Absence of retinoids can induce motoneuron disease in the adult rat and a retinoid defect is present in motoneuron disease patients

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Summary

We generated retinoid-deficient adult rats by the removal of retinoids from their diet. We show that their motoneurons undergo neurodegeneration and that there is an accumulation of neurofilaments and an increase in astrocytosis, which is associated with motoneuron disease. These effects are mediated through the retinoic acid receptor α. The same receptor deficit is found in motoneurons from patients suffering from spontaneous amyotrophic lateral sclerosis. Furthermore, we show that there is a loss of expression of the retinaldehyde dehydrogenase enzyme II in motoneurons. Therefore, we propose that a defect in the retinoid signalling pathway is in part be responsible for some types of motoneuron disease.

Key words: Motoneuron, Retinoic acid, Neurodegeneration

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the loss of motoneurons in the motor cortex, brain stem and spinal cord, which leads to weakness and atrophy (Delisle and Carpenter, 1984; Mulder et al., 1986). ALS occurs in both sporadic (90% of all cases) and familial forms (10% of all cases) (Jackson and Bryan, 1998). In 20% of familial ALS, mutations have been found in the Cu, Zn superoxide dismutase gene (SOD1) (Rosen, 1993; Deng et al., 1993). The genes involved in the sporadic cases have yet to be identified. However, since both the sporadic and familial forms are clinically similar this suggests that they both operate through the same pathways.

A feature of ALS is an abnormal accumulation of neurofilaments (NF) in the cell bodies and axons of motoneurons (Carpenter, 1968; Hirano et al., 1984a; Chou and Fakadej, 1971; Hirano et al., 1984b). NF are members of the family of intermediate filament proteins (IF). NF consist of three proteins known as light (NF-L, 68kDa), medium (NF-M, 95 kDa) and heavy (NF-M, 115 kDa). Overexpression of NF-L in mice results in degeneration and loss of motoneurons (Lee et al., 1994), and injection of NF protein into cultured neurons causes pathological changes that are observed in motoneuron disease (Straube-West et al., 1996). One other pathology that is associated with ALS is reactive astrocytosis. Astrocytes replicate and express increased amounts of glial fibrillary acidic protein (GFAP) in response to neuronal damage (Eddleston and Mucke, 1993; Montgomery, 1994).

One unexplored pathway through which ALS may occur is through a defect in the retinoid signalling pathway since retinoid-deficient diets can induce nerve lesions (Hughes et al., 1924; Irving and Richards, 1938). Retinoic acids (RAs) are synthesised in a two-step process. Firstly alcohol dehydrogenases act on retinols to synthesise retinals (Duester, 1998). RAs are then made from the retinals by retinaldehyde dehydrogenases (Raldhs). Three Raldhs have being identified that show a restricted tissue distribution in the embryo (Niederreither et al., 2002).

RA is important for the birth, survival and function of neurons (Wuarin and Sidell, 1991; Quinn and De Boni, 1991). RA can stimulate both neurite number and length (Maden, 1998; Corcoran and Maden, 1999; Corcoran et al., 2000). The LIM homeodomain gene *islet-1* expressed by motoneurons can be regulated by RA, and *raldh-2* is expressed by these neurons (Sockanathan and Jessell, 1998).

Cellular effects of RA are mediated by binding to ligand-activated nuclear transcription factors. There are two classes of receptors: RA receptors (RARs), which are activated both by all-trans-RA (tRA) and 9-cis-RA (9-cis-RA); and the retinoid X receptors (RXRs), which are activated only by 9-cis-RA (Kastner et al., 1994; Kliewer et al., 1994). There are three subtypes of each receptor: α, β and γ . In addition, there are multiple isoforms of each subtype owing to alternative splicing and differential promoter usage (Leid et al., 1992). RARs mediate gene expression by forming heterodimers with RXRs, whereas RXRs can mediate gene expression either as homodimers or by forming heterodimers with orphan receptors, which are also members of the nuclear receptor superfamily, examples of which include LXR and NGFI-B (Mangelsdorf and Evans, 1995).

In order to generate retinoid deficiency, the genes that encode the RA synthesising enzymes can be deleted. However, gene deletion of *raldh-2* results in embryonic lethality (Niederreither et al., 1999); hence the effects of retinoids on motoneuron survival cannot be studied. Another approach is to create conditional mutants of *raldh-2*; however it cannot be

guaranteed that *raldh-2* can be deleted in all motoneurons in which it is expressed, thus *raldh-2* expression may mask any phenotype. Since Raldh-2 requires substrates in order to make RA, an alternative approach is to deprive the adult of retinoids to prevent formation of RA, which, in effect, creates the equivalent of a conditional mutant. Thus Raldh-2 function is compromised in all cells that express it, including motoneurons, allowing its role to be assessed. Therefore, analogous to a gene deletion study, any phenotype observed must be a due to a lack of Raldh-2 function. We have generated adult retinoid-deficient rats by a dietary deficiency of retinoids and investigated whether there is an effect on their motoneurons. Our results support a role for the retinoid-signalling pathway in the survival of motoneurons, and a defect in this pathway leads to motoneuron disease in the adult rat. In patients suffering from spontaneous motoneuron disease, the retinoid signalling pathway was also found to be defective, suggesting that it may be one of the causes of the disease.

Materials and Methods

Generation of retinoid-deficient adult rats and rotation behaviour

Once the rats (Wistar) were weaned they were fed on a normal diet (controls) or a commercially available vitamin-A-free diet (Special Diet Services) *ad libidum*. No other vitamins were absent from this diet. Animals were weighed every other day and a growth plateau was reached in both groups of rats after 4-5 weeks. After 6 months of constant monitoring and observation, a group of control (n=5) and A– rats (n=8) were killed by perfusion with 4% paraformaldehyde/0.5% glutaraldehyde and the tissues prepared for in situ hybridisation and immunocytochemistry. Another group of controls (n=5) and A– rats (n=8) was kept for 1 year and then killed. The number of rotations that the rats could perform was measured on a rotorod apparatus for 5 minutes. Immunohistochemistry and western blotting was carried out as previously described (Corcoran and Ferretti, 1999). The NF200 and GFAP antibodies were obtained from Sigma.

Human tissue

Post mortem lumbar spinal cord tissue was obtained from 10 cases of spontaneous motoneuron disease and 10 aged-matched controls. The tissue was fixed in 4% PFA, wax embedded and 10 μM sections were cut.

In situ hybridisation

In situ hybridisation was carried out as previously described (Corcoran et al., 2000). RNA-species-specific probes were generated from gene-specific PCR products. Every fifth slide containing two to three sections and five slides were analysed for each probe used.

Identification and counting of motoneurons

For both rats and humans, motoneurons were identified and counted as previously described (Sockanathan and Jessell, 1998). Spinal cord sections were examined at 100× magnification. Images of both left and right ventral horns where the motoneurons are located were captured and analysed by Image Pro Plus software. In order to count all the motoneurons, the motoneurons were selected on the basis of their size (35 μm in diameter and above) and automatically counted using the Image Pro Plus software. For in situ hybridisation analysis, motoneurons (35 μm in diameter and above) with a blue signal above background were selected as positive and automatically counted by Image Pro Plus software. In addition, for quantitative in situ hybridisation analysis the above-background digoxigenin signals of *raldh-2*, *RARα* and *islet-1* were measured in the motoneurons compared to the above-background digoxigenin signal of *gapdh* in motoneurons using the Image Pro Plus software. Sigma plot software was used for statistical analysis.

RT-PCR

RNA was extracted and reverse transcribed as previously described (Corcoran et al., 2000). Quantitative PCR was carried out using species-specific primers on a Roche Lightcycler. The primers used were human gapdh, forward 211 aagggtcatcatctctgcc 229, and reverse, 376 ttccacgataccaaagttgtc 356; human Raldh-2, forward 55 gttccctgtctataatccagcc 76, and reverse 204 gtccccttttctgaagcatc 185; human RARα, forward 84 tctgagagctacacgctgac 103, and reverse 275 cttaatgatgcacttggtggag 254; human islet-1, forward 667 ggtctggtttcaaaacaagcg 687, and reverse 828 ttagcctgtaagccaccgtc 809. Cycling parameters were: denaturing 95°C, 1 second; annealing 55°C, 1 second; and extension 72°C 1 second for 30 cycles.

Results

HPLC measurements were performed on the blood of all animals, the 6-month rats fed on the retinoid-deficient diet had little or no retinol, whereas the 1-year retinoid-deficient rats had no retinol compared to the normal fed rats (Fig. 1A,B). At 6 months, the retinoid-deficient rats could be distinguished from normally fed rats by their inability to extend their hindlimbs when held by the tail (Fig. 2A,B). In a rotorod test, the retinoid-deficient animals (n=5) were only able to perform 52% of the rotations compared to the normal fed rats (n=8), P<0.05 students t-test.

Spinal cord sections were examined by staining for neuronal intermediate filaments with the antibody NF200 and the motoneurons identified by their location in the ventral horns. Neurofilaments had accumulated in the cell body of the motoneurons in the lumbar and cervical regions of retinoid-deficient spinal cords (Fig. 3B,D) compared to the same

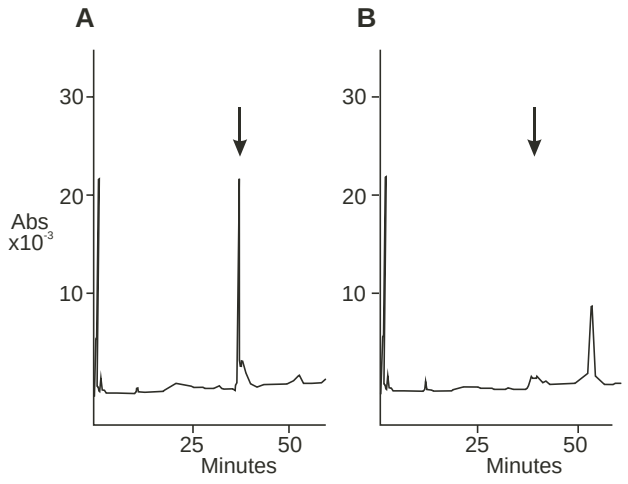

Fig. 1. HPLC analysis of blood of rats. (A) 1-year-old normally fed rats; (B) 1-year-old retinoid-deficient rats. Arrows indicate retinol peak. The amount of retinol in the normally fed rat is 665 ng/ml blood; in the retinoid-deficient rat no retinol could be detected. Similar data were obtained from four other control rats and seven retinoid-deficient rats.

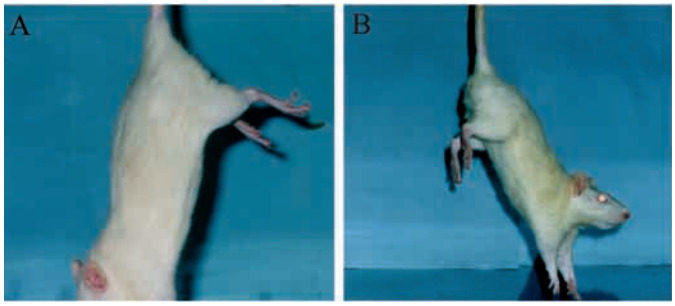

Fig. 2. Effect of a retinoid-deficient diet on adult rats. (A) 6-month-old normally fed rat; (B) 6-month-old retinoid-deficient rat. Normal rats (A) extend their hindlimbs when they are held by the tail, whereas retinoid-deficient rats retract their hindlimbs. *n*=5-8.

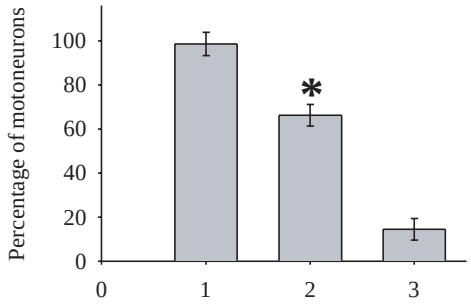

Fig. 4. A graph showing the percentage loss of motoneurons in the lumbar cord of the retinoid-deficient rats compared with the normally fed rats. Columns: 1, normally fed rats; 2, retinoid-deficient rats; 3, motoneurons with vacuolations in the retinoid-deficient rat. Error bar=s.e.m. There was a significant difference between the percentage of motoneurons in the normal and retinoid-deficient rats of **P*<0.01. Students *t*-test, *n*=5-8.

regions of the spinal cord of the normally fed rat (Fig. 3A,C). Also in the retinoid-deficient lumbar cord there was accumulation of the neurofilament in the axons (Fig. 3B). In the six-month-old retinoid-deficient rats, the motoneurons of the lumbar cord had more vacuolar lesions (Fig. 3B) than the motoneurons located in the cervical cord (Fig. 3D). In the normal rat no vacuolar lesions were seen in the motoneurons at either level of the spinal cord examined (Fig. 3A,C). There were 34% less motoneurons in the lumbar cord of the retinoid-deficient rats compared with the normally fed rats (Fig. 4 columns 1 and 2). Of the surviving motoneurons in the retinoid-deficient rat, 14% had vacuoles (Fig. 4 column 3). There was an increase in reactive astrocytosis in the lumbar cord of the retinoid-deficient rats (Fig. 5B,C, lane 2) compared with the lumbar cord of the normal rat (Fig. 5A,C, lane 1). After 1 year of a retinoid-deficient diet there was a dramatic loss of NF200 expression in the cell bodies of the surviving motoneurons of the retinoid-deficient rat compared with the motoneurons of the normally fed rats (data not shown).

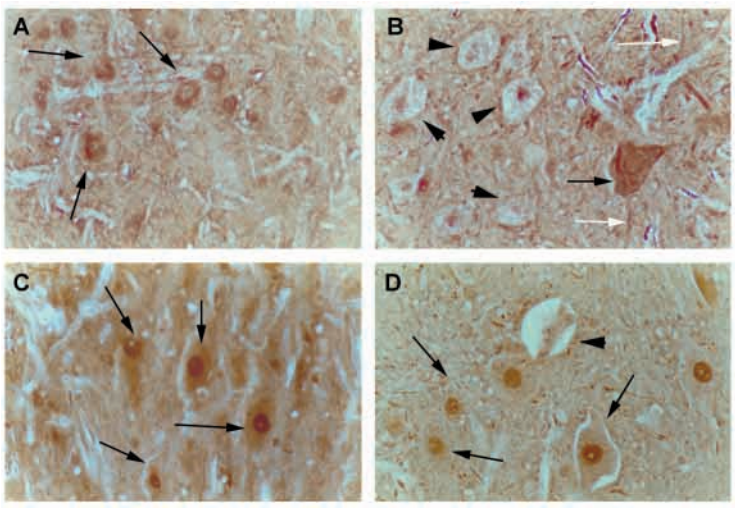

Fig. 3. Expression of NF200 in lumbar (A,B) and cervical cord (C,D). (A) Lumbar cord of a 6-month-old normally fed rat. (B) Lumbar cord of a 6-month-old retinoid-deficient rat. (C) Cervical cord of a 6-month-old normally fed rat. (D) Cervical cord of 6-month-old retinoid-deficient rat. Black arrows and arrowheads indicate motoneurons. The arrowheads indicate motoneurons with vacuolar lesions. The white arrow indicates accumulation of neurofilament in the axons. Similar data were obtained from four other normal fed rats and seven other retinoid-deficient rats.

We next investigated which components of the retinoid signalling pathway were perturbed. In situ hybridisation with the three *RAR*s, and three *raldh*s showed that only *RARα* and *raldh-2* were expressed in the motoneurons. Although *RARα* was depleted in the motoneurons of the lumbar cord of the 6-month-old retinoid-deficient rats compared to the equivalent regions of the cord in the control rats (Fig. 6A,B,E, columns 1 and 2) *raldh-2* did not vary between the normally fed and retinoid-deficient rats (Fig. 6C-E, columns 1 and 2). Similar results were obtained from the 6 month cervical cord and the 1 year cords of both normal and retinoid-deficient rats (data not shown). This suggests that although a loss in *RARα* expression is due to a lack of retinoids, the enzyme that makes these retinoids is not itself regulated by RA in the motoneurons.

We next analysed the components of the retinoid signalling pathway identified in the retinoid-deficient rats in post mortem lumbar spinal cord tissue from spontaneous cases of motoneuron disease. Using real time RT-PCR, we quantified the amount of *RARα*, RA enzymes and *islet-1* expression compared with *gapdh* in human lumbar spinal cord from motoneuron-diseased and normal patients. All three transcripts were depleted in the diseased compared with the normal cord: RARα, 37%; *Raldh-2*, 22%; *islet-1*, 42%; *P*<0.01. In order to count the number of motoneurons expressing these transcripts and the level of their expression in situ hybridisation was performed using gene-species-specific probes (Fig. 7A-F). The percentage of motoneurons in control and diseased patients expressing *islet-1* was 61% and 48%, respectively (Fig. 6, columns 1 and 2). There was a 37% loss of motoneurons expressing *RARα* in patients suffering from the disease compared with normal samples (Fig. 8, columns 3 and 4). We finally asked if there was a defect in the expression of *raldh-2*. In the non-diseased patients 56% of the motoneurons expressed this enzyme (Fig. 8, column 5) compared with 16% of the motoneurons in the diseased patients (Fig. 8, column 6), suggesting a decrease in expression of the enzyme in the diseased state.

Lastly, we quantified the in situ hybridisation signal of the transcripts in the surviving motoneurons to answer the

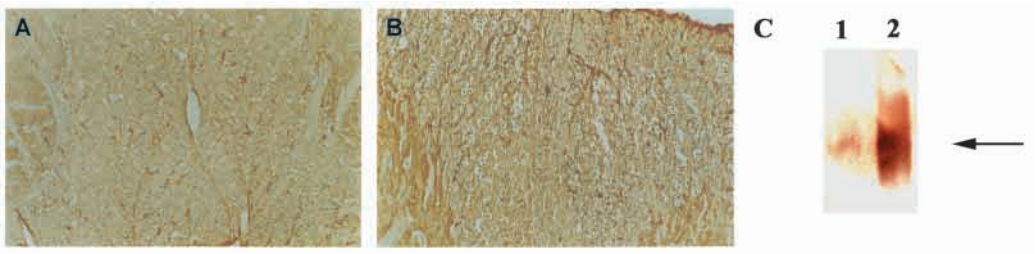

Fig. 5. Reactive astrocytosis in the lumbar cord of 6-month-old rats. Expression of GFAP in astrocytes. (A) Normal lumbar cord; (B) retinoid-deficient lumbar cord; (C) western blot of lumbar cord of 6-month-old rats, lanes: 1, normal lumbar cord lane 2, retinoid-deficient lumbar cord. The arrow indicates a protein band of the correct size. Similar data were obtained from four other normally fed rats and seven other retinoid-deficient rats.

question of whether the retinoid-signalling pathway was depleted in them. *Islet-1* expression was decreased by 56%, *RARα* by 31% and *raldh-2* by 49% in the diseased motoneurons compared with their expression in non-diseased motoneurons, $P<0.05$ (Fig. 9 columns 1-6).

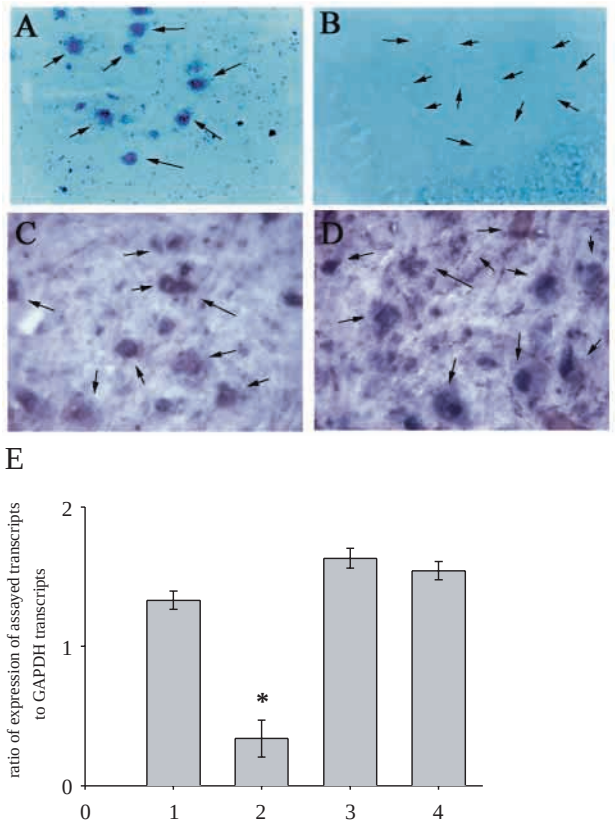

Fig. 6. Expression of *RARα* and *raldh*-2 in motoneurons of lumbar cord of the of 6-month-old adult rat. In situ hybridization of A, *RARα* expression in motoneurons of normal fed rat; B, *RARα* expression in motoneurons of retinoid deficient rat; C, *raldh*-2 expression in motoneurons of normal fed rat; D, *raldh*-2 expression in motoneurons of retinoid deficient rat; E, quantification of the in situ signals of *RARα* (columns 1 and 2) and *raldh*-2 (columns 3 and 4) expression compared with *gapdh* in motoneurons. Columns: 1 and 3, normally fed rats; 2 and 4, retinoid-deficient rats. Error bar=s.e.m. There was a significant difference in *RARα* expression **P*<0.01 but not *raldh-2* expression between normally fed and retinoid-deficient rats. Students *t*-test, $n=5$-8. Arrows indicate motoneurons.

Discussion

Neurodegeneration in the retinoid-deficient rat and motoneuron disease patients have a similar pathology

In the motoneurons of the adult retinoid-deficient rat, there was an accumulation of neurofilament, vacuolations of the motoneurons and an increase in astrocytosis, which are all phenotypes observed in both human sporadic (Carpenter, 1968; Hirano et al., 1984a) and SOD1-mediated familial ALS (Hirano et al., 1984b; Rouleau et al., 1996; Shibata et al., 1996). This suggests that the motoneurons in both the rat and the human disease undergo a similar mechanism of

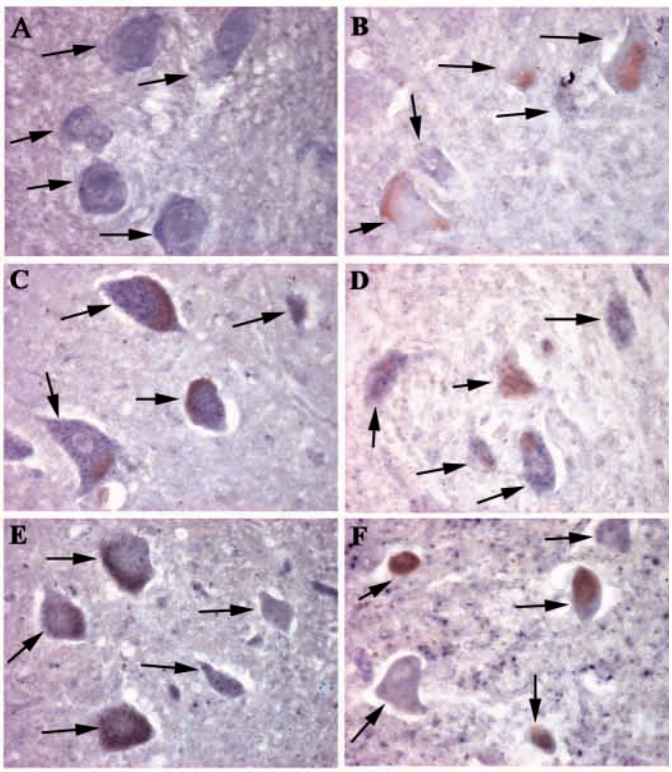

Fig. 7. Expression of *islet-1* and components of the retinoid signalling pathway by in situ hybridisation in the lumbar cord of a normal (A,C,E) and an aged-matched patient suffering from spontaneous motoneuron disease (B,D,F). A,B, *islet-1* expression; C,D, *RARα* expression; E,F, *raldh-2* expression. Arrows indicate motoneurons. The brown deposit is lipofuscin, which is expressed by aged neurons. This does not interfere with the quantitative analysis. The same data was obtained from nine other patients.

degeneration. These effects were not as dramatic in the cervical cord; this may be due to the fact that the animals were kept only until 1 year of age.

Disruption of the retinoid signalling pathway in the adult rat leads to a loss of motoneurons

The major retinoid signalling pathway defect in the retinoid-deficient rat is the loss of *RARα* expression in the motoneurons: no expression of either *RARβ* or *RARγ* was detected by in situ analysis or RT-PCR. RA has been shown to regulate *RARα* (Leroy et al., 1991) and appears to be critical for the survival of the motoneurons. The data presented here shows that the retinoid signalling pathway is critical for the survival of neurons of the adult CNS. This has been previously shown for the embryonic CNS, where in addition it is required for the differentiation of neurons (Wuarin and Sidell, 1991; Quinn and De Boni, 1991). Therefore, the role of the retinoid signalling pathway in neuron survival in the embryonic CNS is conserved in the adult CNS.

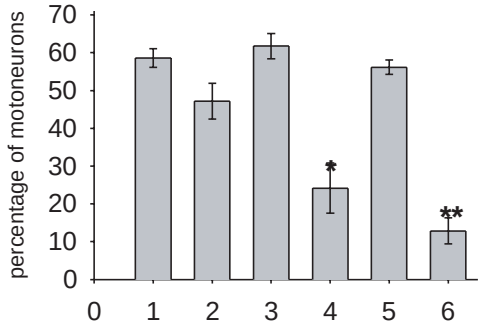

Fig. 8. Graph showing the percentage of motoneurons in the lumbar cord expressing *islet-1* and components of the retinoid signalling pathway in aged-matched normal and motoneuron disease patients. Columns: 1, *islet-1*-positive motoneurons in normal cord; 2, *islet-1*-positive motoneurons in diseased cord; 3, *RARα*-positive motoneurons in normal cord; 4. *RARα*-positive motoneurons in diseased cord; 5, *raldh-2*-positive motoneurons in normal cord; 6, *raldh-2*-positive motoneurons in diseased cord. Error bar=s.e.m. There was no significant difference between the percentage of *islet-1* motoneurons in normal and diseased aged-matched samples (columns 1 and 2). There was a significant difference between the number of *RARα*-positive motoneurons (columns 3 and 4) **P*<0.01 and between the number of *raldh-2*-positive motoneurons (columns 5 and 6) ***P*<0.001 of normal and diseased aged-matched samples. Students *t*-test, *n*=10.

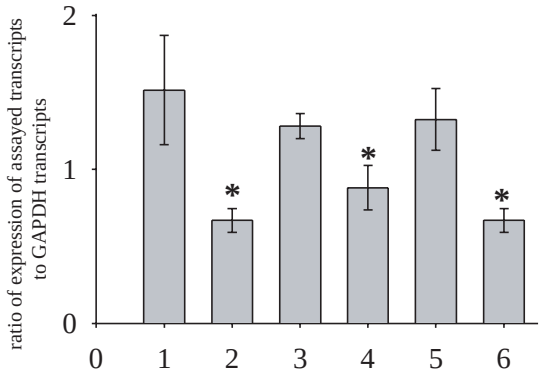

Fig. 9. Quantification of in situ hybridisation of *islet-1* and components of the retinoid signalling pathway in the motoneurons in aged-matched normal and motoneuron disease patients compared to *gapdh.* Columns: 1, *islet-1* expression in motoneurons of normal cord; 2, *islet-1* expression in motoneurons of diseased cord; 3, *RARα* expression in motoneurons of normal cord; 4, *RARα* expression in motoneurons of diseased cord; 5, *raldh-2* expression in motoneurons of normal cord; 6, *raldh-2* expression in motoneurons of diseased cord. Error bar=s.e.m. There was a significant difference between *islet-1* expression (columns 1 and 2), *RARα* expression (columns 3 and 4) and between *raldh-2* expression (columns 5 and 6) **P*<0.05 of normal and diseased aged matched samples. Students *t*-test, *n*=10.

Motoneuron defects have not been reported in *RARα*-null mutant mice but to our knowledge such analysis has not been carried out. However, one of the major problems with such studies has been the functional redundancy between the receptors, thus masking their potential role in development and as well as their functions in the adult. The approach we have taken to overcome such functional redundancy is to create retinoid-deficient animals by a dietary deficiency of retinoids. This has a distinct advantage over the RAR gene deletion studies because the receptors are normally expressed during development when the embryos receive adequate amounts of retinoids, hence development is not perturbed. However, once the animals are retinoid-deprived, only the retinoid signalling pathway that is normally expressed in cells is altered. At such a late developmental stage it is unlikely that one receptor can substitute for another.

Furthermore we are asking which RARs are involved in the survival of normal adult motoneurons, since other RARs may be involved in the survival of developing motoneurons. Such dual functions of the same molecule have been shown before. For instance, in the developing nervous system, NGF is required for the survival of the developing peripheral neurons, whereas in the adult the peripheral neurons do not require NGF for their survival but it has been shown to be involved in neurite outgrowth (Lindsay, 1988). Hence, the generation of retinoid-deficient animals may lead to the discovery of novel roles for other retinoid receptors in the adult.

A retinoid signalling defect is present in patients with spontaneous motoneuron disease

In human spontaneous motoneuron disease, by counting the number of motoneurons, we found a decrease in the number of *RARα*-positive neurons and *raldh-2*-positive neurons in the diseased state compared with control samples. As well as the loss of motoneurons, all three transcripts assayed, *RARα*, *islet-1* and *raldh-2*, were dramatically reduced in the diseased motoneurons compared with the control tissue samples. The same loss in *RARα* in motoneurons of diseased patients was observed as in retinoid-deficient rats. In addition there was a loss of *islet-1* expression in the motoneurons, suggesting that, as in the embryonic CNS, RA regulates this gene in the adult.

Therefore, our results suggest that a cause of the disease in humans is retinoid signalling defect. This is further supported by our observations that motoneuron disease in the rat is a consequence of a retinoid signalling pathway defect since the animals were fed a retinoid-deficient diet, thus the loss of motoneurons must be a consequence of lack of retinoids. The

loss of motoneurons does not precede the loss of retinoid signalling. Most if not all the motoneurons in the retinoid-deficient rat had lost *RARα* expression. This loss of expression included those motoneurons that had vacuolations and were therefore destined to undergo cell death. Also if the defect in the retinoid signalling pathway was a consequence of motoneuron disease then in the surviving motoneurons of both the retinoid-deficient rat and human motoneuron-diseased samples, there would be a downregulation of both *RARα* and *raldh-2* compared with the controls. However, it is only in the human-diseased samples that both transcripts are downregulated.

RA synthesis may be a key factor in motoneuron disease

The sequence of events leading to motoneuron disease is likely to be loss of *raldh-2* expression, followed by the depletion of cellular retinoids. This would result in the loss of *RARα* activation and expression. Eventually a downregulation of *islet-1* would occur, which is either before or after, an increase in neurofilament expression and the consequent motoneuron cell death. It is unlikely that *RARα* can regulate *raldh-2* expression since it makes the ligand, which RARα requires in order to activate gene transcription. Indeed it has already been shown that *raldh-2* slightly precedes the expression of *RARβ* in differentiating limbs (Niederreither et al., 1997).

Factors that regulate *raldh-2* may be associated with motoneuron disease. One of the factors is unlikely to be RA itself, since in the retinoid-deficient rat model there was no difference in the levels of *raldh-2* in their motoneurons compared with the normally fed rats. This suggests that RA does not regulate *raldh-2* expression in the adult rat motoneurons. Therefore, in both the retinoid deficient and normally fed rats, the factors that regulate *raldh-2* are probably still present. By contrast, in human motoneuron disease it is these factors that regulate *raldh-2* that are absent since the enzyme is downregulated in diseased motoneurons compared with normal samples. What may these factors be? Interestingly it has been proposed that neurotrophins such as NT-3 may be useful for treating motoneuron disease (Haase et al., 1998; Haase et al., 1997; Sagot et al., 1998), and we have already shown that a related neurotrophin, NGF, can activate *raldh-2* transcription (Corcoran and Maden, 1999). It will be of great interest to identify the factors that regulate *raldh-2* expression and to see if these are also deficient in motoneuron disease patients. Since the same pathology is seen in spontaneous and familial cases of motoneuron disease, it will also be of interest to ask if retinoids can regulate genes involved in familial forms of the disease. Recently it has been shown that the SOD1 promoter contains a binding site for the orphan receptor peroxisome proliferator-activated receptor (PPAR), which can be induced to bind to the promoter by both RA and 9-cis RA (Yoo et al., 1999). This provides further support for the involvement of the retinoid signalling pathway in human motoneuron disease.

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