Pituitary Adenylyl Cyclase-Activating Peptides and α -Amidation in Olfactory Neurogenesis and Neuronal Survival *In Vitro*

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We investigated the role of amidated neuropeptides, and specifically pituitary adenylyl cyclase-activating polypeptide (PACAP), in olfactory neurogenesis and olfactory receptor neuronal survival. Using both immunohistochemistry and *in situ* hybridization, we find that both peptidylglycine α -amidating monooxygenase (PAM), the enzyme responsible for amidation and therefore activation of all amidated neuropeptides, and amidated PACAP are expressed in developing and adult olfactory epithelium. Amidated PACAP is highly expressed in proliferative basal cells and in immature olfactory neurons. The PACAP-specific receptor PAC₁ receptor is also expressed in this population, establishing that these cells can be PACAP responsive. Experiments were conducted to determine whether amidated neuropeptides, such as PACAP38, might function in olfactory neurogenesis and neuronal survival. Addition of

PACAP38 to olfactory cultures increased the number of neurons to >250% of control and stimulated neuronal proliferation and survival. In primary olfactory cultures, pharmacologically decreased PAM activity, as well as neutralization of PACAP38, caused neuron-specific loss that was reversed by PACAP38. Mottled (Brindled) mice, which lack a functional ATP7A copper transporter and serve as a model for Menkes disease, provided an *in vivo* partial loss-of-function PAM knock-out. These mice had decreased amidated PACAP production and concomitant decreased numbers of olfactory receptor neurons. These data establish amidated peptides and specifically PACAP as having important roles in proliferation in the olfactory system and suggest that a similar function exists *in vivo*.

Key words: PAM; PACAP38; olfaction; neurogenesis; Mottled (Brindled) mice; Menkes disease

The olfactory system is one of the few neuronal systems capable of continuous regeneration. The epithelium contains globose basal cells, immature and mature olfactory receptor neurons, and sustentacular cells (Fig. 1A) (Morrison and Costanzo, 1990, 1992; Uraih and Maronpot, 1990). Basal cells generate new neurons that repopulate the olfactory epithelium throughout life (Schultz, 1960; Graziadei and Monti-Graziadei, 1983; Calof and Chikaraishi, 1989; Morrison and Moran, 1995; Huard et al., 1998). Neurogenesis also occurs in the rostral migratory stream, hippocampus, and retina, but factors that regulate regeneration have not been well established (Gage et al., 1998; Gage, 2000; Tropepe et al., 2000).

In many tissues amidated neuropeptides function as proliferative or survival factors (Wettstein et al., 1995; Waschek et al., 1998; Shioda et al., 1998; Wakade and Leontiv, 1998; Wysolmerski and Stewart, 1998). Pituitary adenylyl cyclase-activating polypeptide (PACAP) belongs to the vasoactive intestinal peptide (VIP)/glucagon peptide family (Miyata et al., 1989, 1990; Ogi et

al., 1990). PACAP is expressed in the embryonic neural tube and autonomic and sensory ganglia, suggesting a neurodevelopmental role (Nielsen et al., 1998; Waschek et al., 1998). PACAP can activate cellular pathways to generate neuroproliferative, survival, or trophic signals (Arimura et al., 1994; DiCicco-Bloom, 1996; Lu and DiCicco-Bloom, 1997; Wakade and Leontiv, 1998; Vaudry et al., 1999). However, whether PACAP or other neurotrophic peptides participate in neuronal regeneration processes in the mature nervous system is unknown.

To assess the role of amidated neuropeptides in development, the levels of the amidating enzyme peptidylglycine α -amidating monooxygenase (PAM) can be measured. Over half of the known neuropeptides require α -amidation for bioactivity (Fig. 1B) (Eipper et al., 1992; Cuttitta, 1993; Kulathila et al., 1999). PAM, the only enzyme known to amidate peptides, contains two enzymatic domains, peptidylglycine- α -hydroxylating monooxygenase (PHM) and peptidyl- α -hydroxyglycine α -amidating lyase (PAL) (Eipper et al., 1993). The PHM domain catalyzes the copper-, ascorbate-, and molecular oxygen-dependent α -hydroxylation of glycine-extended peptides; the PAL domain converts these intermediates into the final α -amidated products (Fig. 1C). Because PAM amidates all peptide-X-Gly substrates, PAM serves as a global marker for neuropeptide production.

The significance of amidated peptides is revealed by knock-out studies. Mice lacking PAM do not exist, and elimination of PHM activity in *Drosophila* is embryonic lethal (Kolhekar et al., 1997). Alternative models can be used to investigate the role of PAM. Because PAM requires copper, *in vivo* and *in vitro* models of altered copper availability are used to examine neuropeptide function. For example, the Mottled (Brindled) mouse (Mo^{br})

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Figure 1. Schematic of olfactory epithelium and PAM enzymatic reaction. *A*, The adult olfactory epithelium. The olfactory epithelium is separated from the lamina propria (*LP*) by the basal lamina (dotted line). Proliferating basal cells (*BC*) give rise to immature olfactory receptor neurons (*IN*), which develop into mature neurons (*MN*). Mature neurons demonstrate dendritic extension to the olfactory lumen and axonal extension through the basal lamina to the olfactory bulb. Sustentacular cells (*SC*) span the olfactory epithelium. *B*, Localization of PAM to the secretory pathway. PAM is a transmembrane protein that functions within the lumen of the secretory pathway and is generally localized to the *trans*-Golgi network (*TGN*) and large dense core vesicles (*LDCV*). PAM requires copper (*Cu*) and ascorbate; cytosolic copper is transported into the secretory pathway via two P-type ATPase copper transporters, Menkes protein (*MNK*, *ATP7A*) and Wilson's disease (*ATP7B*) (Suzuki and Gitlin, 1999). Chaperone proteins make cytosolic copper available for superoxide dismutase (*SOD*) and mitochondrial cytochrome-*c* oxidase (*CCO*). *C*, PAM amidation reaction. The PHM and PAL domains are located within the lumen of the secretory pathway. PHM converts glycine-extended neuropeptide precursors into α -hydroxyglycine intermediates in a copper (*Cu*)-, ascorbate-, and oxygen (O_2)-dependent manner. This intermediate is converted to the final α -amidated product by PAL. *A*, Exon A; *CD*, cytoplasmic domain.

(Lyon and Searle, 1990) contains mutations in the Menkes copper transporter (*ATP7A*) that limit copper transport into the secretory pathway (Suzuki and Gitlin, 1999).

In this study, we used both *in vivo* and *in vitro* models to study amidated peptides, specifically PACAP38, in olfactory neuronal

development. Expression of PAM, PACAP38, and PAC₁ receptor was found in the olfactory epithelium. *In vitro* experiments support an important role for PACAP peptides in neurogenesis and survival. Studies that examined the olfactory epithelium in Mo^{br} mice were consistent with these observations. We propose that PACAP peptides are among several PAM products essential for adult olfactory neurogenesis.

MATERIALS AND METHODS

Experimental animals and tissue preparation. All experimental protocols were approved by the Johns Hopkins University Institutional Animal Care and Use Committee, and all applicable guidelines from the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* were followed. Timed-pregnant or adult Sprague Dawley rats at appropriate ages were obtained from Harlan Sprague Dawley (Indianapolis, IN); embryonic day 0 (E0) is defined as the date of conception. Adult rats were harvested at 5 weeks. Mottled (Brindled) C57BL/6 heterozygous female and C57BL/6 male mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred to obtain Mottled (Brindled) male offspring.

Antibodies and affinity purification. Affinity purification of rabbit polyclonal PAM antibodies 1761 and 629 (Maltese and Eipper, 1992) was performed using 1 mg of recombinant PHM or Exon A, respectively. The proteins were coupled to 2 ml of Affi-Gel 15 resin (Bio-Rad, Hercules, CA) for 2 hr at room temperature; the resins were blocked with 0.1 M ethanolamine HCl, pH 8.0, for 1 hr and washed three times in 0.1 M NaOAc and 1 M NaCl, pH 4.0, followed by 0.1 M Tris-HCl and 1 M NaCl, pH 8.0. Antibody was enriched by precipitation of serum with 45% ammonium sulfate; the precipitate was dialyzed into 100 mM Na-phosphate, pH 7.4, bound to the affinity resin (5 ml serum/2 ml resin) for 3 hr at 4°C, washed, and eluted with 0.2 M glycine, 0.1 M NaCl, and 0.1% Triton X-100, pH 2.3. Affinity-purified antibody was dialyzed overnight into 100 mM Na-phosphate, pH 7.4, and recovery was checked by ELISA. Affinity-purified PHM antibody 1761 (rPAM-1; residues 37–382) was diluted 1:200 for use. Blocking experiments were performed by incubating antibody 1761 in 100 μ g/ml PHM for 1 hr in PBS before slide incubation. Affinity-purified Exon A antibody 629 (rPAM-1; residues 409–496) was diluted 1:200, and the immunodepleted serum was used as a control. Polyclonal PACAP38 antibody (Peninsula Laboratories, Belmont, CA) was diluted 1:6000 for use. Immunodepletion of PACAP38 serum was performed using PACAP(31–38)-amide (Peninsula Laboratories) linked to Affi-Gel 10 resin (Bio-Rad). As assayed by ELISA, the PACAP38 antibody was able to recognize PACAP(31–38)-amide (Peninsula Laboratories) at a 1:27,000 dilution, whereas PACAP(31–38)-Gly (synthesized by Dr. Henry T. Keutman, Massachusetts General Hospital) was not recognized even at a 1:500 dilution. Affinity-purified PAC₁ receptor antibody (antisera ERIQ; 1:2000) was used as described previously (Braas and May, 1999). Monoclonal neuron-specific tubulin (NST) antibody (1:2000) was from BabCo (TuJ1; Richmond, CA); monoclonal glial fibrillary acidic protein (GFAP) antibody (1:1000) was from Chemicon (Temecula, CA). Goat polyclonal antibody to olfactory marker protein (OMP; 1:5000) (Keller and Margolis, 1975) was a generous gift from Dr. Frank Margolis, University of Maryland. Rabbit polyclonal antibody to OE-1 (1:125) was a generous gift from Dr. Randy Reed, The Johns Hopkins University School of Medicine.

Immunocytochemistry. For general immunohistochemistry, animals were anesthetized with Xylaket (25 ml of Ketamine HCl from a stock solution of 100 mg/ml, 2.5 ml of Xylazine from a stock solution of 100 mg/ml, and 14.2 ml of EtOH brought up to a final volume of 100 ml in 0.9% sodium chloride) and perfused with PBS followed by Bouin's fixative. The tissues were dissected, post-fixed in Bouin's solution at 4°C overnight, washed, cryoprotected in 20% sucrose, and embedded in Tissue-Tek (Sakura Finetek, Inc., Torrance, CA). Cryosections (16 μ m) were thaw-mounted onto Superfrost plus slides (Fischer Scientific, Pittsburgh, PA); immunohistochemistry was performed following the Vectastain Elite ABC procedure (Vector Laboratories, Burlingame, CA). Briefly, the sections were rinsed, permeabilized in 0.05% SDS, blocked in 4% normal serum and 1% BSA, and incubated with diluted antiserum at 4°C overnight. The slides were rinsed and incubated subsequently in biotinylated secondary antibody for 30 min, followed by the avidin–biotin complex for 30 min. The peroxidase reaction was catalyzed using 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide as substrates. Rat embryo paraffin sections (12 μ m) were obtained from No-

vagen (Madison, WI). The sections were cleared of paraffin, rehydrated, and immunocytochemically processed as described above. Slides were viewed using a Zeiss Axioskop microscope, and the images were captured digitally.

Cell counts on immunostained tissue were performed on serial sections for immunostained and cresyl violet-stained material. Eight to 10 sections were used per animal per experiment; cell counts were performed on 10 regions of turbinate epithelium per animal. All immunopositive cells were counted within a defined region; total cells were enumerated over the same region stained with cresyl violet.

PACAP38 immunostaining was performed as described above with antibody diluted 1:6000 but processed using the TSA-Indirect Immunohistochemistry Amplification Kit (NEN, Boston, MA). PAC₁ receptor staining was performed exactly as described previously using an indocarbocyanine (Cy3)-conjugated secondary antibody and was viewed by confocal microscopy (Braas and May, 1999). For culture preparations, cells were fixed in 4% paraformaldehyde, rinsed, permeabilized in 0.075% Triton X-100, blocked in 5% normal goat serum and 0.25% BSA, and incubated in primary antibody diluted in 3% normal goat serum and 0.25% BSA. After rinsing, the cells were incubated in species-specific Cy3- or FITC-conjugated secondary antibody, rinsed, and mounted in antifade reagent.

In situ hybridization histochemistry. *In situ* hybridization histochemistry on Bouin's fixed tissue sections (18 μ m) was performed according to protocols in the *Boehringer Mannheim Nonradioactive In situ Hybridization Application Manual*. A plasmid containing the PAM 3'-untranslated region (rPAM-1 nucleotides 3231–3886) was used to generate antisense probe after digestion with *Sma*I and transcription with T7 polymerase (Boehringer Mannheim, Indianapolis, IN) and sense probe after digestion with *Eco*RI and transcription with T3 polymerase (Boehringer Mannheim). The PACAP antisense probe was generated by *Xba*I linearization and T3 transcription; the sense probe was generated by *Hind*III linearization and T7 transcription (Braas and May, 1999).

Protein extracts and Western blotting. Tissues from decapitated animals were harvested and prepared as described previously (Ciccotosto et al., 1999). Supernatants were assayed for protein concentration using the bicinchoninic acid protein reagent kit (Pierce, Rockford, IL).

Northern blot analysis and reverse transcription-PCR. For Northern analyses, total RNA (10 μ g) isolated using RNA Stat-60 (RNA Stat, Friendswood, TX) was fractionated by gel electrophoresis (1.0% agarose) and transferred onto Nytran membranes (Schleicher & Schuell, Keene, NH) by capillary action. Blots were UV-cross-linked, prehybridized, and hybridized overnight at 65°C with a radiolabeled random-primed cDNA (1×10^{-6} cpm/ml) for PAM (1.3 kb rat PAM fragment; nucleotides 356–1682) (Stoffers et al., 1991) or S26 (Vincent et al., 1993) in SDS-PIPES buffer [1.5% (w/v) PIPES, pH 6.8, 5% (w/v) SDS, 50 mM NaH₂PO₄, 1 mM EDTA, and 100 mM NaCl] containing 150 μ g/ml denatured herring sperm. Blots were washed at high stringency and apposed to autoradiographic film for 24–48 hr. Olfactory epithelial RNA (2 μ g) was also reverse transcribed using oligo-dT primers; the cDNA templates were amplified for PAC₁ receptor expression as described previously (Braas and May, 1999).

PAM enzyme activity assays. Aliquots of NaTES-mannitol-Triton X-100 extracts were assayed for PHM and PAL enzymatic activity as described previously (Husten et al., 1993). All samples were serially diluted (twofold) for assay to ensure linearity; all assays were performed in duplicate.

Primary olfactory neuron cultures. Cultures were prepared as described previously (Ronnett et al., 1991) with modifications. For each experiment, tissues from 10–20 litters of pups were dissociated enzymatically, and the cells were plated at a density of 1×10^{-6} cells/cm² onto tissue culture slides (Nunc, Naperville, IL) coated with laminin (25 μ g/ml; Collaborative Research, Bedford, MA) in modified Eagle's medium containing D-valine (MDV; Life Technologies, Gaithersburg, MD). The cells were cultured in MDV containing 15% dialyzed FBS, gentamycin, kanamycin, and NGF (25 ng/ml). Treatments were performed in MDV media adjusted to contain 0.5% dialyzed FBS and 2.5 ng/ml NGF. Similar medium was used for peptide additions with bromodeoxyuridine (BrdU; 20 μ M) labeling. Cell counts were performed using random high-power fields. All experiments were performed a minimum of three times, and results were tabulated by an independent observer. Statistics were performed using an unpaired Student's *t* test comparing samples of unequal variance. Data represent the mean $\pm$ SEM.

3'-Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling. Cultures were treated with peptide for 48 hr, rinsed, and

fixed in methanol/acetone (1:1). Cells were then treated with 3% H₂O₂ and incubated in TdT buffer (33 mM Tris-HCl, pH 7.2, 140 mM Na-cacodylate, and 1 mM CoCl₂) containing 0.12 U/ μ l terminal transferase (Sigma, St. Louis, MO) and a 1:200 dilution of biotin-16-2'-deoxyuridine-5'-triphosphate (Boehringer Mannheim). The cultures were rinsed, blocked with 3% normal horse serum, and processed using the Vectastain Elite ABC Kit (Vector Laboratories).

RESULTS

PAM is expressed and functional in developing and adult olfactory epithelium

PAM is required for the biosynthesis of all bioactive amidated neuropeptides (Eipper et al., 1992). Although PAM is present at high levels throughout the developing and adult nervous system (Schaefer et al., 1992; Zhang et al., 1997), its expression has not been characterized in the olfactory epithelium. To initiate our studies on the potential roles of amidated neuropeptides during olfactory epithelial development and regeneration, we examined the distribution of PAM-1, the most common splice variant, in the adult and embryonic (E12–E19) rat olfactory epithelium.

In the rat olfactory epithelium, neurogenesis begins at E12 and continues throughout the life span of the organism; however, only in the adult animal is the epithelium organized into more distinct proliferative and neuronal layers (Farbman, 1992). PAM immunoreactivity was first evident at E12 in scattered cells of the olfactory epithelium (data not shown) and was quite prevalent by E13 (Fig. 2*A*). At this stage, PAM protein was expressed widely in many different cell types of the developing epithelium, as well as in the underlying lamina propria. PAM expression remained elevated through E19 (data not shown). Thus, PAM is well positioned to generate bioactive amidated neuropeptides in the olfactory epithelium during embryogenesis.

PAM expression was persistent and widespread throughout all regions of the adult olfactory neuroepithelium, including regions containing immature and mature neurons and sustentacular cells (Fig. 2*B,C*). PAM immunostaining was localized typically to one side of the nucleus and appropriately resembles the localization pattern observed with an antibody against a TGN protein (data not shown). PAM staining in the proliferating basal cell layer appeared less intense. The specificity of the staining reaction was confirmed by incubating sections with either blocked antibody (E13, Fig. 2*E*) or immunodepleted antibody (Adult, Fig. 2*E*).

These immunocytochemical localization data correlated well with complementary studies assessing other parameters of PAM expression in the olfactory epithelium. By *in situ* hybridization, the levels of PAM mRNA appeared nearly uniform in most cells of the adult olfactory epithelium, including the basal-most precursor cells (Fig. 2*D,F*, antisense panel). The sense control demonstrated no signal (Fig. 2*F*, sense panel). This contrasted with the data demonstrating a relative attenuation of PAM immunoreactivity in olfactory basal cells (Fig. 2*B*). The immunohistochemical results alone suggested either diminished PAM mRNA levels and expression or increased PAM protein secretion or turnover, in basally positioned cells. However, in view of the *in situ* hybridization data demonstrating prominent expression of PAM mRNA in these cells, altered protein storage or turnover is the more likely explanation. Thus, PAM is synthesized in the three principal cell types of the olfactory epithelium to generate bioactive peptides with autocrine and/or paracrine functions.

Additional studies were conducted to gain a better understanding of the potential roles of PAM, and therefore amidated neuropeptides, in the olfactory epithelium. Northern blot analysis demonstrated PAM mRNA expression in the olfactory epithe-

Figure 2. PAM expression in the developing and adult olfactory epithelium. *A*, Immunohistochemistry on E13 paraffin section using an affinity-purified antibody against Exon A of PAM. The separation between the developing olfactory epithelium and lamina propria is indicated by a dotted line. *B*, Bouin's fixed adult (*Ad*) rat olfactory epithelium immunostained using an affinity-purified antibody against PHM. Neurons (*n*) and sustentacular cells (*s*) are indicated. *C*, High magnification view of the

lumen at all developmental stages (Fig. 3*A*); the most prevalent transcript was 4.2 kb in size corresponding to the mRNA encoding PAM-1 (Stoffers et al., 1991). The appearance of specific PAM forms after post-translational processing of full-length PAM protein, for example, can be indicative of the cellular sites of biological activity (Ouafik et al., 1992). If PAM does not undergo endoproteolytic cleavage to release the PHM and PAL domains from the transmembrane anchor, it may function in a cell-autonomous manner. Conversely, the endoproteolytic production of soluble enzyme allows PAM secretion and action on extracellular substrates produced by other cells.

By Western blot analysis, PAM protein was produced at all stages of olfactory epithelial development, from E13 through adulthood (Fig. 3*B,C*). At each time point, the PAM protein was extensively endoproteolytically processed, as evidenced by the lack of full-length 120 kDa PAM-1 protein and the preponderance of active 44 and 46 kDa PHM forms (Husten et al., 1993). This was demonstrated directly in PAM enzymatic activity assays. PHM activity levels in detergent extracts of olfactory epithelium (Fig. 3*D*) were comparable with those in AtT-20 corticotrope and COS-7 tumor cells. Assays for PAL activity yielded a similar pattern of expression (data not shown), indicating that the two essential steps for α -amidation are fully functional, allowing bioactive peptide production.

Amidated PACAP38 is expressed in embryonic and adult olfactory epithelium

The prevalence and persistent expression of active PAM in the olfactory epithelium suggested prominent roles for amidated peptides in olfactory epithelial development and function (Mains et al., 1991; Takahashi et al., 1997; Cicciotosto et al., 1999). Amidated peptide expression in the olfactory epithelium has not been systematically examined. We first investigated the expression of PACAP peptides because of their established roles as neurotrophic factors in a number of neuronal systems (Arimura et al., 1994; Dicicco-Bloom, 1996). Because PACAP38 is the more prevalent of the two amidated PACAP forms in most nervous tissues, we examined PACAP expression in the adult olfactory epithelium using a C-terminal antibody specific for amidated (bioactive) PACAP38 (Fig. 4*A*). Similar to PAM, amidated PACAP38 immunoreactivity was widespread in the adult olfactory epithelium (Fig. 4*B*). PACAP38 immunoreactivity was present in all neuronal layers, although immunoreactivity was highest in basal cells (Fig. 4*C*). The sustentacular cell layer, which expressed PAM, demonstrated no detectable PACAP38 immunoreactivity, suggesting that expression of other amidated peptides may be more prevalent in this cell type. Staining in adjacent tissue sections was eliminated using immunodepleted antibody (Fig. 4*D*).

The wide distribution of PACAP38 immunoreactivity in the olfactory epithelial cell layers correlated with the PACAP mRNA pattern detected by *in situ* hybridization histochemistry (Fig. 4*E*).

adult olfactory epithelium demonstrating PAM localization to the perinuclear area within the cell bodies of neuronal (*n*) and sustentacular (*s*) cells. *D*, *In situ* hybridization of adult olfactory epithelium using an antisense probe directed against the 3'-untranslated region of PAM. Signal is apparent in all regions of the olfactory epithelium, including the basal layer of cells. *E*, Immunohistochemistry specificity control using immunodepleted antibodies against Exon A (E13) or PHM (*Ad*). *F*, High magnification of adult olfactory epithelium for antisense (*AS*) and sense (*S*) control *in situ* hybridization. Scale bars: *A*, *B*, *D*, 25 μ m; *C*, 12.5 μ m. Box lengths: *E*, *F*, 50 μ m.

Figure 3. Expression of PAM forms and activity in the olfactory epithelium. *A*, Northern blot analysis. Total RNA from olfactory epithelium (10 μg) was probed with cDNA against a PAM 1.3 kb fragment. Standardization was performed using S26. Scion Image Analysis yielded density ratios of PAM/S26 for E13 (0.16), postnatal day 2 (P2; 0.27), 2.5 weeks (0.34), Adult (0.41), and 14 months (0.56). Analysis was repeated three times with similar results. *B*, Western blot of PAM protein. Western blots were performed using 20 μg of protein extract from the olfactory epithelium (OE) of rats of the indicated ages. The blot was probed with affinity-purified antibody against Exon A. *C*, Left, Schematic identifying the antigen used to generate the Exon A antibody (horizontal bar), as well as full-length PAM-1 (PAM-1; 120 kDa), membrane PAL (PAL_m; 70 kDa), and soluble PHM (PHM_s; 44–46 kDa) and PAL (PAL_s; 50 kDa). Arrows indicate major proteolytic cleavage sites. Right, Adult olfactory epithelium probed with the Exon A antibody (ExonA) and immunodepleted antibody (Ctrl). *D*, PHM enzymatic activity. Enzyme activity was calculated as picomoles of substrate produced per microgram of protein per hour. Error bars indicate SE. Four independent sets of extracts were assayed. *mo*, Months; *wk*, weeks.

As with PAM mRNA expression, PACAP mRNA demonstrated a more uniform localization pattern in the neuronal cell populations than did PACAP38 peptide immunoreactivity. This is most apparent in the basal cell population. The antibody used in the immunocytochemical studies was amide specific and visualized

only mature product peptide. Differences between PACAP peptide and mRNA distribution patterns in mature neurons and basal cells reflect differences in peptide synthesis, storage, and secretion. Sustentacular cells contained neither PACAP38 immunoreactivity nor PACAP mRNA. The control sense riboprobe failed to yield any reaction product in adjacent sections (Fig. 4*F*). Knowing that PAM was expressed in the embryonic olfactory epithelium (Fig. 2*A*), we evaluated embryonic expression of PACAP38 (Fig. 4*G*). PACAP38 was present throughout the developing olfactory epithelium and the underlying lamina propria. On the basis of its widespread and early expression in embryonic olfactory epithelium, PACAP38 is positioned to play a role in olfactory receptor neuronal development.

The PACAP-selective PAC₁ receptor is expressed in olfactory epithelium

A prerequisite for PACAP to function in the olfactory epithelium is PAC₁ receptor expression in a target cell population. Immunocytochemical localization of the PAC₁ receptor demonstrated staining in the innermost layers of the olfactory epithelium that represent populations of basal cells and developing neurons (Fig. 4*H*). Punctate regions of PAC₁ receptor immunoreactivity were also apparent in more apical regions, although these sites were less well defined. Adjacent sections stained using immunodepleted antibody yielded no signal (Fig. 4*I*). We also evaluated PAC₁ receptor expression using reverse transcription-PCR (data not shown). Alternative splicing of the PAC₁ receptor in regions encoding the N-terminal extracellular domain (short vs very short variants) and the third cytoplasmic loop (HIP and HOP cassettes) determines PACAP-binding selectivity and intracellular receptor coupling. Using primers flanking these splice sites, we identified both short and very short variants of the PAC₁ receptor containing either one or no cassette (null form) insert into the third cytoplasmic domain in both neonatal and adult olfactory epithelium. Although the characterization and functional implications of these receptor isoforms remain to be fully elucidated, the expression of both PACAP and PAC₁ receptors in the olfactory epithelium strongly support a physiological role for PACAP in olfactory receptor development and function.

PACAP stimulates olfactory neurogenesis and neuron survival *in vitro*

Because of the distribution and neurotrophic properties of the PACAP signaling pathway, we explored a role for PACAP in olfactory neuron precursor proliferation. For these studies, primary dissociated olfactory neuron cultures prepared from postnatal day 2 rat pups were used. Although these cultures contain predominantly olfactory receptor neurons, basal cells and glial elements from the lamina propria are also present (Barber and Lindsay, 1982; Ronnett et al., 1991). By the use of PAM or amidated PACAP38 antisera in colocalization studies with NST, both PAM and PACAP38 immunoreactivities were localized to olfactory neurons (Fig. 5*A*), recapitulating their *in vivo* distributions. Over 90% of the NST-positive cells in the primary cultures demonstrated PAM and PACAP38 immunoreactivity. A very small fraction of GFAP-positive cells also exhibited PAM and PACAP staining (Fig. 5*B*). PAM immunoreactivity was concentrated in the cell soma, whereas amidated PACAP38 was located preferentially in vesicular structures in fibers and the cell soma. This pattern is analogous to that in previous studies using transfected cells, with PAM localized predominantly in the TGN and amidated neuropeptides in dispersed vesicular structures (Milgram et al., 1992).

Figure 4. PACAP expression in the adult olfactory epithelium. *A*, PACAP-processing schematic. Rat ProPACAP is cleaved to yield the glycine-extended peptide PACAP(1–38)-Gly. PAM converts this intermediate into the final amidated product PACAP38, which is specifically recognized by the antibody. PACAP27 can be produced in certain cells by endoproteolytic cleavage at an additional amidation site within ProPACAP. *B*, PACAP expression. Adult rat olfactory epithelium (Bouin's fixed) was probed with antibody specific for amidated PACAP38. High levels of expression are apparent in the basal cells (arrow), as well as the neuronal region of the olfactory epithelium. The dotted line represents the separation between olfactory epithelium and lamina propria. *C*, PACAP in basal cells. High-magnification view of PACAP38-stained basal cells. *D*, Immunodepleted PACAP38 antiserum yielding no staining of the olfactory epithelium. *E*, *In situ* hybridization using an antisense probe specific for proPACAP. *F*, *In situ* specificity control. The sense probe yielded no staining. *G*, PACAP38 immunostaining of E13 rat

To determine the potential effects of PACAP38 on olfactory neurogenesis, the primary olfactory cultures were treated with different concentrations of PACAP38 (Arimura et al., 1994), and NST- or GFAP-positive cells were quantified. PACAP38 promoted a dose-dependent increase in the number of neurons (maximally 270% over control) without affecting the total number of glial cells (Fig. 5C). In contrast, similar concentrations of VIP failed to elicit these responses, suggesting that the responses were mediated preferentially after PAC₁ receptor activation.

To establish that these changes in the olfactory neuronal population reflected in part neuronal proliferation *in vitro*, the PACAP38-treated cultures were incubated in medium containing BrdU for increasing periods of time to label dividing neuronal precursors (Fig. 5D). As early as 2 hr after PACAP38 addition, BrdU labeling of NST-positive cells was increased over threefold compared with that in control untreated cultures (Memberg and Hall, 1995; Luskin et al., 1997); a higher PACAP38-induced neuronal labeling index was apparent throughout the entire experimental time course. Approximately sixteen percent of the neuronal population in the cultures normally enter a proliferative phase during the time period examined; the early increase in BrdU labeling observed after PACAP38 addition (2 hr) suggested that PACAP38 promoted precursor cell transition into S phase of the cell cycle (Nowakowski et al., 1989).

To assess whether the PACAP38-induced changes in neuronal population were also caused by survival processes, PACAP38-treated cultures were analyzed by 3'-terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL). We found that PACAP38 reduced the number of apoptotic neurons by 42% (Fig. 5E). The ability of PACAP38 to stimulate neuronal proliferation and survival correlated well with the localization of the PAC₁ receptor to populations of basal cells and maturing neurons *in vivo* and *in vitro*.

Endogenous olfactory epithelial PACAP production regulates neuronal regeneration

Our localization studies indicate that PACAP38 and its receptor are positioned to act in an autocrine or paracrine manner to affect olfactory neurogenesis. Therefore, several experiments were conducted to examine the roles of endogenous peptide production in modulating the neuronal population. Treatment of olfactory cultures with the PACAP-selective antagonist PACAP(6–38) (Robberecht et al., 1992) diminished the number of NST-positive cells by 20% ($p < 0.05$) without altering the glial cell population (Fig. 6A). Similarly, incubation of cells with a neutralizing antibody specific for amidated PACAP38 induced a 35% loss of NST-positive cells, which was reversed by the addition of 10 nM PACAP38 to the cultures (Fig. 6B; $p < 0.001$). Control nonimmune IgG had no effect on neuronal number. These results suggested that endogenous PACAP peptides were one of several neuroregulators contributing to olfactory neurogenesis.

Inactivation of PAM results in specific olfactory neuronal loss *in vitro*

To establish a more comprehensive measure of the role of neuropeptides in the processes of neuronal development and regen-

olfactory epithelium. *H*, Immunofluorescent staining of adult olfactory epithelium with PAC₁ receptor antibody. Asterisks mark immunostaining in clusters of basally positioned cells; the dotted line indicates the separation between olfactory epithelium and lamina propria. *I*, Immunodepleted PACAP38 antiserum yielding no staining of adult olfactory epithelium. Scale bars: *G*, 50 μ m; *B*, *E*, *F*, *H*, *I*, 25 μ m; *C*, *D*, 12.5 μ m.

PACAP38-treated wells (PAC). The total number of BrdU-positive cells is listed per 50 neurons counted. *E*, PACAP38 stimulates neuronal survival. Incubation of cells with PACAP38 for 2 d reduced the number of cells undergoing apoptosis by 42% as assayed by TUNEL labeling (** $p < 0.001$). *nn*, Number of neurons; *pos*, positive. Scale bars: *panel A*, 12.5 μ m; *panel B*, 25 μ m.

eration, we targeted PAM for inactivation to abrogate all amidated peptide production in these cultures. This was accomplished by adding the relatively selective copper chelator diethyldithiocarbamate (DDC) to the primary cultures. DDC effectively inhibits neuropeptide amidation within the concentration range tested without causing nonspecific chemical injury to the olfactory epithelium (Mains et al., 1986; Deamer and Genter, 1995). DDC yielded a dose-dependent decrease in NST-positive cells, with no loss of glial cells (Fig. 7*A*, *top*). The DDC treatment decreased neuropeptide amidation in a similar manner, as evidenced by a loss of amidated PACAP38 immunoreactivity in the glial cells (Fig. 7*A*, *bottom*). Comparable results were obtained when cells were incubated with 20–100 μ M bathocuproine disulfonic acid (BCS), a Cu(I)-selective chelator. The highest dose of BCS induced a $62 \pm 3\%$ loss of NST-positive olfactory neurons, with no decrease in glial cell numbers (data not shown).

Although glial cells were not affected at the drug concentrations used, there remained the possibility that the observed neuronal cell loss was a consequence of decreased copper availability to cytosolic or mitochondrial copper-dependent enzymes and not caused by diminished bioactive amidated peptide synthesis. To address these concerns, synthetic PACAP38 peptide and DDC were added to the cultures concurrently. The dose of DDC selected resulted in substantial neuronal loss (Fig. 7*B*). Addition of PACAP38 to the DDC-treated cultures significantly increased the number of NST-positive neurons to 47% of the value found in untreated control cultures ($p < 0.001$). These studies illustrate the importance of PACAP peptides in olfactory neuron development. The inability to achieve complete recovery of NST-positive cells with PACAP38 may reflect DDC inactivation of other endogenous amidated neurotrophic peptides essential to the maintenance and genesis of olfactory neurons.

Figure 5. Effect of PACAP38 on olfactory neurogenesis. *Panels A, B*, Dual immunofluorescence localization is shown. *Panel A*, Primary olfactory cultures demonstrate colocalization of PAM and amidated PACAP38 to NST-positive cells (affinity-purified PHM antibody). *Top*, A cell double labeled for PAM (left) and NST (right) is shown. *Bottom*, A cell double labeled for PACAP (left) and NST (right) is shown. *Panel B*, PAM (top left) and amidated PACAP38 (bottom left) were also colocalized to GFAP-positive cells (right). *Panel C*, PACAP38 increases neuronal number. Primary olfactory cultures were incubated for 2 d with 10^{-10} to 10^{-8} M PACAP38. The number of neurons (NST-positive cells) and glia (GFAP-positive cells) was counted. The experiment was replicated four times, and results were verified by an independent observer. VIP (10^{-8} M) had no effect on the number of neurons or glia. *Panel D*, PACAP38 increases BrdU labeling of olfactory neurons. Primary olfactory cultures were incubated with BrdU and 10^{-8} M PACAP38 for 2, 6, 18, and 24 hr. NST-positive cells were counted for BrdU-positive nuclei in control untreated wells (*C*) or

Olfactory neuron number is diminished in the Mottled (Brindled) mouse model of Menkes disease

Because decreased neuropeptide amidation *in vitro* caused a decrease in olfactory neuron number, we wanted to investigate whether a decrease in PAM activity *in vivo* would result in a similar decrease in neuronal number in the olfactory epithelium. Because PAM knock-out animals are not available, an alternate *in vivo* model was sought. In Mo^{br} mice, mutant Menkes (MNK) protein cannot transport copper into the secretory pathway, and secreted enzymes like tyrosinase cannot function because of lack of copper (Suzuki and Gitlin, 1999; Petris et al., 2000). Affected males display severe neurological abnormalities, are hypopigmented, and generally die within 15 d of birth (Kodama, 1993).

We first obtained olfactory epithelium from Mo^{br} mice and control male littermates to determine whether decreased function of the MNK protein decreased the ability of PAM to amidate neuropeptides. We attempted to quantify this change with a radioimmunoassay for amidated (therefore bioactive) PACAP38. PACAP38 levels were 1.83 ± 0.21 fmol/ μ g of protein in extracts prepared from the olfactory tissue of control mice, and 1.49 ± 0.11 fmol/ μ g of protein in extracts prepared from Mo^{br} mice, although variability among animals was noted. The 20% decrease in amidated PACAP38 immunoreactivity did not reach statistical significance ($p = 0.22$). The presence of PACAP38 in the lamina propria, where its production may not be dependent on Menkes protein, and variability in our dissection of olfactory tissue from young mice may contribute to our inability to quantify the clear decrement in PACAP38 levels observed immunohistochemically. To address this issue, direct examination of the olfactory epithelium of control and Mo^{br} mice was performed (Fig. 8). Immunocytochemical staining of amidated, active PACAP38 was greatly

Figure 6. Role of endogenous PACAP38 in olfactory neuron survival. *A*, PACAP38 antagonist decreases olfactory neuron number. Incubation of primary cultures with the PACAP38 antagonist PACAP(6–38) [PAC(6–38)] for 2 d caused a 15–20% loss of neurons, without altering glial cell number (* $p < 0.05$). *B*, Neutralizing antibody against PACAP38 induces neuronal loss. Incubation of primary cultures with anti-PACAP38 antibody (α PAC) at 1:250 dilution for 1 d caused a 35% decrease in NST-positive cells relative to control (** $p < 0.001$). This loss can be prevented by coaddition of PACAP38 (α PAC + PAC). Nonimmune control rabbit serum (IgG) had no effect on neuronal number.

diminished in the olfactory epithelium of Mo^{br} mice, consistent with decreased copper availability for PAM function in the Mo^{br} mice (Fig. 8*A,B*).

Because peptide amidation was impaired in the Mo^{br} mouse olfactory epithelium, we used several neuronal-specific markers to compare the olfactory neuronal populations in Mo^{br} and control mice. Immunocytochemical staining for NST was diminished in the olfactory epithelium and underlying axon bundles in the Mo^{br} mice (Fig. 8*C,D*). NST identifies immature neurons and mitotically active cells, as well as a subpopulation of mature olfactory neurons (Memberg and Hall, 1995; Luskin et al., 1997; Roskams et al., 1998). The Mo^{br} mice had fewer neurons overall compared with the wild-type littermate controls, resulting in decreased olfactory epithelium thickness. In addition, there were areas in the epithelium devoid of NST-positive cells. On the basis of cresyl violet cell counts, there were $40 \pm 5\%$ fewer neurons in the Mo^{br} mouse olfactory epithelium than in littermate controls. Western analyses demonstrated a 36% decrease in NST levels in Mo^{br} olfactory epithelium extracts relative to controls (Fig. 8*C,D*, inset). Similarly, immunostaining for OE-1, a transcription factor that identifies all cells of the olfactory neuron lineage (Wang et al., 1997), revealed a $32 \pm 2\%$ decrease in neuronal cell number in Mo^{br} mice compared with littermate controls (Fig. 8*E,F*). In contrast, immunostaining for OMP, which identifies mature olfactory neurons (Keller and Margolis, 1975), indicated that OMP levels remained relatively constant in the Mo^{br} mice. Consistent with this, OMP protein levels were unchanged in Mo^{br} mice, as determined by densitometric analysis of Western blots (Fig.

Figure 7. Effect of PACAP38 on loss of olfactory neurons because of copper chelation. *A*, DDC induces dose-dependent neuron loss. *Top*, Primary cultures were treated for 2 d with the indicated concentration of DDC. Neurons (NST-positive cells) and glial cells (GFAP-positive cells) were counted. *Bottom*, Immunofluorescence using the amidated PACAP38 antibody identified loss of PACAP amidation in surviving glial cells with DDC treatment. *B*, PACAP38 rescues a subpopulation of DDC-treated neurons. Primary cultures treated with 0.2 μ M DDC had significantly decreased numbers of neurons. Coaddition of 10^{-8} M PACAP38 (PAC) with DDC rescued $\sim 35\%$ of the neurons (unpaired Student's *t* test of PACAP treatment relative to treatment with 0.2 μ M DDC alone, ** $p < 0.001$).

8*G,H*, inset). However, the OMP-positive cells were disorganized in Mo^{br} mice compared with their littermate controls (Fig. 8*G,H*). Thus, Mo^{br} mice exhibited a moderately reduced immature neuronal population, with the surviving immature neurons retaining the capacity to mature.

DISCUSSION

This study provides the first evidence of the involvement of neuropeptides in olfactory neurogenesis and neuronal survival and specifically identifies PACAP38 as an amidated neuropeptide that regulates olfactory neuronal precursor proliferation and survival *in vitro*. PAM is required for the production of bioactive amidated neuropeptides. The localization of PAM to basal cells, olfactory neurons, and sustentacular cells demonstrates that amidated neuropeptides, which may function in an autocrine or paracrine manner, can be generated by a variety of cells in the olfactory epithelium. PACAP38 is similarly expressed in neurons

Figure 8. Immunohistochemical analysis of the olfactory epithelium in Mottled (Brindled) mice. *Panels A, B*, PACAP38 immunostaining. Cryostat sections from Mottled and wild-type littermates were immunostained

and basal cells of the olfactory epithelium and can stimulate the proliferation and survival of olfactory precursor cells and neurons *in vitro*. The loss of neurons in primary olfactory cultures treated with PACAP38 antagonist or neutralizing antibody confirms a role for PACAP38 in neurogenesis. Decreased numbers of immature neurons in the *Mo^{br}* mice also support a role for amidated neuropeptides *in vivo*. The loss of neurons in copper-depleted cultures suggests that amidated neuropeptides, such as PACAP38, may be responsible in part for the pathophysiology underlying Menkes disease.

Specific amidated neuropeptides have been suggested to function as neurotrophic factors (Leslie, 1993). Parathyroid hormone-related peptide serves as both an autocrine survival factor and a growth signal to cultured cerebellar granule cells (Wysolmerski and Stewart, 1998). VIP and PACAP regulate the mitosis, survival, and differentiation of cultured sympathetic ganglion neurons and embryonic sensory neurons *in vivo* (Arimura et al., 1994; Diccio-Bloom, 1996). Neuropeptide Y (NPY) and galanin mRNAs are upregulated after peripheral sensory nerve axotomy, suggesting a role in neuronal survival (Hokfelt et al., 1994). In contrast, few studies examined neuropeptides in the olfactory epithelium. NPY was found in ensheathing cells of olfactory axon bundles (Ubink et al., 1994), and calcitonin gene-related peptide was demonstrated in neurons in developing mouse olfactory epithelium (Baker, 1990; Denis-Donini et al., 1993).

Several challenges exist in studying amidated neuropeptide function and may explain the delay in identifying neuropeptides in olfactory neuronal development. Neuropeptides occur in families, and one member can substitute functionally for another (Strand, 1999). Neuropeptide levels *in vivo* are low, making detection difficult. Because PAM is the only enzyme that catalyzes peptide amidation, it serves as a useful marker to identify sites of amidated neuropeptide synthesis. Because high levels of PAM transcripts were found in the nervous system during early development, including the rat spinal cord (E10), hippocampus, thalamus, cerebellum, and the ventricular zone of the hypothalamus (Zhang et al., 1997), amidated neuropeptides may have many roles. In agreement with this prediction, deletion of *Drosophila* PHM resulted in an embryonic-lethal phenotype (Kolhekar et al., 1997).

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for PACAP38. Staining was reduced throughout the epithelium of the Mottled mice and most prominently decreased in the basally located cells (arrows). *Panels C, D*, NST expression. Color development was extended to permit visualization of low-level NST staining in the *Mo^{br}* mouse. NST immunoreactivity was decreased in neurons throughout the epithelium in *Mo^{br}* animals compared with male littermate controls. *Inset*, Western blots for NST performed using pooled samples from *Mo^{br}* and control animals, 20 μ g/lane, with the band of appropriate molecular weight identified. NST protein levels are decreased in mottled animals. *Panels E, F*, OE-1 immunostaining. OE-1 identifies all cells of the olfactory neuronal lineage, including basal cells, immature neurons, and mature neurons. *Mo^{br}* mice demonstrate 32% fewer OE-1-positive cells than do littermate controls. *Panels G, H*, OMP immunostaining. Immunoreactivity for OMP, a mature neuronal marker, was decreased in the *Mo^{br}* mice compared with control animals. Decreased staining was also apparent in the underlying axon bundles. *Inset*, Western blot analysis of OMP demonstrating equivalent levels of expression in *Mo^{br}* and control mice and confirmed by densitometry, with the band of appropriate molecular weight identified. C, Control mice; M, *Mo^{br}* mice.

Localization of PAM and PACAP indicates a role for amidated neuropeptides in olfactory epithelium function

We examined PAM and PACAP38 in developing and adult olfactory tissues. Between E12–E18 (Farbman, 1992) and adulthood, we found high expression of PAM protein and enzymatic activity throughout the olfactory epithelium. In the adult, PAM was expressed in many olfactory receptor neurons and basal cells, but in only a subpopulation of the sustentacular cells. The determination of PAM enzymatic activity and distribution indicates that a high level of amidated neuropeptide production can be supported in the olfactory epithelium, with multiple neuropeptides being formed by different cell types or at different developmental stages.

Amidated, bioactive PACAP38 expression was widespread in both the developing and adult olfactory epithelium, demonstrating that PAM was functional. In the adult rat, PACAP38 was expressed in the majority of immature and mature neurons, with highest expression in the proliferating basal cells. Interestingly, PACAP38 expression was not apparent in the sustentacular cell layer. A subpopulation of sustentacular cells expresses PAM, suggesting the presence of another amidated neuropeptide in these cells. In other systems PACAP38 functions in an autocrine or paracrine manner to regulate cell division or survival (DiCicco-Bloom et al., 1998), suggesting that PACAP38 may confer similar neurogenic or survival effects on the olfactory neuron population. Expression of PAC₁ receptor in basal cells and immature neurons supports a role for PACAP38 during this phase of olfactory neurogenesis. In addition, PACAP38 expressed in the mature olfactory receptor neurons could be exported to the nerve terminals adjacent to the olfactory bulb, where the PAC₁ receptor is expressed (Hashimoto et al., 1996). PACAP38 may function there as either a neurotransmitter or a neurotrophic factor. The broader distribution of PAM compared with PACAP38 suggests that PACAP38 is not the only amidated neuropeptide produced within the olfactory epithelium.

PACAP38 stimulates olfactory neurogenesis and survival

Using primary olfactory cultures, we demonstrated that PACAP38 increased neurogenesis *in vitro*. The rapid increase in BrdU labeling suggests that PACAP38 functions, in part, to promote a rapid switch from cell cycle quiescence to DNA synthesis and division (Nowakowski et al., 1989). To our knowledge, this ability of PACAP38 to induce a switch into S phase is the first demonstration of such a function of PACAP38 in neuronal proliferation. PACAP38 also promoted olfactory neuron survival, as evidenced by a dramatic decrease in apoptotic cells assayed by TUNEL staining. PACAP38, which is expressed both in the embryonic and adult animal, may promote neurogenesis and neuronal survival during embryonic olfactory development as well as during normal regeneration of the adult olfactory epithelium.

Several strategies confirmed the importance of PACAP38 for olfactory neurogenesis or survival. Addition of a PACAP38 antagonist resulted in neuronal loss *in vitro*. Neutralizing antibody to PACAP38 similarly reduced neuronal numbers, and this effect was reversed by co-incubation with PACAP38. Decreased levels of this peptide would be predicted to result in decreased olfactory neuronal numbers, as seen in the *Mo^{br}* mice.

To determine whether decreased PAM activity was causally related to the neuronal loss observed in the *Mo^{br}* mice, we used

primary olfactory cultures. Loss of PAM enzymatic activity, achieved via DDC and BCS treatments, correlated with a dose-dependent decrease in neuronal survival, similar to the *in vivo* effects demonstrated in the *Mo^{br}* mice. These effects appear to be specific for amidated neuropeptide function, because PACAP38 could partially rescue challenged neurons, and suggest that PACAP38 is one of perhaps several amidated neuropeptides needed for olfactory neuronal survival.

Mo^{br} mice demonstrate immature neuron loss in the olfactory epithelium

Because PAM knock-out animals do not exist and there are no commercially available PAM-specific inhibitors, we used the copper dependence of PAM to alter its enzymatic function in the olfactory epithelium. *Mo^{br}* mice serve as an animal model for Menkes disease and demonstrate similar neurological abnormalities (Fraser et al., 1953; Hunt, 1974; Prins and Van den Hamer, 1979; Lyon and Searle, 1990). Menkes disease is an X-linked recessive disorder that causes severe mental retardation, neurodegeneration, autonomic dysfunction, and death in affected males at 3–4 years of age (Menkes et al., 1962; Harris and Gitlin, 1996; Mercer, 1998). The molecular defect underlying Menkes disease is loss of the Menkes protein (MNK, ATP7A), a P-type ATPase that transports copper from the cytoplasm into the secretory pathway (indicated in Fig. 1*B*). Loss of functional MNK protein results in lowered serum and tissue levels of copper secondary to decreased copper transport into the bloodstream by intestinal mucosal cells (Danks et al., 1972; Chelly et al., 1993; Mercer et al., 1993; Vulpe et al., 1993; Prohaska and Bailey, 1995; Prohaska et al., 1997; Harrison and Dameron, 1999).

The causes of the neuronal abnormalities of Menkes disease are not understood but may involve the altered function of copper-dependent enzymes such as PAM, dopamine- β -monooxygenase (catecholamine biosynthesis), and tyrosinase (melanin formation), which function within the secretory pathway of neurons. We used the *Mo^{br}* mice as a partial loss-of-function PAM knock-out to examine the effects of decreased copper availability in the secretory pathway of olfactory epithelial cells.

In the *Mo^{br}* animals, numbers of immature olfactory neurons were significantly decreased; however, the neurons that persisted through the immature stage retained the capacity to differentiate. The normal level of OMP within the olfactory epithelium may be secondary to the survival effects of PACAP38, as well as caused by other chronic compensatory changes. Thus, causes for the neuropathology underlying Menkes disease may involve a developmental decrease in the ability to generate new neurons and/or the inability of a subpopulation of immature neurons to survive into maturity. The ability of neurons to achieve maturity in the *Mo^{br}* mice indicates that other copper-dependent enzymes such as cytochrome-*c* oxidase and superoxide dismutase must retain at least a moderate level of enzymatic activity and thus would not account for all of the changes seen in the *Mo^{br}* mice.

PACAP38 functions via both PACAP-specific PAC₁ receptors and VIP- and PACAP-specific VPAC receptors (Rawlings, 1994). PAC₁ receptors are present as at least 12 different isoforms coupling either to the cAMP pathway alone or jointly to the cAMP and IP₃ pathways. Both the IP₃ and cAMP pathways have been associated with cellular growth and survival. This system should serve as a model to examine the isoforms of the PAC₁ receptor present in the olfactory epithelium, their localization, and the second messenger pathways used to promote the proliferative and survival effects demonstrated on olfactory neurons.

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