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Huntingtin-associated protein (HAP1): Discrete neuronal localizations in the brain resemble those of neuronal nitric oxide synthase

(Huntington disease/excitotoxicity/dystrophin/muscular dystrophy/glutamate)

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ABSTRACT **Huntington disease stems from a mutation of the protein huntingtin and is characterized by selective loss of discrete neuronal populations in the brain. Despite a massive loss of neurons in the corpus striatum, NO-generating neurons are intact. We recently identified a brain-specific protein that associates with huntingtin and is designated huntingtin-associated protein (HAP1). We now describe selective neuronal localizations of HAP1. *In situ* hybridization studies reveal a resemblance of HAP1 and neuronal nitric oxide synthase (nNOS) mRNA localizations with dramatic enrichment of both in the pedunculopontine nuclei, the accessory olfactory bulb, and the supraoptic nucleus of the hypothalamus. Both nNOS and HAP1 are enriched in subcellular fractions containing synaptic vesicles. Immunocytochemical studies indicate colocalizations of HAP1 and nNOS in some neurons. The possible relationship of HAP1 and nNOS in the brain is reminiscent of the relationship of dystrophin and nNOS in skeletal muscle and suggests a role of NO in Huntington disease, analogous to its postulated role in Duchenne muscular dystrophy.**

Huntington disease (HD) is an autosomal dominant neurodegenerative disease characterized by massive neuronal loss in some brain regions, while certain neuronal populations are resistant to damage (1, 2). For instance, up to 90% of neurons in the corpus striatum may be deleted, but neurons that stain for NADPH diaphorase are relatively preserved (3, 4). The discovery that NADPH diaphorase staining reflects neuronal nitric oxide synthase (nNOS) indicated that some property of nitric oxide synthase (NOS) neurons affords protection against neurotoxic mechanisms in HD (5–7). Various mechanisms have been proposed to account for the neuronal destruction in HD, including excitotoxicity elicited by glutamate and free radical damage (8–11). Excitotoxic damage by glutamate involving *N*-methyl-D-asparate receptors appears to involve the release of NO, as NOS inhibitors block excitotoxic neuronal damage (12, 13). Excitotoxic neuronal loss is also greatly diminished in mice with targeted disruption of the gene for nNOS (14).

Huntingtin is the protein encoded by IT-15, the gene whose expanded CAG repeats account for HD (15). Huntingtin occurs in numerous organs throughout the body and the brain with no discrete localizations that correspond to the selective neuropathology of HD (16–19). Recently, we identified a huntingtin-associated protein (HAP1) that is localized exclusively in the brain (20). HAP1 binds to huntingtin in the region of the glutamine repeats, and the extent of binding parallels their expansion, whose length is associated with the age of onset of the disease, especially for juvenile-onset individuals with 60 or more CAG repeats (21–23). In the present study, we demonstrate selective neuronal localizations of HAP1 mRNA and protein that resemble those of nNOS.

MATERIALS AND METHODS

***In Situ* Hybridization.** The antisense RNA probes were made for *in situ* hybridization on rat brain tissue sections. The cDNA for rat HAP1 (507 bp) was used to generate antisense RNA probes with [^{33}P]UTP labeling (DuPont/NEN). Sense RNA probes provided negative controls. Tissue sections (16 μm) were fixed in 4% paraformaldehyde and rinsed in PBS. Sections were then rinsed in 0.1 M triethanolamine, acetylated in 0.25% acetic anhydride for 10 min, and dehydrated in a graded series of ethanol solutions. Hybridization was performed with 10^6 cpm/100 μl of probe in 50% formamide, 10% dextran sulfate, 0.3 M $MgCl_2$, 10 mM Tris (pH 8), 1× Denhardt's solution, 0.5 mg/ml tRNA, and 10 mM dithiothreitol overnight at 55°C. Excess cRNA probe was removed by digestion with RNase A (20 μg/ml) for 30 min and washed at a final stringency of 0.1× standard saline citrate at 60°C for 30 min. Slides were hand-dipped in Kodak NTB2 emulsion, exposed for 1 week at 4°C, and developed and stained with Giemsa stain (Sigma). NADPH diaphorase staining of rat brain sections was conducted as described (5, 6).

Subcellular Fractionation and Western Blot Analysis. Human and monkey brain regional tissues were homogenized in PBS buffer containing proteinase inhibitors (1 μM pepstatin A/0.1 μM aprotinin/0.1 mM phenylmethylsulfonyl fluoride/10 μM leupeptin). Protein samples (80 μg) were denatured in SDS sample buffer at 100°C for 5 min before loading onto an 8% acrylamide SDS gel. Subcellular fractions of monkey cerebral cortex were prepared essentially as described (16). Tissue homogenate was centrifuged for 10 min at 800 × *g* to produce a pellet (P1). The supernatant (S1) was centrifuged at 9200 × *g* for 15 min to produce a pellet and a supernatant (S2). The pellet was washed by resuspension in the original volume of homogenization buffer and centrifugation for 15 min at 10,000 × *g* (P2). S2 was centrifuged at 165,000 × *g* for 2 hr to give pellet (P3) and soluble (S3) fractions. The P2 fraction was resuspended in a small volume of buffered sucrose and hypotonically lysed by addition of 9 vol of ice-cold water containing the above protease inhibitors and homogenization in a glass–Teflon homogenizer (three strokes). The P2

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Abbreviations: HD, Huntington disease; HAP1, huntingtin-associated protein; HLP, HAP-like protein; nNOS, neuronal nitric oxide synthase.

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lysate was brought to 4 mM Hepes (pH 7.4) by addition of a small volume of a concentrated solution and was centrifuged for 20 min at 25,000 × *g* to yield the lysate pellet (LP1) and lysate supernatant (LS1). The LS1 fraction was then centrifuged at 165,000 × *g* for 2 hr to give a crude synaptic vesicle pellet (LP2) and supernatant (LS2). Equal amounts of protein from each fraction (100 μg per lane) were resolved in an SDS/3–12% acrylamide gel. A nitrocellulose filter was treated with 5% dry milk in PBS for 1–3 hr at room temperature. A blot with transferred proteins was cut into 4 strips and probed with antibodies for huntingtin (AP-78) (16), nNOS (Transduction Laboratories, Lexington, KY), HAP1 (20), and synaptophysin (Sigma) for detecting individual proteins, respectively. After washing with PBS three times each for 15 min, filters were processed for enhanced chemiluminescence (ECL kit, Amersham).

Immunocytochemistry. HEK 293 cells transfected with rHAP1A cDNA (20) and mouse neuroblastoma cell line NIE115 were fixed on glass slides at room temperature for 15 min with 4% paraformaldehyde/0.4% Triton X-100 in PBS for 30 min with washes in PBS following each treatment. Slides were then blocked in 5% normal goat serum in PBS for 1 hr and incubated with antibodies diluted 1:200 in PBS for 24 hr at 4°C. Monkeys were perfused with 4% paraformaldehyde in 100 mM sodium phosphate buffer (pH 7.3). Tissue blocks were protected with 20% glycerol in PBS and stored at −80°C. Blocks were cut into sections (40-μm thick) using a sliding microtome. Some tissue sections were stored in antifreeze solution [50 mM sodium phosphate (pH 7.4)/1% polyvinylpyrrolidone-40/30% sucrose/30% ethylene glycol] at −10°C until use. Sections were then rinsed with 50 mM sodium phosphate buffer (pH 7.3) and processed as described above. Antibodies for glutathione *S*-transferase (GST)-rHAP1 (1 μg/ml) were affinity purified with Affi-Gel 15 (Bio-Rad) linked with immunogen, the GST-HAP1 fusion protein that was produced and purified as described (20). Antibodies for nNOS (1:1000 dilution) were obtained from Transduction Laboratories. ABC and DAB detecting kits (Vector Laboratories) or immunofluorescence was used to visualize immunoreactive signals.

RESULTS

We conducted *in situ* hybridization to localize mRNA for HAP1 and nNOS in rat brain (Fig. 1; Table 1). HAP1 localizations are strikingly discrete. The most intense message expression occurs in the accessory olfactory bulb and the pedunculopontine nuclei. The olfactory bulb itself also has high expression of message. In the hypothalamus, the supraoptic nucleus is intensely labeled. Substantial expression occurs in the basal forebrain, including the islands of Calleja, as well as in the dentate gyrus of the hippocampus, with much less labeling in CA1–3 layers. The superior and inferior colliculi are intensely labeled. Within the inferior colliculus, superficial layers are most intensely labeled, with negligible labeling in the central portion. In the cerebellum, light labeling is evident in the granule cell and Purkinje cell layers. In the lower brain stem, the nucleus of the tractus solitarius displays substantial labeling.

High power magnifications reveal that HAP1 silver grains are exclusively associated with neurons with no labeling of glia (Fig. 2). In the corpus striatum, labeling occurs in scattered neurons in contrast to the accessory olfactory bulb where virtually all neurons appear to be heavily labeled. Within the cerebral cortex and dentate gyrus almost all neurons also are labeled.

The HAP1 localizations are similar in many brain regions to those of nNOS mRNA and protein (5, 24, 25) and in the present study using NADPH diaphorase staining (Fig. 1; Table 1). The pedunculopontine nuclei, the supraoptic nucleus of the hypothalamus, and the accessory olfactory bulb are the most intensely labeled structures for nNOS mRNA. The olfactory bulb is also heavily labeled. Within the hippocampus, the dentate gyrus displays substantially more label than layers CA1–3. Immunohistochemistry and *in situ* hybridization have established the superior and inferior colliculi as sites with intense nNOS labeling most prominent in the superficial rather than central zones (5). Discrepancy between HAP1 and nNOS occurs in the cerebellum, where granule cell layers are intensely labeled for nNOS, while HAP1 levels are less prominent (data not shown). Also, in normal rats the Purkinje cells do not display nNOS mRNA or protein (5, 6). In the cerebral cortex and basal forebrain, neuronal labeling for nNOS is substantially more scattered than for HAP1.

To study the distribution of HAP1 protein, we conducted Western blot analysis of monkey and human brain regions

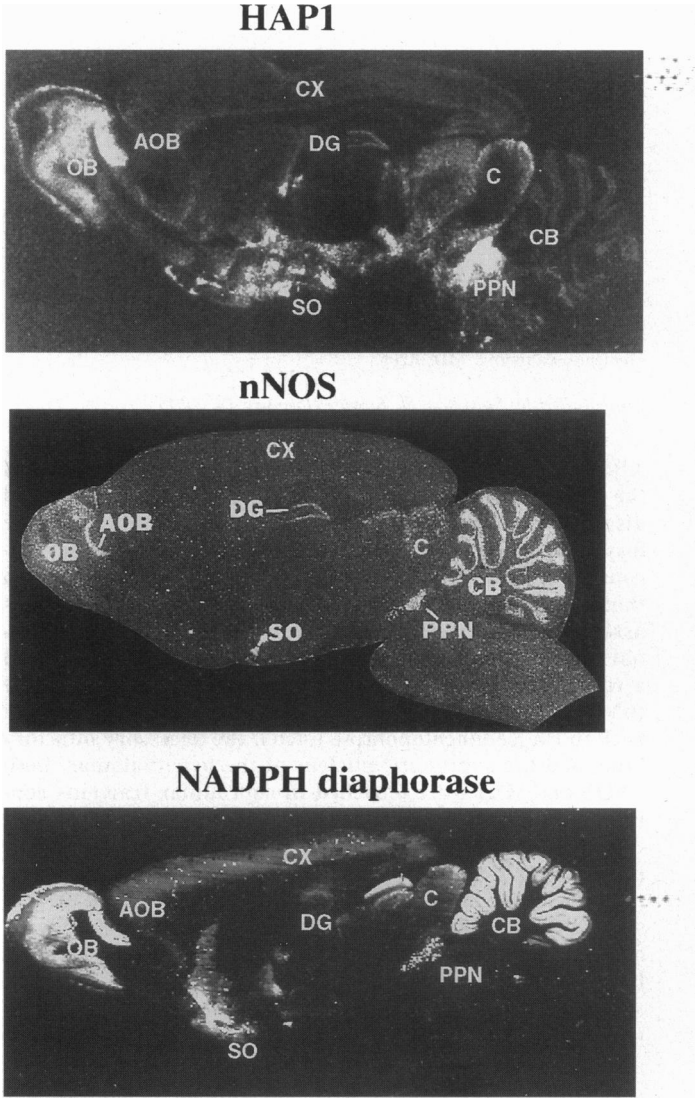

FIG. 1. Similar localizations of HAP1 and nNOS mRNA expression in many regions of rat brain. A dark-field image of a sagittal section of adult rat brain shows strong labeling for HAP1 in olfactory bulb (OB), accessory olfactory bulb (AOB), dentate gyrus of the hippocampus (DG), superior and inferior colliculi (C), pedunculopontine nucleus (PPN), and supraoptic nucleus (SO). Moderate labeling occurs in cerebral cortex (Cx) and cerebellum (CB). nNOS mRNA expression in rat brain was detected as described (5). NADPH diaphorase staining reveals the strongest labeling in OB, AOB, PPN, SO, and CB. Labeling is also evident in C and DG. A comparison of mRNA expression patterns for HAP1 and nNOS is shown in Table 1.

Table 1. Distribution in rat brain of HAP1 and nNOS mRNA

	HAP1	nNOS
Olfactory bulb	+++	+++
Accessory olfactory bulb	++++	++++
Cerebral cortex	++	(++)
Frontal cortex, deep layers	+++	(++)
Corpus striatum	+	(++)
Medium-sized neurons	+	±
Large neurons	++	++
Basal forebrain	+++	(++)
Islands of Calleja	+++	+++
Hippocampus		
Dentate gyrus	+++	+++
CA1	++	++
Thalamus	±	±
Hypothalamus	+++	(++)
Supraoptic nucleus	++++	++++
Superior colliculus	++	++
Inferior colliculus		
Superficial	+++	+++
Central nucleus	±	±
Cerebellum		
Purkinje cell layer	+	—
Granule cell layer	++	++
Pontine nucleus	±	±
Pedunculopontine nucleus	++++	++++
Reticular formation	(++)	(++)
Nucleus of the tractus solitarius	+++	++

Values in parentheses indicate scattered neurons within a structure. Data are presented as relative intensity of hybridization based on our present study and previous studies (5, 24, 25).

using an antibody generated against a HAP1 fusion protein (Fig. 3). Whereas in rat brain three immunoreactive bands are evident (20), in monkey and in human a single prominent band at 68 kDa occurs in all brain regions (though in monkey cortex a less intense band of about 46 kDa is also evident). We observe marked regional variations. In monkey brain intense bands occur in the cerebral cortex and hippocampus, whereas immunoreactivity is barely detectable in the pons and medulla. The vagus nerve, lung, adrenal, skeletal muscle, and spleen lack immunoreactivity, confirming our earlier observations

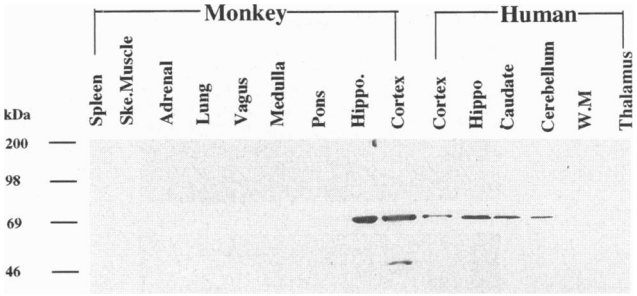

FIG. 3. Western blot analysis of HAP1 in monkey and human brain regions. Ske. Muscle, skeletal muscle; Hippo, hippocampus; Cortex, cerebral cortex; W.M., white matter.

that HAP1 is a brain-specific protein (20). In human brain the hippocampus and caudate display the most intense labeling, whereas levels in the cerebral cortex and cerebellum are somewhat lower and no labeling occurs in thalamus or white matter.

Immunohistochemistry confirms the neuronal localizations of HAP1 (Fig. 4). In monkey brain, negligible staining occurs in white matter, while the cerebral cortex and corpus striatum display substantial immunoreactivity, with higher levels of staining in caudate than cerebral cortex. In the striatum, a larger proportion of neurons display protein immunoreactivity than display mRNA. No staining is evident in sections preabsorbed with antigen.

We compared the subcellular localization of huntingtin, HAP1, and nNOS using a differential centrifugation procedure (Fig. 5). In this procedure, a crude nuclear fraction (P1) is obtained by centrifugation at 800 × *g* for 10 min, and the supernatant is centrifuged at 10,000 × *g* for 15 min to provide a P2 fraction, whose supernatant is centrifuged at 165,000 × *g* for 2 hr, yielding P3 pellet and the soluble supernatant (S3) fraction. The P2 fraction contains primarily mitochondria and synaptosomes. After hypotonic lysis of P2, we obtain an LP1 pellet by centrifugation at 25,000 × *g* for 20 min, while centrifugation of the supernatant of this fraction at 165,000 × *g* for 2 hr provides the LP2 pellet and the soluble supernatant LS2 fraction.

As previously reported, huntingtin is present in both soluble and particulate fractions. Thus, equal densities are evident in

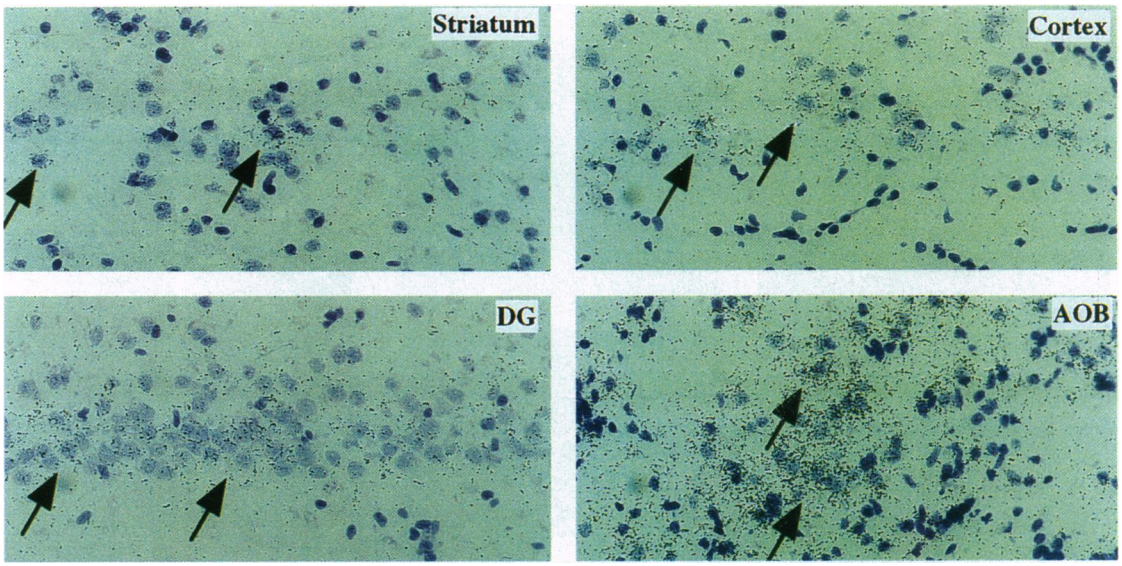

FIG. 2. HAP1 mRNA localized at high power (×400). *In situ* hybridization was conducted with HAP1 probes, and the slide was dipped in emulsion and counterstained with Giemsa for striatum, cerebral cortex (Cortex), dentate gyrus (DG), and accessory olfactory bulb (AOB). Arrows indicate that hybridization signals (silver grains) are over cell bodies of neurons (light blue) but not glial cells or cells in the white matter (dark blue).

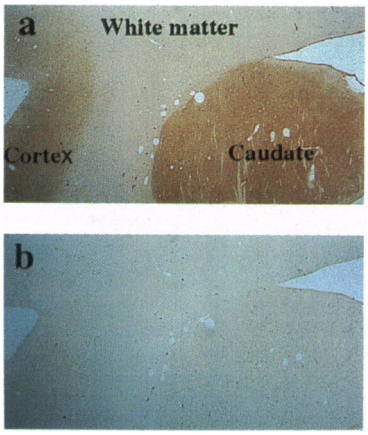

FIG. 4. Immunocytochemical localization of HAP1 in monkey brain tissue sections. (*a*) Section probed with antibody for HAP1. (*b*) Section probed with antibodies preabsorbed with antigen.

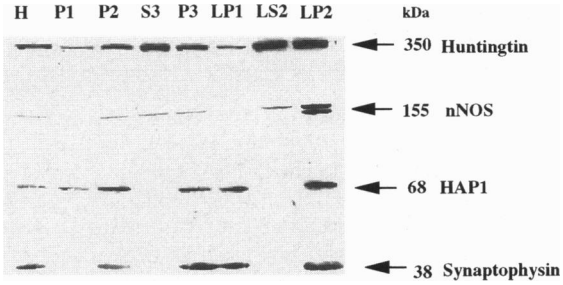

FIG. 5. Subcellular localization of HAP1 in monkey cerebral cortex extracts. Fractions are marked as H, homogenate, and P1, P2, S3, P3, LP1, LS2, and LP2, which are described in the text. A blot was probed with antibodies for huntingtin, nNOS, HAP1, and synaptophysin.

LP2 and LS2 fractions. This contrasts with synaptophysin, a protein localized to membranes of synaptic vesicles, which is highly enriched in LP2 and not evident in LS2. Like synaptophysin, HAP1 is exclusively particulate and not evident in S3 or LS2 but heavily enriched in LP2. nNOS distribution closely resembles that of HAP1 except for the occurrence of some nNOS in the soluble fractions S3 and LS2, corresponding to the known occurrence of nNOS in cytoplasmic fractions (26). Both HAP1 and nNOS are most enriched in LP2.

To further explore the intracellular distribution of HAP1, we examined its localization by immunohistochemistry in HEK 293 cells transfected with HAP1 (Fig. 6*a*) and in NIE115 cells that possess endogenous HAP1 (Fig. 6*c*). In both cell types, HAP1 does not occur in the nucleus and does not appear to be selectively associated with the plasma membrane. Rather, it is concentrated in granular elements scattered throughout the cytoplasm. In monkey striatum, scattered large neurons in the corpus striatum contain greater densities of HAP1 immunoreactivity than medium-sized neurons (Fig. 6*d*). In these large neurons, we also observe granular HAP1 immunoreactivity with a cytoplasmic distribution and no association with the nucleus or plasma membrane (Fig. 6*e*). Double labeling of the monkey striatum with NADPH diaphorase staining for nNOS (5–7) and antibodies for HAP1 shows that some large neurons labeled with antibodies against HAP1 are also labeled by staining for NADPH diaphorase. Some medium-sized neurons are also double labeled. The products of double labeling are predominantly in the cytoplasmic region (Fig. 6 *f* and *g*). More neurons display HAP1 than diaphorase staining.

DISCUSSION

In the present study, we confirm our earlier finding that HAP1 occurs exclusively in the brain. We also show that it is selectively associated with neurons. The major finding is that the localization of HAP1 resembles that of nNOS. The most prominent localizations of both HAP1 and nNOS mRNA are the accessory olfactory bulb and the pedunculopontine nuclei, with dense labeling in the olfactory bulb, supraoptic nucleus, and nucleus of the tractus solitarius. The concatenation of structures enriched in HAP1 and nNOS is unusual. However, their localizations are not identical. In the basal forebrain and

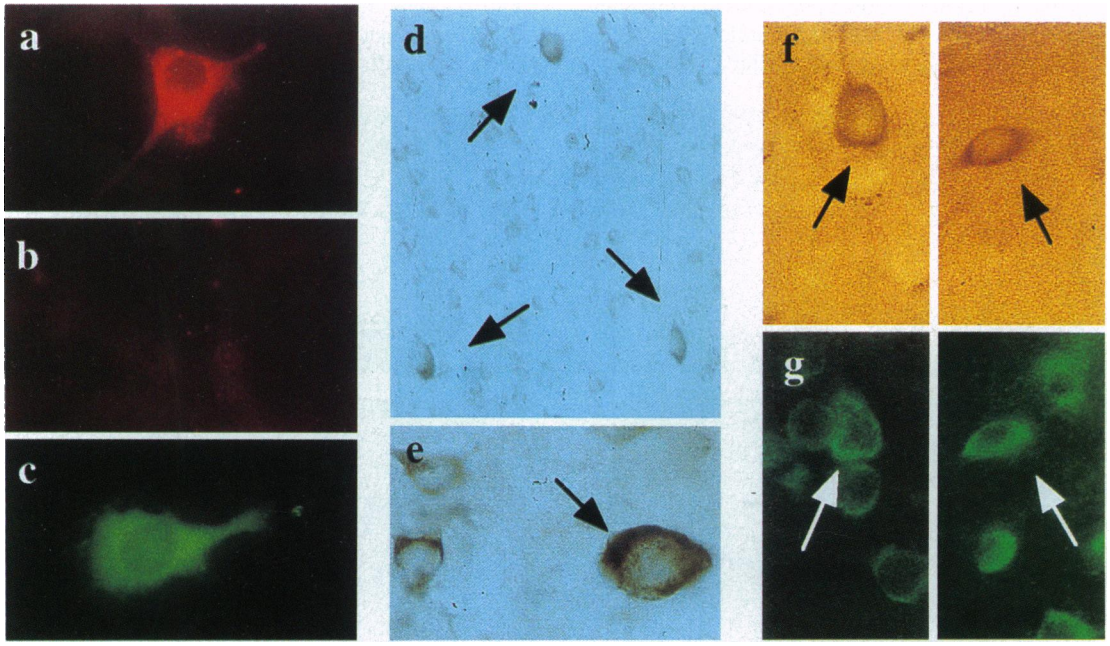

FIG. 6. Localization of HAP1. (*a*) HEK cells transfected with rHAP1A cDNA [shown by rhodamine (red) (×400)]. (*b* and *c*) Untransfected HEK 293 cells (*b*) and NIE115 mouse neuroblastoma cells [shown by fluorescein isothiocyanate (green) (×400)]. (*d*) Monkey caudate (×200). (*e*) Monkey caudate (×600). Immunoreactivity for HAP1 is predominantly in the cytosolic region and not in plasma membrane and nucleus (*a*, *c*, and *e*). (*f* and *g*) Double staining of interneurons in caudate (×400) with NADPH diaphorase (*f*) (brown) and antibody for HAP1 (*g*) (green). Some neurons in caudate stain for both NADPH diaphorase and HAP1 (indicated by arrows). The fluorescence in the doubly labeled neurons is partly obscured by the NADPH diaphorase reaction product.

cerebral cortex, nNOS appears in scattered neurons while HAP1 is more widely expressed. In the cerebellum, Purkinje cells contain HAP1, while in normal rat brain they do not possess nNOS. Interestingly, in some conditions Purkinje cells can express nNOS. For instance, following treatment with ibogaine, a psychotomimetic drug that is neurotoxic, many Purkinje cells are destroyed, while the remaining intact Purkinje cells express nNOS abundantly (27). Immunohistochemical studies reveal localizations of citrulline, the product of NOS activity, that are essentially the same as those of nNOS with no staining in Purkinje cells in normal animals (28). However, in mice with targeted deletion of nNOS, prominent citrulline staining is evident in Purkinje cells though it vanishes from most other neuronal populations (M. Eliasson and S.H.S., unpublished results). Purkinje cells do not normally contain endothelial NOS or inducible NOS, suggesting that a novel form of NOS with neuronal localizations may be responsible for the observed staining, consistent with recent molecular biological evidence for such novel forms of the enzyme (H.-Y. Yun, V. L. Dawson, and T.M.D., unpublished results).

In addition to HAP1, we identified another protein designated HAP-like protein (HLP), which possesses 80% amino acid sequence identity to HAP1 but which does not bind to huntingtin (20). Our antiserum, developed against a fusion protein of HAP1, may also interact with HLP, so that immunohistochemical localizations we have observed might reflect HLP as well as HAP1. The *in situ* hybridization patterns are specific for HAP1. We have not yet established the regional or intracellular localizations of HLP.

At a cellular level, HAP1 has been detected in neuronal cells and some fiber extensions. HAP1 appears to be associated with granular structures that occur in the cytoplasm. The subcellular localization of HAP1 in the LP2 fraction could reflect an association with synaptic vesicles or cytoskeletal elements. This would fit with evidence for an association of huntingtin with the cytoskeleton and with synaptic vesicles (16–19). Double labeling of neurons with NADPH diaphorase and HAP1 indicates colocalizations of nNOS and HAP1 in the cytoplasm of a subpopulation of neurons.

The relationship between HAP1 and nNOS might be relevant to the pathophysiology of HD. A variety of evidence suggests that neuronal loss in HD is due to excitotoxic damage (8–11). Intrastriatal injections of malonate and systemic administration of 3-nitropropionic acid (3-NP) produce striatal lesions that closely resemble the histologic, neurochemical, and clinical features of HD and involve excitotoxic mechanisms consequent to energetic disturbances (29–33). Excitotoxic neuronal loss caused by these toxins is greatly diminished in mice with a targeted disruption of the gene for nNOS or treated with 7-nitroindazole, a selective nNOS inhibitor (14, 34). Thus, neuronally derived NO may participate in the pathophysiology of HD.

The possible relationship of nNOS with HAP1 is reminiscent of the association of nNOS and dystrophin (35). Dystrophin is an extremely large cytoskeletal protein of skeletal muscle, whose mutation is responsible for the symptoms of Duchenne muscular dystrophy (36, 37). Subcellular fractionation reveals an intimate association of nNOS and dystrophin (35, 38). Dystrophin or one of its associated proteins is responsible for the predominantly particulate localization of nNOS in skeletal muscle. Thus, in *mdx* mice, which lack dystrophin, nNOS is fully soluble, and in human muscular dystrophy, nNOS is depleted from skeletal muscle (35). A relationship between NO and the symptoms of muscular dystrophy is further suggested by the selective involvement of fast skeletal muscle fibers in the disease process and the selective association of nNOS with the fast fibers (39, 40).

By analogy with the dystrophin–nNOS link, there may exist a complex of huntingtin, HAP1, and nNOS in certain neurons in the brain. nNOS is present only in a subpopulation of HAP1 neurons. Similarly, HAP1 is present only in a subpopulation of huntingtin-containing neurons. There is evidence for association of huntingtin with a protein complex that includes calmodulin (41). Calmodulin is also involved in the regulation of nNOS (42). In HD, it is conceivable that interactions among these proteins are altered. We have already established that HAP1 binds more avidly to mutated than to wild-type huntingtin (20). Perhaps as a result of altered interactions among these proteins, NO production is increased or occurs in aberrant intracellular sites, facilitating neuronal damage.

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