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# Continuous supply of the neurotrophins BDNF and NT-3 improve chick motor neuron survival in vivo

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**Abstract**

Following neurogenesis, motor neurons undergo a phase of large-scale neuronal loss. During this period, the motor neurons are responsive to specific trophic factors for their survival. Several neurotrophic factors, including the neurotrophins BDNF and NT-3, have survival effects although no single factor has been shown to support the survival of all motor neurons. It is unclear whether this is due to factor deprivation during the study or whether there are distinct neuronal subpopulations dependent on different factor requirements. In this study, we have used an expression system to supply a continuous source of BDNF and/or NT-3 to the developing motor neurons in the chick. Continuous supply of BDNF resulted in the survival of 40% of the motor neurons normally lost between embryonic day 6 and embryonic day 10, whereas NT-3 supported 36% of the motor neurons normally lost. In combination, BDNF and NT-3 supported 62% of the motor neurons normally lost indicating that there is some redundancy in neurotrophin requirements. Our results show that a continuous supply of neurotrophins is more effective in promoting motor neuron survival than intermittent administration, particularly for NT-3. However, even with continuous administration of both factors in combination we are unable to support all motor neurons that would normally undergo neuronal degeneration.


*Keywords:* Neurotrophic; Chick embryo; Spinal cord

The neurotrophins BDNF and NT-3 are known to be potent trophic factors for cultured spinal motor neurons derived from chick embryos during the period of naturally occurring neuronal cell death (NOCD) (Becker et al., 1998). Such responsiveness is supported by the expression of the corresponding receptors TrkB and TrkC in embryonic spinal motor neurons. However, the same trophic response has not been observed in developing chick motor neurons in vivo. While, BDNF has been found to rescue one-third of those motor neurons that normally succumb during NOCD (Oppenheim et al., 1992; Calderó et al., 1998), NT-3 has not been shown to significantly rescue spinal motor neurons during this period (Calderó et al., 1998). Furthermore, no single neurotrophic factor has been observed to rescue the entire spinal motor neuron population from NOCD. Whether this phenomenon results from specific subsets of motor neurons within the spinal cord being unresponsive to the supplied factors or whether it is an artefact of intermittent withdrawal, is not known.

Previous studies that have examined the trophic effects of neurotrophins on developing spinal motor neurons have involved intermittent administration during the experimental period (Oppenheim et al., 1992; Calderó et al., 1998). However, the interval between administrations in these studies has usually exceeded both the known half-life of the model neurotrophin NGF and the observed time frame during, which neurons succumb to NGF deprivation (Tria et al., 1994; Deckwerth and Johnson, 1993). Therefore, it remains uncertain as to whether the lack of survival is a true indication of the trophic responsiveness of motor neurons or whether it is the end result of intermittent factor deprivation. There is increasing evidence that neurotrophic factors work

*Abbreviations:* BDNF, brain derived neurotrophic factor; NT-3, neurotrophin-3; E6, embryonic day 6; NOCD, naturally occurring neuronal cell death

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in combination on gross cell populations to elicit a complete survival response (Kato and Lindsay, 1994; Mitsumoto et al., 1994). Within the PNS neurotrophic factors may act sequentially, with cells switching their trophic requirements. When isolated from stages prior to target innervation, cultured murine trigeminal ganglion neurons die unless BDNF or NT-3 is present in the culture medium. When isolated at later periods, trophic responsiveness to BDNF and NT-3 is lost in favour of NGF (Paul and Davies, 1995). Such apparent shifts in trophic response are supported by a corresponding change in Trk receptor expression from TrkB and TrkC to TrkA in these populations in vivo (Pinon et al., 1996). Similarly, TrkC is also expressed early in the target innervation of superior cervical sympathetic ganglia. However, at later stages TrkC expression decreases as the neurons begin to express TrkA (Birren et al., 1993; Wyatt and Davies, 1995). In vitro studies have shown that dorsal root ganglia are responsive to BDNF, NT-3 and NGF early in development but subsequently narrow their trophic requirements to NGF (Memberg and Hall, 1996; Lefcort et al., 1996). Early in post-natal development a substantial proportion of the dorsal root ganglia population undergo a further shift in trophic responsiveness towards GDNF dependence (Molliver et al., 1997).

In this study, we employed a neuroepithelial cell line genetically modified to produce either BDNF or NT-3 to deliver a continuous supply of these neurotrophins to the developing spinal cord. We aimed to determine the extent to which continuous supplementation of BDNF and NT-3 individually could rescue motor neurons from NOCD in vivo. BDNF and NT-3 was also supplied in combination to determine whether the addition of both factors could elicit a greater survival response. The effect of the neurotrophins on motor neuron survival was examined both after the main phase of neuronal degeneration and at a stage several days later to determine whether single factors could maintain their trophic support or whether the trophic requirements showed temporal changes.

## 1. Experimental procedures

### 1.1. Transient transfection of 2.3D cell line

The 2.3D neuroepithelial cell line (Bartlett et al., 1988) was transiently transfected with pcDNA3 construct, pcDNA3/BDNF, pcDNA3/NT-3 or a combination of pcDNA3BDNF/pcDNA3NT-3. The pcDNA3/BDNF and NT-3 constructs were generated by PCR amplification of the preproBDNF and preproNT-3 cDNA followed by ligation into the pcDNA3 vector. Constructs were checked for accuracy of sequence and correct orientation of insertion by sequencing prior to use in experiments. A calcium phosphate method was used for the transfection (Chen and Okayama, 1998). Briefly, 2.3D cells were grown to near confluency then diluted to a 1 in 15 ratio on the day prior to transfection and plated in 5 ml DMEM/10%FCS in 25 $cm^2$ tissue culture flasks (Greiner) in a 10% $CO_2$ incubator at 37 °C. Ten micrograms of vector DNA was precipitated in ethanol overnight at −20 °C prior to transfection. On the day of transfection, the precipitated DNA was centrifuged at 13,000 × *g* and washed with 70% ethanol to remove salts. The pellet was air-dried in a laminar flow hood then resuspended in 450 μl sterile water. Fifty microliters of 2.5 M $CaCl_2$ was added to the resuspended DNA to make a final $CaCl_2$ concentration of 0.25 M. Five-hundred microliters of 2×HeBS (0.28 M NaCl, 50 mM HEPES, 1.5 mM $Na_2HPO_4$, pH 7.05) was added to a sterile 15 ml conical tube (Falcon). The DNA/$CaCl_2$ solution was added dropwise to the tube while a mechanical pipettor with a 1 ml sterile pipette attached was used to blow air through the HeBS solution to ensure constant mixing of the two solutions. After the addition of all the DNA/$CaCl_2$, the sample was vortexed, then incubated for 20 min at room temperature. The DNA precipitate solution was added to the culture flask containing the 2.3D cells and incubated at 37 °C in a humidified incubator for 16 h. After 16 h, the media was removed and the cells washed with 5 ml of 1×PBS. Five millilitres of fresh DMEM/10% FCS was added and the cells incubated for a further 2 days prior to harvesting of cells and injection into the developing chick embryo. Media was checked for the presence of growth factor using a DRG bioassay (results not shown).

### 1.2. In ovo embryo manipulation

All experiments were approved by Monash University School of Biological Sciences Animal Ethics Committee and were conducted in accordance with Australian Code of Practice for the care and use of animals for scientific purposes. All embryo manipulation was carried out in a laminar flow cabinet using sterile techniques. A window was created in the blunt end of the shell of an embryonic day 5 (E5) white leghorn chick using the method previously described by Oppenheim et al. (1988). The outer membrane was removed and the embryo gently manipulated so that the optic tectum was accessible. Care was taken to avoid any haemorrhaging of the blood vessels surrounding the embryo. Recombinant cells (∼20,000) containing the pcDNA3 vector alone, the pcDNA3/BDNF vector, the pcDNA3/NT-3, or a combination of the two factor secreting cells were pressure-injected into the ventricular space of the optic tectum in a volume of 100 μl. Following surgery, the aperture was sealed with tape and the eggs returned to the egg incubator.

### 1.3. Tissue processing

Spinal tissue was harvested from the chick embryo at daily intervals between E6 and 15 for analysis of motor neuron survival and at E10 and 15 for factor supplementation analysis. The embryo was removed from the egg,