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Original Article

Genetic disruption of the growth hormone receptor does not influence motoneuron survival in the developing mouse

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ABSTRACT In the rodent central nervous system (CNS) during the five days prior to birth, both growth hormone (GH) and its receptor (GHR) undergo transient increases in expression to levels considerably higher than those found postnatally. This increase in expression coincides with the period of neuronal programmed cell death (PCD) in the developing CNS. To evaluate the involvement of growth hormone in the process of PCD, we have quantified the number of motoneurons in the spinal cord and brain stem of wild type and littermate GHR-deficient mice at the beginning and end of the neuronal PCD period. We found no change in motoneuron survival in either the brachial or lumbar lateral motor columns of the spinal cord or in the trochlear, trigeminal, facial or hypoglossal nuclei in the brain stem. We also found no significant differences in spinal cord volume, muscle fiber diameter, or body weight of GHR-deficient fetal mice when compared to their littermate controls. Therefore, despite considerable *in vitro* evidence for GH action on neurons and glia, genetic disruption of GHR signalling has no effect on prenatal motoneuron number in the mouse, under normal physiological conditions. This may be a result of compensation by the signalling of other neurotrophic cytokines.

KEY WORDS: *growth hormone receptor, motoneuron, apoptosis, nervous system*

Introduction

Neuronal proliferation in response to growth hormone (GH) was first reported over sixty years ago (Zamenhof, 1941; Zamenhof *et al.*, 1966), yet the role of GH in development of the central nervous system (CNS) remains obscure. This is because patients lacking the GH receptor (GHR) appear to behave normally (Krieger, 1980), although they do show a significant loss of spatial discrimination speed and efficiency compared to their normal relatives (Kranzler *et al.*, 1998). Recent investigations showing GH to be neuroprotective against ischaemic brain injury (Scheepens *et al.*, 2001), capable of attenuating trauma-induced depression of spinal cord evoked potentials (Winkler *et al.*, 2000), and able to ameliorate motor dysfunction resulting from spinal cord injury (Hanci *et al.*, 1994) have renewed interest in the putative role of GH in the CNS. These *in vivo* studies are concordant with demonstrations of *in vitro* actions of GH on neurons and glial cells, including a 300% increase in cell number and Bcl-2 expression in fetal rat brain slices (Cacicedo, 1999; Ajo, 2001) and an increase in counts of nestin positive neurons in primary cultures of fetal cerebro-cortical cells (Sanchez Franco *et al.,* 1999).

A potential role for GH in development of the CNS is supported by the widespread distribution of GH and its receptor within the developing CNS (Garcia-Aragon *et al.*, 1992; Lobie *et al.*, 1993). Both GH and GHR are present in the adult CNS of rodents, chickens, rabbits, primates and humans (Kyle *et al.*, 1981; Hojvat *et al.*, 1982a; Render *et al.*, 1995; Ramesh *et al.*, 2000; Harvey *et al.*, 2001), and are widespread throughout the CNS in both fetal and neonatal animals (Garcia-Aragon *et al.*, 1992; Lobie *et al.*, 1993; Harvey *et al.*, 2001). In the rat, GHR expression in the CNS increases from embryonic day 14 (E14) to a maximum at E18, then declines postnatally (Garcia-Aragon *et al.*, 1992). Sites of increased expression include the frontal lobe, thalamus, brainstem, choroid plexus, hippocampus, hypothalamus, inferior colliculus and motoneurons of the spinal cord (Garcia-Aragon *et al.*, 1992; Lobie *et al.*, 1993). Increased expression of GHR in motoneurons is supported by the presence of mRNA transcripts in pig motoneu-

Abbreviations used in this paper: bGH, bovine growth hormone; CNS, central nervous system; E, embryonic day; GH, growth hormone; GHR, growth hormone receptor; LMC, lateral motor column; MT, metallothionine; P, postnatal day; PCD, programmed cell death.

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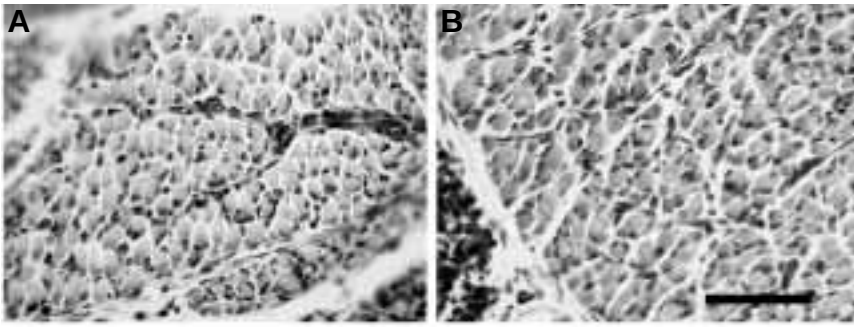

Fig. 1. Disruption of GH signalling in GHR-deficient mice does not affect gross morphology of skeletal muscle prior to birth. *Shown are transverse sections through wild type* **(A)** *and GHR-deficient* **(B)** *erector spinae muscles at E18.5, showing no change in gross muscle structure or organisation. Arrows indicate the peripheral location of the muscle fibre nuclei, which is the same for wild type and mutant fibres. Scale bar, 60 μm.*

rons (Kolle *et al.*, 1998) and has been reported on both spinal cord and cranial motoneuron populations in the developing chick (Harvey *et al.*, 2001).

This increased expression of GH receptors in the fetal rat brain, reaching a maximum around E18, is paralleled by increased expression of GH itself within the CNS which also reaches a peak just prior to birth (Hojvat *et al.*, 1982a). In the rat and primate CNS, the highest concentrations of GH are found in the amygdala, hypothalamus, caudate putamen, brain stem and spinal cord, although detectable levels have been observed in all areas studied (Hojvat *et al.*, 1982a; Lechan *et al.*, 1983). Motoneuron pools in the ventral horn of the brachial and lumbar spinal cord of the chick are also highly GH immunoreactive (Harvey *et al.*, 2001). This GH is thought to be of local origin, since explants from the CNS, totally removed from the influence of the pituitary, have been found to release immunoassayable GH for 16 – 30 days in culture and hypophysectomized rats continue to display normal levels of GH in the CNS despite the removal of the pituitary (Hojvat *et al.*, 1982a). *In situ* hybridization and RT-PCR studies have confirmed the brain as a site of extrapituitary gene expression (Gossard *et al.*, 1987; Harvey *et al.*, 2000).

The expression of GH and its receptor at peak levels during the prenatal period from E14–E18 coincides with the process of programmed cell death (PCD). PCD is a naturally occurring process whereby excess neurons created during early CNS development are removed, leaving a functional framework of viable cells. This cell death is programmed both in the sense that is occurs at a reproducible stage of development and that it involves the activation of signal transduction mechanisms dedicated to killing the cell (Henderson, 1996). It has been best studied in the neuromotor system where approximately 50% of motoneurons undergo PCD between E13 and birth (Lance-Jones, 1982; Banks *et al.*, 2001). The decision as to whether a particular neuron survives or dies is thought to be dependent upon its access to survival-promoting neurotrophic factors derived from both target and support cells (Oppenheim, 1991; Henderson, 1996; Lemke, 2001).

Given that GH can act as an anti-apoptotic agent in non-neuronal cells (Jeay *et al.*, 2001), strongly increases Bcl-2 expression in explants of fetal rat brain (Ajo, 2001) and is neuroprotective in response to ischemic brain injury (Scheepens *et al.*, 2001), it is plausible that GH acts at this time as a neurotrophic factor to promote the survival of a proportion of neurons by opposing PCD. This would be analogous to the actions of the related cytokines ciliary neurotrophic factor and leukemia inhibitory factor, both of which have been shown to promote neuronal survival in knock-out mice by paracrine or autocrine means (DeChiara *et al.*, 1995; Sendtner *et al.*, 1996).

Testing the hypothesis that GH decreases PCD during development of the fetal CNS requires a suitable model, which is known to be GH responsive. Chen and colleagues (Chen *et al.*, 1997) found an increase in lumbar motoneuron size in adult mice over expressing the bovine GH transgene. In particular, GH over expression was found to coordinately increase nuclear size, however, no attempt was made to quantitate motoneuron number. Accordingly, we have used the GH receptor knockout mouse (Zhou *et al.*, 1997) to determine whether the absence of GH action in the prenatal period accentuates PCD of spinal motoneurons. Recent investigations have shown that the trophic dependence of specific populations of motoneurons is not uniform, but rather, motoneurons from cranial, brachial and lumbar motor pools have distinct trophic requirements (Novak *et al.*, 2000; Oppenheim *et al.*, 2001). Therefore, the current study has investigated the survival of motoneurons in the brachial and lumbar lateral motor columns and several cranial nuclei before and after PCD.

TABLE 1

BODY MASS, SPINAL CORD VOLUME AND MUSCLE FIBER DIAMETER IN WILD-TYPE AND GHR-DEFICIENT MICE DURING DEVELOPMENT

	Wildtype ± SD	GHR-Deficient ± SD	P Value	No. of Litter Pairs (n)
Body Mass (g)				
E18.5	0.9717 ± 0.1401	0.94 ± 0.1185	0.51	6
P2	2.105 ± 0.2339	1.958 ± 0.2647	0.21	4
Spinal Cord Volume (μm³)				
E13.5 Brachial	0.3625 ± 0.02331	0.3427 ± 0.03367	0.11	5
E18.5 Brachial	1.427 ± 0.2327	1.353 ± 0.1084	0.23	6
E18.5 Lumbar	1.160 ± 0.2155	1.098 ± 0.1984	0.47	5
P2 Lumbar	2.085 ± 0.2822	1.915 ± 0.2674	0.46	4
Muscle Fiber Diameter (μm)				
E18	45.61 ± 4.125*	45.37 ± 6.326Δ	0.91	6

Each value represents the mean ± SD from at least 4 litter matched pairs of wild-type (WT) and GHR-deficient mice, *P* values calculated from paired Student's t-test. Calculated means are from 180* fibers and 172Δ fibers from litter matched pairs of WT and GHR-mutant mice.

Results

Mass, Muscle and Spinal Cord Development in GHR-Deficient Mice

In agreement with previous studies, the current investigation found no significant differences in body mass of developing GHR-deficient animals from E18 through to P2 (Table 1; Zhou *et al.*, 1997). Spinal cord volume of both the brachial and lumbar spinal cords were also determined to define any morphological change in spinal cord development, which may impact on motoneuron development or survival. No significant change was observed in spinal cord volume at E13.5, E18.5 or at P2 (Table 1).

Insofar as GHR is present in skeletal muscle (Frick *et al.*, 1990) and that GH over expression has a potent hypertrophic effect on muscle (Guler *et al.*, 1988), its absence could conceivably have secondary effects on skeletal muscle development which in turn could affect its physiological function. We therefore characterized muscle differentiation in GHR-deficient mice and compared it to that in wild-type littermates at E18.5.

We found no significant difference in gross organisation of muscle fibres or fibre density in cross sections of skeletal muscle (Fig. 1). The cross-sectional area of muscle fibres in the wild type did not differ significantly from that of the GHR-mutant (Table 1). Moreover, the proportion of nuclei placed centrally, compared to the outer edge of muscle fibres did not differ between wild type and GHR -deficient mice (Fig. 1). In addition, we have found no changes in either motor axonal branching or arborization of their nerve terminal endings in the diaphragm of GHR-deficient and wild type mice (data not shown). Together, these results indicate that GH is not an essential regulator of spinal cord or muscle development, nor does it appear to affect the innervation patterns of skeletal muscle, during mid to late embryonic development.

Formation and Gross Morphology of Motoneurons in the Brachial and Lumbar Lateral Motor Columns in GHR-Deficient Mice

Motoneurons of the spinal cord are generated and undergo PCD in a rostral-caudal direction throughout the spinal cord, with apoptotic cells becoming evident from E11.5 in the brachial LMC of the mouse (Yamamoto and Henderson, 1999). We found that the brachial LMC could be readily distinguished in sections from E13.5 in both wild type and GHR-mutant mice (Fig. 2). The appearance and position of the brachial LMC and motoneuron nuclei did not differ between wild type and GHR-deficient mice at E13.5 (Fig. 2). We then examined the morphology of brachial and lumbar LMCs and their motoneurons, towards the end of the PCD period (E18.5 to P2). Again, there was no change in spinal cord appearance or motoneuron location with these LMCs at either E18.5 or P2 (Fig. 2).

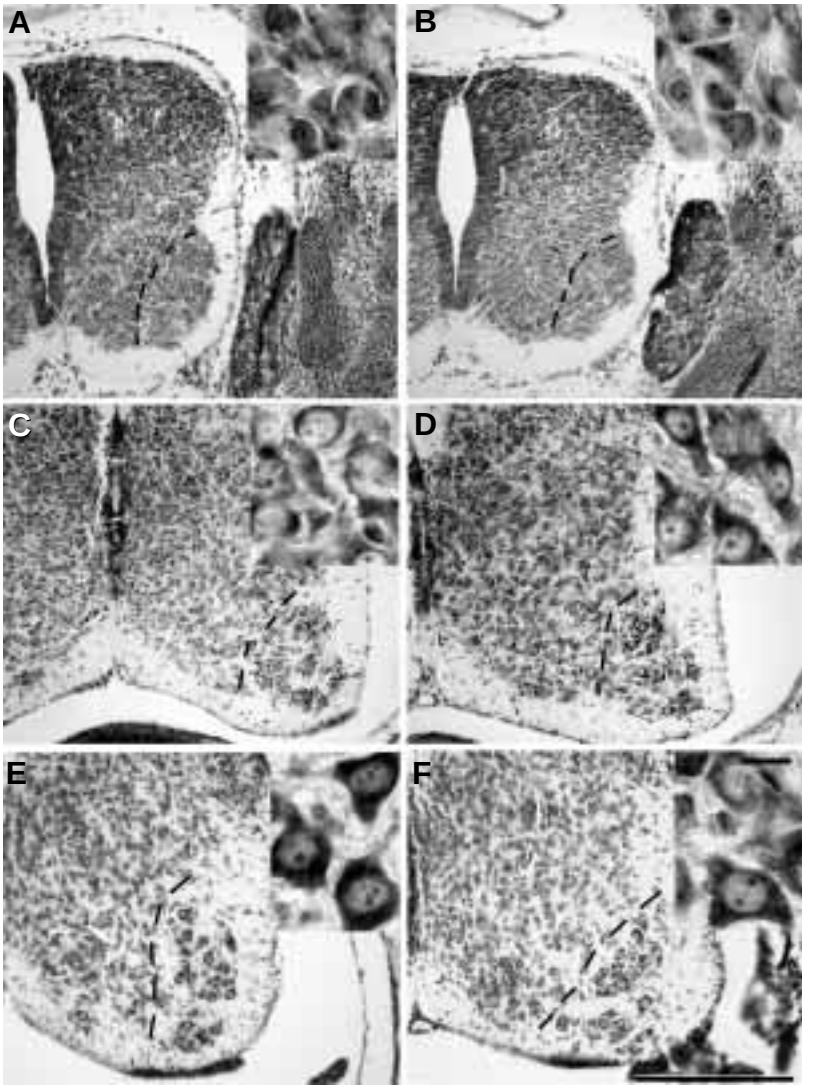

Fig. 2. Morphology of the brachial and lumbar LMCs appears normal in wild type and GHR-deficient mice. *Displayed are light micrographs of the brachial* **(A-D)** *and lumbar* **(E,F)** *spinal cords showing the gross appearance and location of the lateral motor columns (LMCs) and motoneurons within GHR-deficient mice and wild type litter mates during the period of programmed cell death. Displayed are transverse sections through the brachial LMC at embryonic day 13.5 (E13.5; A, wild type; B, mutant), E18.5 (C, wild type; D, mutant) and lumbar LMC at postnatal day 2 (P2; E, wild type; F, mutant). Dashed lines indicate the medial border of the LMC. Insets show higher magnification of motoneurons located within the LMCs. Scale bars, 200 μm; 10 μm for insets.*

Survival and Maturation of Brachial and Lumbar LMC Motoneurons in GHR-Deficient Mice

Our central aim was to test if GH, signalling through its receptor, acts as a neurotrophic factor by opposing PCD of motoneurons during embryonic development (see introduction). To test this, we counted the number of motoneurons in the brachial and lumbar LMCs at the beginning (E13.5), end (E18.5) of the PCD period for motoneurons, and two days after the end (P2) in GHR-deficient mice and their wild type litter mates. We observed no significant difference in motoneuron number between GHR-deficient and wild type littermate mice at these ages (Fig. 3).

Given that over expression of GH in mice causes an increase in adult motoneuron size (Chen *et al.*, 1997), we determined if the lack of GH signalling affected the maturation of spinal cord motoneurons during the period of PCD. We measured the nuclear area of motoneurons, which is directly proportional to cell soma size for spinal cord neurons (Holley *et al.*, 1982a,b; Oppenheim *et al.*, 1982; McIlwain, 1991; Sato *et al.*, 1994; Chen *et al.*, 1997). At E13.5 motoneuron nuclear area in the brachial LMC was significantly smaller in GHR-deficient mice compared to litter mate controls (13% decrease, $P < 0.0001$, linear regression; Fig. 4), although the magnitude of the change was not large, it was noticeable (see Fig. 5). By the end of the PCD period at E18.5,

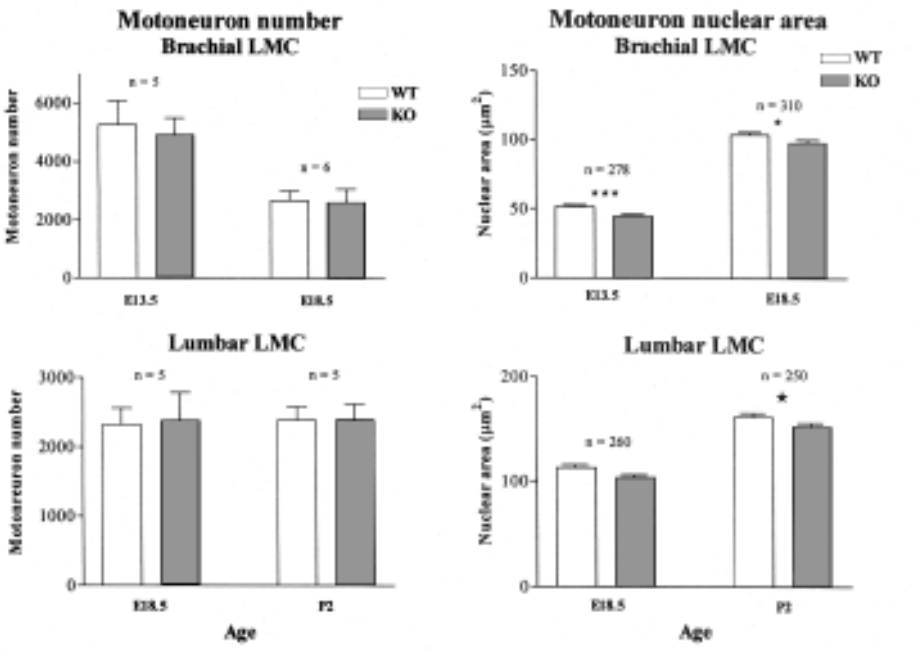

Fig. 3. (Left) Disruption of GH signalling does not affect the number of brachial and lumbar LMC motoneurons. *Shown are the means ± SD of brachial and lumbar LMC motoneuron numbers in wild type (open bars) and GHR-deficient (grey bars) mice, at E13.5, E18.5 and P2. The n values indicate the number of wild type and mutant litter matched pairs analysed. Statistical analysis was undertaken by the Student paired t test.*

Fig. 4. (Right) Disruption of GH signalling results in a decrease in spinal cord motoneuron nuclear area during embryonic development. *Shown are the means ± SEM of nuclear areas of wild type (open bars) and GHR-deficient (grey bars) mice at E13.5, and E18.5 for brachial LMC motoneurons, as well as at E18.5 and P2 for lumbar LMC motoneurons. The n value indicates the combined total number of nuclei measured across five pairs (mutant and their litter matched controls) for each age indicated. The asterisks indicate the level of significance (E13.5 P<0.001; E18.5 P=0.0102; P2 P = 0.0089). Statistical significance was conducted using the multiple linear regression model.*

brachial LMC motoneuron nuclear area remained significantly smaller in the GHR-mutants (6% decrease, P = 0.012, linear regression; Fig. 4). A similar trend was also observed for lumbar LMC motoneuron nuclear areas. Specifically, motoneuron nuclear areas appeared to be slightly, but not significantly smaller (P = 0.08, linear regression; Fig. 4) at E18.5, and became significantly smaller by P2 in GHR-deficient mice compared to litter mate controls (6% decrease, P = 0.0089, linear regression; Fig. 4). Together, these results suggest that GH has a small and significant influence on spinal cord motoneuron maturation during embryonic development (E13.5 to P2).

TABLE 2

MOTONEURON NUMBERS IN THE TROCHLEAR, TRIGEMINAL, FACIAL AND HYPOGLOSSAL MOTOR NUCLEI IN WILD-TYPE MICE AND GROWTH HORMONE RECEPTOR-DEFICIENT MICE AT E18.5

	Wildtype	GHR-Deficient	% Change	*P* values
(IV) Trochlear	197 ± 77	224 ± 51	+13	0.33219
(V) Trigeminal motor	832 ± 37	890 ± 203	+7	0.6960
(VII) Facial	5322 ± 764	5008 ± 851	-6	0.7193
(XII) Hypoglossal	2139 ± 5	2087 ± 426	-3	0.8536

Values represent the mean ± S.D. number of motoneurons. Percentages refer to the change in motoneuron number with respect to wild-type mice. Data is from three litter matched pairs of GHR-knockout and wild type control mice (n=3).

Morphology and Number of Motoneurons in Cranial Motor Nuclei in GHR-Deficient Mice

Recent studies have shown that distinct motoneuron pools within the CNS require different trophic factors to mediate their survival during PCD (Novak *et al.*, 2000; Oppenheim *et al.*, 2001). This raises the possibility that GH acting through its receptor (GHR) may have selected trophic actions on discreet populations of motoneurons. We examined morphology and number of motoneurons of the Trochlear (cranial nerve IV), Trigeminal (V), Facial (VII) and Hypoglossal (XII) nuclei of the brain stem in GHR-deficient mice and their littermate controls at E18.5. First, we observed that there was no difference in the gross morphology of these nuclei and the appearance of motoneurons found within these nuclei (Fig. 6). We then counted the number of motoneurons found within these nuclei at E18.5, and again observed no significant difference between wild type and GHR-deficient mice (Table 2).

Analysis of GH Over-Expressing Transgenic MT-1bGH Mice reveal that the MT-1 Promoter is not active in the Embryonic Nervous System

Since motoneurons in adult MT-1bGH transgenic mice have been shown to significantly increase in size (Chen *et al.*, 1997), we used this line to investigate the effects of GH over expression on motoneuron size and number, at the end of the PCD period (E18.5). We found no difference in lumbar motoneuron size or number in MT-1bGH mice when compared to their wild type littermate controls at E18.5 (data not shown). This finding was in contrast to the finding that GH over expression increases adult motoneuron size (Chen *et al.,* 1997), and was at odds with our findings of reduced embryonic motoneuron size in GHR-knockout mice. We then investigated the

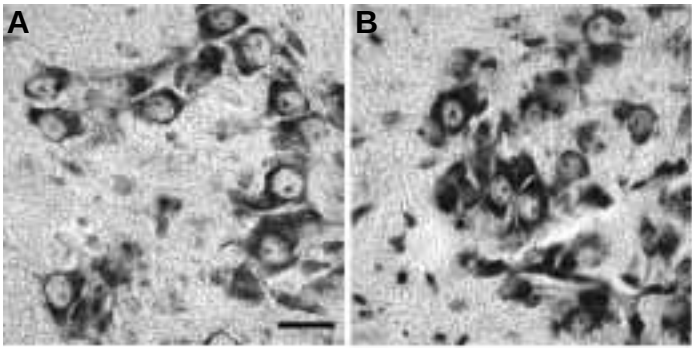

Fig. 5. A slight change in motoneuron nuclei size was noted in GHR-disrupted mice. *Shown are the motoneurons found within the brachial LMC of GHR-deficient* **(B)** *and their wild-type litter mates* **(A)** *at E18.5. Motoneurons are characterised by a dark stained cytoplasm, pale nucleus and darkly stained nucleoli. Scale bar, 20 μm.*

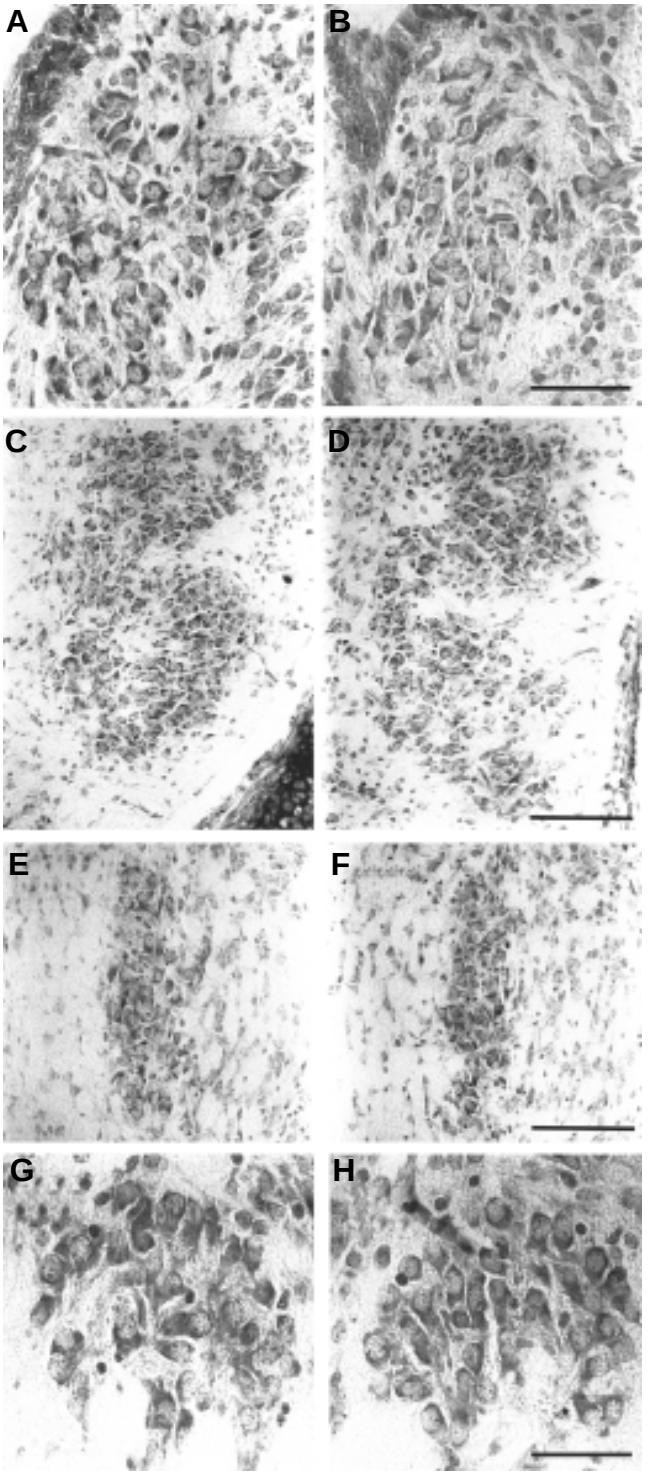

Fig. 6. The morphology of neurons found within cranial motor nuclei in GHR-deficient mice was unaltered. *Shown are a series of transverse sections taken through the Hypoglossal* **(A,B)**, *Facial* **(C,D)**, *Trigeminal* **(E,F)** *and Trochlear* **(G,H)** *motor nuclei from GHR-deficient mice (right column) and their litter mate controls (left column), at E18.5. The appearance of the nuclei and motoneurons found within them does not differ between wild type (A,C,E and G) and GHR-deficient mice (B,D,F and H). Scale bars: 50 μm for A,B,G and H; 100 μm for C,D,E and F.*

Fig. 7. Lack of expression of bGH in the CNS of *MTbGH* transgenic mice, at E18.5 (fetal). *Depicted is an immunoblot with 50 μg cytosolic protein per lane, showing 22 kDa immunoreactive bands that represent the presence of bGH in fetal liver (FeLivbGH), adult liver (AdLivbGH), and lower levels in adult brain (AdBrainbGH), from* MTbGH *transgenic mice. In contrast, there were no 22 kDa immunoreactive bands in extracts of* MTbGH *fetal brains (FEbrainbGH). The anti-bGH anitbody did not cross react with mouse GH as immunoreactive bands from brain (FeBrainWT) or liver (FeLiverWT) extracts taken from E18.5 wild type litter mates were not observed. The specificity of anti-bGH for recombinant purified bGH is also shown (bGH).*

expression of bGH embryonic CNS (brain + spinal cord) and in liver, from E18.5 MT-1bGH embryos by immunoblot. We found high levels of the transgene expressed in both fetal and adult liver (estimated 5 μg/g) but not in the fetal CNS, although the transgene was expressed in adult brain (Fig. 7). As the permeability of the neonatal blood brain barrier to GH is limited (Hojvat *et al.,* 1982a), it appears unlikely that the bGH would access the developing CNS at this age. This explanation could account for our failure to observe any changes to motoneurons in the MT-1bGH mice, but raises the need to create new lines of GH-transgenic mice, where GH is over expressed in the CNS during embryonic development.

Discussion

The aim of the present study was to test the hypothesis that GH is acting as a neurotrophic factor for motoneurons during PCD. This hypothesis was formulated from previous observations that showed transient increases in GH and GHR expression in the CNS coincident with the period of PCD (Hojvat *et al.*, 1982b; Lance-Jones, 1982; Garcia-Aragon *et al.*, 1992) and from *in vitro* and *in vivo* observations suggesting GH is a promoter of neuronal survival (Cacicedo, 1999; Ajo, 2001). In this study the role of GH in the control of PCD of motoneurons has been investigated by quantification of motoneuron number at the beginning of PCD, and following PCD in the GHR-deficient mouse, the best available model for investigation of GH action *in vivo* (Kopchick *et al.*, 1999; Kopchick and Laron, 1999).

The results show that while there is no change in mass, spinal cord volume or motoneuron number between mice deficient in the GHR and wild-type littermates, there does appear to be a small, but statistically significant difference in motoneuron size. Specifically, our results demonstrate that at the beginning of motoneuron PCD (E13.5) and following PCD (E18.5 and P2), motoneuron nuclear area, and by direct inference the neurons themselves, are smaller compared to littermate controls. The significance of our findings is discussed below.

Growth Hormone has No Obvious Prenatal Role in Peripheral Development

Alteration in muscle fibre diameter or spinal cord volume can result in indirect effects on motoneuron survival and/or size

(Finkelstein *et al.*, 1991). Our study has found no alteration in body mass, muscle fibre diameter and spinal cord volume in the GHR-deficient mouse prior to and including postnatal day 2. The absence of peripheral (innervated tissue) alteration by GH prior to birth allows for morphological analysis of direct actions of GH on the developing CNS, rather than effects secondary to peripheral action. In addition, these findings are the first quantification of spinal cord volume and muscle fibre diameter of the GHR-mutant mouse prior to birth. This is of interest as the prenatal role of GH remains under debate (Pantaleon *et al.*, 1997).

Disruption of the GH Receptor does not alter the Survival of Motoneurons

The results presented here demonstrate that disruption of GHR signalling does not alter the survival of motoneurons in the developing mouse. Motoneuron number immediately following PCD did not differ between GHR-knockout and wild type mice in lumbar, brachial or cranial motoneuron pools. In support of this we also observed no gross changes in motor innervation patterns or changes to the morphologies of neuromuscular junctions between wild-type and GHR-deficient mice. These findings demonstrate the contrast, which has emerged between the survival-promoting effects of GH seen *in vitro* (see introduction) and the lack of alteration in motoneuron number evident in the CNS of GHR-deficient mice. Given this contrast, are the findings presented here enough to eliminate a role for GH in PCD? Previous investigations into motoneuron survival using genetically modified mice suggest PCD is a robust process capable of compensating for the loss of factors involved in its regulation. This compensatory capacity is a result of the ability of complex organisms to maintain homeostasis through the use of multiple pathways involved in the control of the same function. An excellent example of this is the pro-apoptotic TGF-β family, where inactivation of all TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3) simultaneously, but not any of them individually (Goumans and Mummery, 2000), results in an increase in neuronal survival (Krieglstein *et al.*, 2000).

There are a number of potential mechanisms for such compensation in the GHR-knockout mouse. Prolactin receptors are most closely homologous to the GH receptor, and are known to have cross-reactivity with GH (Mustafa *et al.*, 1994; Ramesh *et al.*, 2000) and are widely expressed in the rodent brain (Royster *et al.,* 1995). Therefore, a compensatory response from the PRL receptor may, in part, account for the lack of observed effect of GH receptor knockout on motoneuron survival. This possibility is supported by the finding that deficiency in GH and PRL signalling combined, but not individually, results in loss of fertility, indicating mutual compensation for loss of action (Bartke *et al.*, 1999).

A related mechanism of compensation is through paralagous cytokine signalling pathways. The GHR is a class one cytokine receptor, and its signalling pathways are common with those of neurotrophic class one cytokine receptors, such as CNTF and LIF, as well as those of neurotrophins such as BDNF and NT-4 (Kaplan and Miller, 2000). The three main signalling pathways employed by these neurotrophic factors (and GH) are the mitogen activated protein kinase (MAP), phosphatidyl inositol 3-kinase (PI3K) and the janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways (Smit, 1999; Dolcet *et al.*, 2001). Therefore, the reduction in second messenger signalling as a result of GHR disruption may be compensated by neurotrophic factors utilizing common signalling pathways, particularly LIF and CTNF (Sendtner *et al.*, 1996).

The fact that we observed no change in motoneuron number with only a small change in size, may suggest that GH-GHR signaling may be required outside the period of naturally occurring motoneuron cell death, and/or be revealed under abnormal physiological conditions. For example, during normal postnatal development, CNS GH and GHR levels drop dramatically some 24 to 48hrs after birth, (Hojvat *et al.*, 1982a; Lobie *et al.*, 1993). However, Scheepens and colleagues (Scheepens *et al.*, 2001) have shown that under conditions of induced hypoxia levels of GH levels were dramatically increased in regions of neuronal cell loss. Furthermore, they showed that injection of GH into the CNS after moderate hypoxic-ischemica brain injury, GH acted as a neurotrophic agent to restrict the extent of neuronal death in selected regions of the cortex (frontoparietal), hippocampus and thalamus (Scheepens *et al.*, 2001). Whether or not GHR is upregulated in a similar fashion under such conditions and that disruption to GH-GHR signaling can attenuate such neuronal loss remain to be determined. Moreover, whether motoneurons would respond to injections of GH under hypoxic conditions also remain to be investigated.

The Maturation of Motoneurons is retarded in GHR-Deficient Mice

This study has shown that motoneurons of GHR-deficient mice have a small, but statistically significant reduction in nuclear area as compared to wild-type controls. A variety of morphometric studies have demonstrated that the nuclear area of motoneurons is known to vary directly with motoneuron cell size (McIlwain, 1991; Chen *et al.*, 1997), therefore indicating a small reduction in the size of motoneurons in GHR-knockout mice. Interpretation of these results must be conducted with some caution, as histological processing causes significant insult to delicate embryonic/neonatal tissue and results in tissue shrinkage (Braendgaard and Gundersen, 1986). However, wild type and GHR-knockout litter-matched pairs were processed with identical reagents at the same time and quantified blind. Thus, the relative comparison of litter-matched GHR-deficient and wild type is statistically valid. The finding of reduced nuclear area within the GHR-mutant mouse supports a previous report of increased motoneuron size in postnatal GH over expressing mice (Chen *et al.*, 1997).

Previously, a decrease in motoneuron size, but no change in motoneuron number has been noted in newborn NT-3 knockout mice (Woolley *et al.*, 1999). *In vitro,* NT-3 increases expression of choline acetyl transferase, leading to accelerated accumulation of synaptic vesicle specific proteins and increasing the aggregation of acetylcholine receptors on myotubes (Wong *et al.*, 1993; Wang *et al.*, 1995; Braun *et al.*, 1996). These studies into the actions of NT-3 have led to the suggestion that NT-3 promotes the maturation of motoneurons and the development of neuromuscular connections (Woolley *et al.*, 1999). It is not yet known whether GH plays a similar role in neural development of motoneurons to NT-3.

Materials and Methods

Animals

GHR knockout mice have functional disruption of the GHR gene resulting in complete lack of GHR signalling (Zhou *et al.*, 1997). These animals present phenotypically with substantially reduced postnatal growth

(~50% of wild type at 6 weeks), increased systemic GH and reduced levels of systemic IGF-1. However, these deficits do not become apparent until after the second week postnatally, at birth GHR-deficient mice are not distinguishable from their wild-type littermates (Zhou *et al.*, 1997).

Litter matched mouse embryos carrying two normal copies (wild-type), or two copies of the disrupted GHR gene (homozygote mutant) were used in this study. These embryos were obtained from matings of mice heterozygous for the GHR mutant allele (Zhou *et al.*, 1997). The heterozygous mice used to generate the embryos for this study were maintained on defined 129OLA/BalbC genetic background. The age of the embryos was determined from the day on which a vaginal plug was identified, designated embryonic day E0. Pregnant females of appropriate gestational age were anaesthetised with Nembutal (30 mg) and killed by cervical dislocation. Embryos were then placed into ice cold phosphate buffered saline pH 7.4 (PBS). Post-natal animals were anaesthetised on ice and killed by cervical dislocation at post-natal day 2 (P2), where day of birth was recorded as P0. Wild-type and homozygote embryos were identified by PCR analysis of tail or hind limb DNA as previously described (Chandrashekar *et al.*, 1999).

GH over expressing transgenic mice have a genomic insertion of a 2.6kb DNA fragment consisting of the bovine GH cDNA (bGH) fused to a mouse metallothionine (MT-1) promoter (Hammer *et al.*, 1985). These mice (MTbGH) have previously been used to investigate the effect of GH over expression on motoneuron development in postnatal mice (Chen *et al.*, 1997). This strain has a greater than ten fold elevation in serum GH concentration. It is on a C56/B6SJL genetic background, maintained as heterozygotes. MTbGH transgenic (heterozygote) and wild type litter matched embryo pairs were obtained by the mating of wild type (+/+) females with MTbGH heterozygote males (+/+*) resulting in embryos of either +/+ or +/+* genotype. The genotype of MTbGH transgenic mice was determined by the presence of the transgene (MTbGH) by PCR amplification using a sense primer from the metallothionine promoter (5'-CTGAGTACCTTCTCCTCACTTAC-3'), and an antisense primer specific to bovine growth hormone cDNA (5'-AGCCCAAAGCTCTGACACCATC-3') to amplify a unique 400 base pair band. Details of tail extraction, and reaction conditions have been previously reported (Chandrashekar *et al.*, 1999; Hanley and Merlie, 1991). MTbGH and wild type embryos were then processed as described above. All animal procedures have been approved by the University of Queensland's Animal Ethics Committee.

Motoneuron Counts and Morphometric Analyses

Spinal cords and brain stems from E13.5 and E18.5 mice were dissected, fixed in 10% neutral buffered formalin (pH 7.4), dehydrated in ethanol and toluene, and paraffin embedded for transverse sections. Serial 6 to 12μm thick sections were cut through cranial nuclei and through the brachial and lumbar regions of the spinal cord, and stained with 1% thionine in sodium acetate buffer. After dehydration and mounting, numbers of motoneurons in the cranial nuclei, brachial and lumbar lateral motor columns (LMCs) were counted using previously established methods (Clark and Oppenheim, 1995). This counting method does not require either stereology or correction factors, and has been shown to produce results which differ by no more than 5% from methods using unbiased correction factors (Clark and Oppenheim, 1995), and to be within 2% of results obtained using the physical dissector method (Clark and Oppenheim, 1995; Banks *et al.*, 2001).

The brachial and lumbar lateral motor columns, located in the ventral horn of the gray matter, extend from the fifth cervical dorsal root ganglia (DRG) to the first thoracic DRG and from the twelfth thoracic DRG to the fifth lumbar DRG, respectively. The starting and finishing positions of the lateral motor columns in the spinal cord were found using a combination of the LMC thickening, presence of the dorsal root ganglia and morphology of the vertebrae. In addition to these anatomical markers, the lateral motor column enlargement was used to aid precise identification of commencement and termination of the lateral motor columns. The locations of the cranial nuclei (Trochlear (IV), Trigeminal (V), Facial (VII) and Hyopglossal (XII)) were determined by their relative positions and appearance within the mouse brain stem. Motoneurons in every 5th (cranial) or 10th (LMC) section were counted, divided by the total number of sections counted, and multiplied by the number of sections containing the cranial motor nuclei or LMC. Only those motoneurons with dark staining cytoplasm, pale nucleus and dark staining nucleoli were included in the counts. It has been reported that identification of motoneurons on the basis of these criteria ensures that the same neuron is counted in adjacent sections less than 2% of the time (Clark and Oppenheim, 1995). The genotype was not made available to the researcher conducting the counts until they were complete.

Spinal cord volume and nuclear areas were obtained using previously established methods (Holley *et al.*, 1982a; b; Oppenheim *et al.*, 1982; Sato *et al.*, 1994; Chen *et al.*, 1997; Banks *et al.*, 2001). Nuclear area was determined by integrating the area outlined by a tracing of the projected images of the nucleus using Scion Image Beta 4.0.2 image analysis software program. Only motor neurons that displayed a pale nucleus with dark nucleoli, and were localised in histological sections through the centre of the motor column or motor nucleus were selected for measurement. The spinal cord areas were calculated using Cavalieri's principle (Gundersen and Jensen, 1987). The cross sectional area of the spinal cord was obtained from every 10th section using Scion image. Average spinal cord cross-sectional area was calculated from these sample measurements and multiplied by the length of the motor pool (thickness of sections multiplied by total number of sections that contained the motor pool) to obtain the spinal cord volume.

Immunoblot Analysis

Immunoblots were carried out in 15% acrylamide/bis (29:1) gels as described previously for the ovary (Tam *et al.*, 2001), except that NIADD anti-oGH-2 (National Pituitary Program, USA, www.humc.edu) was used at 1:1000 in Tween-20 in PBS, and the blot was blocked with 3% non-fat milk powder in 0.1 % Tween 20 in PBS. This allowed detection of less than 100 ng bovine GH using enhanced chemiluminescence detection, according to the manufacturers' instructions (Pierce Supersignal Wes Pico Chemiluminesscent Kit, Pierce Rockford, ILL. USA). Samples were normalized for protein (50 μg) using the Pierce BCA protein assay.

Immunohistochemistry

Mouse embryo diaphragms were processed for whole-mount immunohistochemistry with a combination of rabbit antibodies to synaptophysin (Dako Corporation, Carpinteria, USA) and neurofliament (Sigma, St. Louis, MO USA), followed by FITC-conjugated rabbit secondary antibodies (Slienus; AMRAD. Melbourne, Australia) and rhodamine α-bungarotoxin, as previously described (Banks *et al.*, 2001).

Statistical Analyses

Motoneuron quantification and morphometric analyses were performed on three litter matched pairs of wild type and mutant embryos for cranial motor nuclei, and on at least five litter matched pairs of wild type and mutant embryos for brachial and lumbar spinal segments, at the various embryonic ages studied. Paired two-tailed Students t-tests were used for the statistical analysis of motoneuron number, spinal cord volume, and animal mass. Nuclear area measurements were analyzed using a multiple linear regression model (PC-SAS version 8, Cary USA). Differences were regarded as statistically significant if the probability was less than 5% (P<0.05).

Conclusion

The expression of GH and GHR in the CNS of a wide range of species, ranging from sea lampreys to humans indicates that the role of GH in CNS development has been conserved on an evolutionary basis. However what this role is remains to be determined. In the present study surprisingly no significant influence of GH on motoneuron survival was evident, although the robustness and redundancy in control of PCD cannot exclude a role for GH as a neurotrophic factor, either under normal or

abnormal physiological conditions. The finding of reduced nuclear area in the GHR KO mouse may provide an indication that GH is involved in the growth and maturation of motoneurons, although the magnitude of the effect is limited.

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