different mechanism of action than strictly as an anti-apoptotic agent.

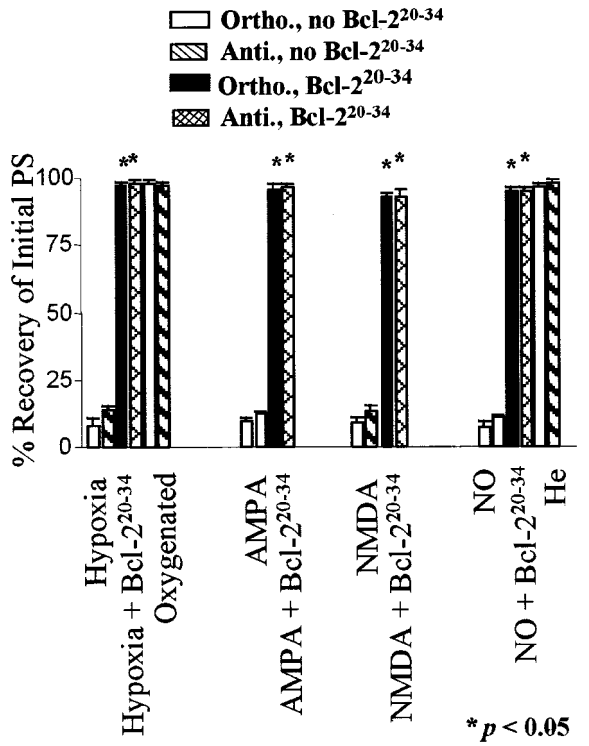

FIG. 4. Treatment with Bcl-2^{20-34} (150 μM) protects against CA1 injury from hypoxia, AMPA (25 μM), NMDA (20 μM) and nitric oxide (150 μM). Exposure time for hypoxia was 20 min. Exposure to AMPA and NMDA was for 8 min. Exposure to nitric oxide was for 10 min (with 2 min pre-exposure to helium saturated ACSF prior to nitric oxide treatment). Values are mean ± s.e. $*p < 0.05$, Wilcoxon rank-sums test.

In the present study, the Bcl-2^{20-34} peptide sequence was applied extracellularly in the perfusion fluid. This protection with extracellular application suggests that the peptide fragment is likely to be entering the cell, although the exact mechanism is not fully understood. However, several studies have shown that there is increased cellular membrane permeability following trauma[20] and ischemic injury,[21] which may enhance peptide entry.

Protection with Bcl-2^{20-34} was also found against hypoxia, which is important as a secondary mechanism of injury following trauma. Additionally, excellent protection with Bcl-2^{20-34} treatment was observed against the injurious effects of exposure to NMDA, nitric oxide or the non-NMDA agonist, AMPA. The protection against non-NMDA induced injury with Bcl-2^{20-34} treatment is in keeping with a prior study showing increased susceptibility to AMPA induced damage to cortical cells with reduced Bcl-2 expression.[22] This robust protection with Bcl-2^{20-34} against multiple excitotoxic pathways suggests that the mechanism of protection occurs at a distal point downstream in the cascade of cellular injury.

Although the molecular effects of the Bcl-2^{20-34} sequence are not known, the Bcl-2 protein has been postulated to produce several different mechanisms of protection. Among these, overexpression of Bcl-2 produces enhanced mitochondrial calcium sequestration.[23] In addition, Bcl-2 has been found to reduce cellular redox potential without major alteration of cellular antioxidant enzymes.[24] The Bcl-2 protein has also been shown to modulate the mitochondrial permeability transition pore complex, thereby decreasing ionic flux induced by anoxia and toxins.[25] Further studies are needed to determine if the neuroprotection provided by Bcl-2^{20-34} occurs through these mechanisms.

Conclusion

This study demonstrates that the Bcl-2^{20-34} peptide sequence provides significant neuroprotection of CA1 electrophysiological function against the injurious effects of fluid percussion trauma and hypoxia through direct neural effects. The protection seen with Bcl-2^{20-34} extended to CA1 orthodromic and antidromic evoked response, and to the ability to induce LTP in CA1 pyramidal neurons following trauma. With traumatic brain injury, the ability to induce LTP is frequently lost,[15] and this deficit within CA1 neurons is thought to be a major contributor to memory problems seen with head trauma.[10,11] These findings of rapidly occurring, robust neuroprotection, suggest that the Bcl-2^{20-34} peptide sequence plays a critical role in the mechanism of Bcl-2 protection. In addition, these findings suggest that manipulation of this peptide sequence may represent a possible therapeutic strategy in the treatment of head trauma and stroke.

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