THE JOURNAL OF COMPARATIVE NEUROLOGY 505:235–248 (2007)

Conserved Neurochemical Pathways Involved in Hypothalamic Control of Energy Homeostasis

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ABSTRACT

The melanocortin system, which includes α-melanocyte-stimulating hormone (α-MSH) and its endogenous antagonist, agouti-related protein (AgRP), is fundamental for the central control of energy homeostasis in mammals. Recent studies have demonstrated that many neuropeptides involved in the control of ingestive behavior and energy expenditure, including melanocortins, are also expressed and functional in teleost fishes. To test the hypothesis that the underlying neural pathways involved in energy homeostasis are conserved throughout vertebrate evolution, the neuroanatomical distribution of α-MSH in relation to AgRP was mapped in a teleost (zebrafish, *Danio rerio*) by double-label immunocytochemistry. Zebrafish α-MSH- and AgRP-immunoreactive (ir) cells are found in discrete populations in the ventral periventricular hypothalamus, the proposed arcuate homologue in teleosts. Major ascending projections are similar for both peptides, and dense ir-fibers innervate preoptic and ventral telencephalic nuclei homologous to paraventricular, lateral septal, and amygdala nuclei in mammals. Furthermore, α-MSH and AgRP-ir somata and fibers are pronounced at 5 days post fertilization when yolk reserves are depleted and larvae begin to feed actively, which supports the functional significance of these peptides for feeding behavior. The conservation of melanocortin peptide function and projection pathways further support zebrafish as an excellent genetic model system to investigate basic mechanisms involved in the central regulation of energy homeostasis. J. Comp. Neurol. 505:235–248, 2007.

Indexing terms: α-melanocyte-stimulating hormone; agouti-related protein; melanocortin; zebrafish (*Danio rerio*); teleost; obesity

The melanocortin system is an integral component of the central mechanisms that control energy homeostasis in mammals. α-Melanocyte-stimulating hormone (α-MSH) is one peptide derived from the hormone precursor pro-opiomelanocortin (POMC), which is synthesized mainly in neurons within the arcuate nucleus of the hypothalamus as well as a smaller population in the caudal brainstem. Arcuate α-MSH-containing neurons send dense projections to the paraventricular nucleus of the hypothalamus (PVN) and other nuclei known to be involved in the control of energy balance and function primarily through the melanocortin-4 receptor (MC4R; one of five related G-coupled melanocortin receptors), to tonically inhibit food intake (Cone, 1999). Furthermore, agouti-related protein (AgRP), made exclusively in arcuate neurons, projects in parallel to a subset of nuclei innervated by α-MSH and functions not only as its endogenous antagonist but also as an inverse agonist of the MC4R (for review, see Lechan and Fekete, 2006). Perturbations on either side of this system (i.e., deletion of MC4R or POMC genes, overexpression of AgRP, or specific ablation of AgRP and POMC

Grant sponsor: National Institutes of Health; Grant numbers: R56DK075721 (to R.D.C.) and postdoctoral training grant 5 T32 DK007680 (to P.M.F.); Grant sponsor: Bristol-Myers Squibb Freedom to Discover Award (to R.D.C.).

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Received 25 January 2007; Revised 23 April 2007; Accepted 11 June 2007

DOI 10.1002/cne.21447

Published online in Wiley InterScience (www.interscience.wiley.com).

neurons) result in a severe disruption of energy balance leading to obesity and related diseases (for review, see Cone, 2005; Gropp et al., 2005). Thus, at the level of the MC4R, a complex agonist-antagonist interaction that regulates levels of energy intake and expenditure is required to maintain functional energy homeostasis.

The fundamental importance of this regulatory system across vertebrates is exemplified by a remarkable conservation of the melanocortins in primary sequence, structure, receptor pharmacology, and function over evolutionary time. Several recent papers have reviewed what is currently known about melanocortin (POMC, MCRs) gene sequence and expression and function in energy homeostasis in fishes (Schioth et al., 2005; Volkoff et al., 2005; Metz et al., 2006; Takahashi and Kawauchi, 2006).

A few lines of evidence have demonstrated that these peptides have a similar functional role in vivo in fishes compared with mammals. Like mammals, AgRP mRNA expression in the hypothalamus of goldfish and zebrafish is upregulated after fasting (Cerda-Reverter and Peter, 2003; Song et al., 2003). In goldfish, central injections of NDP- α-MSH and MTII (an MC4R agonist) and HS024, an MC4R antagonist, decrease and increase, respectively, food intake in a dose-dependent manner (Cerda-Reverter

Abbreviations

α-MSH	α-melanocyte-stimulating hormone
A	anterior thalamic nucleus
AgRP	agouti-related protein
APN	accessory pretectal nucleus
ATN	anterior tuberal nucleus
Cantd	anterior commissure, dorsal part
Cantv	anterior commissure, ventral part
CC	cerebellar crest
CCe	corpus cerebelli
Chab	habenular commissure
Chor	horizontal commissure
CM	corpus mammilare
CP	central posterior thalamic nucleus
CPN	central pretectal nucleus
Cpop	postoptic commissure
Cpost	posterior commissure
Ctec	tectal commissure
D	dorsal telencephalic area
Dc	central zone of D
Dd	dorsal zone of D
DIL	diffuse nucleus of the inferior hypothalamic lobe
DiV	diencephalic ventricle
Dl	lateral zone of D
Dm	medial zone of D
DON	descending octaval tract
DOT	dorsomedial optic tract
Dp	posterior zone of D
DP	dorsal posterior thalamic nucleus
DT	dorsal thalamus
DTN	dorsal tegmental nucleus
E	epithalamus (pineal)
EG	eminentia granularis
ENv	entopeduncular nucleus, ventral part
FR	fasciculus retroflexus
Ha	habenula
Had	dorsal habenular nucleus
Hav	ventral habenular nucleus
Hc	caudal zone of periventricular hypothalamus/ caudal hypothalamus
Hd	dorsal zone of periventricular hypothalamus
Hi	intermediate hypothalamus
Hr	rostral hypothalamus
Hv	ventral zone of periventricular hypothalamus
Hy	hypothalamus
IMRF	intermediate reticular formation
IO	inferior olive
IR	inferior raphe
IRF	inferior reticular formation
LFB	lateral forebrain bundle
LH	lateral hypothalamic nucleus
LLF	lateral longitudinal fascicle
LR	lateral recess
LX	lobus vagus
MFB	medial forebrain bundle
MLF	medial longitudinal fascicle
MO	medulla oblongata
MON	medial octavolateralis nucleus
MOT	medial olfactory tract
NIII	oculomotor nucleus
NT	nucleus taeniae
NLV	nucleus lateralis valvulae
NMLF	nucleus of MLF
NXm	vagal motor nucleus
OB	olfactory bulb
OT	optic tract
PG	preglomerular nucleus
PGa	anterior preglomerular nucleus
PGl	lateral preglomerular nucleus
PGm	medial preglomerular nucleus
PGZ	periventricular gray zone of optic tectum
Pit	pituitary
PM	magnocellular preoptic nucleus
PO	posterior pretectal nucleus
POA	preoptic area
PP	periventricular pretectal nucleus
PPa	parvocellular preoptic nucleus, anterior part
PPp	parvocellular preoptic nucleus, posterior part
PPT	paracommissural pretectum
PSp	parvocellular superficial pretectal nucleus
PT	posterior tuberculum
PTG	pretectal tegmentum
PTN	posterior tuberal nucleus
R	rostrolateral nucleus
RT	rostral tegmental nucleus
RV	rhombencephalic ventricle
Sc	suprachiasmatic nucleus
SD	saccus dorsalis
SG	subglomerular nucleus
21 SRF	superior reticular formation
T	telencephalon
Teg	tegmentum
Tel V	telencephalic ventricle
TeO	tectum opticum
TeV	tectal ventricle
TL	torus longitudinalis
Tla	torus lateralis
TPp	periventricular nucleus of posterior tuberculum
SO	secondary octaval population
TS	torus semicircularis
TSc	central nucleus of torus semicircularis
TSvl	ventrolateral nucleus of torus semicircularis
TTB	tractus tectobulbaris
V	ventral telencephalic area
Val	lateral division of valvula cerebelli
Vas	vascular lecuna of area postrema
Vc	central nucleus of ventral telencephalic area
Vd	dorsal nucleus of ventral telencephalic area
VIII	octaval nerve
Vl	lateral nucleus of ventral telencephalic area
VL	ventrolateral thalamic nucleus
VM	ventromedial thalamic nucleus
VOT	ventrolateral optic tract
Vp	postcommissural nucleus of V
Vs	supracommissural nucleus of V
VT	ventral thalamus
Vv	ventral nucleus of V
X	vagal nerve
ZL	zona limitans

et al., 2003a,b). Furthermore, transgenic overexpression of AgRP in zebrafish causes an obesity syndrome comparable to that seen in mammals, including a significant increase in weight and total triglyceride content (Song and Cone, 2007). A natural obesity phenotype occurs in the cobalt trout, in which the site of α-MSH production in the pituitary does not develop. Levels of α-MSH in the hypothalamus, however, were not investigated, and obesity is thought to be a function of depressed lipid mobilization (Yada et al., 2002).

Although several studies have documented the expression of POMC and AgRP by in situ hybridization (ISH) (Cerda-Reverter and Peter, 2003; Cerda-Reverter et al., 2003a; Song et al., 2003; de Souza et al., 2005) or detected α-MSH immunoreactivity (ir) (Kishida et al., 1988; Vallarino et al., 1989; Pandolfi et al., 2003; Amano et al., 2005) in the lateral tuberal hypothalamus of teleosts, no studies have investigated the distribution of AgRP-ir somata and projections or, most importantly, the relationship of AgRP to α-MSH-ir in any teleost species or, for that matter, in any nonmammalian vertebrate. In this study, we tested the hypothesis that neurochemical pathways involved in energy homeostasis are conserved throughout vertebrate evolution by detailed neuroanatomical mapping of two central players, α-MSH and AgRP, in the brain of adult zebrafish, *Danio rerio*, by double-label immunocytochemistry. We also characterized α-MSH and AgRP-ir pathways in larvae at 5 days post fertilization (5dpf), when fish are free-swimming and begin to feed actively. The projection patterns of these neurons in relation to homologous brain areas in mammals are highlighted in order to support the use of teleost fishes, and zebrafish in particular, as appropriate genetic models to investigate the mechanisms underlying the central control of energy homeostasis common to all vertebrates.

MATERIALS AND METHODS

Animals

Adult wild-type zebrafish, *Danio rerio*, were raised and maintained in recirculated aquaria at 28.5°C under a 14: 10-hour light/dark cycle and fed a mixture of live brine shrimp and fish food twice daily. Embryos were collected soon after spawning and raised in zebrafish embryonic E3 media in deep-welled Petri dishes maintained at 28.5°C until 5dpf. Ten sexually mature adults (mixed sexes) and 40 larvae (5dpf) were used in this study. Experimental protocols were approved by the OHSU institutional animal care and use committee.

Immunocytochemistry

Adults were deeply anesthetized in MS222 (tricaine methanesulfonate; Sigma, St. Louis, MO) and transcardially perfused with teleost Ringer's solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.2). Brains were dissected out and postfixed for 1 hour at room temperature (RT) in the same fixative. Upon removal of the brain after perfusion of the adult, the pituitary was generally lost, as it is deeply embedded in the base of the skull. Larvae were anesthetized on ice and fixed whole for 1 hour at RT. After fixation, adult brains and larvae were washed in PB and cryoprotected in 30% sucrose in PB overnight at 4°C. Adult brains and larvae were embedded in Tissue-TeK OCT medium (Sakura Finetek, Torrance, CA) in Tissue-Tek intermediate cryomolds and stored at −80°C until sectioned on a cryostat in the transverse, sagittal, or horizontal plane at 20 μm (adults) or 16 μm (larvae) and collected onto Superfrost Plus slides (Fisher Scientific, Fair Lawn, NJ).

The immunocytochemical labeling protocol is as follows: slides were washed 10 minutes in 0.1 M phosphate-buffered saline (PBS; pH 7.2), blocked for 1 hour in PBS + 2% bovine serum albumin (BSA) + 0.3% Triton-X-100 (PBST), incubated overnight at RT (~15 hour) in primary antibody solution (sheep anti- α-MSH diluted 1:30,000 and rabbit anti-AgRP diluted 1:2,000 in PBST), washed 3 × 10 minutes in PBS + 0.5% BSA, incubated for 2 hours at RT with secondary antibodies (anti-sheep Alexa Fluor 594 [red] and anti-rabbit Alexa Fluor 488 [green]; Molecular Probes, Eugene, OR] diluted 1:200 in PBST], washed 4 × 10 minutes in PBS, and coverslipped with SlowFade Gold with DAPI (Molecular Probes) nuclear counterstain to provide cytoarchitectonic detail (blue).

Experiments were also performed by using single antibodies. Label specificity was determined by processing alternate sections of the same brain without the primary antibodies and by preabsorption of the antibodies overnight with synthetic peptide (10 μM; AgRP, Phoenix Pharmaceuticals, San Diego, CA; α-MSH, American Peptide, Sunnyvale, CA). The sheep α-MSH antiserum (Chemicon, Temecula, CA, # AB5087; lot # 25050629) was prepared against the synthetic peptide for α-MSH (sequence identical in mammals and teleosts) conjugated to bovine thyroglobulin. The specificity of this antibody was previously reported by radioimmunoassay (RIA) and by preadsorption with its respective antigen for immunocytochemistry on brain slices (Elias et al., 1998). The rabbit AgRP antiserum (Phoenix Pharmacueticals # H-003-53; lot # 00481) was made against a synthetic peptide representing amino acids 83–132-NH_2 from human AgRP and shows no cross-reactivity with α-MSH, or mammalian leptin, orexin A/B, neuropeptide Y, or melanocyte-concentrating hormone (MCH) in RIA (manufacturer's technical information). The specificity of this antiserum has also been documented by its detection of a single band of 17 kDa molecular weight by Western blot in brain tissue and by preadsorption with its respective antigen for immunocytochemistry on brain slices (Legradi and Lechan, 1999; Mirabella et al., 2004).

Photomicroscopy

Micrographs were taken on a Leica DM4000 B compound microscope outfitted with epifluorescence and captured with a Leica DFC 340 FX camera. Maximum signal-to-noise ratio was optimized, and immunofluorescence was captured as monochrome on three separate channels, color-merged, and overlaid by using Leica camera software. Images were labeled and compiled into plates, and in some instances contrast was enhanced and debris and artifact was removed in Adobe Photoshop 7.0. Line drawings were based on a representative double-labeled adult brain sectioned in a plane that nearly matched the zebrafish brain atlas (Wullimann et al., 1996).

Neuroanatomical nomenclature

Neuroanatomical terminology followed Wullimann et al. (1996). Several papers cited within the present study use terminology from Peter and Gill (1975) when hypothalamic areas are described. Teleost cytoarchitecture and

nomenclature used in Wullimann et al. (1996) was proposed by Braford and Northcutt (1983) and is cross-referenced therein to terms used by Peter and Gill (1975), i.e., anterior, posterior, and inferior parts of the lateral tuberal hypothalamus (NLTa/p/i) described in Peter and Gill all correspond to Hv in Braford and Northcutt. Nomenclature and localization of different regions in the larval brain followed Wullimann and Puelles (1999).

RESULTS

Distribution of α-MSH and AgRP-ir in the adult zebrafish brain

Ascending pathways. The distribution of α-MSH and AgRP cell bodies and fibers is summarized in the line drawings seen in Figure 1. α-MSH-immunoreactive (-ir) somata were localized in the anterior part of the ventral periventricular hypothalamus (Hv; Figs 1E; 4A,B) and also in a region just lateral to Hv (Figs. 1F, 2D, 4B). This lateral nucleus is a diffuse population of cells undefined in the zebrafish brain atlas by Wullimann et al. (1996) but appears to correspond to the lateral division of the lateral tuberal nucleus (NLT-l) defined by Peter and Gill (1975) in the goldfish. In contrast, AgRP-ir somata were localized only medially in the posterior Hv (Figs. 1G, 2B, 4C). Figure 1 (A–G) shows the ascending projections of both α-MSH and AgRP-ir cells throughout the forebrain. Fibers from α-MSH and AgRP-ir somata in the ventral hypothalamus project laterally and dorsally and course through the lateral hypothalamus (LH) and around the periphery of the anterior tuberal nucleus (ATN) and dorsal periventricular hypothalamus (Hd; Figs. 1F,G, 2A–C).

Both peptides follow a similar distribution where dorsal fiber tracts innervate posterior tuberal nuclei (PTN, TPp) and several thalamic nuclei that line the third ventricle (ventromedial [VM]; central and dorsal posterior [CP and DP]). Densest fiber projections course anteriorly along the ventricle, through the caudal suprachiasmatic nucleus (Sc) and posterior parvocellular preoptic area (PPp), with dense terminals and varicosities around the lateral aspect of the magnocellular preoptic nucleus (PM; Figs. 1D, 2C, 3D). PM is perhaps the only nucleus in the brain with noticeably more dense AgRP-ir fibers and terminals compared with α-MSH-ir (Fig. 3D). Whereas both peptides show dense ir-fibers through the anterior parvocellular preoptic area (PPa; Fig. 3B,C) that extend into the postcommissural nucleus of area ventralis of the telencephalon (Vp), only α-MSH-ir fibers are found in abundance in the medial zone of area dorsalis of the telencephalon (Dm); α-MSH-ir fibers project dorsomedially along the telencephalic ventricle and are robust throughout this region of Dm (Fig. 1B–D). At the anterior part of PPa, ir-fibers extend around the anterior commissure and in the supracommissural nucleus of area ventralis (Vs; Figs. 1B, 3B). Heavy innervation of α-MSH-ir fibers and, to a lesser degree, AgRP are found throughout the ventral nucleus and into the ventral part of the dorsal nucleus of area ventralis (Vv and Vd; Figs. 1A, 3A). Whereas both peptides follow similar distribution patterns throughout the hypothalamus, thalamus, and ventral telencephalon, α-MSH-ir fiber pathways are generally more prominent and well defined outside of those areas. In addition to the Dm population, α-MSH-ir fibers are consistently found just outside the periventricular gray zone (PGZ) in the optic tectum (TeO) (Figs. 1G–I, 2A). Diffuse and fine-caliber AgRP-ir fibers can be found in these areas and throughout the dorsal telencephalon and olfactory bulbs but are not restricted into defined boundaries, as is seen with α-MSH-ir.

Descending pathways. Just caudal to Hv, a high and moderate density of α-MSH and AgRP-ir fibers, respectively, project dorsally through the caudal periventricular hypothalamus (Hc; Figs. 1H, 2A, 4D), continue medially, and then course laterally along the tectal ventricle through the dorsal tegmental nucleus (DTN) into the torus semicircularis (TS; Fig. 1H,I). Few fibers are found in Hd and the inferior lobe of the hypothalamus (e.g., DIL). Figure 2 (A–C) demonstrates the extent of α-MSH and AgRP-ir projections in a midsagittal plane. Both peptides send projections through the midbrain tegmentum where one group of fibers continues dorsally along the tectal and rhombencephalic ventricle and the other extends ventrally, primarily along the reticular formation (IRF) into the spinal cord. Caudal to the midbrain, α-MSH-ir fiber tracts are robust in comparison with AgRP-ir. In the hindbrain, α-MSH-ir fibers specifically follow the inferior reticular formation (IRF), whereas AgRP-ir is more diffuse and scattered outside the IRF, and both peptides project fibers into the vagal motor nucleus (NXm). AgRP-ir fibers, however, are more prevalent in the facial and vagal lobes (LX) in comparison with α-MSH-ir (Fig. 1J,K).

Distribution of α-MSH and AgRP-ir in the larval zebrafish brain

At 5dpf, pronounced α-MSH and AgRP-ir somata and fiber projections are consistently detected across individuals. As in the adult, α-MSH and AgRP-ir somata are differentially distributed in the hypothalamus. α-MSH-ir cell bodies are located in the rostral hypothalamus (Hr; Fig. 5C) as well as the lateral part of the intermediate hypothalamus (Hi; Fig. 6C,D), whereas AgRP-ir somata are located in the ventral Hi (Fig. 6D,E). Thus, this population of α-MSH and AgRP-ir somata likely define the developing Hv in the larval brain. Ascending projections are similar between both peptides, which run lateral along the medial cell masses of the Hr and within the lateral forebrain bundle (LFB) into the preoptic area (POA; Figs. 5, 6A,B). However, a greater number of α-MSH-ir fibers extend dorsally into the posterior tubercle area (PT), thalamus, and TeO (Fig. 5). Figure 6A shows the highly specific distribution pattern of α-MSH-ir fibers in the forebrain and the surprisingly extensive distribution throughout the hindbrain. Neither the pineal nor the pituitary were readily preserved in the preparation of adult material but were easily preserved and identifiable in larvae. The pars intermedia lobe of the pituitary was intensely labeled by anti-α-MSH (Fig. 6F,G). Both α-MSH and AgRP-ir fibers follow the same pattern around the caudal hypothalamus (Hc) and appear to innervate this part of the pituitary (Fig. 6F,G). The epiphysis (E), or pineal, is highly immunoreactive to AgRP but not α-MSH antibodies (Fig. 5A,B).

Antibody specificity

In both adult and larval tissue, staining was completely absent when the α-MSH antibody was preabsorbed with its peptide counterpart. In the olfactory bulbs (not shown) and the anterior ventral telencephalon (arrowheads, Fig.

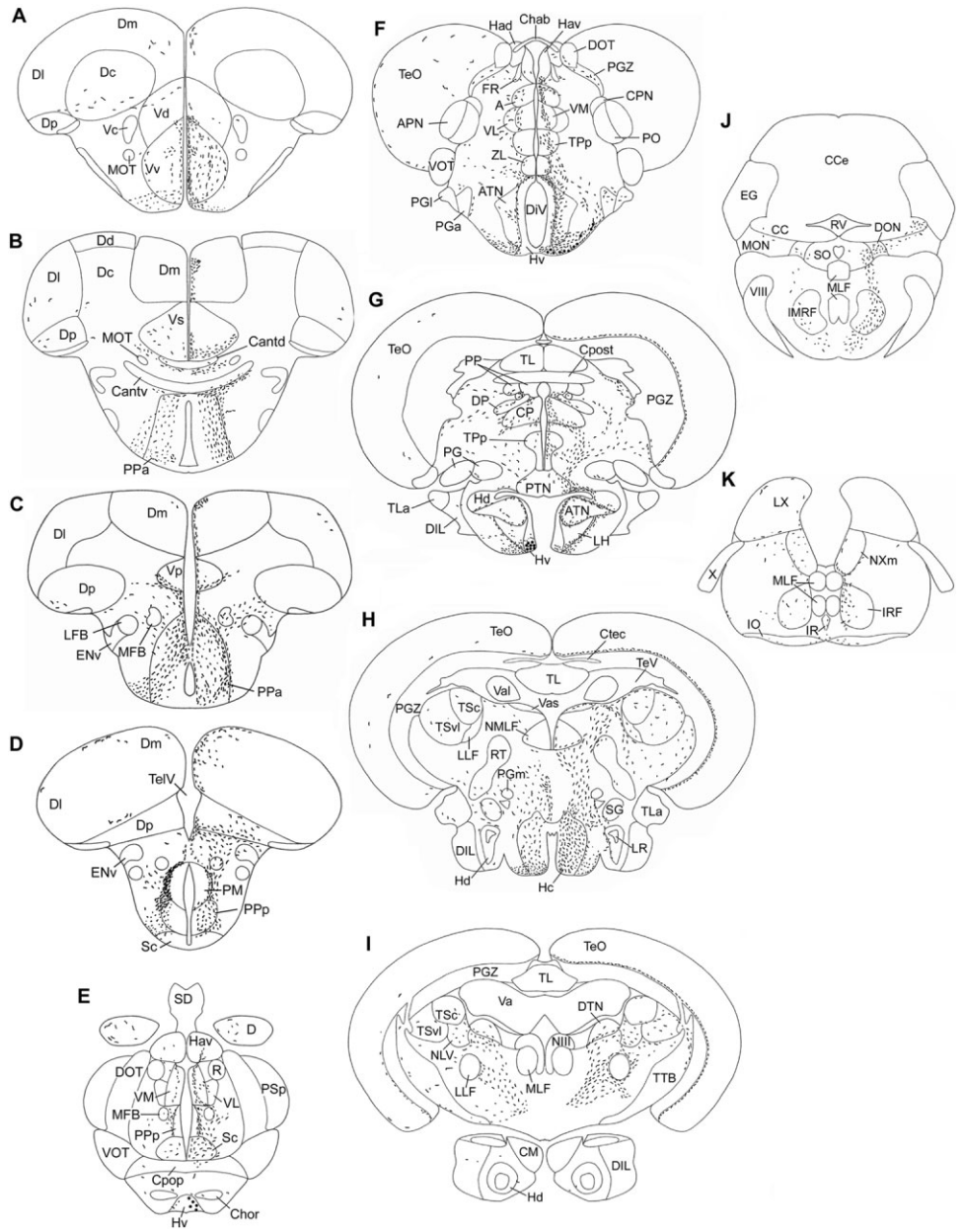


Fig. 1. Representative line drawings of rostral to caudal transverse sections throughout the adult zebrafish brain (from Wullimann et al. 1996), which demonstrate the distribution of α-MSH (right half) and AgRP (left half) -ir somata (large dots) and fiber projections. **A–G:** Ascending projections. **H–K:** Descending pathways. α-MSH-ir somata are localized in the anterior part of the ventral periventricular hypothalamus (Hv; E) and in and just lateral to the intermediate part of Hv (F). AgRP-ir somata are localized more medially in the caudal Hv (G). Both cell types have parallel projections along the thalamic midline (E–G) and into the posterior parvocellular (PPp), magnocellular (PM), and anterior parvocellular (PPa) preoptic nuclei (B–E). Fibers continue anteriorly into the postcommissural (Vp), supracommissural (Vs), and ventral (Vv) and dorsal (Vd) nuclei of area ventralis of the telencephalon (A–C). Only α-MSH-ir cell bodies have a robust projection into the medial nucleus of area dorsalis of the telencephalon (Dm; B–D). Descending projections cover much of the midbrain tegmentum, and α-MSH-ir fibers are concentrated just outside of the periventricular gray zone (PGZ) of the optic tectum (TeO; H,I). Throughout the hindbrain, robust α-MSH-ir fibers follow the reticular formation (IRF; J,K). AgRP-ir fibers are diffuse throughout the hindbrain, and both peptides innervate the vagal motor nucleus (NXm), whereas AgRP-ir fibers are more prevalent in the vagal lobe (LX; K). For abbreviations, see list.

3A,B) thick (nonbeaded) bundles of fiber tracts were labeled by the AgRP antibody in both larvae and adults. After preabsorption with excess AgRP peptide (10 μM), these bundles persisted whereas no beaded and varicose fibers and ir-cell bodies were labeled. These thick bundles, easily distinguishable from the AgRP-specific label by size, staining intensity, and location, appear to be primary olfactory projections, as glomeruli in the olfactory bulb

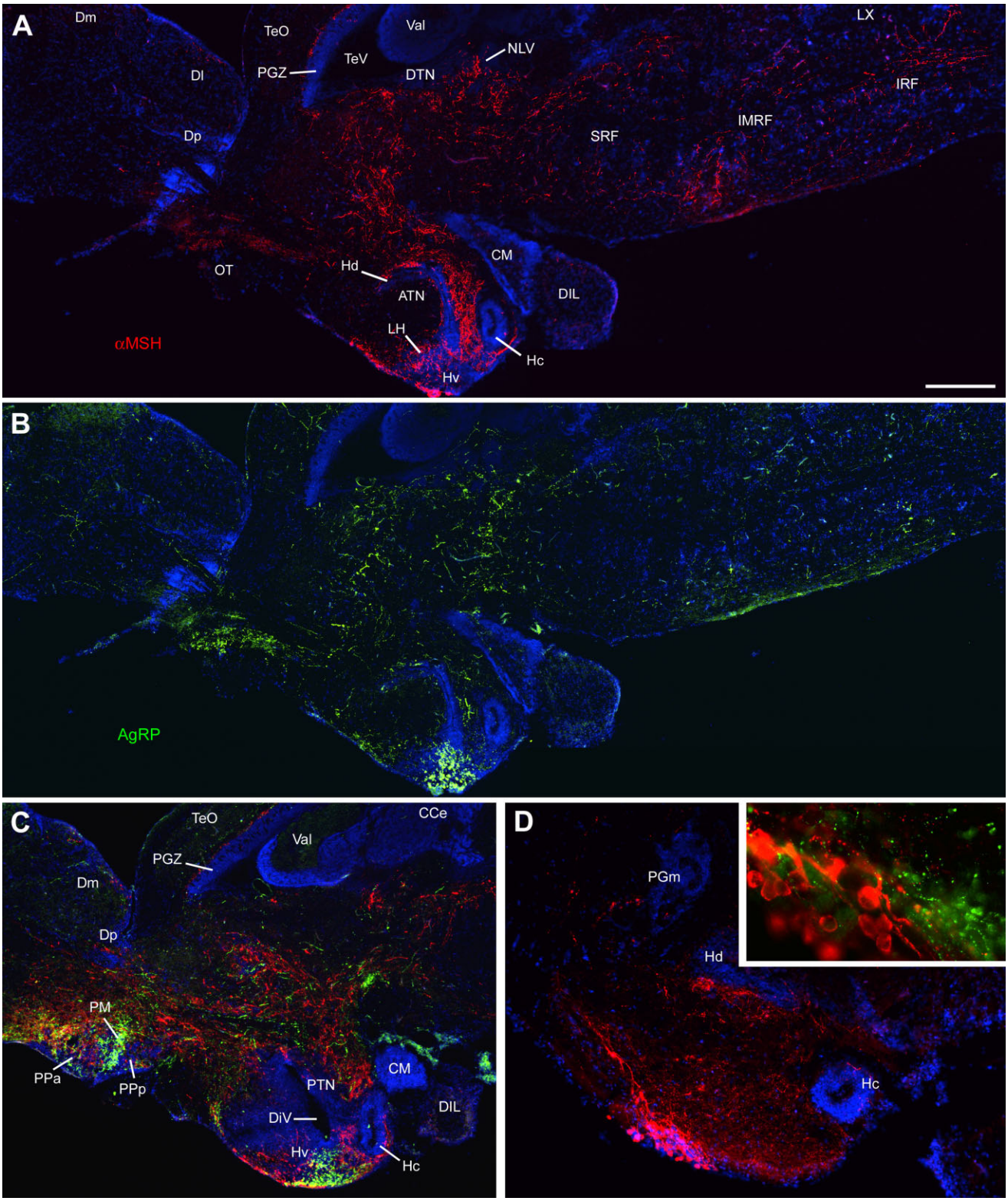


Fig. 2. Photomicrographs of parasagittal views of adult zebrafish brain illustrating the major ascending and descending projections of α-MSH (red) and AgRP (green) -ir cell bodies. **A,B:** Same section double-labeled with anti-α-MSH (A) and anti-AgRP (B) with DAPI counterstain shows location of AgRP-ir somata in Hv. **C:** Double-labeled medial parasagittal section illustrating major innervation of preoptic nuclei (PPp, PM, PPa). Note high density of AgRP-ir fibers terminating in the magnocellular preoptic area (PM). **D:** More lateral section through the ventral hypothalamus shows clusters of α-MSH-ir somata; higher magnification of cells in D with close apposition of AgRP-ir fibers and terminals (inset). For abbreviations, see list. Scale bar in A = 200 μm for A–C; 100 μm for D; 25 μm for inset to D.

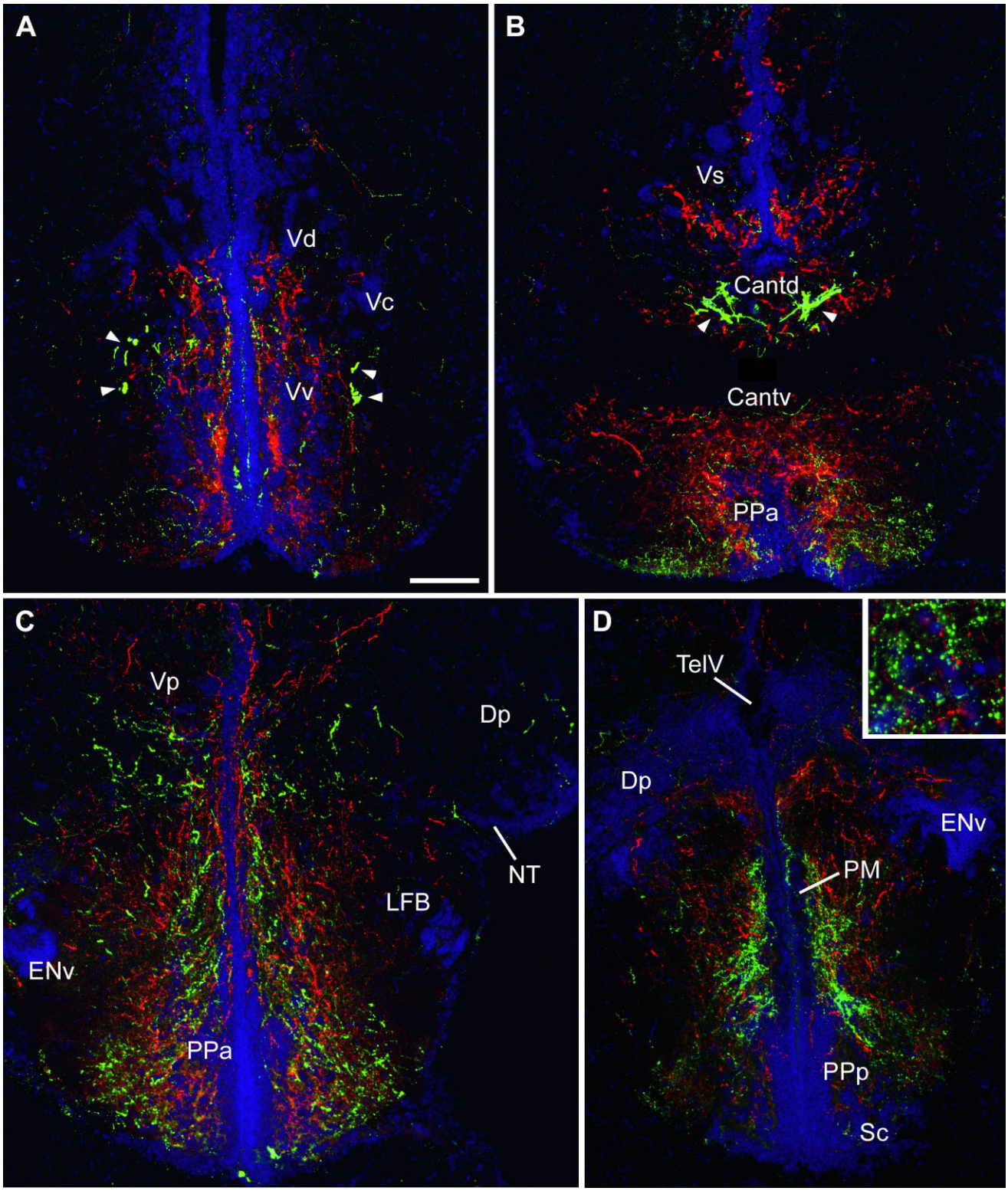


Fig. 3. AgRP (green) and α-MSH (red) -ir in the preoptic area and ventral telencephalon of adult zebrafish. **A:** AgRP and α-MSH-ir in ventral (Vv) and dorsal (Vd) nuclei of area ventralis at the level of Figure 1A. **B:** Immunoreactivity in the anterior parvocellular preoptic area (PPa) and supracommissural nucleus of area ventralis (Vs) at a level near Figure 1B. Arrowheads in A and B denote non-AgRP specific labeling of primary olfactory projections in medial olfactory tract (see MOT, Fig. 1A,B). **C:** Immunoreactivity throughout the PPa and into the postcommissural nucleus of area ventralis (Vp) at the level of Figure 1C. **D:** Very dense AgRP-ir fiber complex and terminals intermixed with α-MSH-ir along the lateral aspect of the magnocellular preoptic nucleus (PM) at the level of Figure 1D. Inset shows higher magnification of abundant AgRP and α-MSH-ir terminals. For abbreviations, see list. Scale bar in A = 100 μm for A–D; 25 μm for inset to D.

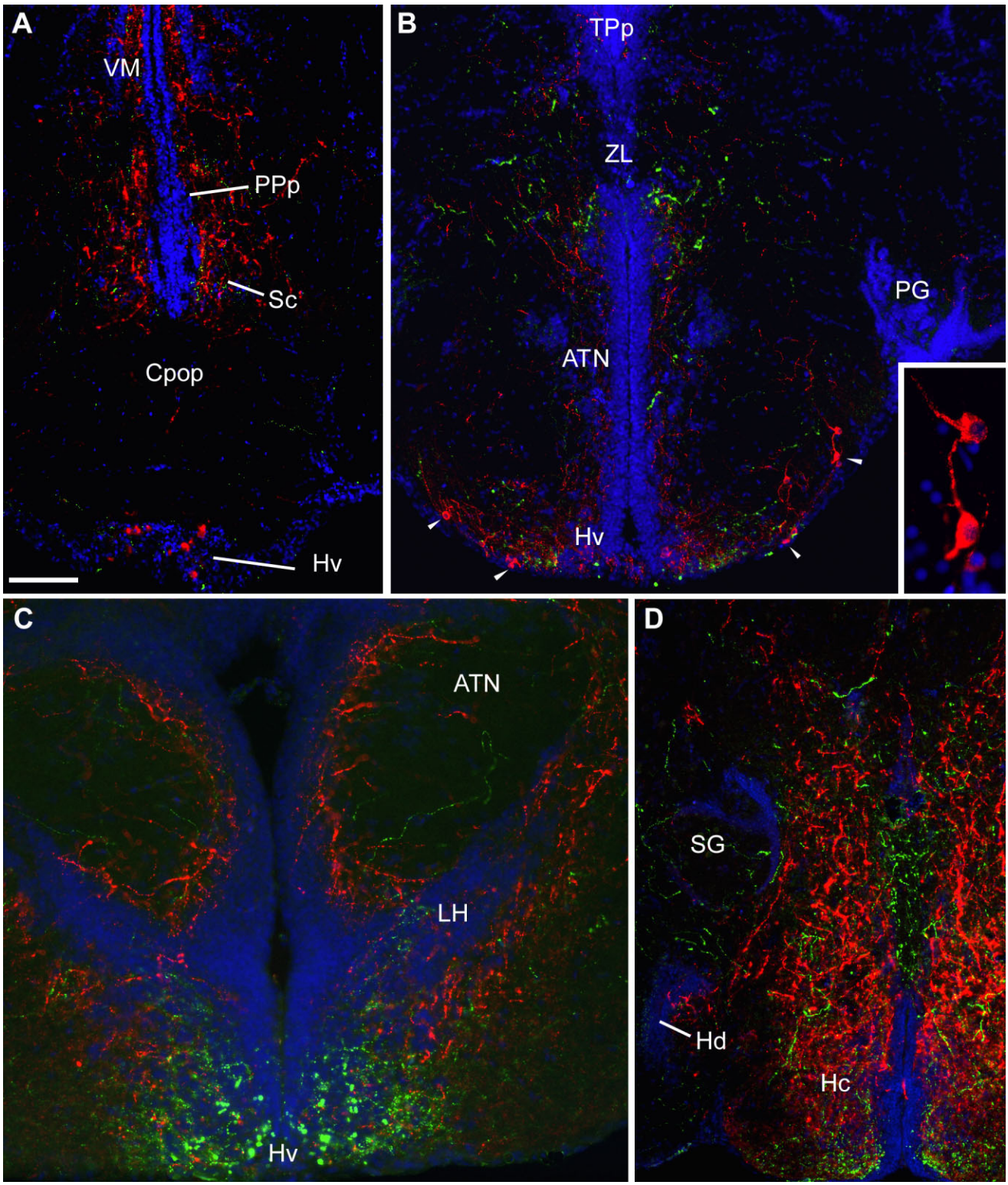


Fig. 4. Localization of AgRP (green) and α-MSH (red) -ir somata and fibers throughout the hypothalamus of adult zebrafish. **A:** α-MSH-ir somata are first seen in the rostral Hv at the level of Figure 1E. Fibers are also found throughout the caudal suprachiasmatic nucleus (Sc) and PPp into the ventromedial thalamus (VM). **B:** Localization of α-MSH-ir somata (arrowheads) within and lateral to Hv at the level of Figure 1E. Both α-MSH and AgRP-ir fibers are located in Hv and course dorsally along the Hv and medial anterior tuberal nucleus (ATN) interface toward the periventricular posterior tuberculum nucleus (TPp). High magnification of two interconnected cell bodies lateral to Hv (inset). **C:** Localization of AgRP-ir somata in the mediocaudal Hv at the level of Figure 1G. Both AgRP and α-MSH-ir fibers course through the lateral hypothalamic nucleus (LH) and around the periphery of the ATN. **D:** AgRP and α-MSH-ir fibers in the caudal periventricular hypothalamus (Hc) at the level of Figure 1H. For abbreviations, see list. Scale bar in A = 100 μm for A,B,D; 50 μm for C; 25 μm for inset to B.

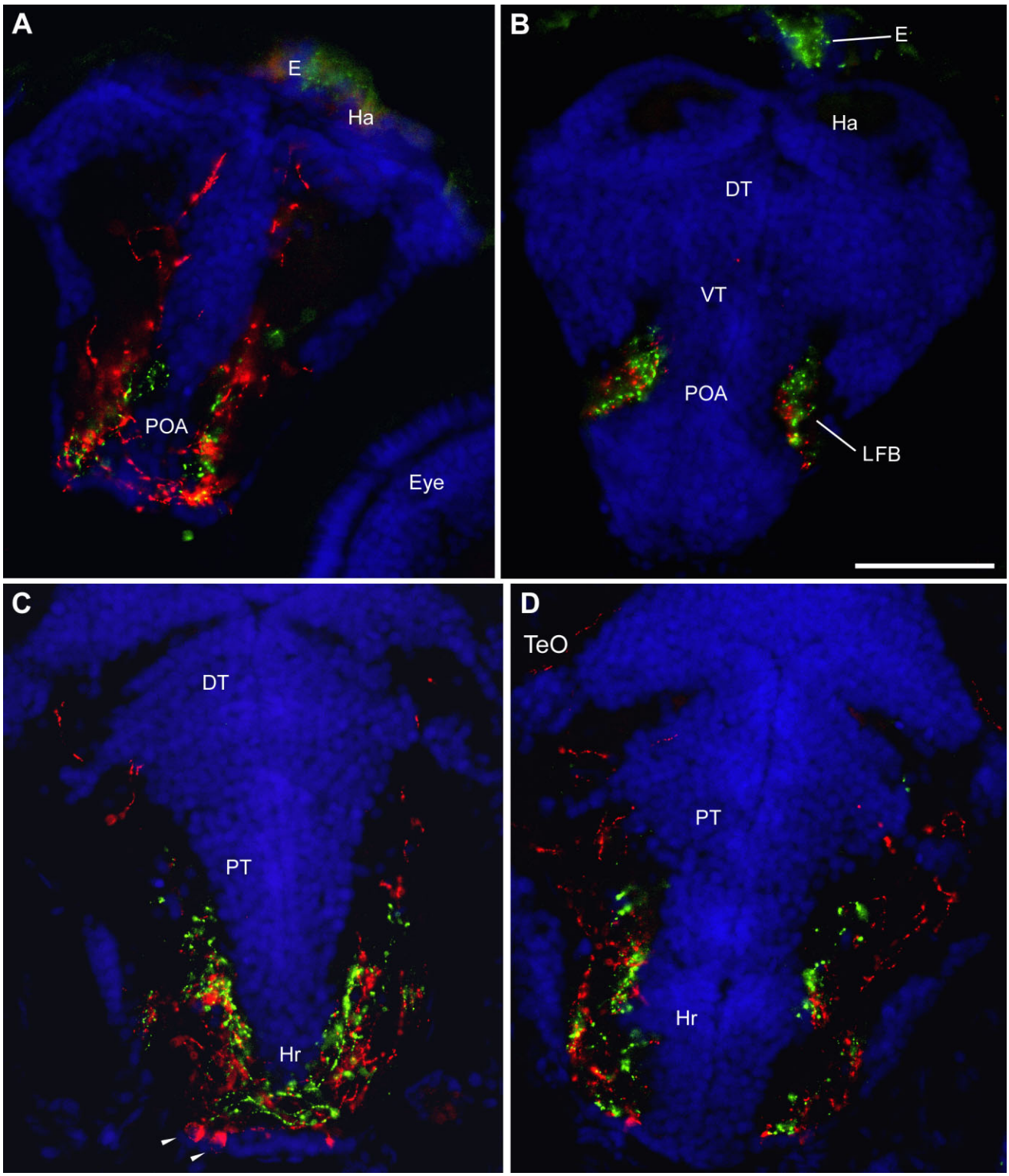
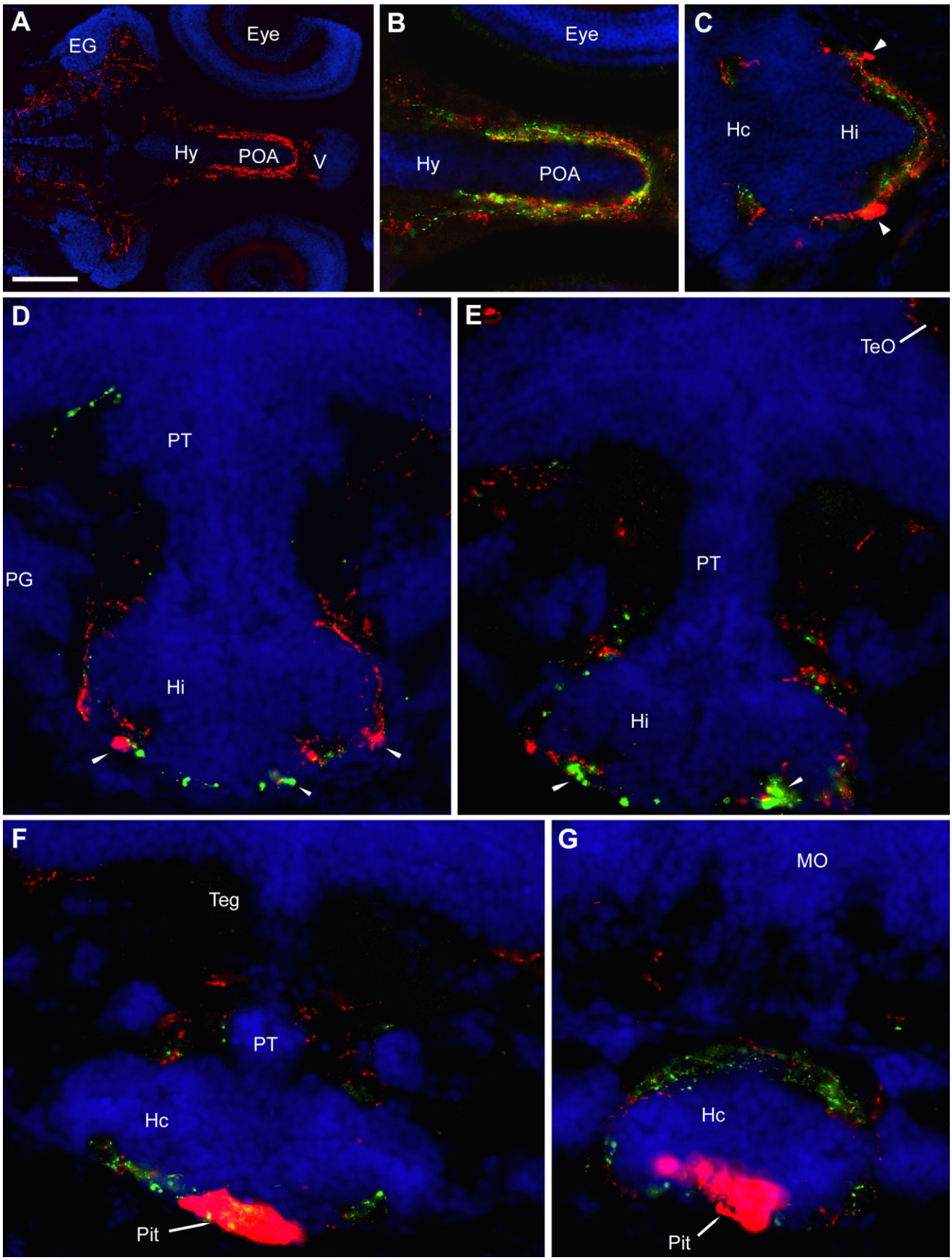


Fig. 5. Localization of AgRP (green) and α-MSH (red)-ir somata and fibers throughout the rostral hypothalamus (Hr) and preoptic area (POA) of larval zebrafish. **A,B:** AgRP and α-MSH-ir fiber projections at rostral (A) and caudal (B) levels of the POA. Both peptides follow the lateral forebrain bundle (LFB) to reach the POA (B). AgRP-ir is consistently found within the epiphysis (E), or pineal. **C,D:** α-MSH-ir cell bodies (arrowheads) in the rostral Hr (A). AgRP-ir fibers mostly remain in the Hr, whereas α-MSH-ir fibers extend into the posterior tuberculum (PT), dorsal thalamus (DT), and optic tectum (TeO). For abbreviations, see list. Scale bar = 50 μm in B (applies to A–D).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TeO
PT
Hi
F
Teg
PT
Hc
Pit
G
MO
Hc
Pit

Figure 6

were intensely labeled (data not shown) and the staining pattern is identical to that found in a previous study that used preabsorbed α-MSH and substance P antisera (α-MSH was different from the one used in the present study) to delineate olfactory projections in trout (see Becerra et al., 1994 and references within for similar cases).

DISCUSSION

In this study we provide a detailed comparison of neuronal projections of the POMC-derived peptide, α-MSH, in relation to its endogenous antagonist, AgRP, throughout the brain of a teleost fish. Although the projections of these two neuropeptides emanate from somata in distinct regions of the ventral periventricular hypothalamus (Hv), ascending fibers run parallel and appear to innervate many of the same nuclei. This study has therefore provided a neuroanatomical template for the location where these two peptides are potentially interacting (see below). A precise mapping of AgRP and α-MSH-ir in the adult zebrafish brain then allowed an accurate comparison with the brain of the developing larva in which both α-MSH and AgRP-ir somata and prominent fiber projections are already present. Thus, these antibodies provide good markers for the development of specific hypothalamic areas.

Comparisons of AgRP and α-MSH-ir with other teleosts

Only one study to date has localized AgRP mRNA to precise brain regions in a teleost (goldfish; Cerda-Reverter and Peter, 2003) and found expression in nuclei in the ventral portion of the posterior area of the tuberal hypothalamus (equivalent areas to the posterior Hv of Braford and Northcutt, 1983). Thus, localization of AgRP-ir somata in the present study corresponds well to AgRP mRNA expression by in situ hybridization (ISH) in goldfish, which are in the same family (Cyprinidae) as zebrafish. Furthermore, the localization of AgRP-ir cell bodies in 5dpf larvae is corroborated by AgRP mRNA detection by whole mount ISH (Song et al., 2003). The early larval stage of zebrafish is defined at 3–7 dpf (Kimmel et al., 1995), and at 5 dpf, larvae begin to feed actively on live food. Although we did not sample earlier than 5dpf, AgRP mRNA expression is seen as early as embryonic 2 dpf (Song et al., 2003). Thus, both the gene and peptide are expressed at developmental time points when regulation of energy expenditure is needed. Interestingly, AgRP-ir was found in the pineal, where a second AgRP gene, AgRP2, is exclusively expressed (Song and Cone, unpublished data); we suspect that the AgRP antibody is cross-reacting with the protein product of this related molecule, because no AgRP mRNA was detected in the zebrafish pineal (Song et al., 2003). This expression appears to be specific and is likely in photoreceptor cells, as no fibers are labeled that would otherwise indicate expression in projection neurons. Furthermore, AgRP2 mRNA is regulated by the light cycle, reaching highest levels just prior to onset of the light period (Song and Cone, unpublished data).

Several studies have consistently identified α-MSH-ir cells in the nucleus lateral tuberis of the hypothalamus (NLT; equivalent to Hv of Braford and Northcutt, 1983) of teleosts (two species of trout, *Salmo fario* and *Salmo gairdneri*, Chinese grass carp *Ctenopharyngodon idellus*, a cichlid, *Cichlasoma dimerus*, and the barfin flounder, *Verasper moseri*) with various antisera (Kishida et al., 1988; Vallarino et al., 1989; Pandolfi et al., 2003; Amano et al., 2005), but few describe the precise fiber distribution outside of the diencephalon, although high-performance liquid chromatography (HPLC)-RIA has identified α-MSH in all major brain areas in trout and carp (Kishida et al., 1988; Vallarino et al., 1989). We found α-MSH-ir somata in the anterior and medial Hv, as well as in an area just lateral to Hv, unlabeled in the zebrafish atlas (Wullimann et al., 1996), but probably comparable to the NLT-l (pars lateralis) in Peter and Gill (1975). Kishida et al. (1988) also report a population of α-MSH-ir cells in this area, and in carp but not trout (*S. fario*), approximately half of these express melanin-concentrating hormone-ir. In contrast, this lateral population was not reported in another species of trout (*S. gairdneri*; Vallarino et al., 1989) or in the barfin flounder (Amano et al., 2005). In all of the above studies, antisera specific to α-MSH were used, and therefore species and/or condition differences may result in variable expression of α-MSH-ir in this area.

Interestingly, in the African lungfish, an ancestral lobe-finned fish, α-MSH-ir somata were detected in the preoptic area (POA) in addition to the pituitary and caudoventral hypothalamus, whereas adrenocorticotropic hormone (ACTH)-ir was undetectable (Vallarino et al., 1992). A recent study demonstrated subfunctionalization of duplicated POMC genes in the highly derived teleost pufferfish *Tetraodon*, in which POMCα is expressed in the ventral hypothalamus and pituitary and POMCβ is only expressed in the POA and weakly in the pituitary. Also, an antibody to ACTH labeled cells in regions where both genes are expressed (de Souza et al., 2005). α-MSH-ir somata, however, have not been identified in the POA of teleosts, and therefore if the POMCβ gene is expressed in the POA of other teleosts, peptide expression may be limited to ACTH. Other than *Tetraodon*, ACTH-ir cells were found in the POA of the common carp (Metz et al., 2004) but not in other species such as goldfish, salmon, and eel (Olivereau and Olivereau, 1990).

Similar to our findings, α-MSH-ir somata in the hypothalamus are detected at 5 days post hatching in the barfin flounder, just prior to the onset of feeding (Amano et al., 2005). In *Cichlasoma*, α-MSH-ir cells are detected at 6 days after hatching in the NLT and also in the nucleus periventricularis posterior (NPP; equivalent in part to the dorsal periventricular hypothalamus [Hd] of Braford and Northcutt, 1983). However, cells in the NPP are not seen

Fig. 6. Localization of AgRP (green) and α-MSH (red) -ir somata and fibers throughout the intermediate (Hi) and caudal (Hc) hypothalamus and pituitary of larval zebrafish. **A:** Horizontal section illustrating the robust α-MSH-ir forebrain pathway and extensive projections in the hindbrain (rostral is to the right). **B:** Higher magnification of anterior fiber bundles through forebrain in A, double-labeled to show parallel AgRP-ir pathway. AgRP-ir is not comparable to α-MSH-ir in the hindbrain. **C:** α-MSH-ir somata and parallel AgRP-ir fibers in a horizontal section through the ventral hypothalamus **D,E:** Transverse sections through two levels of Hi showing ventrally localized α-MSH (D) and AgRP (E) -ir somata (arrowheads) and fibers in the developing Hv. **F,G:** Transverse sections through two levels of Hc showing parallel α-MSH and AgRP-ir fibers that appear to project to α-MSH-ir pituicytes. For abbreviations, see list. Scale bar in A = 100 μm for A; 50 μm for B,C; 25 μm for D–G.

in juveniles or adults, and both these populations are similarly located in MCH-ir cells in the cichlid (Pandolfi et al., 2003).

Relationship of AgRP and α-MSH-ir to melanocortin 4 receptor (goldfish)

Central melanocortin signaling occurs, in part, through the MC4R, and AgRP is a potent antagonist as well as an inverse agonist at the receptor because it contains its own inhibitory activity of MC4R independent of blocking α-MSH activity (Ollmann et al., 1997). Only one study to date has mapped the precise neuroanatomical distribution of MC4R mRNA in a teleost, the goldfish, by ISH (Cerda-Reverter et al., 2003a). When one compares the innervation patterns of both AgRP and α-MSH-ir projections in zebrafish, there is a striking overlap of nuclei that express MC4R mRNA in the goldfish brain. Both α-MSH and AgRP-ir somata send dense fiber projections through the ventral (Hv) and dorsal (Hd) periventricular and anterior tuberal (ATN) hypothalamus, ventromedial nucleus of the thalamus (VM), suprachiasmatic nucleus (Sc), parvo- and magnocellular preoptic nuclei (PPp, PPa, PM), and ventral nucleus of the ventral telencephalon (Vv), which all express MC4R mRNA in equivalent nuclei in the closely related goldfish. The most concentrated region of AgRP-ir fibers was found in the lateral aspect of the PM, and in goldfish, MC4R expression coincides precisely in this part of the magnocellular nucleus.

Although the question was not addressed by ISH, MC4R mRNA was also detected by reverse transcriptase-polymerase chain reaction (RT-PCR) followed by Southern blot hybridization in optic tectum, medulla, and spinal cord (Cerda-Reverter et al., 2003a). AgRP and α-MSH-ir were differentially expressed in very few areas in the forebrain and midbrain. The medial nucleus of the dorsal telencephalon (Dm) was one area that showed a robust α-MSH-ir projection but only sparse AgRP-ir. This nucleus in goldfish has profuse MC4R mRNA but more anteriorly; however, MC4R mRNA was also found in an adjacent area (Dd; dorsal nucleus of dorsal telencephalon), topologically closer to α-MSH-ir in Dm in zebrafish. Thus, Dm in zebrafish may represent an area that lacks antagonism of MC4R by AgRP. In the medulla, AgRP-ir fibers are generally of finer caliber and more diffuse (in several areas) in comparison with α-MSH-ir fibers, which can easily be followed but are largely restricted throughout the reticular formation and into the spinal cord. In contrast, some robust AgRP-ir fibers are seen in the vagal lobe (LX), whereas both fiber types are found just ventral in the vagal motor nucleus (nXm). Thus, in the hindbrain there appears to be less overlap and potential "cross-talk" between the two opposing peptides.

Comparisons to mammalian melanocortin neuroanatomy and homologous nuclei

It has been proposed that the lateral tuberal nucleus (NLT), which is encompassed largely by Hv, is homologous to the mammalian arcuate nucleus (ARC; Cerda-Reverter et al., 2003a). Because both AgRP and POMC are specific markers for ARC in mammals, this appears to be the case. Kishida et al. (1988) also suggested that α-MSH-ir cells in the ventral periventricular hypothalamus of fish may be homologous to POMC neurons in the ARC. Similar to what we found in zebrafish hypothalamus, α-MSH and AgRP-ir cell groups are differentially expressed in the arcuate nucleus of mammals, including humans, where α-MSH and AgRP-ir cells are found in the lateral and medial divisions of the arcuate, respectively (Elias et al., 1998; Hahn et al., 1998). Furthermore, remarkable similarities in α-MSH and AgRP-ir projection pathways are found between teleosts and mammals when homologous nuclei are compared. In mammals, a major target of AgRP- and α-MSH-containing ARC neurons is the paraventricular nucleus of the hypothalamus (PVN). There is a greater abundance of AgRP-ir fibers and terminals in the PVN compared with POMC/α-MSH-ir (Bagnol et al., 1999; Fekete et al., 2000). Many of these fibers terminate on thyrotropin-releasing hormone (TRH)-containing neurons, and although all of these neurons that are innervated by α-MSH are also contacted by AgRP, other TRH-ir neurons are innervated by AgRP alone (Lechan and Fekete, 2006). The magnocellular nucleus of the preoptic area (PM) is thought to be the teleost homologue of the PVN due to its abundance of neurons that express arginine vasotocin (AVT; vasopressin homologue; Gilchriest et al., 2000; Goodson and Bass, 2001; Mukuda et al., 2005), the orphan nuclear receptor Nurr1 (Kapsimali et al., 2001), and, in some cases, corticotrophin-releasing factor (CRF; Olivereau et al., 1988; Olivereau and Olivereau, 1988; Okawara et al., 1992; Ando et al., 1999), as well as TRH, as seen in zebrafish (Diaz et al., 2002); all of these substances are expressed in the mammalian PVN.

To support this homology further, we demonstrate heavy innervation of the PM by α-MSH and AgRP-ir fibers; also, as is the case in mammals, AgRP-ir is notably more dense in this area. AgRP and α-MSH/POMC-ir neurons project in parallel along the third ventricle in mammals and send dense fibers that largely line the periphery of the ventromedial nucleus (VMH) into the dorsomedial nucleus (DMH) and innervate periventricular thalamic and preoptic nuclei, the lateral septum, the bed nucleus of the stria terminalis, and amygdala nuclei (Jacobowitz and O'Donohue, 1978; Broberger et al., 1998; Bagnol et al., 1999; Haskell-Luevano et al., 1999); for review, see Cone, 2005). Similarly, our results show that prominent AgRP and α-MSH-ir fibers run along the third ventricle and the periventricular thalamus and innervate homologous nuclei of the lateral septum, Vv, bed nucleus and basal amygdala, Vs and Vp, and pallial amygdala, Dm, (Braford, 1995; Northcutt, 1995, 2006; Wullimann and Mueller, 2004) and encircle the potential VMH homologue, ATN, (Forlano et al., 2005; Goodson, 2005). Similarly, in zebrafish α-MSH-ir cells send strong projections throughout the midbrain, along the dorsal tegmentum and periaqueductal gray as well as the ventral tegmentum; as in mammals, these tracts follow the reticular formation into the hindbrain (and spinal cord) and are much heavier than AgRP (Bagnol et al., 1999). We did not, however, find evidence of a medullary population of α-MSH/POMC neurons such as that seen in mammals.

Thus, there is ample evidence that melanocortins have not only retained an ancestral function but have also retained similar neural pathways of action, as projections in teleosts are comparable to homologous mammalian circuitry. Furthermore, the common occurrence in several homologous nuclei of fibers containing melanocortin agonists and antagonists, and even the relative density of these fiber types, appear to be conserved between mammals and teleost fish. These data suggest that the mechanisms by which the melanocortin circuitry regulates en-

ergy homeostasis are quite ancient and originated in the earliest vertebrates. The identification of highly conserved neurochemical pathways in zebrafish highlights their use as appropriate models to investigate the mechanisms underlying the central control of energy homeostasis common across diverse vertebrate groups.

ACKNOWLEDGMENTS

We thank Youngsup Song for technical advice, Rob Duncan for fish care, and Andrew Bass for discussions of teleost neuroanatomy.

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