IDENTIFICATION OF THE SITES OF EXPRESSION OF TRIPLE A SYNDROME mRNA IN THE RAT USING IN SITU HYBRIDISATION

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Abstract—Triple A syndrome is characterised by achalasia, alacrima, adrenocorticotropic-resistant adrenal insufficiency and a variable and progressive neurological phenotype. It is caused by mutations in a gene that is normally referred to as the triple A syndrome gene (AAAS) and which has recently been shown to encode a nuclear pore protein named ALADIN (alacrima, achalasia, adrenal insufficiency neurologic disorder). In this study we performed in situ hybridisation with radioactive oligonucleotide probes in the adult and developing rat and present the first detailed map of AAAS mRNA expression. Consistent with a role for AAAS in adrenal function, we detected high levels of its mRNA in the adrenal cortex. On the other hand hepatocytes, enteric smooth muscle and fibroblasts had relatively little or no detectable AAAS mRNA. In both the peripheral and central nervous systems, AAAS mRNA was abundantly expressed. Neurons in sensory and sympathetic ganglia expressed high levels. CNS expression was highest in neurons of the cerebral cortex, cerebellum, hippocampus, motor-associated nuclei of the brainstem including cranial nerve nuclei, and ventral horn of the spinal cord. Although neuronal expression of AAAS mRNA was striking, non-neuronal cells including those of the circumventricular organs and fibrous astrocytes also expressed AAAS mRNA. Within the developing embryo, the highest levels of expression were found in neural tissues. These findings indicate a widespread but not ubiquitous or uniform expression of AAAS mRNA in the rat. Robust expression in neural systems associated with cognitive, motor and sensory functions is consistent with the myriad of symptoms experienced by patients with triple A syndrome. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: triple A syndrome, ALADIN, embryo, nuclear pore complex, neuropathy.

Triple A syndrome (AAAS; also known as Allgrove syndrome, OMIM 231550) is a rare autosomal recessive disorder characterised by alacrima (absence of tears), achalasia of the cardia and adrenal failure (Allgrove et al., 1978). About 60% of patients also suffer progressive neurological symptoms (Grant et al., 1993; Huebner et al., 1999). Impairment of the CNS is manifested as progressive mental retardation, optic atrophy, cerebellar ataxia and clumsiness, sensorineural deafness, hyperreflexia, Parkinsonism and dementia (Clark and Weber, 1998). Bulbar dysarthria is reported in about 25% of patients. Impairment of the peripheral nervous system leads to muscle hypotonia, weakness, pes cavus, sensory impairment and, in approximately 30% of patients manifested as postural hypotension, impaired sweating, impaired cardiovascular reflexes, cardiac dysrhythmias, unequal pupils and impotence (Clark and Weber, 1998; Huebner et al., 2002; Clark, 2003). Whilst the adrenal disorder may be life threatening if unrecognised, it is often the neurological manifestations which make this a severely disabling disease for which no current therapies are available (Allgrove et al., 1978; Grant et al., 1993; Clark and Weber, 1998).

Using genome scanning, the AAAS gene was localised to a single locus on chromosome 12q13 (Weber et al., 1996; Stratakis et al., 1997). The causative gene mutated in almost all cases of AAAS was recently identified (AAAS) and its protein product given the name ALADIN (alacrima, achalasia, adrenal insufficiency neurologic disorder; Tullo-Pelet et al., 2000; Handschug et al., 2001). This 60 kD protein has a central 170 amino acid domain composed of four WD (tryptophan–aspartic acid) repeats and was demonstrated to be a component of the nuclear pore complex (NPC; Dregger et al., 2001; Cronshaw et al., 2002). Initial studies of disease-associated AAAS mutations suggest that mutated ALADIN proteins fail to localise to NPCs and are found predominantly in the cytoplasm (Cronshaw and Matunis, 2003). It has been proposed that ALADIN shuttles on and off NPCs and plays a dynamic role in nucleocytoplasmic transport. AAAS mutations would thus disrupt the localisation and transport of other proteins, with the exact effects being tissue or cell specific (Cronshaw and Matunis, 2003). Modifying genes and/or environmental factors are also likely to play a role and contribute to the phenotypic variability seen between patients (Huebner et al., 2002).

Few studies have investigated the tissue distribution or expression of AAAS. Northern blot analyses have shown ubiquitous but uneven expression in human tissues with high relative expression in the pituitary and adrenal glands, gastrointestinal structures and foetal lung. Expression in the brain was highest in the cerebellum (Tullo-Pelet et al., 2000; Handschug et al., 2001; Huebner et al., 2002). How-
ever no detailed study of cellular localisation has been conducted. We have therefore investigated the sites of AAAS mRNA expression in the nervous system and other tissues in the rat using in situ hybridisation (ISH). This more detailed analysis is important in order to elucidate possible functions of the ALADIN protein and pathogenesis of AAAS.

**EXPERIMENTAL PROCEDURES**

**Tissue preparation**

One pregnant female Wistar rat carrying 18.5 day post-fertilisation embryos and adult male Wistar rats (n = 3) were used for this study. All animals were humanely killed according to UK Home Office guidelines governing the ethical use of animals. The minimum number of animals to establish reliable results and perform analysis was used and their suffering minimized. Embryos and adult male rat tissues were rapidly frozen on dry ice. Sections (8 –12 μm) were cut with a cryostat, thaw-mounted onto Superfrost Plus slides (BDH Chemicals, Poole, UK) and stored at −80 °C until use. Our protocol for ISH using radiolabeled oligonucleotide probes has previously been described in detail (Michael et al., 1997). Before hybridisation, sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and stored at −80 °C until use. Our protocol for ISH using radiolabeled oligonucleotide probes has previously been described in detail (Michael et al., 1997). Before hybridisation, sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and stored at −80 °C until use.

**Preparation of probes**

Four antisense DNA oligonucleotide probes (1–4) were designed to be complimentary to the rat AAAS mRNA sequence in the NCBI database (accession number XM_217063; complimentary to nucleotides 776–809, 1024–1057, 1296–1329, 1329–1362 respectively). The oligonucleotide sequences were as follows:

- **probe 1**: 5’ ACTCGAAAGACAGCGGAAGGATTTGAGCC AGGA
- **probe 2**: 5’ CGAGTCTCCTCCTCTCATGAGGGCTGAA TTGT
- **probe 3**: 5’ AAAGTACAGGGATGTGGGATTCCGGCC TGTT
- **probe 4**: 5’ CACTGGGCTAAAGCGTGGAATTGCGGT GACA

Homology analysis using BLASTn searches of the databases available through the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) identified no known rat sequences to which these probes were likely to hybridise other than AAAS. Radioactive probes were made by end-labeling the oligonucleotides (Sigma Genosys, Poole, UK) with 35S-dATP (Perkin Elmer, Boston, MA, USA) using terminal deoxynucleotidyl transferase (Promega, Southampton, UK).

**ISH procedure**

Probes were used either singly in reactions or in combination in order to enhance the ISH signal. Probes 1 and 3 were selected for such combined use as individually these gave the best signal to noise ratios.

Hybridisation was performed overnight at 37 °C. Following posthybridization washes, dehydration through alcohols and drying, slides were apposed to Kodak BioMax MR-1 film (Sigma, Poole, UK). Film was developed after 6 –14 days and slides were then coated in autoradiographic emulsion (LM-1; Amersham, Chalfont St. Giles, UK) and left for 4 –6 weeks before development. Sections were counterstained for Nissl substance using Toluidine Blue, dehydrated and mounted from xylene using DPX mountant (BDH).

Controls were performed to confirm specificity of the AAAS mRNA signal. To ensure that ISH detected RNA, RNase pretreatment of tissue sections was conducted to degrade RNA before hybridisation. Sections were incubated 30 min at 37 °C with 20 μg/ml RNase A (Sigma) in a buffer containing 10 mM Tris–HCl, 1 mM EDTA, 0.5 M NaCl, pH 8.0. After fixing for 5 min in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, the slides were processed for subsequent hybridisation. Sense sequence probes and the addition of unlabelled probe at 1000 times the concentration of the radioactive probe were used to estimate background levels in tissues. Comparison of the signal obtained using probes to different portions of the AAAS mRNA gave further evidence for specificity of the signals.

**Imaging**

Film autoradiograms were scanned. Emulsion-coated sections were visualised using bright field and epi-polarized illumination to view the counterstain and silver grains representing AAAS mRNA hybridisation signal respectively. Photographs of emulsion coated sections were taken using a Hamamatsu C4742-95 digital camera (Herrsching, Germany) attached to the microscope and HiPic software to capture images. Abode Photoshop was used to assemble images.

**RESULTS**

**Control studies**

AAAS mRNA expression was examined using ISH with oligonucleotide probes. Several control studies confirmed the specificity of labelling. Four oligonucleotides to different regions of the AAAS mRNA produced identical patterns of expression. Sense oligonucleotide controls produced light, homogeneous non-specific labelling over sec-

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**Fig. 1.** Specificity of AAAS mRNA ISH signal. Ventral horn sections are shown using epipolarised illumination for visualisation of silver grains in emulsion autoradiography (A, C) and Toluidine Blue counterstain for cellular profiles (B, D). (A, B) Moderate to high levels of AAAS mRNA are detected using specific AAAS antisense oligonucleotide probes with ISH. Grains are localised mainly over the cytoplasm of cells but occasionally appear to be associated with the nucleus (arrows). (C, D) An excess of nonradioactive oligonucleotide effectively competes with specific hybridisation of radioactive probe and produces low to background levels of labelling with grains not generally associated with cells in the sections. Scale bar=32 μm.
tions generally, although in a few tissues such as the liver high concentrations of silver grains were observed over scattered cells. Similar labelling observed with antisense probes was considered non-specific. Because ISH signal was often observed to concentrate over the nuclei of cells (see below; examples are shown in Figs. 1, 3, 5), controls were performed in which the hybridisation reaction resulted in a loss of specific signal such that few or no silver grains were observed over cells (Fig. 1).

**General distribution of AAAS mRNA expression outside the nervous system**

Various tissues throughout the body were analysed to assess the overall distribution of AAAS mRNA. Epithelial cells of the tongue and oesophagus as well as the glandular and duct epithelium of the submandibular gland expressed low to moderate levels. Expression in the lacrimal gland was not observed. In the pancreas, the exocrine portion expressed moderate levels whereas the endocrine pancreas had levels only slightly above background. In the liver, hepatocytes had only low levels of the mRNA. Muscle and connective tissue components in these tissues did not express AAAS mRNA above background. In the lymph node, low to moderate levels were detected with germinal centers exhibiting increased signal compared with other regions. The spleen did not have labelling above control levels. The thymus in contrast had moderate expression. The highest level of expression in any tissue was found in the pituitary gland (Fig. 2B). Relatively robust expression was also observed in the adrenal gland (Fig. 2A). Expression was not uniform within these tissues however. In the pituitary gland whereas the anterior and intermediate lobes had very high expression, the posterior pituitary gland did not show significant levels above back-
ground (Fig. 2B). Pituicytes observed in emulsion-coated sections were negative (data not shown). The adrenal cortex as a whole had much higher levels of AAAS mRNA than the adrenal medulla (Fig. 2A). Whereas all zones of the adrenal cortex had high levels and a homogeneous expression pattern, the adrenal medulla had a patchy appearance such that some collections of cells had moderate levels of expression and others had lower levels (Fig. 3A, B).

**Expression of AAAS mRNA in the peripheral nervous system**

In the enteric nervous system, most ganglion cells of the neural plexuses of the oesophagus, duodenum and colon did not express AAAS mRNA. However, occasional enteric neurons were found that expressed the mRNA at low to moderate levels (Fig. 3E–G).

In contrast, high levels of AAAS mRNA were expressed in the sympathetic superior cervical ganglion and sensory dorsal root and nodose ganglia (Fig. 2C, D; Fig. 3C, D, H–J). AAAS mRNA signal was often concentrated over nuclei of ganglion cells although this distribution was not observed when sections clearly went through the nucleus of a cell (arrowheads in Fig. 3H–J). We conclude from this and the results of control studies that such AAAS ISH signal represents AAAS mRNA enriched in the perinuclear region of cells. No significant levels of AAAS mRNA were found over Schwann cells in autonomic or sensory nerves. Smooth muscle cells of the jugular vein sectioned alongside the superior cervical ganglion were also negative (arrowhead in Fig. 2C).

**Expression of AAAS mRNA in the CNS: Film autoradiography**

Film autoradiograms showed that AAAS mRNA was widely expressed in the CNS (Fig. 2E–K). In the forebrain, expression in the cerebral cortex was particularly notable with all cortical areas examined having moderate to high signals. In areas of high neuronal density such as layer II of the piriform cortex (Fig. 2E–G), signal on autoradiographic film appeared very high. In the olfactory tubercle, the densocellular layer and the adjacent islands of Calleja could be distinguished from surrounding tissue on autoradiographic film (Fig. 2E). Such signal appeared of lower intensity than the adjacent piriform cortex. Very high levels of labelling similarly characterised closely packed cells of both the CA1-3 pyramidal cell and granule cell layers of hippocampus. In the caudate putamen and thalamus, overall levels of AAAS mRNA were slightly lower than in the cortex. The medial habenula showed a moderate to high signal. Several parts of the hypothalamus including the paraventricular, supraoptic, suprachiasmatic and ventromedial nuclei as well as the mammillary bodies could be distinguished on films due to their moderate intensity of signal relative to the lower surrounding levels.

Throughout the midbrain and brainstem, AAAS mRNA continued to be abundantly expressed. Levels differed between cell groups. The labelling in the superior colliculus ranged from moderate to high and labelling of the substantia nigra pars compacta and oculomotor nucleus was moderate (Fig. 2H). In the same section, the red nucleus could just be distinguished above surrounding tissue levels. The cerebellum, particularly the wide banding pattern of the granule cell layer, had one of the highest levels of expression of AAAS mRNA of any region in the CNS (Fig. 2I). The pontine nuclei had moderate intensity signal and in the medulla the inferior olivary nucleus was evident as a banding pattern above the pyramidal tract (Fig. 2J).

In the spinal cord, the dorsal and ventral horns had higher levels of AAAS mRNA signal than surrounding white matter (Fig. 2K).

**Expression of AAAS mRNA in neuronal groups of the CNS: emulsion autoradiography**

Analysis of labelling intensity of individual neurons was performed on the sections after processing for emulsion autoradiography. Such examination indicated that the majority of pyramidal cells in all cortical regions and layers expressed AAAS mRNA at moderate to high levels. The large pyramidal cells of layer V were consistently highly labelled as were pyramidal neurons in layer II of the piriform cortex and those of the entorhinal cortex. Non-pyramidal neuron populations appeared more variable in their expression although many were also observed with moderate to high levels of signal (Fig. 4A–C). In layer IV of the cortex where many neurons are non-pyramidal, most displayed moderate signals. In the olfactory tubercle, the modified pyramidal cells of the densocellular layer as well as the cells in the islands of Calleja had moderate labelling.

Cellular analysis of the hippocampal formation indicated that pyramidal cells of CA1-3 had moderate to high levels of signal. Less signal was generally observed over individual granule cells in the dentate gyrus. In the hilar region of the dentate gyrus, a region which contains principal or ‘mossy’ cells and classes of inhibitory interneurons, the expression of AAAS mRNA was found to be heterogeneous with most neurons expressing moderate to high levels and a minority expressing no or only low levels of the mRNA. Scattered interneurons of the stratum lucidum and stratum oriens of the hippocampal formation also showed some variability in expression levels. Whilst the majority of these interneurons had little or no labelling, moderate levels were detected in some.

Cells of the caudate putamen and thalamus generally had moderate levels of AAAS mRNA signal. Large cells in the caudate putamen, likely to be cholinergic interneurons, ranged in labelling from low to moderate level. The closely packed cells of the medial habenula showed moderate cellular labelling. Neurons of the suprachiasmatic, the ventromedial hypothalamic and arcuate nuclei and the mammillary body showed moderate levels, slightly higher than other surrounding cell groups. Cells in the medial parvicellular part of the paraventricular nucleus had generally lower levels of signal compared with the larger cells of the lateral magnocellular part. However a wide variation in the level of expression between cells was noted (Fig. 4G–L). Cells of the supraoptic nucleus were more uniformly labelled at a moderate to high intensity.
In the midbrain and brainstem many neurons expressed AAAS mRNA. Moderate labelling of most cells with a neuronal morphology was observed in the superior and inferior colliculi. Cells in the dopaminergic ventral tegmental area and substantia nigra pars compacta, as well as the serotonergic and noradrenergic systems of the raphe nuclei and locus coeruleus respectively also expressed moderate levels. Neurons of the brainstem retic-
ular formation expressed AAAS mRNA. Very large neurons of the gigantocellular reticular nucleus were conspicuous due to their size but also for the fact that they had especially high levels of labelling. Smaller neurons in nuclei associated with control of voluntary movement including the pontine and inferior olivary nuclei expressed moderate levels of AAAS mRNA. Expression in the red nucleus was evident but not striking in film autoradiograms as mentioned previously; however the widely separated large neurons of the magnocellular part were found to be highly labelled in emulsion coated sections (Fig. 5A–C). Similarly, neurons of the cranial nerve motor nuclei including the oculomotor, facial and hypoglossal nerve nuclei expressed high levels of AAAS mRNA (Fig. 5D–F). In the cerebellum, labelling over individual granule cells varied with some cells being moderately labelled and others having little or no silver grains. Expression was evident in Purkinje cells with most having low to moderate levels (Fig. 5G–I).

In the spinal cord, high levels of AAAS mRNA expression were detected in ventral horn motoneurons (Fig. 1A, B). Neurons in the dorsal horn and intermediate grey laminae including lamina X around the central canal showed moderate expression.

Non-neuronal expression of AAAS mRNA in the CNS

In general, ependymal epithelium lining the ventricles of the brain had only weak labelling for AAAS mRNA (Fig. 6A–D). One exception was noted however. Low to moderate levels of AAAS mRNA were expressed by the tanyocyte ependyma lining the third ventricle dorsal to the median eminence. Interestingly in our examination of ependymal expression we observed moderate expression of AAAS mRNA in the subventricular zone (SVZ) of the lateral ventricle (Fig. 2E, 6A–C). The SVZ is the largest germinal zone of the adult mammalian brain and contains mainly migrating neuroblasts as well as smaller populations of glial and proliferating cells (Yasuoka et al., 2004).
Circumventricular organs including the subfornical organ, median eminence, subcommissural organ, pineal gland, area postrema and the choroid plexus had moderate to high expression of AAAS mRNA (Figs. 2 and 6A–C).

Small cells throughout grey matter areas of the brain and spinal cord were sometimes observed to express low levels of AAAS mRNA. These cells are likely to be astrocytes based on their size and location. ISH signal in white matter tracts was close to background levels on film autoradiograms. However on close examination of emulsion-coated tissues, some scattered cells were observed to express low to moderate levels of AAAS mRNA. These cells were observed in most fibre tracts including the corpus callosum, internal capsule, optic tract, lateral lemniscus and medial cerebellar peduncle. Labelled cells were particularly evident in the ventral and lateral funiculi of the spinal cord. (Fig. 6D–F) This expression was predominantly localised to a class of cells with multiple processes radiating from the cell body and are likely to be fibrous astrocytes (Bodega et al., 1986). Smaller white matter cells with oval nuclei and no obvious Toluidine Blue-stained processes, presumed to be oligodendrocytes, were not usually labelled.

Expression of AAAS mRNA in the E18 embryo

In the embryonic day 18 rat, AAAS mRNA had widespread expression in many tissues (Fig. 7A). Expression was particularly striking in neural structures including the developing brain, peripheral ganglia including the trigeminal ganglion (Fig. 7B, C) and retina. In the cerebral cortex of

Fig. 5. Localisation of AAAS mRNA in motor-associated nuclei of the brainstem and the cerebellum. Sets of three images are presented. AAAS mRNA silver grain signal is shown in the first panel (A, D, G), Toluidine counterstain in the middle (B, E, H) and overlaid silver grains in the third panel (C, F, I). The very large neurons of the magnocellular division of the red nucleus (A–C) show high levels of AAAS mRNA. Similarly the motor neurons of the facial nerve nucleus (D–F) are intensely labelled. Arrows in A–F indicate examples of such cells. Occasionally silver grains above background levels can be observed to be localised to very small cells likely to be glia (arrowheads in D–F). In the cerebellum (G–I) most cells in the granule cell layer (GL) have moderate labelling although labelling of individual cells varies. Purkinje cells also exhibit somewhat variable labelling. A relatively heavily labelled Purkinje cell is indicated by the arrows. Scale bars = 32 μm.
the developing brain at this embryonic age, the distribution of AAAS mRNA labelling had a banded pattern reflecting expression in both neuroepithelium and postmitotic neurons that have migrated to the cortical plate (inner and outer bands respectively at the asterisk in Fig. 7A). Low to moderate levels were found in the liver and musculature. Epithelial tissues including those found in the submandibular gland, vibrissae, primordia of the teeth, developing lung, kidney and gut had moderate to high levels. The Harderian gland, an accessory lacrimal gland found in the rat, had high levels of signal. Other lacrimal glands could not be identified in the sections processed. Although expression was also of moderate to high level in perichondral tissue, as seen surrounding Meckel’s cartilage (Fig. 7A), expression in the cartilage of developing endochondral bone was not observed (Fig. 7B, C).

**DISCUSSION**

In this study we present the first detailed description of AAAS mRNA distribution in the rat, with particular emphasis on its CNS distribution. Previous studies using northern and dot blots reported expression of the human AAAS mRNA with highest levels in the pituitary, adrenal, cerebellum and pancreas (Huebner et al., 2002; Smith et al., 1999). Our results confirm a fairly ubiquitous but uneven distribution of AAAS mRNA and indicate that there are not large interspecies differences. We observed high expression levels in the pituitary and adrenal glands in addition to neurons of sensory and sympathetic ganglia, the cerebellum, cerebral cortex, hippocampus and motor systems of the brain and spinal cord.

The widespread expression in both neuronal and non-neuronal cells indicates that the AAAS product, ALADIN, is likely to play a fundamental role in cellular function. This is consistent with its identification as a part of the NPC. The fact that ALADIN is a WD repeat domain-containing protein suggests that it acts as a scaffold for multiprotein assemblies at the NPC (Smith et al., 1999; Cronshaw and Matunis, 2003). Disease-associated mutations in AAAS disturb ALADIN localisation at the cytoplasmic face of the NPC and/or affect the...
WD repeat domain and thereby its putative interaction with as yet unidentified binding partners. Many of the regions that displayed high levels of AAAS expression can be linked to symptoms associated with AAAS. For example, we observed high levels of AAAS mRNA expression in the adrenal cortex. Dysfunction of the adrenal cortex in AAAS leads to the hallmark feature of adrenocorticotropin-resistant glucocorticoid deficiency supported by the finding of degenerative changes in the adrenal zona fasciculata and reticularis (Grant et al., 1993; Clark, 2003). Likewise AAAS mRNA expression was high in the superior cervical sympathetic ganglion. If this is the case for the sympathetic ganglia in general, such expression could underlie autonomic dysfunction seen in the AAAS, as well as contributing to the presentation of achalasia and alacrima. In other parts of the nervous system, some of the highest levels of AAAS expression occurred in sensory neurons (dorsal root ganglion [DRG] cells) and in spinal and bulbar motoneurons. This is consistent with additional peripheral nervous system manifestations in AAAS, including reduced motor and sensory nerve conduction velocity, sensory impairment, hypotonia, bulbar dysarthria, muscle weakness and atrophy (Grant et al., 1993; Gazarian et al., 1995; Huebner et al., 1999). Interestingly, in the large soma of motoneurons and DRG cells, the signal for AAAS mRNA appeared to be localised in close proximity to the cell nucleus. The reason for this is not known, but it may indicate spatial regulation of the AAAS mRNA so that it is translated close to its functional locale. In the CNS the highest levels of AAAS expression were seen in the cerebral cortex, hippocampus and cerebellum. These findings correlate with the observation that approximately 51% of patients have mental retardation and 13% have ataxia (Huebner et al., 1999). Moderate levels of AAAS mRNA were observed in other central nuclei likely to contribute to motor dysfunction including the pontine and inferior olivary nuclei, the red nucleus and substantia nigra. The localisation of AAAS mRNA in fibrous astrocytes suggests that a dysfunction of these cells in white matter tracts also may contribute to the pathogenesis of neurological deficits in AAAS.

There are several examples of apparent discordance between AAAS mRNA expression and AAAS symptomatology. On the one hand, high levels of expression do not always correlate with pathology. In the adrenal gland, expression in the zona glomerulosa which produces mineralocorticoids does not appear significantly different from that found in other cortical layers. Although glucocorticoid insufficiency is common in AAAS patients, some also have impaired mineralocorticoid responses (Grant et al., 1993; Kimber et al., 2003). In addition, we have found abundant AAAS mRNA is present in the anterior and intermediate lobes of the pituitary in agreement with the findings of Tulio-Pelet and colleagues (2000). However, no fully established defects in pituitary function have been described. An occasional observation of short stature in affected patients is unexplained and may relate to the growth hormone axis (Clark and Weber, 1998). On the other hand, only low levels of expression of AAAS were observed in some systems which often are affected. In the enteric nervous system little expression was observed, despite achalasia being a cardinal sign of the AAAS. Interestingly however, a small number of enteric neurons were found to express low to moderate levels of AAAS mRNA. As it is
thought that achalasia may be due to defects in nitric oxide releasing neurons of the myenteric plexus (Khelif et al., 2003), there remains the possibility that in the enteric nervous system, AAAS mRNA is selectively expressed by nitricergic neurons. Further study is necessary to characterize the phenotype of the AAAS expressing enteric neurons. Similarly in the adult little expression was observed in lacrimal tissue, although it is affected in AAAS.

There are several further explanations for the apparent mismatches between expression of AAAS mRNA and symptomaticity described for AAAS. First, it is unknown whether levels of mRNA correspond with those of translated, functional ALADIN protein. There are currently no suitable antisera for immunocytochemical studies, but when they become available studies of protein levels of ALADIN, its subcellular localization and regulation will be important.

Second, the heterogeneity of expression levels observed may relate to an important enigma seen in AAAS, namely that there is no clear genotype-phenotype correlation. Frequency, age of onset and severity of clinical symptoms is highly variable even between patients with the same AAAS mutation (Huebner et al., 2002). Although our distribution studies suggest that cells with high levels of AAAS mRNA (i.e. in the adrenal cortex and many neural systems) are particularly vulnerable in AAAS, other modifying factors and genes are likely to interact to determine penetrance and progression of the disease. In addition, subtle interactions between affected systems may occur. As such, altered input from the autonomic nervous system to the lacrimal glands and oesophageal musculature may contribute to the ultimate expression of symptoms related to alacrima and achalasia.

Finally, developmental deficiency in ALADIN function may also play a role in pathogenesis of AAAS. Little is known about the role of ALADIN in development, but our preliminary study of late embryonic rat tissues revealed widespread expression, with particularly high levels in brain, retina and epithelial tissues. Our finding of moderate expression of AAAS mRNA in the perichondrium of Meckel’s cartilage and the tooth primordium may relate to the findings of facial dysmorphism and susceptibility to caries which have been observed in some patients with AAAS (Clark and Weber, 1998). Alacrima in AAAS is thought to result from hypoplasia of the lacrimal gland and is the most consistent and earliest feature of the syndrome (Clark, 2003). Despite the lack of expression in adult lacrimal tissue, we have found high levels of expression in the E18 rat Harderian gland, an accessory lacrimal gland. This suggests that ALADIN may play a significant role in the development of these glands with deficits being manifest later.

We have described the expression of AAAS mRNA in the rat with particular emphasis on its expression in the nervous system. Further understanding of the molecules with which ALADIN interacts and its role in the NPC is necessary to understand how dysfunction of this protein leads to symptoms in AAAS. Such work is also likely to shed light on other neurological disorders.

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