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Journal
The Journal of Comparative Neurology, 522(17)

ISSN
1550-7149

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Publication Date
2014-12-01

DOI
10.1002/cne.23642

Peer reviewed

Identification of Neuropeptide Receptors Expressed by Melanin-Concentrating Hormone Neurons

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ABSTRACT

Melanin-concentrating hormone (MCH) is a 19-amino-acid cyclic neuropeptide that acts in rodents via the MCH receptor 1 (MCHR1) to regulate a wide variety of physiological functions. MCH is produced by a distinct population of neurons located in the lateral hypothalamus (LH) and zona incerta (ZI), but MCHR1 mRNA is widely expressed throughout the brain. The physiological responses and behaviors regulated by the MCH system have been investigated, but less is known about how MCH neurons are regulated. The effects of most classical neurotransmitters on MCH neurons have been studied, but those of most neuropeptides are poorly understood. To gain insight into how neuropeptides regulate the MCH system, we investigated which neuropeptide receptors are expressed by MCH neurons by using double in situ hybridization. In all, 20 receptors, selected based on either a suspected interaction with the MCH system or demonstrated high expression levels in the LH and ZI, were tested to determine whether they are expressed by MCH neurons. Overall, 11 neuropeptide receptors were found to exhibit significant colocalization with MCH neurons: nociceptin/orphanin FQ opioid receptor (NOP), MCHR1, both orexin receptors (ORX), somatostatin receptors 1 and 2 (SSTR1, SSTR2), kisspeptin recepotor (KissR1), neurotensin receptor 1 (NTSR1), neuropeptide S receptor (NPSR), cholecystokinin receptor A (CCKAR), and the κ-opioid receptor (KOR). Among these receptors, six have never before been linked to the MCH system. Surprisingly, several receptors thought to regulate MCH neurons displayed minimal colocalization with MCH, suggesting that they may not directly regulate the MCH system. J. Comp. Neurol. 522:3817–3833, 2014.

INDEXING TERMS: MCH; in situ hybridization; hypothalamus; neuroanatomy; neuropeptide receptors

Melanin-concentrating hormone (MCH) is a cyclic neuropeptide that activates one G-protein-coupled receptor (GPCR) in rodents, MCHR1. MCH is expressed almost exclusively in the lateral hypothalamus (LH) and zona incerta (ZI) (Bittencourt et al., 1992). Although MCH expression is limited to these areas, MCH-expressing neurons project to and MCHR1 is expressed in a wide variety of regions throughout the brain (Bittencourt et al., 1992; Saito et al., 2001), with a particularly high density in the nucleus accumbens shell (NAc shell), hippocampus, locus coeruleus (LC), basolateral amygdala (BLA), and throughout the cortex. This distribution pattern indicates that the MCH system may be involved in a wide variety of physiological functions.

MCH was implicated in the regulation of feeding when it was discovered that central injection of MCH stimulates food intake (Qu et al., 1996) and that MCH precursor mRNA is overexpressed upon fasting and in ob/ob mice (Qu et al., 1996). Mice lacking the MCH precursor were later found to be hypophagic and to exhibit reduced body fat (Shimada et al., 1998), further supporting a role for MCH in the central regulation of feeding and metabolism. MCHR1 antagonists were subsequently developed for potential therapeutic use in the treatment of obesity (Borowsky et al., 2002; Kowalski et al., 2004; Takekawa et al., 2002). It was discovered that MCHR1 antagonists also exert anxiolytic and

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Grant sponsor: National Institutes of Health; Grant number: DA024746; Grant sponsor: NARSAD Established Investigator Award; Grant sponsor: Tourette's Syndrome Foundation.

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Received August 30, 2012; Revised June 17, 2014;
Accepted June 24, 2014.
DOI 10.1002/cne.23642
Published online June 24, 2014 in Wiley Online Library (wileyonlinelibrary.com)

antidepressant effects (Borowsky et al., 2002), which led to the investigation of other physiological functions of the MCH system. In recent years, MCH has been implicated in the regulation of metabolism, reward, anxiety and depression-like behaviors, sleep, learning and memory, and seizure threshold, among other functions (Chaki et al., 2005; Chung et al., 2009; Garcia-Fuster et al., 2012; Lagos et al., 2009; Lee et al., 2011; Parks et al., 2010; Qu et al., 1996; Roy et al., 2007; Shimada et al., 1998; Verret et al., 2003).

MCH is regarded as an inhibitory peptide, because MCHR1 couples to G_i/G_o and typically inhibits the activity of postsynaptic target neurons (Gao and van den Pol, 2001, 2002). Its downstream effects on a number of brain nuclei have been characterized (Chung et al., 2009; Georgescu et al., 2005; Lagos et al., 2009; Torterolo et al., 2009), as have the physiological effects of MCHR1 antagonism. Despite its involvement in a number of critical functions and behaviors, relatively little is understood about how activity of the MCH system is regulated. Several studies have investigated the effects of neurotransmitters on MCH neurons (Bayer et al., 2005; Gao et al., 2003; Guyon et al., 2009; Huang and van den Pol, 2007; Parsons and Hirasawa, 2011; van den Pol et al., 2004) both in brain slices and using dissociated neurons, but most of these studies have focused primarily on the principal excitatory and inhibitory (GABA, glutamate, acetylcholine) or monoamine neurotransmitters. Despite the extensive network of projections received by the LH and ZI (Hahn and Swanson, 2010), the effects of only a few neuropeptides on MCH neurons are known (Guyon et al., 2009; Parsons and Hirasawa, 2011; van den Pol et al., 2004)

One leading model for how MCH neurons are controlled is that they function as "second-order neurons," which receive projections from neuropeptide Y (NPY)- and α-melanocyte-stimulating hormone (α-MSH)-expressing neurons in the arcuate nucleus (Guyon et al., 2009; Saito and Nagasaki, 2008) to control feeding behavior. According to this hypothesis, NPY- and α-MSH-producing neurons receive energy homeostasis signals from the periphery and then activate or inhibit MCH and the nearby orexin/hypocretin neurons in the LH, which in turn relay signals throughout the brain to modulate food-seeking behavior and metabolism (Guyon et al., 2009). NPY is orexigenic, whereas α-MSH inhibits food intake. Supporting this model is the observation that both NPY and proopiomelanocortin (POMC) neurons project to MCH and orexin/hypocretin neurons (Elias et al., 1998) and the fact that central injection of α-MSH inhibits MCH but not NPY induced food intake (Tritos et al., 1998). Although NPY has been demonstrated to inhibit MCH neuron activity both presynaptically and postsynaptically, α-MSH has been reported to have no effect (van den Pol et al., 2004). Aside from this putative circuit with α-MSH and NPY that may modulate food intake, how neuropeptides may control other functions of the MCH system is not well understood.

To gain insight into how MCH neurons are regulated and in an attempt to find new circuits that modulate the activity of MCH neurons, we have performed a double in situ hybridization study aimed at identifying neuropeptide receptors expressed by MCH neurons. From a search of the existing literature, we selected several neuropeptide receptors that are expressed at high levels in the LH and ZI and performed double in situ hybridization to determine whether they colocalize with MCH precursor mRNA. We also focused on neuropeptide receptors that have been either demonstrated or proposed in the literature to interact with MCH neurons. The in situ hybridization experiments described here detect the presence of specific mRNAs and cannot determine whether functional receptors are expressed by MCH neurons or whether they are presynaptic or postsynaptic. However, a high degree of colocalization strongly suggests that a given receptor may regulate the MCH system, and we have previously used a similar approach to identify receptors that regulate MCH neurons (Parks et al., 2014). This study will provide an extensive map of neuropeptide receptors expressed by MCH neurons and will serve as a basis for future studies into the functional regulation of the MCH system.

MATERIALS AND METHODS

Animals

Brains collected from adult male Sprague-Dawley rats (Charles River, San Diego, CA; 200−400 g) were used for in situ hybridization experiments. Animals were group housed under controlled conditions (temperature 21°C ± 2°C; 12 hr light−dark cycle, lights on at 6:00 AM) with free access to water and food. All animal procedures were approved by the Institutional Animal Care and Use Committee, University of California, Irvine.

Tissue preparation and prehybridization

Tissues were prepared as described previously (Clark et al., 2001), with minor modifications. While under deep CO_2-induced anesthesia, animals were decapitated, and brains were rapidly removed and frozen in in −20°C 2-methylbutane. Brains were stored at −80°C until cryostat sectioning into 20-μm coronal sections and mounted onto Fisher SuperFrost slides. Every other section was collected. Tissue sections covering the entirety of MCH-expressing regions were fixed with cold

4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 1 hr. After fixation, tissues were rinsed in 0.1 M PB, washed briefly with water, treated with with proteinase K (0.1 μg/ml) for 10 minutes, acetylated, and dehydrated through ascending concentrations of ethanol and air dried. Sections were then stored at −20°C until hybridization experiments.

Probes and cRNA synthesis

The complete coding sequences (open reading frame; ORF) of the following genes were cloned into pBluescript vector:MCH precursor (pMCH), somatostatin receptors 1 and 2 (SSTR1, SSTR2), neurotensin receptor 1 (NTSR1), MCHR1, melanocortin receptors 3 and 4 (MC3, MC4R), galanin receptor 1 (GalR1), nociceptin receptor (NOP), cholecystokinin receptors A and B (CCKAR, CCKBR), neuropeptide S receptor (NPSR), orexin receptors 1 and 2 (OX1R, OX2R), and kisspeptin receptor (KissR). All genes were cloned using rat cDNA sequences. Rat corticotrophin-releasing factor receptor 1 and 2 (CRFR1, CRFR2) probes were provided by Drs. Paul Sawchenko and Tali Baram (Eghbal-Ahmadi et al., 1997), and rat δ-, κ-, and μ-opioid receptor (DOR, KOR, MOR) probes were provided by Dr. Francis M. Leslie (Winzer-Serhan et al., 2003). Rat NPY receptor 5 (NPY5R) was provided by Dr. Herbert Herzog (Garvan Institute of Medical Research; see Parker and Herzog, 1999).

The MCH precursor was digested with SmaI or HindIII and cleaned by phenol-chloroform extraction prior to probe synthesis. Antisense or sense cRNA was generated using T7 or T3 polymerase, respectively, and labeled with digoxigenin (DIG) by using a DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN). Labeled probes were separated from unincorporated nucleotides by using Sephadex G-50 columns (Roche Applied Science).

For double in situ hybridization, antisense and sense cRNA of SSTR1, SSTR2, NTSR1, MCHR1, MC3R, MC4R, GalR1, NOP, MOR, KOR, DOR, CCKAR, CCKBR, NPSR, OX1R, OX2R, KissR, NPY5R, CRFR1, and CRFR2 were generated as described by Clark et al. (2001) using T7, T3, or SP6 RNA polymerase (Promega, Madison, WI) in the presence of ^{35}S-UTP (Perkin Elmer, Norwalk, CT). Radiolabeled probes were separated from unincorporated nucleotides by using Sephadex G-50 columns (Roche Applied Science).

IN SITU HYBRIDIZATION

Hybridization was performed as previously described, with minor modifications (Clark et al., 2001). Prehybridized slides were removed from −20°C and warmed to room temperature. Next, slides were incubated at 60°C for 16−20 hours with 0.5 ng/ml DIG-labeled MCH antisense probe mixed with $1-2 \times 10^7$ cpm/ml of an ^{35}S-labeled sense or antisense probe for one of the above-mentioned GPCRs in hybridization buffer (50% formamide, 10% dextran sulfate, 0.3 M NaCl, 0.02% polyvinylpyrolidone, 0.02% bovine serum albumin, 0.02% Ficoll, 10 mM dithiothreitol [DTT], 10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0, and 500 μg/ml yeast tRNA). After hybridization, sections were treated with 20 μg/ml RNaseA for 30 minutes at 37°C and washed in decreasing salinity (from 2× to 0.1× SSC, 5−10 minutes each), followed by a 30-minute incubation in 0.1× SSC at 68°C and a brief rinse in room temperature 0.1× SSC. Next, sections were rinsed in genius buffer 1 (GB1; 100 mM Tris-HCl, 150 mM NaCl, pH 7.5) briefly and then incubated in blocking buffer (GB1 + 5% nonfat dry milk and 0.25% Triton X-100) for 30−60 minutes at room temperature. Slides were then incubated at 37°C for 3 hours in blocking buffer with 1 ml alkaline phosphatase-conjugated sheep anti-DIG antibody (Roche Diagnostics) diluted 1:7,500. Sections were then washed with GB1 and treated with 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 0.09 mg/ml) and nitroblue tetrazolium salt (NBT; 0.25mg/ml) for 20−60 minutes at room temperature while shielded from light. Sections were washed, dehydrated in ascending concentrations of ethanol, air dried, and exposed to β-Max film (Kodak, Rochester, NY) for up to 7 days to determine strength of signal. Slides were then dipped in 0.3% parlodion dissolved in isoamylacetate and dried overnight. Sections were dipped in NTB emulsion (Kodak) and stored in the dark at 4°C for 4−8 weeks before development with D-19 (Kodak), followed by fixative. A subset of DIG-hybridized sections (Fig.1) was counterstained with nuclear fast red (Vector Laboratories, Burlingame CA).

Data analysis

Emulsion-dipped sections were analyzed with a light microscope (Axioskop 40; Carl Zeiss, Goettingen, Germany). Images were captured with Spot camera software version 2.22 (Diagnostic Instruments, Sterling Heights, MI) and optimized for brightness, contrast, and sharpness in Adobe Photoshop 10.0 (Adobe Systems, San Jose, CA). Minor artifacts (debris, air bubbles) were retouched, but silver grains or DIG signal were never altered. Figures were arranged in ACD Canvas 11.0 (ACD Systems, Seattle, WA). Several images display selected regions of MCH expression: the perifornical region of the lateral hypothalamus (pfLH), the lateral portion of the LH, and the ZI, which were identified by comparison with an adult rat brain atlas (Paxinos and

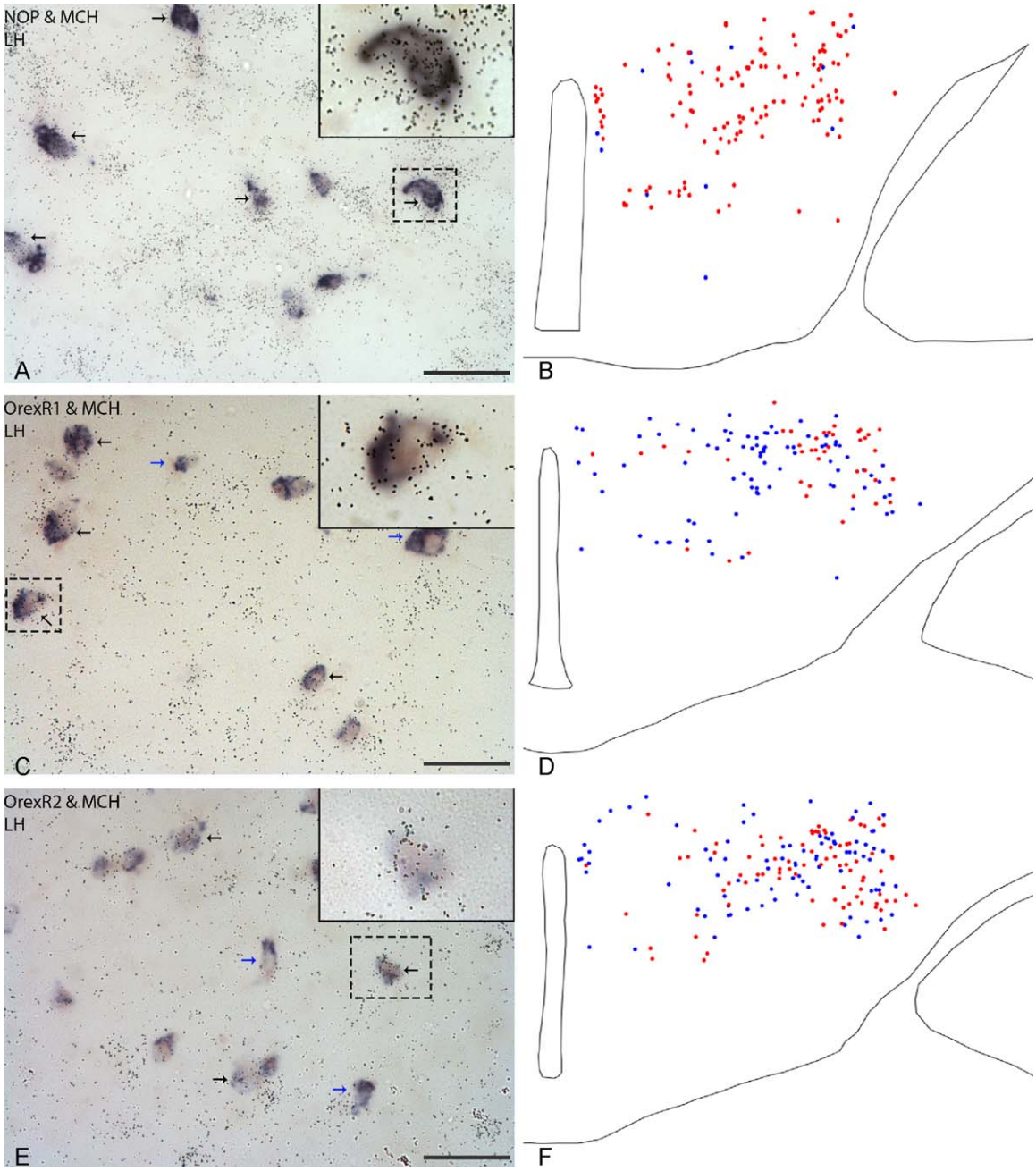

Figure 1. Double in situ hybridization of pMCH and NOP or orexin receptor mRNA. Microscopy images of sections hybridized with antisense ^{35}S-labeled riboprobes (black grains) for NOP, OX1R, or OX2R and DIG-labeled pMCH (purple) in the lateral region of the lateral hypothalamus (LH). **A:** NOP colocalizes with most MCH neurons. **B:** Diagram illustrating distribution of NOP-positive MCH neurons. **C:** OX1R colocalizes with some MCH neurons in the LH **D:** Diagram illustrating distribution of OX1R-positive MCH neurons. **E:** OX2R colocalizes with MCH neurons. **F:** Diagram illustrating distribution of OX2R-positive MCH neurons. Black arrows display colocalization, and blue arrows point toward representative MCH neurons that do not colocalize. **Insets** in top right of some panels represent high-power microscopy images of the region within the rectangle. In the diagrams, red dots represent MCH neurons that colocalize with the corresponding receptor mRNA (NOP, OX1R, OX2R), and blue dots signify MCH neurons that do not demonstrate colocalization. Scale bars = 50 μm.

Watson, 2009). To ensure proper localization of cells labeled as "pfLH," images were taken only from cells very near the fornix, and those of the LH were taken only from obviously lateral regions of MCH expression.

For selected slices, all MCH-DIG-positive cells were manually examined under high magnification for the presence of silver grains. Cells that displayed at least five to 10 times more silver grains within their borders

TABLE 1.
Percentage of MCH Neurons Found To Express Each Neuropepide Receptor[1]

Probe	Percentage colocalization
NOP	87.47 ± 3.11 (n = 3,417)
MCHR1	64.77 ± 5.46 (n = 3,837)
OrexR1	36.16 ± 2.04 (n = 1,996)
OrexR2	51.34 ± 5.48 (n = 2,016)
SSTR1	49.3 ± 2.89 (n = 2,166)
SSTR2	36.73 ± 9.49 (n = 3,080)
KissR1	48.14 ± 3.14 (n = 6,066)
NTSR1	30.43 ± 4.22 (n = 3,480)
NPSR	30.16 ± 4.66 (n = 3,224)
CCKAR	17.97 ± 2.62 (n = 3,266)
CCKBR	1.57 ± 0.24 (n = 2,252)
KOR	12.22 ± 3.86 (n = 1,971)
MOR	5.76 ± 2.24 (n = 2,347)
DOR	5.23 ± 0.72 (n = 1,659)
CRF1	8.53 ±0.53 (n = 2,142)
CRF2	5.72 ± 1.52 (n = 1,591)
GalR1	5.20 ± 0.24 (n = 2,270)
NPY5R	4.40 ± 0.74 (n = 2,901)
MC3R	4.16 ± 0.50 (n = 2,401)
MC4R	3.60 ± 0.11 (n = 2,023)

[1]N = number of MCH neurons counted. Data are presented as mean ± SEM.

than background were counted as exhibiting colocalization. Background was determined by observing the density of silver grains on MCH-DIG-positive sections hybridized with radiolabeled sense probes. The percentage colocalization is the proportion of MCH-positive cells expressing significant levels of mRNA for the given receptor. For each probe tested, three to six rat brains were scored, and the colocalization levels for each brain were averaged to determine mean and standard error of the mean (SEM). Raw counts of MCH-positive cells scored are reported for each probe. Between about 1,590 and 6,000 MCH-positive cells were counted for each probe to determine colocalization levels. For diagrams illustrating the distribution pattern of receptor mRNA colocalization with MCH neurons, cells were scored in a representative slice using Stereo Investigator software (MBF Bioscience, Williston, VT) and converted to a vector graphic in MathWorks MatLab 7.11.00 (MathWorks, Natick, MA). A low-resolution image of the scored slice was traced in Adobe Illustrator CS 6 (Adobe Systems), and the scored vector image was plotted onto a tracing by aligning reference landmarks.

RESULTS

MCH precursor distribution

MCH neuron distribution was studied by using in situ hybridization. DIG-labeled MCH precursor cRNA was found in the LH and ZI in a pattern very similar to that observed in previous studies (Bittencourt et al., 1992; Hahn, 2010; Swanson et al., 2005). No specific hybridization signals were found in sections hybridized with sense probe.

Nociceptin/orphanin FQ receptor

The nociceptin/orphanin FQ receptor (NOP) was expressed widely throughout the brain, in agreement with reported distribution patterns (Neal et al., 1999), and was expressed at high levels throughout the LH and ZI. NOP colocalized strongly with MCH-expressing neurons in both the LH and the ZI (Fig. 1A); approximately 87% of MCH neurons expressed NOP (Table 1). There was no obvious variation in colocalization levels throughout the subregions of MCH neurons (Fig 1B). High levels of NOP signal were also present in areas of the LH and ZI that did not express MCH (Fig. 1A), indicating that NOP expression is not exclusive to MCH neurons. No specific signal was observed for NOP sense strand control, indicating that the antisense signal corresponds to NOP mRNA.

Orexin/hypocretin receptors

Both orexin and hypocretin receptor (OX1R, OX2R) mRNAs were present at high levels in the LH and ZI and were expressed throughout the brain, in agreement with the published distribution pattern (Marcus et al., 2001). OX1R was coexpressed with MCH neurons throughout the LH and ZI (Fig. 1C,D, Table 1), but appeared to colocalize to a greater degree in the more lateral regions (Fig. 1D). OX2R exhibited a higher degree of colocalization with MCH than did OX1R (Table 1), and colocalization was observed in all MCH-expressing regions (Fig. 1E,F). Sense-labeled control probes gave no specific signal, confirming specificity of the antisense probes.

MCHR1

MCHR1 expression was consistent with that found in previous reports (Hervieu et al., 2000; Saito et al., 2001), with widespread distribution throughout much of the brain. MCHR1 was moderately expressed in both the LH and the ZI, in MCH-positive cells, and also in clusters without MCH expression. Approximately 60% of MCH neurons coexpressed MCHR1 (Fig. 2A,C), with a relatively even distribution throughout all subregions (Fig. 2E). Sense-probe-hybridized sections did not display any specific signal, confirming specificity of the MCHR1 antisense probe.

Kisspeptin receptor

Kisspeptin receptor (KissR) was expressed throughout the brain in a pattern consistent with published

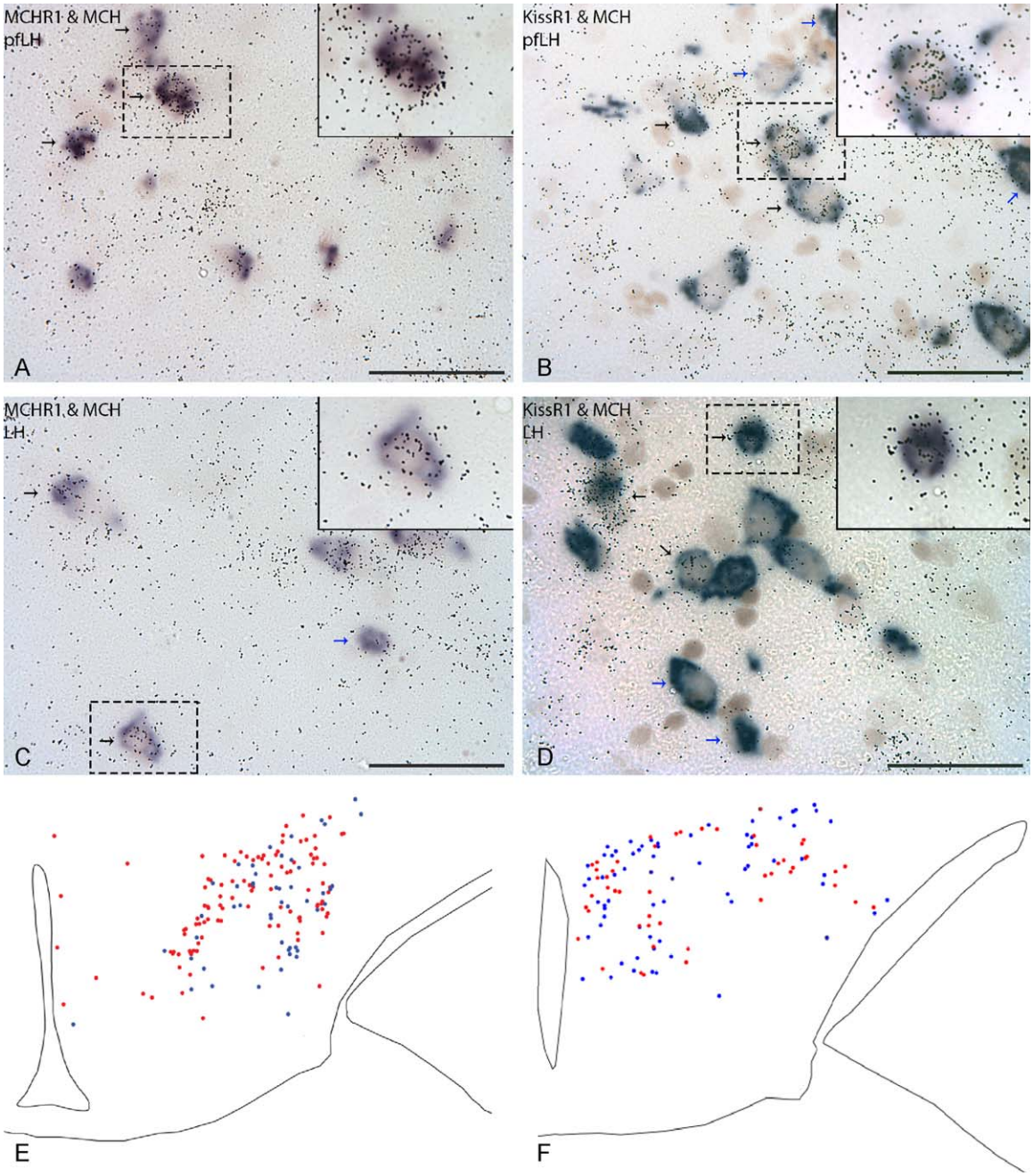

Figure 2. MCHR1 and KissR are expressed by MCH neurons. Double in situ hybridization of pMCH and MCHR1 or KissR1. **A:** MCHR1 in the perifornical lateral hypothalamus (pfLH). **B:** KissR1 in the pfLH. **C:** MCHR1 in the lateral portions of the LH. **D:** KissR1 in in the lateral portions of the LH. **E:** Diagram of the distribution pattern of MCHR1 colocalization. **F:** Distribution of KissR1-expressing MCH neurons. Black arrows show examples of colocalization, and blue arrows point toward representative MCH neurons that do not colocalize with MCHR1 or KissR. **Insets** in top right of some panels represent high-power microscopy images of select MCH neurons within the rectangle. In the diagrams, red dots represent MCH neurons that colocalize with the corresponding receptor mRNA (MCHR1, KissR), and blue dots signify MCH neurons that do not demonstrate colocalization. Scale bars = 50 μm.

reports of KissR distribution (Lee et al., 1999). Expression was high throughout much of the hypothalamus. KissR colocalized with MCH neurons in the ZI, pfLH (Fig. 2B), and LH (Fig. 2D). Approximately 50% of all MCH neurons were found to express KissR (Table 1), with double-labeled cells found throughout all MCH-expressing sugregions (Fig 2F). KissR expression in the LH and ZI was not limited to MCH neurons; dense

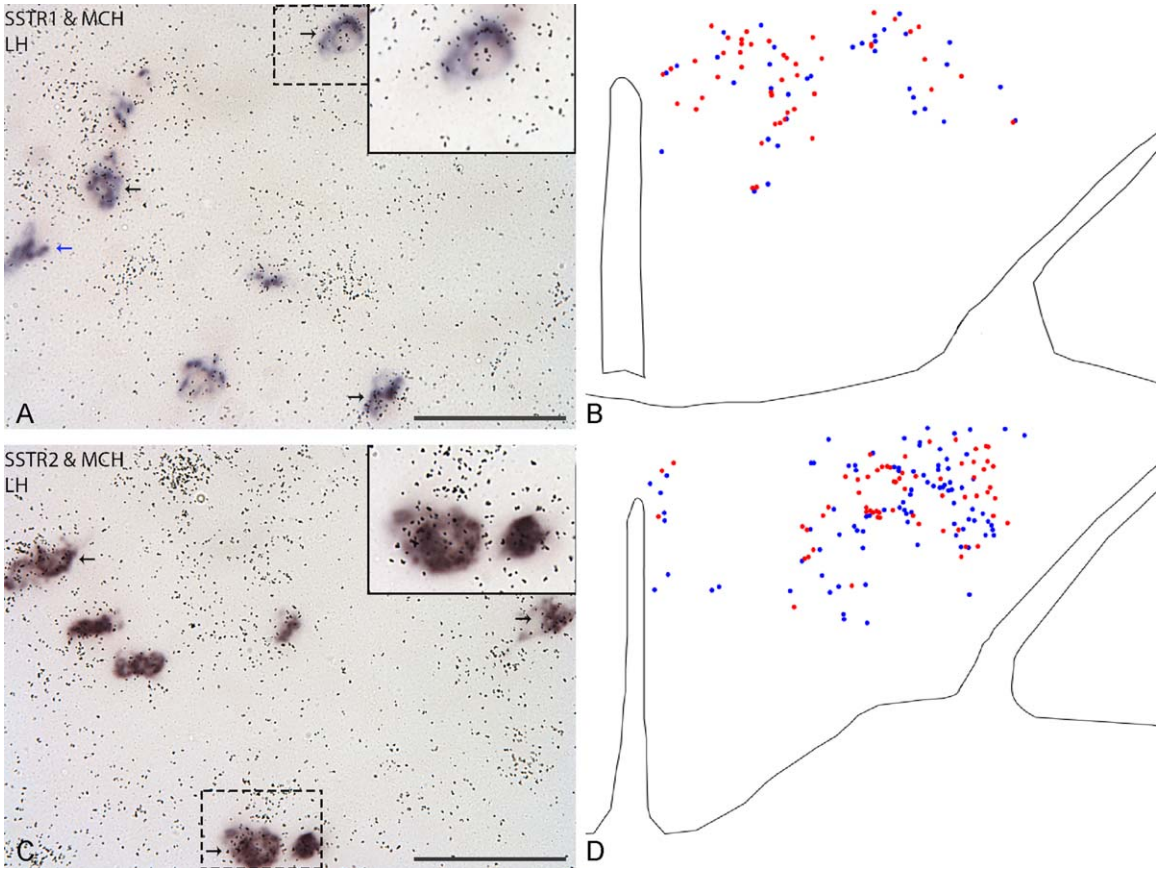

Figure 3. SSTR1 and SSTR2 are expressed by MCH neurons. Double in situ hybridization of pMCH with SSTR1 or SSTR2. **A:** SSTR1 in the lateral regions of the lateral hypothalamus (LH). **B:** Distribution pattern of SSTR1-expressing MCH neurons. **C:** SSTR2 in the LH. **D:** Diagram illustrating distribution of SSTR2 positive MCH neurons. Black arrows show examples of colocalization, and blue arrows point toward representative MCH neurons that do not colocalize with SSTR1 or SSTR2. In the diagrams, red dots represent MCH neurons that colocalize with the corresponding receptor mRNA (SSTR1, SSTR2), and blue dots signify MCH neurons that do not demonstrate colocalization. Scale bars = 50 μm.

labeling was also observed in clusters not expressing MCH. The sense control probe gave no specific signal, confirming the specificity of the antisense probe.

Somatostatin receptors

Somatostatin receptors (SSTR1 and SSTR2) were both found to be highly expressed in the LH and ZI. SSTR1 was expressed at high levels throughout the cortex and hippocampus, with moderate expression in the hypothalamus, whereas SSTR2 was highly expressed in the cortex, with more moderate expression the hippocampus and hypothalamus, which is consistent with published reports of both rat and mouse expression (Breder et al., 1992; Kong et al., 1994). Strong signal for both probes was observed in both MCH-positive and MCH-negative neurons (Fig. 3A,C) in the LH. Approximately 50% of all MCH neurons expressed SSTR1 (Table 1), but less colocalization was found in the lateral portions of the LH than in the pfLH or ZI (Fig. 3B). SSTR2 colocalization was lower than that of SSTR1 (Table 1); approximately 35% of MCH neurons expressed SSTR2. A higher degree of SSTR2 colocalization was observed in the lateral parts of the LH than in more medial subregions (Fig. 3D). Sense control probes exhibited minimal signal, confirming specificity of both antisense probes.

Neurotensin receptor 1

Neurotensin receptor 1 (NTSR1) was highly expressed in the LH and ZI, in agreement with published distribution patterns (Alexander and Leeman, 1998). Nearly one-third of all MCH neurons expressed NTSR1 (Table 1), with roughly similar levels of colocalization found throughout the subregions (Fig. 4A,C,E). Large clusters of NTSR1 signal were found in neurons that did not express MCH mRNA, indicating that NTSR1 is expressed in multiple cell types in the LH and ZI. Sense control

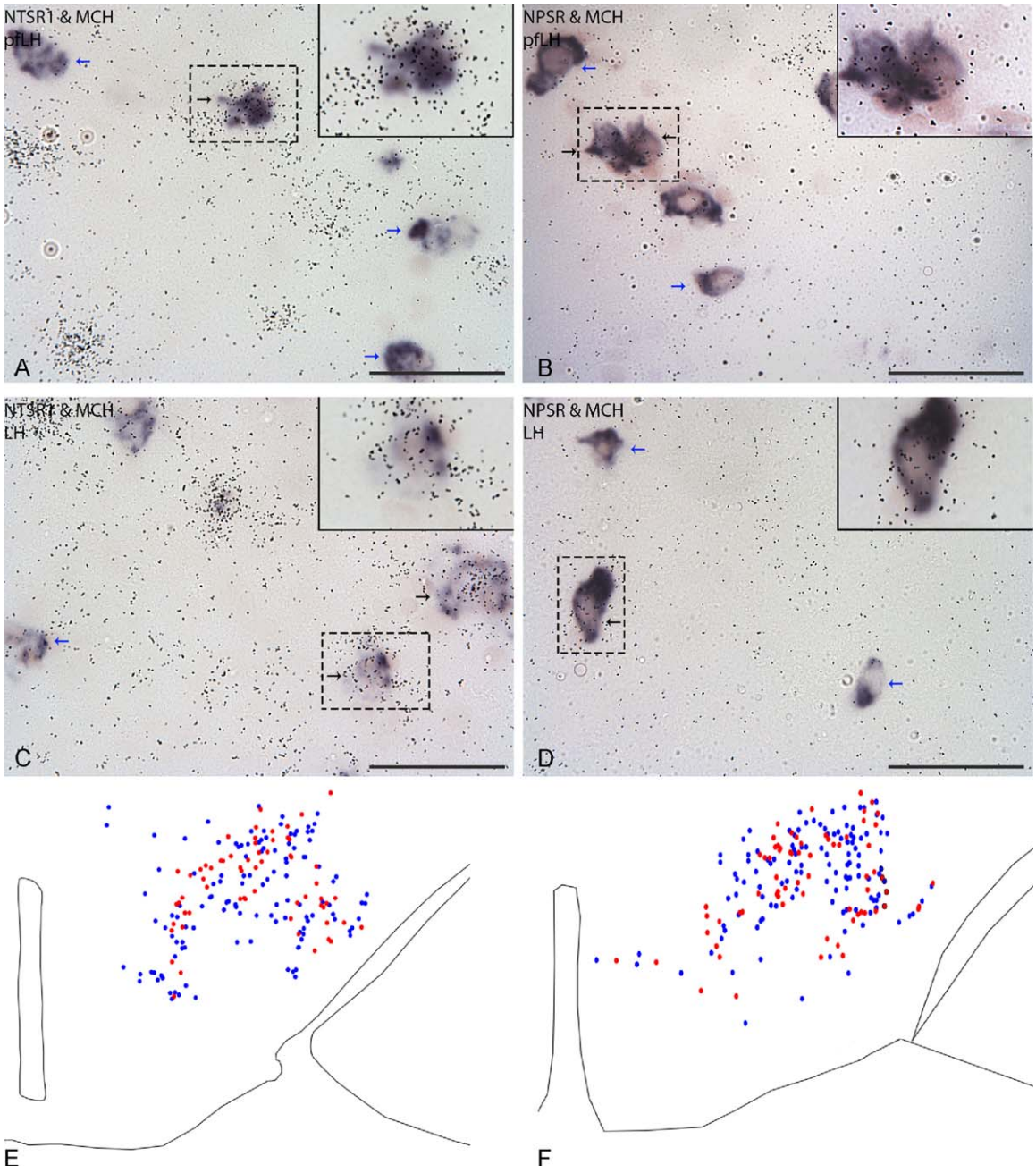

Figure 4. Double in situ hybridization of pMCH with NTSR1 and NPSR. Both NTSR1 and NPSR colocalize with some MCH neurons. **A:** NTSR1 in the perifornical lateral hypothalamus (pfLH). **B:** NPSR in the pfLH. **C:** NTSR1 in lateral portions of the lateral hypothalamus (LH). **D:** NPSR in the LH. **E:** Diagram illustrating distribution of NTSR1-positive MCH neurons. **F:** Distribution of NPSR-expressing MCH neurons. Black arrows show examples of colocalization, and blue arrows point toward representative MCH neurons that do not colocalize with NTSR1 or NPSR. **Insets** in top right of some panels represents high-power microscopy images of select MCH neurons within the rectangle. In the diagrams, red dots represent MCH neurons that colocalize with the corresponding receptor mRNA (NTSR1, NPSR), and blue dots signify MCH neurons that do not demonstrate colocalization. Scale bars = 50 μm.

probe treated sections did not exhibit any specific signal, confirming specificity of the antisense probe.

Neuropeptide S receptor

Neuropeptide S receptor (NPSR) was widely expressed in the LH and ZI, with the densest signal found in the medial portions of the LH. Extensive colocalization was observed in all regions of MCH expression (Fig. 4B,D,F), with a somewhat higher proportion of MCH neurons expressing NPSR in the pfLH than in the lateral parts of the LH (Fig. 4F). Heavy NPSR probe labeling was observed in MCH-negative areas of the LH and ZI, indicating that NPSR expression was not

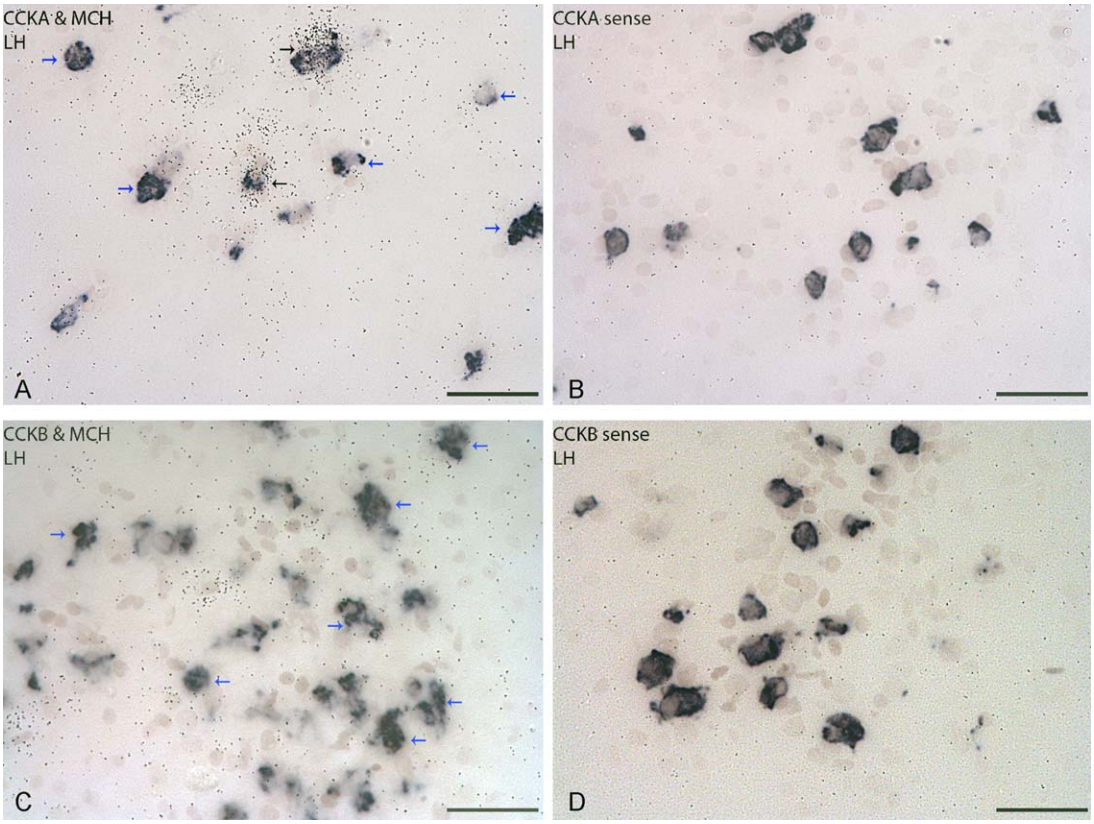

Figure 5. CCKAR weakly colocalizes with MCH. CCKBR does not colocalize with MCH. **A:** CCKAR in the lateral portions of the lateral hypothalamus (LH). **B:** CCKAR sense probe. **C:** CCKBR in the LH. **D:** CCKBR sense probe. Black arrows show examples of colocalization, and blue arrows point toward representative MCH neurons that do not colocalize with CCKA/B receptors. Scale bars = 50 μm.

exclusive to MCH neurons. Sections hybridized with a sense probe exhibited minimal signal, confirming specificity of the antisense probe.

Cholecystokinin receptors

Both cholecystokinin receptors (CCKAR and CCKBR) exhibited distribution patterns consistent with their reported expression (Honda et al., 1993), with moderate CCKAR and low CCKBR expression in the LH and ZI. CCKAR coexpression with MCH was moderate (Fig. 5A, Table 1), whereas CCKBR was not expressed by MCH neurons (Fig. 5C). Interestingly, small populations of MCH neurons exhibited dense CCKAR expression, but most colocalizing cells displayed only moderate CCKAR signal. Sections hybridized with sense strand control probes for CCKAR or CCKBR displayed no signal (Fig. 5B,D), confirming specificity of both antisense probes.

Opioid receptors

Opioid receptor (KOR, MOR, DOR) expressions were all consistent with known distribution patterns (George et al., 1994; Mansour et al., 1987). In the LH and ZI, KOR was expressed at high levels, MOR moderately, and DOR only minimally. KOR exhibited moderate colocalization with MCH (Fig. 6A, Table 1). MOR coexpression with MCH was minimal (Fig. 6B), although a few small clusters of MCH neurons did express MOR in the LH. DOR was found in only a few MCH neurons (Fig. 6C). Sense-probe-treated sections did not exhibit significant labeling for any of the probes.

NPY and melanocortin receptors

NPY and melanocortin receptor (NPY5R, MC3R, MC4R) mRNAs were distributed throughout the brain in patterns consistent with published reports (Kishi et al., 2003; Parker and Herzog, 1999; Roselli-Rehfuss et al., 1993). NPY5R was expressed at high levels in the arcuate nucleus but exhibited low expression levels in the LH and ZI. MC3R and MC4R were expressed in a variety of hypothalamic nuclei but exhibited minimal expression in the LH and ZI. NPY5R colocalized with only a very small number of MCH neurons (Fig. 7A, Table 1), and MC3R and MC4R were generally not detected in MCH neurons (Fig. 7C,D). No signal was given by sense control probes, confirming specificity of the antisense probes.

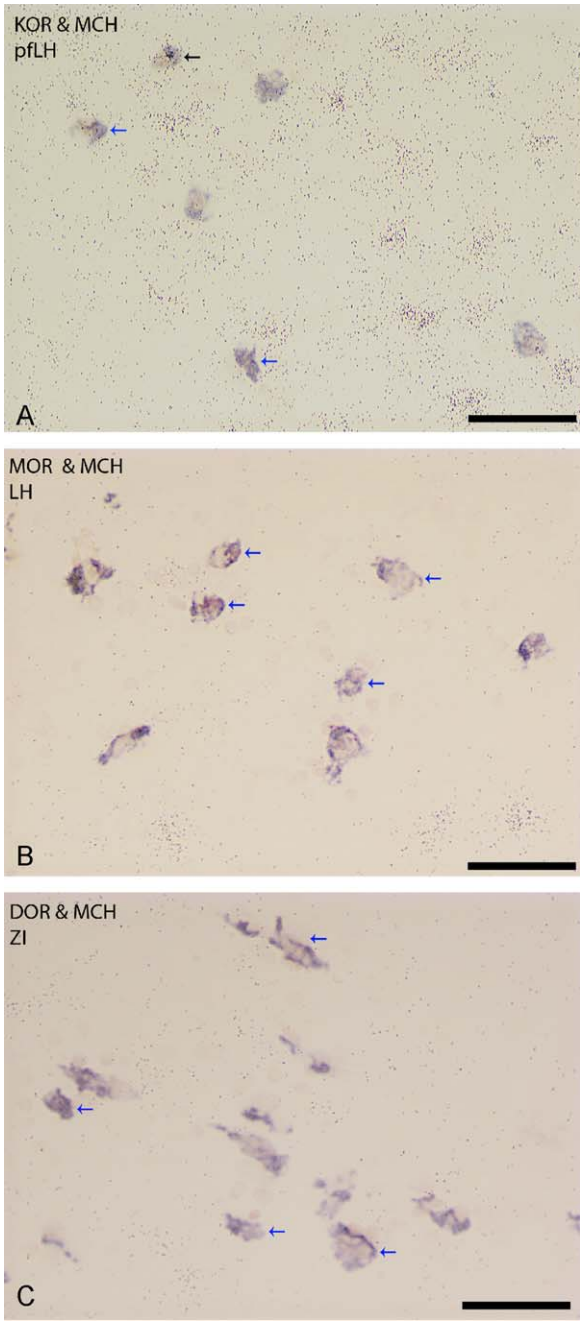

Figure 6. KOR is expressed by some MCH neurons, but MOR and DOR do not colocalize with MCH neurons. **A:** KOR in the perifornical lateral hypothalamus (pfLH). **B:** MOR in the lateral portions of the lateral hypothalamus (LH). **C:** DOR in the zona incerta (ZI). Black arrows show examples of colocalization, and blue arrows point toward representative MCH neurons that do not colocalize. Scale bars = 50 μm.

Galanin receptor 1

Galanin receptor 1 (GalR1) was expressed in a pattern similar to published reports (O'Donnell et al., 1999). GalR1 was highly expressed in the LH and ZI, but minimal colocalization with MCH was detected (Fig. 7B, Table 1).

Corticotropin-releasing factor receptors

Corticotropin-releasing factor receptor 1 (CRFR1) was expressed at moderate levels and CRFR2 at low levels in the LH and ZI, consistent with published distribution patterns (Chalmers et al., 1995). CRFR1 exhibited low levels of colocalization with MCH (Fig. 7E, Table 1), and CRFR2 colocalization with MCH was negligible (Fig. 7F, Table 1).

DISCUSSION

A description of the receptors expressed by MCH neurons is an important step in elucidating the regulatory circuitry of MCH neurons. Because the MCH system is involved in physiological processes such as feeding (Borowsky et al., 2002; Qu et al., 1996; Shimada et al., 1998), metabolism (Chung et al., 2010), anxiety and depression-like behavior (Garcia-Fuster et al., 2012; Roy et al., 2006), addiction (Chung et al., 2009), and sleep (Jego et al., 2013; Konadhode et al., 2013; Verret et al., 2003), identifying how the MCH system is regulated will provide insight into how these fundamental processes operate. In the present study, double in situ hybridization was used to identify neuropeptide receptors that are expressed by MCH neurons in the LH and ZI. MCH expression was detected by using a DIG-labeled ppMCH antisense RNA probe, neuropeptide receptors expression by using ^{35}S-labeled antisense probes.

General

The anatomical distribution of MCH DIG-labeled cells was found to be very similar to that reported from earlier studies showing that MCH precursor expression is mostly limited to the LH and ZI (Bittencourt et al., 1992; Hahn, 2010; Swanson et al., 2005) in the rat. This limited regional distribution stands in contrast to the expansive projection network of MCH neurons (Bittencourt et al., 1992) and widespread distribution of the primary MCH receptor, MCHR1 (Hervieu et al., 2000; Saito et al., 2001). It should be noted that the MCH precursor also gives rise to two other neuropeptides, NGE and NEI (Bittencourt and Celis, 2008), that are not well understood and may be important for the functions of the MCH system. Rats used in this study were not all of uniform size (200–400 g), and it is unknown how this might influence the receptor colocalization levels.

Neuropeptide receptors with high levels of colocalization

The neuropeptide receptor most commonly found to be expressed by MCH neurons is the NOP receptor

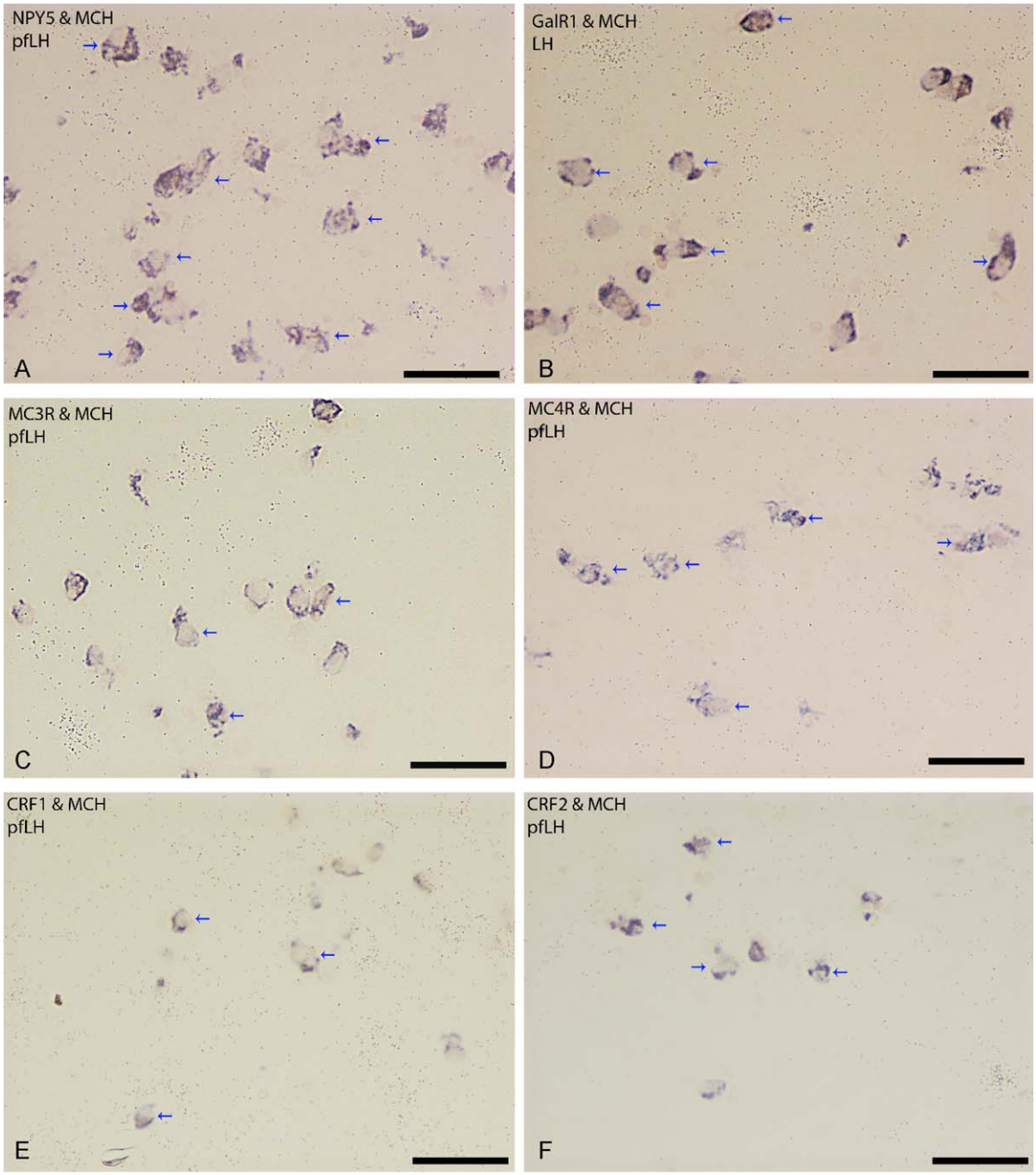

Figure 7. NPY5R, MC3R, MC4R, GalR1, CRF1, and CRF2 do not colocalize with MCH neurons. **A:** NPY5R in the perifornical lateral hypothalamus (pfLH). **B:** GalR1 in the lateral portions of the lateral hypothalamus (LH). **C:** MC3R in the pfLH. **D:** MC4R in the pfLH. **E:** CRF1 in the pfLH. **F:** CRF2 in the pfLH. Blue arrows point toward representative MCH neurons that do not colocalize with receptor mRNA. Scale bars = 50 μm.

(Table 2). Nearly 90% of MCH neurons express NOP, indicating that most MCH neurons may be under the regulation of nociceptin/orphanin FQ (OFQ) and suggesting that OFQ may be critical to the regulation of the MCH system. Indeed, a recent study has indicated that OFQ exerts an inhibitory effect on MCH neurons mediated via GIRK channels (Parsons and Hirasawa, 2011) in brain slices. OFQ has been hypothesized to be released from neighboring orexin/hypocretin neurons (Parsons and Hirasawa, 2011), but the origin of OFQ efferents to MCH neurons is currently unknown. OFQ is anxiolytic and inhibits the rewarding effects of drugs of abuse (Jenck et al., 1997; Kotlinska et al., 2003; Sakoori and Murphy, 2004), whereas MCH is anxiogenic and promotes drug reward

TABLE 2.
Summary of Receptor Colocalization With MCH Neurons

High, >30%	Moderate, 10–30%	Low <10%
NOP	NPSR	MOR
MCHR1	NTSR1	DOR
KissR	CCKAR	CCKBR
SSTR1	KOR	NPY5R
SSTR2		MC3R
OXR1		MC4R
OXR2		GalR1
		CRF1
		CRF2

(Borowsky et al., 2002; Chung et al., 2009; Smith et al., 2006), so it is possible that the inhibition of MCH neurons is one pathway through which OFQ exerts these effects.

The neuropeptide receptor exhibiting the second highest level of colocalization with MCH neurons is MCHR1 itself. Over 60% of MCH neurons expressed significant levels of MCHR1. This is in agreement with previous reports (Gao et al., 2003; Gao and van den Pol, 2002) that the MCH system exerts some form of autoregulation. Application of MCH to MCH neurons is known to depress high voltage-activated (HVA) calcium channels but has no effect on membrane potential or other electrophysiological properties (Gao et al., 2003; Gao and van den Pol, 2002). Whether these receptors are presynaptic autoreceptors or are expressed postsynaptically cannot be determined from the present study, but our data provides further evidence that the MCH system engages in autoregulation.

Kiss is a critical mediator of GnRH release (Han et al., 2005) and is involved in regulating the initiation of puberty (Han et al., 2005; Navarro and Tena-Sempere, 2011) and fertility (Clarkson et al., 2008; Pineda et al., 2010). MCH has been implicated in the regulation of pituitary gonadotropin release (Gonzalez et al., 1997; Tsukamura et al., 2000) and has been demonstrated to inhibit a subpopulation of GnRH-expressing neurons (Wu et al., 2009) and to inhibit their activation by Kiss. Our results indicate that approximately 50% of MCH neurons express KissR1, indicating that Kiss is likely to regulate the activity of the MCH system. Though speculative, this suggests that Kiss may form a circuit where it activates MCH neurons, thereby increasing MCH release at terminals, which would in turn inhibit activity of GnRH neurons. Interestingly, anterograde tracing studies have demonstrated that Kiss-expressing neurons in the arcuate nucleus project to the LH (Yeo and Herbison, 2011), indicating that direct regulation of the MCH system by Kiss may occur. It is worth noting that high levels of KissR expression were also observed in MCH-negative regions of the LH and ZI, indicating that Kiss may also regulate other types of neurons in the LH and ZI.

Approximately half of MCH neurons were found to express SSTR1, and over 35% expressed SSTR2. The colocalization of two different somatostatin receptors with MCH indicates that somatostatin is likely to regulate the MCH neurons. The role of SSTR1 in the nervous system is only beginning to be understood, but some evidence suggests that it may be involved in reward, (Schulz et al., 2000; Semenova et al., 2010; Vasilaki et al., 2004), a function that is well known to involve the MCH system (Chung et al., 2009; Georgescu et al., 2005; Sears et al., 2010). The functions of SSTR2 in the central nervous system are better characterized. SSTR2 modulates some of the same physiological functions as the MCH system, including food intake (Stengel et al., 2010a,b), metabolism, and anxiety and depression-like behavior (Engin et al., 2008; Engin and Treit, 2009). A study reporting that somatostatin binds in the LH in a SSTR2-dependent manner (Videau et al., 2003) supports this putative link between SSTR2 and the MCH system. SSTR2 is generally inhibitory, which may indicate that somatostatin blocks activity of MCH neurons through SSTR2. Central administration of somatostatin is known to be anxiolytic and antidepressant (Engin and Treit, 2009), whereas MCH administration is anxiogenic (Smith et al., 2006) and promotes depression-like behavior (Georgescu et al., 2005), raising the possibility that somatostatin modulates affective states by inhibiting the MCH system.

OXR2 was found to be expressed in a larger proportion of MCH neurons than was OXR1 (Table 1), though both strongly colocalized with the MCH system. Orexin/hypocretin neurons are known to excite MCH neurons (van den Pol et al., 2004). It has been suggested that this excitatory effect is mediated primarily through OXR2, which is consistent with the high degree of OXR2 colocalization found in our study. However, our observation that MCH neurons express both ORX receptors suggests that orexinergic regulation of the MCH system is not mediated entirely by OXR2, thus adding another dimension to the interactions between the two systems.

Neuropeptide receptors with moderate levels of colocalization

Four neuropeptide receptors, NPSR, NTSR1, CCKAR, and KOR, were found to be expressed by 10–30% of MCH neurons (Table 2). The NPS system is involved in arousal, memory, fear and anxiety, and reward responses (Kallupi et al., 2010; Xu et al., 2004). NPSR has recently been demonstrated to modulate cue-induced cocaine relapse responses by activating the

orexin/hypocretin system (Kallupi et al., 2010). In the same study, central injection of NPS was found also to activate neurons in the LH that do not express orexin/hypocretin, which is consistent with the hypothesis that NPS also activates MCH neurons. MCHR1 is strongly expressed throughout the locus coeruleus region (Saito et al., 2001), with an expression pattern that may overlap that of NPS-producing neurons (Xu et al., 2004).

Neurotensin has been linked to physiological functions that overlap with those of the MCH system, such as food intake (Luttinger et al., 1982) and dopamine signaling (Leinninger et al., 2011; Nemeroff et al., 1982), and both may be involved in psychiatric disorders (Caceda et al., 2006; Chung et al., 2011). A recent study indicated that a population of leptin-receptor-expressing neurotensin neurons in the LH innervates orexin/hypocretin neurons and modulates their response to fasting (Leinninger et al., 2011), but these neurons do not project directly to MCH neurons. In light of this, we suggest that neurotensin neurons originating in a different brain region(s) may regulate the MCH system.

We found that some MCH neurons express CCKAR, but practically none express CCKBR. CCKAR couples to G_q/G_s and would be expected to excite MCH neurons upon activation. CCK is involved in a variety of physiological functions, some of which overlap with those of the MCH system. CCK is anxiogenic (de Montigny, 1989; Murray et al., 2006), and icv MCH injections promote anxiety-like behavior (Smith et al., 2006). Paradoxically, CCK has also been reported to suppress feeding (Schick et al., 1986), whereas MCH is orexigenic, so how the two systems might interact to regulate feeding behavior is unclear.

KOR exhibits moderate levels of colocalization ($\sim$10$-$16%). This finding corroborates a previous report indicating that MCH neurons are inhibited by dynorphin (Li and van den Pol, 2006). Although the fraction of MCH neurons sensitive to dynorphin was not determined in that study, it is assumed that a large proportion of MCH neurons tested was inhibited by the peptide. However, we found that KOR colocalizes with only a minority of MCH neurons. This potential discrepancy could result from KOR being expressed by most MCH neurons at levels below our detection threshold. In any case, the present study indicates that some MCH neurons express and may be regulated by KOR.

Neuropeptide receptors with very low levels of colocalization

Eight neuropeptide receptors were found to exhibit very low levels of colocalization with MCH neurons (Table 2). These are MOR, DOR, CCKBR, the two CRF receptors, GalR1, NPY5R, and the two melanocortin receptors (MC3R and MC4R).

Our data indicate that MOR coexpresses with MCH in only a few neurons and that DOR colocalization was almost entirely absent. A recent study has suggested that the orexigenic and reward promoting effects of MCH may require interactions with the endogenous opioid system (Lopez et al., 2011). Our data indicate that, although KOR may regulate MCH neurons, it is unlikely that MOR or DOR does so directly.

Despite the effects of MCH in the regulation of anxiety-like behavior (Borowsky et al., 2002; Lee et al., 2011; Roy et al., 2006), and the distribution of MCHR1 in brain regions involved in stress response (Saito et al., 2001), neither of the two CRF receptors was found to coexpress significantly with MCH neurons.

GalR1 was expressed at high levels in the LH but exhibited minimal colocalization with MCH. These data suggest that GalR1 does not directly modulate the activity of MCH neurons. In agreement with our data, galanin-like peptide (GLP), an endogenous ligand for GalR1, has previously been reported to activate orexin/hypocretin but not MCH-expressing neurons (Kageyama et al., 2006).

Previous authors have hypothesized that the MCH system may function as "second-order neurons" that receive signals from POMC and NPY neurons in the arcuate nucleus and regulate energy homeostasis and emotional states based on inputs received from these neurons (Guyon et al., 2009; Saito and Nagasaki, 2008). NPY5R, MC3R, and MC4R are generally believed to be associated with the central regulation of food intake. In support of this hypothesis, a single-cell RT-PCR study has indicated that both NPY5R and MC4R are expressed by MCH neurons (Harthoorn et al., 2005), and NPY has been reported to inhibit MCH neurons in brain slices (van den Pol et al., 2004). We have found that very few MCH neurons express NPY5R, MC3R, or MC4R. The agreement between the reported receptor expression levels and our detected signal in all brain regions examined indicates that the lack of signal observed in MCH neurons is unlikely to be the result of poor hybridization efficiency or probe labeling. This suggests that POMC neurons do not directly regulate the MCH system through α-MSH. This is supported by studies indicating that MC4R is not coexpressed with MCH in mice expressing GFP coupled to MC4R expression (Cui et al., 2012; Liu et al., 2003) and another reporting that melanocortin receptor agonists do not functionally affect MCH neuron activity (van den Pol et al., 2004). More difficult to understand, however, is our finding that very few MCH neurons express NPY5R, which seems to be in conflict with a report that NPY inhibits

MCH neurons both directly and indirectly (van den Pol et al., 2004). Published studies suggest that NPY5R, MC3R, and MC4R are expressed at only low levels in the LH (Kishi et al., 2003; Parker and Herzog, 1999; Roselli-Rehfuss et al., 1993), which is in agreement with our findings. It is certainly possible that a different NPY receptor is the target of NPY on MCH neurons. It has been reported that NPY4R is expressed by and regulates orexin/hypocretin neurons and is also present in populations of neurons in the LH that are negative for orexin/hypocretin (Campbell et al., 2003), raising the possibility that it may also regulate MCH neurons. It is also possible that NPY5R is expressed in MCH neurons at levels too low to be detected by in situ hybridization but is abundant enough to modulate MCH neuron activity. We can only conclude that our data show that NPY5R, MC3R, and MC4R do not strongly colocalize with MCH, which raises questions about the mechanism underlying how NPY regulates the MCH system and about the relevance of α-MSH to the regulation of the MCH system.

CONCLUSIONS

The present study used an in situ hybridization colocalization analysis of 20 neuropeptide receptors to gain insight into the regulatory circuitry of the MCH system. We found that several receptors believed to regulate MCH neurons are in fact highly expressed by MCH neurons, thus confirming previous reports indicating their involvement with the MCH system. We also found that some receptors for peptides thought to regulate the MCH system did not colocalize with MCH to any significant degree. Additionally, we have identified eight neuropeptide receptors that exhibit little to no colocalization with MCH and are thus unlikely to regulate MCH neurons directly. We have also found that MCH neurons express six neuropeptide receptors that have never before been linked to the MCH system and may represent novel regulatory circuits. These candidate genes can now be studied systematically to better understand the circuitry of MCH system and the numerous physiological processes it regulates.

ACKNOWLEDGMENTS

We are particularly grateful to Krupa Gohil, Rio Hoshi, Jonathan Zhao, Tracy Lu, Quyen Dang, and Nina Rastegar for excellent technical support and to Nayna Sanathara and Dr. Yan Zhang for critical discussion of the manuscript. We also thank Drs. Rainer Reinscheid (UC Irvine), Tali Baram (UC Irvine), Frances Leslie (UC Irvine), Stanley Watson (Michigan), Joel Elmquist, and Herbert Herzog (Garvan Institute of Medical Research) for supplying us with probes. We also acknowledge the Sigma Alpha Mu foundation for support provided to G.S.P. O.C. is holder of the Nelson Chair of Pharmacology.

ROLE OF AUTHORS

GSP designed experiments, performed experiments, analyzed data, and wrote the paper. LW performed experiments. ZW constructed probes. OC designed experiments and wrote the paper.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

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