Oculopharyngeal muscular dystrophy-like nuclear inclusions are present in normal magnocellular neurosecretory neurons of the hypothalamus

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Intranuclear inclusions composed of tubular filaments constitute a pathological hallmark of oculopharyngeal muscular dystrophy (OPMD). Autosomal dominant OPMD is caused by (GCG) repeat expansions in the gene that encodes for poly(A) binding protein nuclear 1 (PABPN1). The mutation results in the expansion of a polyalanine stretch in the N-terminus of the protein. It has been proposed that mutated PABPN1 induces protein aggregation, which in turn causes the formation of the filamentous nuclear inclusions. Here we report the presence of intranuclear inclusions composed of tubular filaments in oxytocin-producing neurons from normal rat hypothalamus. Like OPMD inclusions, the filamentous structures in neurosecretory neurons accumulate PABPN1, poly(A) RNA, ubiquitin and proteasomes. These inclusions do not contain members of Hsp40 and HDJ-2/DNAJ families of chaperones. The proportion of oxytocin-producing neurons that contain inclusions decreases during parturition and lactation (when synthesis and release of oxytocin is maximal) and increases at 1 day post-weaning (when occurs a drastic reduction in the production of the hormone). Thus, PABPN1 filaments in normal neurons are dynamic structures, the appearance of which correlate with changes in cellular activity. These data provide the first physiological evidence that polyalanine expansions are not essential to induce polymerization of PABPN1 into filamentous nuclear inclusions.

INTRODUCTION

Oculopharyngeal muscular dystrophy (OPMD) is an adult-onset disease characterized by progressive eyelid drooping (ptosis), swallowing difficulties (dysphagia) and proximal limb weakness (1). OPMD is usually inherited as an autosomal dominant trait with complete penetrance and without sex preference. The OPMD locus was mapped to chromosome 14q11 (2), and the gene mutated was identified as PABP2 or PABPN1 [poly(A) binding protein nuclear 1] (3). The dominant OPMD mutation consists of short (GCG) expansions causing the lengthening of a polyalanine tract located at the N-terminus of the PABPN1 protein. The PABPN1 binds with high affinity to nascent poly(A) tails, which are post-transcriptionally added to the 3’ ends of all eukaryotic mRNAs, with the single exception of histone messengers (4).

How the pathological repeat expansions in the PABPN1 gene relate to the disease symptoms remains unknown. A hallmark of OPMD consists of intranuclear inclusions formed by the PABPN1 protein and detected exclusively in patient muscle fibers (5,6). When viewed with the electron microscope, the OPMD inclusions are composed of filaments, which have a tubular appearance with a diameter of 8.5 nm and up to 0.25 μm in length (5,6). In addition to PABPN1, the OPMD inclusions accumulate poly(A) RNA, ubiquitin and proteasomes (7).

The presence of abnormal protein deposits in human tissues is a relatively common finding in degenerative diseases. A particular group of disorders associated with aggregation of
abnormal proteins consists of inherited neurodegenerative diseases caused by expansion of CAG/glutamine repeats (reviewed in 8). In these diseases, the CAG repeat expansion results in an expanded polyglutamine tract in otherwise unrelated proteins. In many cases the mutant proteins are detected in the inclusions and formation of the deposits precedes the appearance of symptoms. Although in some experimental systems the toxicity of expanded polyglutamine has been dissociated from the formation of visible nuclear inclusions (8,9), oligomerization of the mutant protein into insoluble molecular aggregates appears to play a key role in neurodegeneration (10).

According to a current view, the intranuclear inclusions detected in both OPMD and polyglutamine diseases are caused by abnormal protein aggregation induced by the mutation (7,11–14). Here we report that intranuclear inclusions composed of tubular filaments are present in a subset of normal rat brain cells, the oxytocin-producing neurons of the hypothalmo-neurohypophysial system. Magnocellular neurosecretory neurons of the hypothalamus play a fundamental role in the maintenance of body homeostasis by controlling the secretion of oxytocin and vasopressin from the pituitary in response to stimuli such as changes in plasma osmolarity, parturition and lactation (15–17). Like OPMD inclusions, the structures present in oxytocin-secreting neurons accumulate PABPN1, poly(A) RNA, ubiquitin and proteasomes. These observations argue that polyalanine expansions are not essential to induce polymerization of PABPN1 into filamentous nuclear inclusions.

RESULTS

Intranuclear tubulo-filamentous inclusions are present in hypothalamic neurons

Electron microscopic examination of the magnocellular neurosecretory neurons in rat hypothalamus revealed the presence of intranuclear bundles of filaments (Fig. 1A). The filaments are arranged parallel to one another, and in cross-section they appear as tubules with 8–10 nm in diameter (Fig. 1B). When observed in thin sections with the electron microscope, the bundles of filaments have diameters ranging between 0.2 and 0.4 μm and are up to 5 μm in length. This type of inclusion was specifically observed in magnocellular neurons from the paraventricular and supraoptic nuclei (SON) of the hypothalamus. It was not found in either glial or endothelial cells or in parvocellular neurons of other hypothalamic areas. Images obtained from resinless thicker sections show a three-dimensional view of the inclusions (Fig. 1C). The packed tubules appear free of coating material and exhibit lateral and terminal connections with the fibers of the nuclear matrix.

The intranuclear inclusions are enriched in PABPN1 and poly(A) RNA

The tubulo-filamentous structures observed in the hypothalamus of normal rat brain are morphologically reminiscent of the intranuclear inclusions associated with oculopharyngeal muscular dystrophy. This prompted us to investigated whether the two types of inclusions share any molecular components. First, immunofluorescence was performed using antibodies specific for PABPN1. All SON neurons showed a predominant nuclear immunolabeling throughout the nucleoplasm excluding the nucleolus, as previously described in other cell types (7,18). Within the nucleus, a fraction of the neurons exhibited a single and strongly immunostained rodlet or cigar-shaped inclusion (Fig. 2A). Although the length of these nuclear rodlets was variable, in some cells they were up to 12 μm long. PABPN1 immunoreactive inclusions were found in all animals examined (more than 20) and they were also detected in murine SON neurons (data not shown). Quantification of the fluorescence signal revealed that the intensity of PABPN1 staining in the nuclear rodlets is ~3-fold higher than in the nucleoplasm (data not shown). Immunelectron microscopy confirmed that the tubulo-filaments forming the inclusions are decorated by anti-PABPN1 antibodies (Fig. 2B). Immunoblotting analysis further revealed that anti-PABPN1 antibodies recognize a single protein band of identical electrophoretic mobility in nuclear extracts prepared from HeLa cells and hypothalamic neurons (Fig. 2C). This strongly suggests that the nuclear inclusions observed in the hypothalamus of normal rats are composed of PABPN1.

Since most trinucleotide repeats associated with disease show both somatic and germline instability (reviewed in 8,19), it was decided to determine whether the PABPN1 gene expressed in the hypothalamus encodes expanded GCG tracts. RNA was

Figure 1. Electron microscopy of SON neurons reveals the presence of intranuclear filaments. (A) This thin section has cut through a bundle of filaments longitudinally. (B) In transverse section the filaments appear as tubes. (C) Bundle of filaments as seen after critical point drying of a thick section. Bar, 100 nm.
extracted from dissected brain tissue containing either the hypothalamic SON or the cerebellum (a region where rodlet-shaped intranuclear inclusions were never observed). From the RNA, cDNA was synthesized and a PCR reaction was performed using a GC-rich PCR system. As can be seen in Figure 2D (lane H), the PCR product was a specific band of 440 bp, as expected. A similar product was amplified from the cerebellum (lane C). Sequencing of these products (Fig. 2E) revealed the exclusive presence of six (GCG) repeats, therefore excluding the possibility that an expanded version of the PABPN1 gene is expressed in the hypothalamus.

Next, the presence of poly(A) RNA in the nuclear inclusions was investigated by in situ hybridization using either oligo(U) or oligo(dT) probes. Both probes label intensely the marginal cytoplasm of neuronal bodies, where the rough endoplasmic reticulum and free polyribosomes are concentrated (Fig. 3B and E). The perinuclear cytoplasm shows irregular areas free of hybridization signal which are likely to correspond to the Golgi complex. The nucleus is also stained, excluding the nucleolus. Within the nucleus, poly(A) RNA appears concentrated in nuclear speckles, as previously reported in other cell types (20,21). In addition, the rodlet-shaped inclusions are brightly stained (Fig. 3B and E). Double labeling experiments using in situ hybridization and immunofluorescence demonstrated that the nuclear inclusions contain both PABPN1 and poly(A) RNA (Fig. 3A–C).

The intranuclear inclusions contain ubiquitinylated proteins and proteasomes

It is commonly accepted that the intranuclear aggregates seen in degenerative diseases result from aberrant properties of the mutated protein leading to tissue deposition. Supporting evidence for this view is provided by the detection of ubiquitin and proteasomes in the aggregates (reviewed in 8). The proteasome is a multimeric enzyme composed of two sub-complexes, the 20S proteolytic core and the 19S regulator. Protein substrates degraded by the proteasome must first be marked by covalent ligation to ubiquitin in order to become targeted for proteolysis (22). When immunofluorescence was performed with an anti-ubiquitin antibody, which recognizes ubiquitin conjugated proteins, the SON neurons displayed a...
diffuse staining pattern throughout the cytoplasm and nucleoplasm, excluding the nucleolus. The rodlet-shaped inclusions were the most intensely stained structures standing out over the diffuse nucleoplasmic signal (Fig. 3D–F). These inclusions were also brightly labeled by an antibody directed against the 19S proteasome, at both light and electron microscopic level (Fig. 4A and B). Although positive, the staining produced by an antibody specific for the 20S proteasome was less intense, presumably due to epitope masking (Fig. 4C). Double-labeling experiments using antibodies directed against PABPN1 and the 19S proteasome (Fig. 5A and B) further confirmed that the PABPN1-containing nuclear rodlets are enriched in proteasomes.

In addition to ubiquitin and proteasomes, inclusion bodies associated with polyglutamine diseases have been described to accumulate members of Hsp40 and HDJ-2/DNAJ families of chaperones (23–25), possibly reflecting proteasome malfunction and consequent insufficient protein degradation in the affected cells (23,25). We therefore performed double-labeling experiments using either anti-PABPN1 and anti-Hsp40 antibodies (Fig. 5C and D), or anti-19 S proteasome subunits and anti-HDJ-2/DNAJ antibodies (Fig. 5E and F). As shown in the figure, neither of these chaperones was detected in the nuclear rodlets. Furthermore, we observed no evidence for overexpression of Hsp40 or HDJ-2/DNAJ chaperones.

Another characteristic of pathological aggregates seen in familial degenerative diseases is resistance to biochemical extraction (7). Therefore, we analyzed the solubility of PABPN1 in SON intranuclear inclusions. The results show that the fraction of PABPN1 protein associated with the rodlet-shaped inclusions is resistant to extraction with either 1 M potassium chloride (Fig. 5G) or 2.5 μg/ml proteinase K (Fig. 5H). Taken together, these results demonstrate that normal PABPN1 protein in SON neurons can form insoluble aggregates containing ubiquitin and proteasomes, but not Hsp40 or HDJ-2/DNAJ chaperones.

Splicing proteins and cytoskeletal components are excluded from SON intranuclear inclusions

Because the nuclei of mammalian cells contain domains, termed nuclear speckles, which are enriched in PABPN1, poly(A) RNA and splicing factors (for a recent review see 26), we asked whether spliceosomal components are also present in the SON intranuclear inclusions. Immunofluorescence experiments showed that antibodies directed against spliceosomal proteins fail to label the nuclear rodlets (Fig. 6A–C and data not shown). Similarly, spliceosomal proteins are excluded from the OPMD inclusions seen in patient muscle tissue (A. Calado and M. Carmo-Fonseca, unpublished data). Thus, with regard to molecular composition PABPN1 inclusions are clearly distinct from nuclear speckles.

Previous studies have demonstrated the presence of actin and tubulin in nuclear inclusions of stressed fibroblasts and certain neuronal types (27–29). To determine whether cytoskeletal components are part of the SON intranuclear inclusions, immunofluorescence was performed using antibodies directed against components of microfilaments, neurofilaments and

Figure 3. The tubulo-filamentous inclusions contain poly(A) RNA and ubiquitin. SON magnocellular neurons were hybridized with a probe complementary to the poly(A) tail of mRNAs (B and E, green staining) and immunolabeled with either anti-PABPN1 or anti-ubiquitin antibodies (A and D, red staining). The yellow staining in C and F represents co-localization of poly(A) RNA, PABPN1 and ubiquitin in the rodlet-shaped inclusions. Bar, 10 μm.
neurotubules. In addition, FITC-conjugated phalloidin was used to visualize polymerized actin. Immunofluorescence with an antibody that recognizes β-tubulin isotype III, which is enriched in neuronal populations, labels microtubular arrays of the cytoplasm, but not the intranuclear inclusions of magnocellular SON neurons (Fig. 6D–F). In contrast, the intranuclear inclusions of hypothalamic parvocellular neurons were intensely immunostained by this antibody (Fig. 6G–I). However, these nuclear inclusions do not contain PABPN1 (Fig. 6G) and have a distinct morphological structure when observed with the electron microscope (30,31). Additional probes that failed to label the PABPN1-containing intranuclear inclusions in SON neurons included phalloidin and antibodies directed against pan-actin, β-tubulin, tau and neurofilament peptides (data not shown).

SON intranuclear inclusions are dynamic structures

Because the supraoptic nucleus consists almost entirely of magnocellular oxytocin and vasopressin neurons, we next performed immunofluorescence experiments using anti-vasopressin and anti-oxytocin antibodies. The data show that rodlet-shaped inclusions are exclusively detected in oxytocin-producing neurons (Fig. 7). This result was confirmed by the examination of more than 1000 neurons, immunolabeled in either squash preparations or semithin sections.

Since the metabolism of oxytocin-producing neurons changes drastically during parturition, lactation and post-weaning (15,16), we asked whether the appearance of nuclear inclusions correlates with cellular activity. To address this question, we estimated the proportion of oxytocin-producing neurons containing rodlet-shaped inclusions labeled by the anti-19S proteasome antibody. For each experimental condition, a total of ~400 neurons were sampled from at least three animals (see Materials and Methods). In female virgin rats, 48% of oxytocin-producing neurons contained inclusions. This proportion decreased to 37% (P < 0.001) and 40% (P < 0.05) after parturition and during lactation, respectively. In contrast, the proportion of neurons with rodlet inclusions increased to 68% (P < 0.001) at 1 day post-weaning. The relative immunostaining intensity produced by anti-PABPN1 antibodies in the nuclear rodlets and in the

![Figure 4](https://humanmoleculargenetics.org/article-abstract/13/03/0525019/833)

Figure 4. The tubulo-filamentous inclusions contain proteasomes. SON magnocellular neurons were immunolabeled with antibodies directed against the 19S regulatory (A and B) and the 20S catalytic (C) subunits of the proteasome. (A) The fluorescence microscope shows brightly stained intranuclear rodlets. Bar, 10 μm. (B and C) Observed in the electron microscope, the filaments are decorated by immunogold particles. Bar, 100 nm.

![Figure 5](https://humanmoleculargenetics.org/article-abstract/13/03/0525019/833)

Figure 5. Chaperones are not present in the SON intranuclear inclusions, which contain insoluble PABPN1. SON magnocellular neurons were double-labeled using antibodies directed against either PABPN1 (A) and the 19S proteasome (B), PABPN1 (C) and Hsp40 (D), or 19S proteasome subunits (E) and HDJ-2/DNAJ (F). Additionally, SON neurons were treated with 0.1% Triton X-100 and 1 M potassium chloride in PBS buffer for 5 min before fixation (G) or 2.5 μg/ml proteinase K for 5 min after formaldehyde fixation (H). The cells were then immunolabeled with anti-PABPN1 antibodies. Bar, 10 μm.
nucleoplasm remained similar in the three groups of animals, suggesting that the level of PABPN1 protein present in the inclusions is not significantly altered.

During parturition and lactation the synthesis and release of oxytocin is greatly stimulated, whereas after cessation of suckling there is a drastic reduction in the production of the hormone (17,32). Thus, our data suggest that formation of intranuclear inclusions is stimulated when oxytocin production is down-regulated.

DISCUSSION

Here we report that intranuclear inclusions similar to those observed in muscle biopsies from patients with OPMD are normally present in the magnocellular oxytocin-producing neurons of the hypothalmo-neurohypophysial system. Both types of inclusions are composed of tubular filaments ~8 nm in diameter, and both accumulate PABPN1, poly(A) RNA, ubiquitin and proteasomes. The major difference resides in the fact that OPMD filaments converge to form tangles or palisades (5,6), whereas neuronal inclusions are shaped like a cigar, with all of the filaments running parallel to one another in the long axis.

The presence of abnormal protein deposits in human tissues is a relatively common finding in degenerative diseases. In particular, a group of inherited neurodegenerative disorders caused by expansion of CAG/glutamine repeats shares with OPMD the presence of intranuclear inclusion bodies (reviewed in 8,9). The CAG repeat expansion results in an expanded polyglutamine tract in the mutant proteins, and in most cases these abnormal proteins are detected in the inclusions. Nuclear inclusions associated with polyglutamine diseases are additionally labeled by anti-ubiquitin, anti-proteasome and anti-chaperone antibodies, suggesting that they contain aberrant proteins recognized and targeted to the ubiquitin-proteasome degradation pathway (23,33). The disease-associated proteins further precipitate in vitro as insoluble fibers, whereas their counterparts from normal individuals are soluble. Based on these data, a model has been proposed in which polyglutamine expansion confers an increased tendency of the mutant protein to misfold and aggregate, giving rise to pathogenic deposits (34).

Surprisingly, our results show that under physiological conditions normal PABPN1 can polymerize into filaments and form intranuclear inclusions that recruit ubiquitin and proteasomes. This implies that polyalanine expansions are dispensable for the oligomerization of PABPN1. In agreement with this observation, the normal PABPN1 protein was recently shown to oligomerize in vitro (13), producing linear filaments of 7 nm in diameter when bound to poly(A) RNA (35). The view that pathological expansions are not necessarily the only cause of molecular aggregation is further supported.

Figure 6. The tubulo-filamentous inclusions do not contain splicing factors or tubulin. Both magnocellular and parvocellular neurons of the hypothalamus were immunolabeled using antibodies directed against 19S proteasomes (A, red staining), PABPN1 (D and G, red staining), the B0 protein of U2 splicing snRNP (B, green staining), and ß-tubulin III (E and H, green staining). (C, F and I) A superimposition of red and green images. Bar, 10 µm.
by the unexpected finding that relatively high expression of wild-type human ataxin-1 transgenes in a Drosophila model induced formation of nuclear inclusions in a variety of cell types (36). Additional evidence that intracellular inclusions can be formed by normal proteins has been observed (37–41).

Interestingly, the nuclear inclusions formed by wild-type PABPN1 in neurosecretory neurons are dynamic structures. The proportion of oxytocin-producing cells with inclusions decreases during parturition and lactation, and increases after weaning. Oxytocin stimulates contractions of the uterine wall during labour and delivery, triggers the milk ejection reflex during lactation and enhances prolactin release from the anterior pituitary, thus stimulating milk production. It is well known that the events of late pregnancy, parturition and lactation stimulate the synthesis and release of oxytocin by magnocellular neurons in the paraventricular and supraoptic nuclei of the hypothalamus, while weaning the pups results in reversal of these effects (15–17). Therefore, PABPN1 nuclear inclusions are more sparse in stimulated cells and become prominent after cessation of the stimulus. This implies that PABPN1 nuclear inclusions in neurosecretory neurons can assemble and disassemble in parallel with changes in cellular activity, despite the fact that these structures are biochemically insoluble. Early observations indicating that in vitro polyglutamine and polyalanine aggregates are difficult to dissolve, and that in vivo the aggregates disperse during mitosis and re-form in the interphase nucleus led to the proposal that abnormal protein aggregates grow irreversibly into inclusion bodies in neurons and muscle because these cells cannot divide. Yet, even Huntington’s aggregates in neurons can be dismantled, provided that expression of the mutant protein is blocked (42). Thus, the results reported here reinforce the view that insoluble intranuclear inclusions can be reverted.

Having shown the presence of non-pathological nuclear inclusions in normal SON neurons, what remains to be understood is why inclusions of similar composition are associated with disease in muscles from OPMD patients. Taking into account several lines of evidence suggesting that mutant proteins with expanded polyglutamine stretches can disrupt gene expression through interactions with specific transcriptional coactivators (8), one possibility is that mutant PABPN1 protein interferes specifically with regulators of muscle gene expression. To test this hypothesis it will be important to determine whether expanded PABPN1 protein in OPMD nuclear inclusions sequesters specific proteins responsible for inducing abnormalities in muscle gene expression.

**MATERIALS AND METHODS**

**Animals**

All experiments were performed on Sprague–Dawley rats, housed in controlled environments (14 h light/10 h dark, 22 ± 2°C) with food and water ad libitum. The animals were supervised and handled according to the approved national guidelines for animal care. The following reproductive stages were examined: (i) non-stimulated female virgin animals at 3 months of age; (ii) parturition at 2 h after the first pup was expelled; (iii) lactation for 10 days; and (iv) post-weaning for 1 day. At least five animals were examined at each stage.
SDS–PAGE and immunoblotting

Nuclear extracts from HeLa cells and SON neurons were prepared as described (43). Briefly, HeLa cells were harvested by scraping, washed in PBS and incubated in buffer A (10 mM HEPEs–KOH pH 7.9 at 4°C, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF) for 10 min on ice. After centrifugation, the pellet was resuspended in buffer C (20 mM HEPEs–KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF) and incubated on ice for 20 min. For the preparation of neuronal nuclear extracts, tissue fragments were dissected from 13 rats as described (44). Approximately 100 mg of pooled SON tissue was dounce-homogenized in the presence of buffer A. The samples were incubated on ice for 10 min, pelleted, resuspended in buffer C and further incubated on ice for 20 min. After the final centrifugation step, nuclear extracts were mixed with SDS–PAGE sample buffer as described (45). Proteins were separated on 12% polyacrylamide–SDS gels, transferred to nitrocellulose membranes and immunoblotted as described (45).

RNA extraction, reverse transcription and PCR

RNA was extracted from tissue samples (hypothalamus and cerebellum) stabilized in RNA-later solution (Qiagen). These were lysed and homogenized using 1 ml of TRizol reagent and the RNA was subsequently extracted according to the manufacturer’s instructions (Gibco BRL, Scotland). A 10 µg aliquot of total RNA was treated with RNase-free DNaseI (Roche) to remove any potential contamination of genomic DNA. cDNA synthesis was carried out using the Transcriptor Reverse Transcriptase enzyme (Roche) following the manufacturer’s protocol. We used 1 µl aliquot of total RNA in a 20 µl reaction containing random hexamers. A 1.5 µl aliquot of the resulting cDNA was added to 50 µl of the PCR reaction, using the GC-rich PCR system (Roche). The following PABPN1-specific primers were used: forward primer: 5’-GATTGCCAAGTAGAATTCC-3’; reverse primer: 5’-GGGGTCGGTCTCAACCA-3’. The PCR products were run on a 3% agarose gel, extracted (Qiagen extraction kit) and sequenced.

Immunocytochemistry

For light and electron immunocytochemistry, the animals were perfused under deep anesthesia with 3.7% formaldehyde (freshly prepared from paraformaldehyde) in PBS, pH 7.4 for 15 min at room temperature. Tissue fragments containing SON were removed from 300 µm vibratome sections and washed in PBS. For immunofluorescence, each tissue fragment was transferred to a drop of PBS on a siliconized slide and squash preparations of dissociated neurons were performed following the procedure previously reported (46). Then, the samples were sequentially treated with 0.5% Triton X-100 in PBS for 15 min, 0.1 M glycine in PBS containing 1% bovine serum albumin (BSA) for 30 min and 0.01% Tween 20 in PBS for 5 min. The samples were incubated for 1 h with the primary antibody containing 1% BSA at room temperature, washed with 0.01% Tween 20 in PBS, incubated for 45 min in the specific secondary antibody conjugated with FITC or TexasRed (Jackson, USA), washed in PBS and mounted with the antifading medium Vectashield (Vector, USA). For immunocytochemistry on semithin sections, tissue blocks containing the SON were dehydrated and embedded in dietylene glycol. Semithin sections, 1 μm thick, were processed for immunofluorescence according to the procedure of Ojeda et al. (47).

For electron microscopy immunocytochemistry, SON fragments were dehydrated in increasing concentrations of methanol at −20°C and embedded in Lowicryl K4M at −20°C. Ultrathin sections were sequentially incubated with 0.1 M glycine in PBS (15 min), 1% normal goat serum in PBS (5 min), and the primary antibodies diluted in PBS containing 0.1 M glycine and 1% BSA (1 h at room temperature). After washing, the sections were incubated with the secondary antibody conjugated with 10 nm gold particles (BioCell, UK) diluted 1:25 in 1% BSA in PBS (45 min at room temperature). After washing, the sections were stained with uranyl acetate and lead citrate. As controls, sections were treated as described but omitting the primary antibody.

The following primary antibodies were used in this study: monoclonal antibody 4G3 (Euro Diagnostica B.V., The Netherlands) directed against the B’ protein of U2 snRNP (48); rabbit polyclonal serum anti-PABPN1 (18); rabbit antibodies anti-ubiquitin (Dako, Denmark), 19S proteasome subunit 6a (Tbp1, Affiniti Labs UK), 20S proteasome (49) and Hsp40 (SPA-400, StressGen); mouse polyclonal serum raised against recombinant human PABPN1 (to be described elsewhere); monoclonal antibodies anti-oxytocin P38 and anti-vasoressin P41 (50), anti-β tubulin III (Sigma, UK) and anti-HDJ-2/DNAJ (Ab-1 clone KA2A5.6, NeoMarkers). Phalloidin-FITC (Sigma, UK) was used to visualize polymerized actin.

The quantitative analysis of nuclear inclusions was performed in squash preparations, double-labeled with the anti-19S proteasome and anti-oxytocin antibodies. The proportion of oxytocin-producing neurons containing rodlet-shaped nuclear inclusions was estimated by direct examination of a series of focal planes through each nucleus. Samples of three or four animals for each control and experimental group were used. A total of 390–470 neurons for each group were counted. Data were analyzed by the StatView 4.5 software, using the statistical tests ANOVA and chi square. Significance was established at P < 0.05.

In situ hybridization

In situ hybridization was performed on squash preparation of SON tissue samples fixed with 3.7% paraformaldehyde. A biotinylated 2′-O-alkyl oligoribonucleotide poly (U) probe containing 20 tandem uridine residues was used (7). Fixed samples were rinsed in 6 × SSPE (0.9 M NaCl, 0.06 M NaH₂PO₄, 6 mM EDTA, pH 7.4) containing 0.01% Tween 20 and incubated for 30 min in 10 µl of 0.5 mg/ml tRNA, 6 × SSPE and 5 × Denhardt’s. Hybridization was performed by adding 10 µl of the poly U probe diluted to 2 µg/µl in 6 × SSPE, 5 × Denhardt’s. Hybridization was carried out for 1 h at room temperature in a humid chamber. After washing (three times for 15 min each in 6 × SSPE; twice for 5 min each in 4 × SSC, 0.1% Tween 20) the hybridization signal was detected with FITC-avidin. Some samples were processed for double labeling experiments combining poly(A) RNA detection with...
immunofluorescence with either anti-proteasome 19S or anti-PABPN1 antibodies. All samples were mounted with Vectashield (Vector, USA).

Confocal microscopy

Samples were examined with a BioRad laser scanning microscope MRC-1024, equipped with an argon ion laser (488 nm) to excite FITC fluorescence and a He–Ne laser (543 nm) to excite Texas Red. For double labeling experiments, images of the same confocal plane were sequentially recorded and pseudocolor images were generated and superimposed. TIFF images were transferred to Adobe Photoshop 5.0 software (Adobe Systems Inc.) for presentation.

Electron microscopy

For conventional ultrastructural examination of SON neurons, the rats were perfused with 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. SON tissue fragments were removed from 300 μm coronal sections of the hypothalamus, rinsed in 0.1 M phosphate buffer, post-fixed in 2% osmium tetroxide, dehydrated in acetone and embedded in Araldite (Durcupan, Fluka, Switzerland). Ultrathin sections stained with uranyl acetate and lead citrate were examined with a Philips EM-208 electron microscope.

The ultrastructural examination of the nuclear matrix was performed using the embedment-free method of Capco et al. (51). The SON tissue sections were fixed in glutaraldehyde and treated with 2% osmium tetroxide for 4 h at 4°C. Then they were washed three times with PBS, dehydrated in ethanol and embedded in diethylene glycol diestearate. Sections were placed on formvar-coated carbon-stabilized grids. The embedding medium was removed with xilol and the sections were dried through the CO2 critical point. The embedment-free sections were examined with the electron microscope using 100 kV.

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