Anatomy of an Endogenous Antagonist: Relationship between Agouti-Related Protein and Proopiomelanocortin in Brain

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Agouti-related protein (AGRP) is a recently discovered orexigenic neuropeptide that inhibits the binding and action of α-melanocyte-stimulating hormone derived from proopiomelanocortin (POMC) at the melanocortin 3 receptor (MC3R) and melanocortin 4 receptor (MC4R) and has been proposed to function primarily as an endogenous melanocortin antagonist. To better understand the interplay between the AGRP and melanocortin signaling systems, we compared their nerve fiber distributions with each other by immunohistochemistry and their perikarya distribution with MC3R and MC4R by double *in situ* hybridization. Although deriving from distinct cell groups, AGRP and melanocortin terminals project to identical brain areas. Both AGRP and melanocortin neurons selectively express the MC3R, which provides a neuroanatomical basis for a dual-input circuit with biological amplification and feedback inhibition. These studies highlight a broader complexity in POMC-mediated behavior in the brain.

Key words: Agouti-related protein; proopiomelanocortin; ingestive behavior; MC3R; MC4R; arcuate nucleus

Naturally occurring antagonists can act either by binding to and sequestering a ligand or by binding to a receptor to prevent its response to another molecule. Unique advantages for biological regulation are provided by the latter mechanism, of which Agouti protein and Agouti-related protein (AGRP) are prime examples (Ollmann et al., 1997, 1998; Shutter et al., 1997). These proteins inhibit the activity of melanocortins, small peptides such as α-melanocyte-stimulating hormone (α-MSH) or adrenocorticotrophic hormone derived from a large precursor, proopiomelanocortin (POMC), that also gives rise to β-endorphin. AGRP binds directly to melanocortin receptors but has little intrinsic signaling activity, and instead functions primarily by inhibiting α-MSH binding (Ollmann et al., 1997; Shutter et al., 1997). Indeed, the melanocortin receptors were originally identified by their ability to activate adenylate cyclase in response to α-MSH. However, recent studies have suggested that physiological modulation of receptor signaling may be accomplished mainly by alteration in the levels of AGRP rather than α-MSH. Starvation and leptin deficiency cause a predominant rise in levels of AGRP mRNA rather than a decrease in POMC mRNA levels in the hypothalamus (Thornton et al., 1997; Mizuno et al., 1998; Mizuno Mobbs, 1999; Wilson et al., 1999). Artificial increases in AGRP achieved pharmacologically or in transgenic animals cause elevated food intake and obesity (Graham et al., 1997; Ollmann et al., 1997; Grill et al., 1998; Rossi et al., 1998).

Because the action of AGRP has only been tested on melanocortin receptors, the question remains, does AGRP work primarily as a melanocortin antagonist, or might it have other functions? One approach to this question is to examine its anatomy vis-a-vis the anatomy of the melanocortins and to determine whether AGRP only exists where melanocortins are found, or whether it is also expressed at other sites independent of either the ligands or the receptor(s) that it is purported to antagonize. To investigate the potential for presynaptic and/or direct crosstalk between AGRP and POMC systems, we examined the colocalization of AGRP or POMC with the MC3R and MC4R using double *in situ* hybridization.

MATERIALS AND METHODS

Animals. Male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 300–350 gm were used in this study. Rats were housed in groups of two or three per cage with food and water available *ad libitum* in a 12 hr light/dark cycle (lights on at 7 A.M.) under conditions of constant temperature and humidity. Animals were allowed to habituate for 1 week before experiments. Protocols for animal exper-

Received May 21, 1999; revised July 15, 1999; accepted July 21, 1999.

This work was supported by National Institutes of Health Grants P01 MH-42251 (from National Institute of Mental Health; to S.J.W.), DK28506 (to G.S.B.), and R01 DK-54032–01 (to I.G.). We thank Sharon Burke and Robert Pavlic for technical assistance and Drs. Serge Campeau and Manuel Lopez-Figueroa for advice and comments.

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This article is published in *The Journal of Neuroscience*, Rapid Communications Section, which publishes brief, peer-reviewed papers online, not in print. Rapid Communications are posted online approximately one month earlier than they would appear if printed. They are listed in the Table of Contents of the next open issue of JNeurosci. Cite this article as: JNeurosci, 1999, 19:RC26 (1–7). The publication date is the date of posting online at www.jneurosci.org.

http://www.jneurosci.org/cgi/content/full/3484

Table 1. Distribution and relative abundance of AGRP- and POMC-immunoreactive fibers and terminals in the rat CNS

Anatomical sites	Agrp	γ-MSH
Telencephalon		
Anterior olfactory nucleus, posterior part	+/−	+
Olfactory tubercle	+/−	+
Cingulate cortex	−	+/−
Infralimbic cortex	+/−	+/−
Ventral orbital cortex	−	+/−
Endopiriform nucleus	+/−	+/−
Accumbens nucleus		
Shell	+	++
Core	+/−	+/−
Substantia innominata	++	+++
Anterior amygdaloid area	+	+
Anterior cortical amygdaloid nucleus	+/−	+/−
Central nucleus		
Medial division	++	++
Lateral division	+	+
Medial amygdaloid nucleus	+	+
Basomedial amygdaloid nucleus		
Anterior part	+	+
Ventral part	−	+/−
Bed nucleus of stria terminalis		
Ventral division	++++	+++
Medial division	+++	++++
Lateral division	+(+)	+++
Supracapsular division	+	+/−
Lateral septum:		
Ventral part	++	+++
Dorsal division	+/−	+/−
Medial septum ventral	+	++
Nucleus of the diagonal band	+	+
Subfornical organ	+	+
Diencephalon		
Medial habenular nucleus	−	+
Paraventricular thalamic nucleus	++(+)	+++
Paratenial thalamic nucleus	+/−	++
Laterodorsal thalamic nucleus, ventrolateral part	−	+/−
Stria terminalis	+	+/−
Nucleus of stria medullaris	+/−	+
Zona incerta	+	+(+)
Strial part of the preoptic area	++	+++
Striohypothalamic nucleus	++(+)	++(+)
Organum vasculosum of the lamina terminalis	++++	+++
Medial preoptic area	++++	+++
Median preoptic nucleus	+++(+)	+++
Anteroventral preoptic nucleus	+++	++(+)
Lateral preoptic area	++	++
Supraoptic nucleus	+	++
Suprachiasmatic nucleus	++	+
Anterior hypothalamic nucleus	++	+++
Paraventricular hypothalamic nucleus		
Magnocellular division	+++++	+++
Parvocellular division	+++	++
Periventricular hypothalamic nucleus	++++	++++
Retrochiasmatic area	++++	++++
Ventromedial hypothalamic nucleus	+	+
Dorsomedial hypothalamic nucleus		
Dorsal part	++	+++
Compact	−	−
Ventral part	+++	++++
Dorsal hypothalamic area	++	+++
Lateroanterior hypothalamic nucleus	+	+++
Lateral hypothalamic area	+++	+++
Ventrolateral hypothalamic nucleus	++	++
Perifornical nucleus	++++	+++
Posterior hypothalamic area	+	++
Arcuate nucleus	+++++	++++
Median eminence, internal part	+++	+++
Median eminence, external part	+	+
Medial tuberal nucleus	++	+++
Supramammillary nucleus	+	++
Mesencephalon		
Substantia nigra compact	−	++
Ventral tegmental area	+/−	++
Intrafascicular nucleus	+/−	+
Interpeduncular nucleus	+/−	+
Rostral linear nucleus raphe	+/−	+
Periacqueductal gray	+(+)	++(+)
Edinger–Westphal nucleus	+/−	+
Dorsal raphe nucleus	+(+)	+++
Precommissural nucleus	+	+++
Commissure of the superior colliculus	+	+
Medial pretectal nucleus	−	+
Deep mesencephalic nucleus	−	++
Anterior pretectal nucleus, ventral part	−	+
Peripedoncular nucleus	−	+
Metencephalon		
Cerebellum	−	−
Parabrachial nucleus, lateral part	++	+++
Parabrachial nucleus, medial part	+/−	+(+)
Locus coeruleus	++	+++
Subcoeruleus nucleus	+/−	+
Motor trigeminal nucleus	++	+++
Mesencephalic trigeminal nucleus	++	+++
Pontine reticular nucleus	+/−	++
Pontine reticular nucleus caudal	−	+
Principal sensory trigminal nucleus, ventrolateral	−	++
Myelencephalon		
Raphe magnus nucleus	−	+(+)
A5 Noradrenaline cells	−	+
Gignatocellular reticular nucleus	−	+
Nucleus of solitary tract, median part	+	++
Nucleus of solitary tract, lateral part	(+)	+(+)
Ambigus nucleus	++	++
Spinal cord (cervical)		
Spinal cord layers 1–7, 10	−	+/−
Lateral funiculus of the spinal cord	−	++
Pituitary gland		
Posterior pituitary	+	−

The density of immunoreactive fibers is estimated and indicated as −, undetectable immunoreactivity; +/−, occasional single fibers; +, light density; ++, moderate; +++, dense; ++++, heavy; and +++++, compact, with parentheses representing intermediate levels.

imentation were approved by the University of Michigan Institutional Animal Care and Use Committee.

Immunohistochemistry. For immunohistochemical studies male unfasted Sprague Dawley rats were perfused via the ascending aorta with a Zamboni's fixative solution, and brains were removed, immersion-fixed, cryoprotected, frozen, and sectioned. Thirty-micrometer-thick free-floating sections were incubated or co-incubated with human AGRP affinity-purified antibody (1:30,000) or γ3-MSH (Lys-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-Pro-Arg-Asn-Ser-Ser-Ser-Ala-Gly-Gly-Ser-Ala-Gln, coupled to thyroglobulin with glutaraldehyde) antibody (1:20,000) following a procedure previously described (Wilson et al., 1999).

In situ *hybridization histochemistry.* For *in situ* hybridization histochemistry male Sprague Dawley rats were killed by rapid decapitation (2 hr after lights on), and brains were removed and frozen. A 345 bp fragment of the rat AGRP cDNA and a 936 bp fragment of the rat POMC cDNA were used to synthesize antisense cRNA probes. The rat MC3R (281–1270) probe was generated by PCR using Pfu polymerase (Statagene, La Jolla, CA) with genomic DNA as a substrate, and the rat MC4R (141–1181) probe was generated by screening a rat EMBL3 genomic library (Clontech, Palo Alto, CA) using the human MC4R as a probe. Digoxigenin-11-UTP (Boehringer Mannheim, Mannheim, Germany) antisense-labeled rat AGRP or POMC probes and [α-^{35}S]UTP and [α-^{35}S]CTP (Amersham, Arlington Heights, IL) antisense-labeled MC3R or MC4R probes were simultaneously hybridized on paraformaldehyde-fixed setions as previously described by Curran and Watson (1995) and Wilson et al. (1999).

The specificity of hybridization was confirmed by control experiments using sense probes or tissue that had been pretreated with ribonuclease A (200 μg/ml) for 1 hr at 37°C before hybridization with antisense probes. No specific hybridization signals were observed in these conditions.

Photomicrography and image analysis. The immunostained and autoradiographic tissue sections were viewed using a Leica (Nussloch, Germany) DMR microscope, and images were captured with an MCID M5 image analysis system (Imaging Research, St. Catherine's Ontario, Canada). Images were prepared with Adobe (Mountain View, CA) Photoshop 4.0 software, and only the contrast or transparency was adjusted. For double *in situ* hybridization, nonradioactive labeling was visualized under bright field as a blue precipitate, and radioactive labeling was identified under dark field by silver grain clusters. Digoxigenin-labeled neurons (AGRP or POMC mRNA-containing neurons) were counted bilaterally and then examined for the presence of silver gains (either MC3R or MC4R). A series of sections spaced 100 μm apart was analyzed for each set of probes. No attempt was made to determine the total number of cells in the arcuate nucleus; therefore, the cell counting data represent a relative percentage of AGRP or POMC cells expressing MC3R rather than an absolute number. The boundaries of nuclei were determined according to the atlases of Kruger et al. (1995) and Paxinos and Watson (1986).

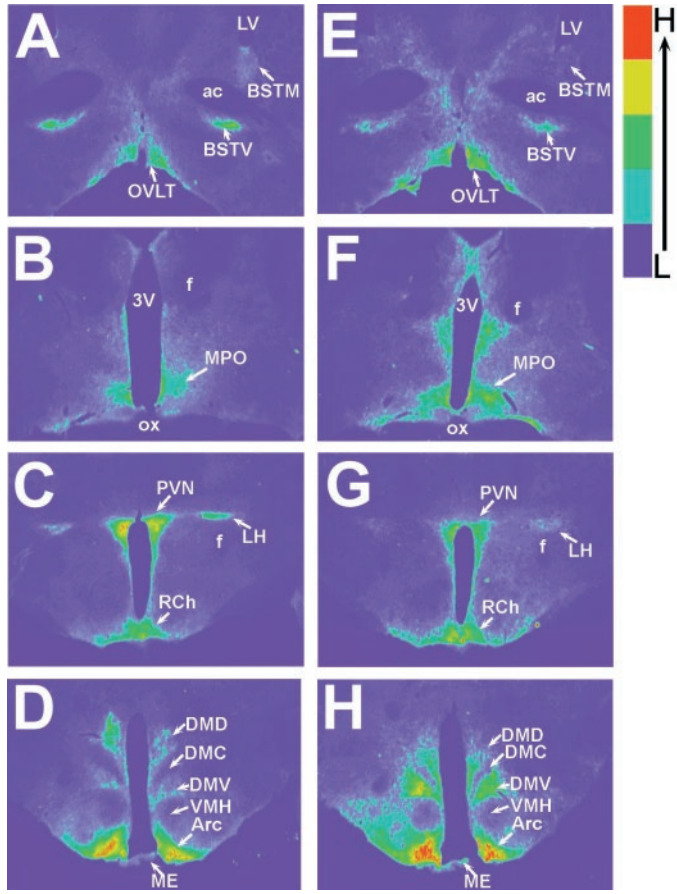

Figure 1. Color scale illustration of AGRP (*A–D*) and POMC (*E–H*) immunoreactivity in the rat brain. Images of coronal brain sections were acquired with NIH Image software. The intensity of the labeling ranges from *blue* (low, *L*) to *red* (high, *H*), which is a summation of density of fibers and intensity of immunostaining per fiber. The distribution patterns of AGRP and POMC are very similar. Note the high intensity of AGRP-immunoreactive fibers in bed nucleus of the stria terminalis, ventral division (*BSTV*; *A*), organum vasculosum lamina terminalis (*OVLT*; *A*), medial preoptic nucleus (*MPO*; *B*), paraventricular hypothalmic nucleus (*PVN*; *C*), retrochiasmatic area (*Rch*; *C*) lateral hypothalamus (*LH*; *C*), and arcuate nucleus (*Arc*; *D*). *ac*, Anterior commissure; *DMD*, dorsomedial hypothalamic nucleus, diffuse; *DMC*, dorsomedial hypothalamic nucleus, compact; *ME*, median eminence; *VMH*, ventromedial hypothalamic nucleus.

RESULTS

Co-distribution of AGRP- and POMC-immunoreactive nerve fibers in the brain

Previous studies by us and other groups have indicated that the POMC and AGRP systems were derived from two distinct cell populations in the arcuate nucleus (Shutter et al., 1997; Hahn et al., 1998; Wilson et al., 1999). In the present study, we compared AGRP and POMC projections in the brain, spinal cord, and pituitary with adjacent sections. Immunoreactive POMC processes were widely distributed throughout the brain (Table 1), exhibiting a projection pattern identical to that previously described for other POMC-derived peptides such as β-endorphin and α-MSH (Watson et al., 1978a,b; Khachaturian et al., 1985). AGRP fibers essentially overlapped with POMC projections. Three major projectional systems of AGRP fibers could be delineated as has been described for the POMC system (Khachaturian et al., 1985): rostral, lateral, and caudal systems. The rostral and lateral projections of AGRP in the forebrain were prominent and closely paralleled the rostral and lateral POMC projections (Fig. 1). Within the same fields, POMC projections were more broadly distributed. AGRP-immunoreactive fibers appeared to be thinner and to have more varicosities than POMC fibers. AGRP terminals were densely packed to form compact immunoreactive patches in some forebrain regions, such as the bed nucleus of the stria terminalis, ventral division, the organum vasculosum of the lamina terminalis, the paraventricular nucleus of the hypothalamus, the arcuate nucleus, the dorsomedial nucleus of the hypothalamus, and the perifornical nucleus and lateral hypothalamus (Figs. 1, 2, 3*E–H*). These findings of the distribution of AGRP immunoreactivity were consistent with the recent reports as described in the mouse, diestrous rat, and fasted monkey (Broberger et al., 1998; Haskell-Luevano et al., 1999). In the caudal projections to the brainstem and spinal cord, POMC innervation was much heavier and broader than that of AGRP, as shown in Table 1. AGRP was greatly reduced or absent from many POMC-

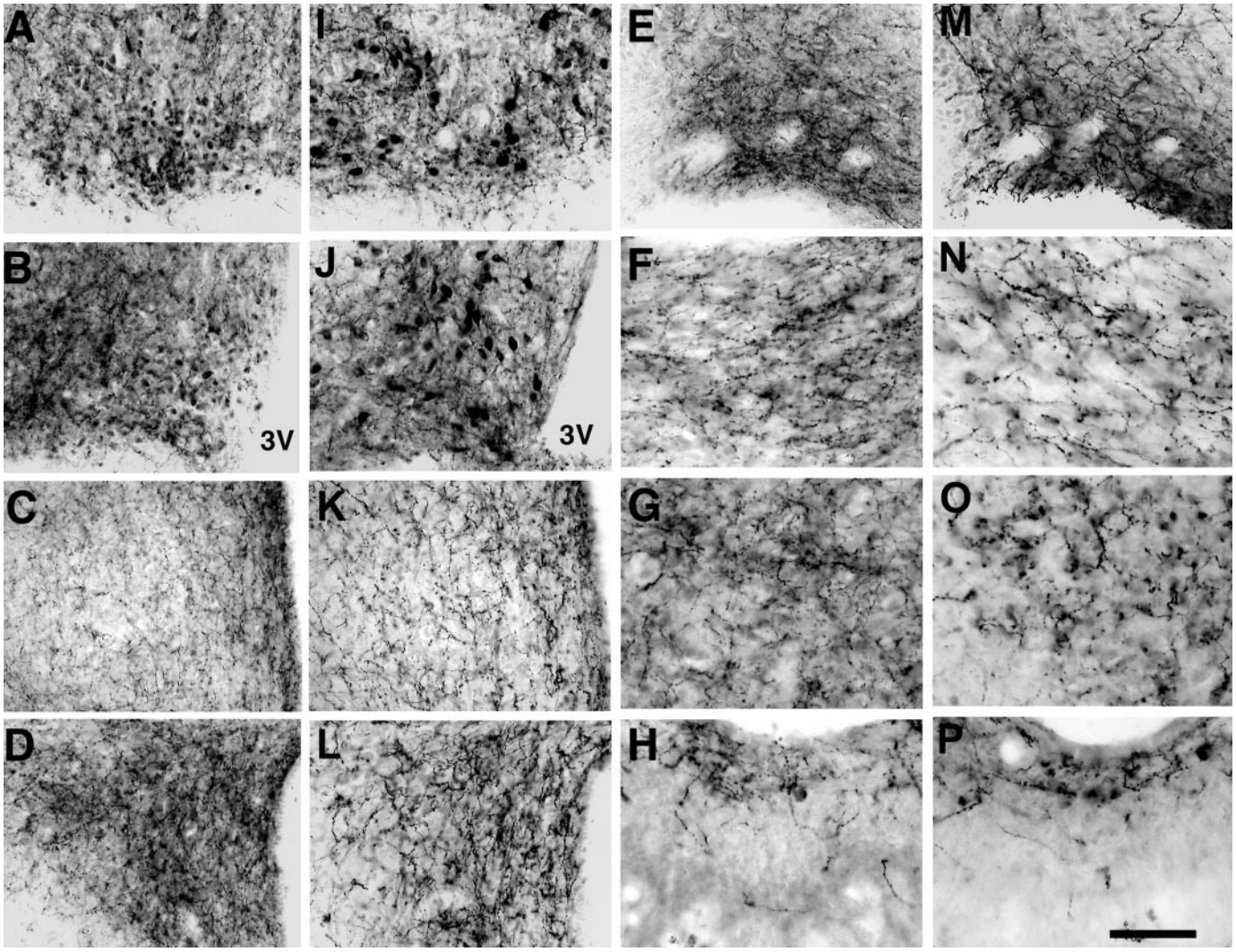

Figure 2. Photomicrographs illustrating AGRP (*A–H*) and POMC (*I–P*) immunohistochemistry in the hypothalamic nuclei. Areas include Rch (*A*, *I*), Arc (*B*, *J*), MPO (*C*, *K*), PVN (*D*, *L*), OVLT (*E, M*), BSTV (*F*, *N*), paraventricular nucleus of the thalamus (*G*, *O*), and ME (H, P). Note the small size of AGRP immunoreactive perikarya versus large POMC immunoreactive perikarya in the Rch and Arc (*A*, *B*, *I*, *J*). The density of AGRP immunoreactive fibers is more prominent than for POMC fibers in the Arc (*B, J*), PVN (*D*, *L*), and BSTV (*F*, *N*). Note the presence of moderate to dense innervation of AGRP and POMC in the internal part of the median eminence. *3V*, Third ventricle; other abbreviations as in Figure 1. Scale bars, 100 μm.

innervated areas in the brainstem and cervical spinal cord (Table 1), suggesting that a substantial portion of the caudal POMC system originates from cells in the nucleus of the solitary tract Bronstein et al., 1992, whereas the rostral and lateral projections of POMC and AGRP probably originate in parallel from the arcuate nucleus of the hypothalamus.

Using double-labeling immunohistochemistry, we found that AGRP fiber boutons were closely apposed to POMC neurons in the arcuate nucleus (Fig. 3*E*). These findings suggest that AGRP and POMC neurons interact locally in their cell body regions and are consistent with the observation that NPY neurons (which always contain AGRP in the arcuate nucleus) make synaptic contact with POMC neurons (Csiffary et al., 1990).

Expression of melanocortin receptors by AGRP or POMC cells

Colocalization of MC3R/MC4R with AGRP and POMC cells was first investigated in the present study. We found that MC3R mRNA was contained in both AGRP and POMC neurons with a rostrocaudal gradient in the arcuate nucleus (Arc). MC3R mRNA was identified in 55% of AGRP neurons in rostral Arc and in 28% for the most caudal Arc sections (Fig. 3*A*) with an average of 44% (all of the Arc sections counted). Similarly, we found that MC3R mRNA was expressed in 43% of POMC neurons in rostral Arc and 13% for the most caudal sections (Fig. 3*B*) with an average of 31% (all Arc sections counted). In contrast, neither AGRP nor POMC cells displayed MC4R mRNA (Fig. 3*C*,*D*).

DISCUSSION

In the present study we extended the recent reports about AGRP projections in the CNS and compared the distribution of AGRP and POMC systems with each other with double immunohistochemistry methods as well as with melanocortin receptor subtypes using double *in situ* hybridization techniques.

Anatomical coregistration of AGRP with POMC peptides demonstrated by our study implies that AGRP might be completely dependent on the melanocortin receptors for its actions.

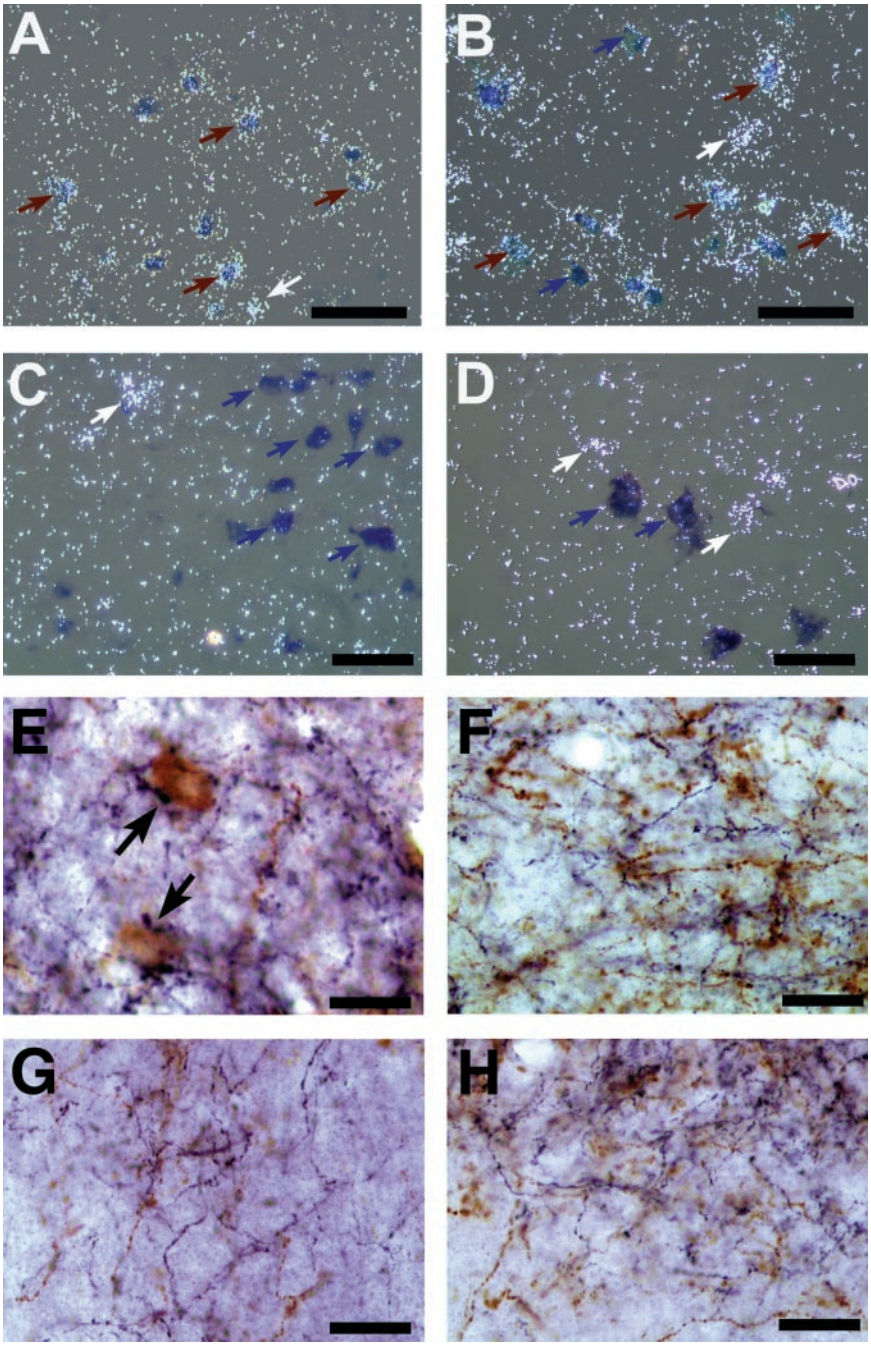

Figure 3. *A–D*, Representative photomicrographs of double *in situ* hybridization histochemistry indicating that AGRP or POMC mRNA colocalize with MC3 receptor mRNA (*A*, *B*), but not with MC4 receptor mRNA (*C*, *D*). The *white* silver grain clusters represent MC3 or MC4 receptor mRNAs, which were detected by ^{35}S-labeled riboprobes. AGRP (*A*, *C*) and POMC (*B*, *D*) mRNAs were visualized by digoxigenin-labeled riboprobes (in *blue*). Thirty-one to 44% of AGRP (*A*) and POMC (*B*) cells expressed MC3R (*red arrows*), whereas none of them displayed MC4R (in *C*, *D*, respectively AGRP and POMC cells). Some MC3R and MC4R cells contained other neurotransmitters than AGRP and POMC (*A–D*, *white arrows*). Some AGRP and POMC cells do not display MC3R and MC4R (*B–D*, *blue arrows*). *E–H*, Double-labeling immunohistochemistry on coronal brain sections showing both AGRP- and POMC-immunoreactive fibers in the arcuate nucleus (*E*), lateral hypothalamus (*F*), central amygdala (*G*), and dorsomedial hypothalamus (*H*). γ3-MSH immunoreactivity was detected by DAB (in *brown*), whereas AGRP immunoreactivity was detected by NiCl amplification (in *black*). Note in *E* POMC-immunoreactive cells being surrounded by AGRP-immunoreactive fibers (*arrows*), suggesting synaptic contact between AGRP fibers and POMC neurons. Scale bars: *A*, *B*, 50 μm; *C*, *D*, *F–H*, 25 μm; *E*, 15 μm.

This is supported by *in vitro* data indicating that AGRP suppresses MSH-induced cAMP accumulation via either MC3R or MC4R (Ollmann et al., 1997). Comparing MCR mRNA distribution and AGRP terminal projections reveals a strong overlap in most areas rich in MC3R mRNA and a subset of areas expressing MC4R mRNA (Roselli-Rehfuss et al., 1993; Mountjoy et al., 1994). In addition, we found a good correlation between AGRP projection fields and the reported MCR binding sites (Tatro, 1990). These observations strongly support the hypothesis that MCRs are the putative receptors for AGRP, although we cannot rule out the possibility of the existence of distinct AGRP receptors that might be distributed identically to MCRs.

The wide distribution of AGRP terminals suggest that AGRP participates in the regulation of food consumption through several hypothalamic structures, including the paraventricular, arcuate, dorsomedial, and lateral hypothalamic nuclei, as well as the amygdala, an area implicated in emotional aspects of feeding behavior. Interestingly, AGRP and POMC-immunoreactive fibers are not found in the ventromedial hypothalamic nucleus, even though this region contains abundant MC3R mRNA and α-MSH binding sites and has been referred to historically as a "satiety center" (Brobeck, 1946). These discrepancies could reflect functional receptor trafficking to the axon terminals. Besides feeding behavior likely mediated by hypothalamic regions, the widespread co-distribution of AGRP and POMC projections in the forebrain and brainstem implicates AGRP in the control of other behaviors or functions attributed to POMC, including stress, thermoregulation, pain, and reproduction (Khachaturian

et al., 1985). We also observed AGRP and POMC fibers in the internal layer of the median eminence. These fibers extend into the posterior lobe of the pituitary and suggest that AGRP could be released into the systemic circulation and function as an endocrine hormone.

The presence of MC3R in both AGRP and POMC neurons demonstrated by the present study suggests that MC3R may mediate the potential interaction between AGRP and POMC systems. Moreover, the colocalization of Mc3R with AGRP and POMC neurons suggest that MC3R may act at the upstream of MC4R in the control of food intake. Because activation of MC3R by melanocortins is stimulatory (i.e., it increases levels of cAMP), and its blockade by AGRP is inhibitory, we would propose two possible roles for MC3R in the POMC and AGRP neuronal circuit. Expression of the MC3R by POMC neurons provides a potential circuit for amplification of AGRP-mediated signals, because AGRP-induced inhibition of POMC neurons via the MC3R would reinforce the postsynaptic effects of AGRP. Furthermore, the expression of the MC3R by AGRP neurons provides a potential circuit for negative autoregulation of POMC-mediated signals, because POMC-induced activation of AGRP neurons via the MC3R would terminate the postsynaptic effects of POMC. Both of these types of actions would tend to reinforce orexigenic behavior but limit signals for satiety and may explain why AGRP or other melanocortin antagonists exert a prolonged effect after intracerebroventrical administration (Grill et al., 1998; Rossi et al., 1998). From an evolutionary perspective, biological amplification of food-seeking behavior and/or feedback inhibition of satiety behavior may offer a selective advantage in situations in which reproductive success is limited by nutrient availability. Given the colocalization of AGRP with NPY (Broberger et al., 1998; Hahn et al., 1998) (D. Bagnol, X.-Y. Lu, and S. J. Watson, unpublished observations) and of POMC and CART (cocaine- and amphetamine-regulated transcript; Elias et al., 1999), it is further suggested that regulation of AGRP and POMC neurons could be accomplished not only via MC3R but also by NPY and CART receptors to coordinate their neurotransmission.

These studies also highlight a broader complexity in POMC-mediated behavior in the brain. POMC mediated behaviors can be elicited through the release of several peptides acting on several classes of receptors, including the activation of μ and δ opioid receptors by β-endorphin. AGRP might allow partial suppression of melanocortin peptide activity although leaving POMC-mediated opioid actions intact. Given that opioid and melanocortin actions can be either synergistic or antagonistic depending on the behavior under study (Khachaturian et al., 1985; Adan and Gispen, 1997), the presence or absence of AGRP activity may serve to alter the balance between these two components of POMC neurotransmission. Thus, POMC-mediated behavior would be under the control of the amount of AGRP as well as the amount and mix of POMC-derived peptides released at the terminals and the presence of melanocortin receptors or opioid receptors in their vicinity. This represents a novel mechanism whereby a multitransmitter neuron may have its impact selectively altered by local antagonism of one of its potentially active products. The full complexity of this type of neuronal regulation has yet to be appreciated. Still, the essential elements of this system include natural agonist–antagonist pairs, parallel neuronal fiber pathways, possible coordination of cross-regulation via shared receptors, and the actions of other co-transmitters. This level of regulation suggests several new principles of neurotransmitter signaling and may apply to other newly discovered endogenous antagonists, such as the recently reported 5-HT-moduline, a naturally occurring peptide antagonist that blocks the action of 5-HT at the 5-HT1B receptor (Massot et al., 1996).

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