Neurons Synthesizing Melanin-Concentrating Hormone Identified by Selective Reporter Gene Expression After Transfection In Vitro: Transmitter Responses

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INTRODUCTION

Neurons that synthesize the peptide melanin-concentrating hormone (MCH) are found scattered throughout the perifornical/lateral hypothalamic (LH) region of the brain and have no obvious identifying morphological characteristics. The cells receive inputs from many sources and project widely throughout the brain and spinal cord (Bittencourt et al. 1992). A wealth of data support the view that MCH neurons may play an important role in food intake and energy homeostasis. Intracerebral administration of MCH evokes feeding in rodents (Qu et al. 1996; Rossi et al. 1997). MCH-deficient mice have reduced body weight and lean mass due to decreased feeding and an enhanced metabolism (Shimada et al. 1998). Similarly, MCH receptor-deficient mice are lean, hyperphagic, and have altered metabolism (Marsh et al. 2002). Expression of MCH and c-fos mRNA increases in MCH neurons in starving animals (Qu et al. 1996) and after leptin administration (Huang et al. 1998). MCH neurons may also play a role in arousal (Marsh et al. 2002), depression (Borowsky et al. 2002), and modulation of memory (Monzon et al. 1999). The neurotransmitter response properties of these neurons have not been reported, in part due to the difficulty in identifying them (Eggermann et al. 2003). Because the MCH neurons make up only a small percentage of the cells in the LH/perifornical area, a method to select live cells of a particular transmitter phenotype prior to recording would be an asset because neurons of different transmitter phenotypes may show substantive differences in cellular physiology.

In this study, we identified the MCH neurons in hypothalamic neuronal culture by transfecting neurons with a green fluorescent protein (GFP) or red fluorescent protein (dsRed2) gene driven by the MCH promoter. We confirmed the selectivity of the reporter gene expression and investigated neuro-modulation of MCH neurons in culture. The gene transfection approach described here may be useful to identify other neuronal phenotypes in complex neuronal cultures for the purpose of cellular recording. The transfection procedure is simple and can be done readily in any neurophysiology laboratory.

METHODS

Construction of dsRed2 and GFP plasmids driven by the MCH promoter

The MCH promoter sequence was synthesized by PCR from a plasmid containing the rat MCH gene, kindly provided by Dr. R. Thompson (Thompson and Watson 1990). Two synthetic oligomers from the 5′ and 3′ end of the MCH promoter were synthesized: 5′-TCAGATCTCGAGTCTAGAGATAACTTCTATTTAATAAGG-3′ and 5′-CTGAGAATTCCTCGTTTGCTCTCCGTAACCAGAGAGAG-3′ and used as primers for PCR amplification. The former oligomer contained the XhoI restriction site and the later an EcoRI site. A 470-bp DNA band was obtained as expected. This PCR product was cloned in a TA-TOPO cloning system and the sequence verified. This promoter-containing plasmid was digested with XhoI and EcoRI, and gel purified to obtain a 460-bp MCH promoter fragment. This fragment was ligated to a promoterless 4.1-kb XhoI and EcoRI digested pDsRed2–1 plasmid or to the 4.2-kb pEGFP1 plasmid (Clontech) to obtain dsRed or GFP plasmids driven by the MCH promoter.

Cell culture, transfection, and immunostaining of MCH

Neuronal cultures were prepared from rat embryos as described previously (Gao and van den Pol 2001). Use of animals was approved by the University Committee on Animal Use. A serum-free culture...
medium contained neurobasal medium (Gibco), 5% B27 supplement (Gibco), 0.5 mM L-glutamine, 100 units/ml penicillin-streptomycin, and 6 g/ml glucose. Neurons were fed twice a week.

MCH-dsRed, MCH-GFP, and control cytomegalovirus (CMV)-GFP constructs were transfected into neurons after 10–14 days in vitro using lipofectamine 2000 (Invitrogen, Gaithersburg, MD) according to the supplier’s recommendations.

Immunostaining of MCH was performed 3 days after the transfection of MCH-dsRed and MCH-GFP constructs. Briefly, neuronal cultures were fixed with 4% of paraformaldehyde, washed in phosphate-buffered saline (PBS) containing 0.4% of Triton X-100, and blocked with 2% normal goat serum in PBS. Cultures were incubated with rabbit primary antibody against MCH that has been previously characterized (Bittencourt et al. 1992) (gift of Dr. P. Sawchenko). The next morning, neurons were incubated with an Alexa 488 or Texas Red-conjugated goat antirabbit IgG (1:100, Molecular Probes).

**Electrophysiological recording**

A L/M EPC-7 amplifier was used to record cultured neurons after 14–21 days in vitro and within 2–7 days after the transfection. The recording chamber was continuously perfused at a 2 ml/min with a bath solution containing (in mM) 150 NaCl, 2.5 KCl, 2 CaCl₂, 10 HEPES, and 10 glucose, pH 7.3 with NaOH. The tip resistance of the recording pipettes was 4–6 MΩ, and the pipette solution contained (in mM) 145 KCl (KMeSO₄), 1 MgCl₂, 10 HEPES, 1.1 EGTA, 2 Mg-ATP, and 0.5 Na₂-GTP, pH 7.3 with KOH. After a gigaohm seal and whole cell access were achieved, the series resistance was between 15 and 30 MΩ and partially (40–60%) compensated by the amplifier. Only those recordings with stable series resistance recorded throughout the experiments were accepted. Passive membrane properties were tested under current clamp. Voltage-dependent calcium currents were monitored as described previously (Gao and van den Pol 2002).

Data were sampled at 10 kHz, filtered at 5 kHz using AxoData 1.2.2 (Axon Instruments), and analyzed with Axograph 3.5 (Axon Instruments) and plotted with Igor Pro software (WaveMetrics, Lake Oswego, OR). Data are expressed as means ± SE.

MCH was from Phoenix Pharmaceuticals (Belmont, CA), neuropeptide Y (NPY) was from Bachem (King of Prussia, PA), 2-amino-5-phosphons-pentanoic acid (AP5), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and bicuculline methobromide (BIC) were from SIGMA-RBI (St. Louis, MO), and TTX was from Alomone Labs. KCl (KMeSO₄), L-glutamate, and and partially (40–60%) compensated by the amplifier. Only those recordings with stable series resistance recorded throughout the experiments were accepted. Passive membrane properties were tested under current clamp. Voltage-dependent calcium currents were monitored as described previously (Gao and van den Pol 2002).

**RESULTS**

**Neurons containing MCH in cultures: specificity of the constructs**

GFP appeared in neurons 18 h after the initial transfection, whereas dsRed took longer, appearing 48 h after the initial transfection. As expected, only a small percentage of LH neurons expressed GFP or dsRed under the influence of MCH promoter. Using 1 µg DNA/ml to transfect neurons, we found that the number of red or green neurons varied between 20 and 60 in each 35-mm petri dish, with about 200,000 total cells per dish. The mean number of neurons expressing the reporter gene (4 experiments) was 46 neurons per 200,000 neurons. As shown in Fig. 1, dsRed or GFP was expressed throughout positive neurons, including in the cell body, dendrites, and dendritic spines and appendages, and in the axon and axon terminals (Fig. 1, C and D). Cells survived for several weeks after transfection, with continuous reporter gene expression. For electrophysiology, we generally used the cells within a few days of transfection.

In the second set of experiments, we confirmed that neurons expressing GFP under the influence of MCH promoter synthesized the MCH peptide. Two days after transfection, green neurons were observed in LH neuronal cultures under fluorescent illumination. We fixed and stained for MCH antigen using a red immunosignal. There were an average of 510 MCH immunoreactive neurons (red in Fig. 2, B and C) in 200,000 neurons as calculated. All green neurons expressing GFP under the influence of the MCH promoter also showed immunocytochemical staining for MCH (Fig. 2, A and C), as shown in Fig. 2C. Additional neurons were immunoreactive for MCH but showed no GFP expression. Based on the number of neurons that were immunoreactive for MCH and the number that expressed the reporter gene, our data suggest that about 10% of the total number of MCH neurons were identified by the GFP or dsRed2 under control of the MCH promoter.

To test further the specificity of the constructs, we transfected neurons from other brain regions that do not contain MCH neurons, i.e., the cortex, with the construct containing dsRed driven by the MCH promoter. We also used a control construct containing GFP driven by a CMV ie1 promoter. As
expected, both MCH-dsRed and CMV-GFP constructs were expressed in the LH neuronal cultures (Fig. 3). There were more green neurons (with CMV promoter) than red (MCH) neurons (Fig. 3, A and B) because the CMV-GFP construct did not have specificity and could express in any type of neuron. In some cases, red and green fluorescent signals could be observed in the same neuron (Fig. 3 C). In contrast, in the cortical cultures, no reporter gene expression was found with the MCH promoter, but expression was detected in control experiments using the CMV promoter (Fig. 3, D–F). In additional experiments, we combined cultures of cortex, hippocampus, striatum, and thalamus and found no reporter gene expression under the MCH promoter. These experiments suggest that transfection with a reporter gene under control of the MCH promoter is selective for hypothalamic neurons that are immunoreactive for MCH. We therefore used this transfection procedure to identify live MCH neurons to study their electrophysiological properties and transmitter responses in vitro. In addition, we compared axon outgrowth of MCH neurons with cerebellar granule cells, both transfected with GFP and both in culture for 5 days. MCH axons were quite long (1,118.3 ± 117.7 μm, n = 11) compared with age-matched granule cell axons (239.2 ± 63.7 μm, n = 11); this was statistically significant (P < 0.05, t-test). The MCH axons continued to elongate, and after 10 days, some were >5,000 um in length.

**Membrane properties of MCH neuron**

In green MCH neurons, passive membrane properties were tested under current clamp. Neurons were held at resting membrane potential, and a series of current pulses (from hyperpolarizing pulses to depolarizing pulses, −20 pA amplitude with increment of 5 pA, 600-ms duration) were injected to the recorded neurons (Fig. 4A). The linear current-voltage (I-V) relationship obtained for each neuron and membrane resistance, determined by calculating the slope, was 1,013 ± 117 MΩ (n = 21). There was no sag (membrane rectification) during a series of hyperpolarizing current injections (Fig. 4A), indicating the probable absence of I_h current parallel to recordings in slices (Eggermann et al. 2003). The resting membrane potential was −57.5 ± 0.6 mV (n = 44), and spontaneous spike frequency was low (<0.25 Hz). The action potential threshold was −31.2 ± 2.4 mV (n = 11).

For control purposes, we compared membrane potential, input resistance, and spike threshold in cells transfected with GFP driven by a CMV promoter and nontransfected controls. No significant difference between the two groups was found, suggesting that GFP expression did not alter cellular physiology; both groups were therefore combined and used as non-MCH controls. The mean resting membrane potential for non-MCH neurons was −56.4 ± 0.9 mV (n = 19), input resistance was 934.0 ± 84.3 MΩ (n = 13), and spike threshold was

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**Fig. 2.** Double labeling of MCH neurons in culture. A: green fluorescent protein (GFP) expression in MCH neuron. B: immunocytochemical staining reveals MCH immunoreactive neurons (in red). C: green (GFP gene) and red (immunopositive) signals overlap in 2 neurons indicated by arrows, suggesting that they are MCH neurons. Scale bars: A–C, 15 μm.
–34.4 ± 1.8 mV (n = 12). None of these three values were different from those obtained from MCH neurons (P > 0.05).

During extended depolarization, spike frequency adaptation was observed in MCH neurons (Fig. 4C, bottom trace). The interval between the first two spikes was 48.3 ± 8.4 ms and increased to an 85.7 ± 14.8-ms interval between the last two spikes (n = 10 neurons). The difference was very significant (t-test; P < 0.001). Action potential firing was depressed by more intense depolarization (Fig. 4C, top trace).

Response of MCH neurons to neurotransmitters

We studied the neurotransmitter responses of identified MCH neurons. Glutamate (10 μM) triggered action potentials and depolarization in MCH neurons (n = 6; Fig. 4D). In contrast, GABA (10 μM) reversibly inhibited MCH neurons (n = 5) by hyperpolarizing the membrane potential (Fig. 4E). All MCH neurons tested showed substantial responses to GABA and glutamate, as expected.

Norepinephrine (NE) is found in axons throughout the brain and in high density in the region of the hypothalamus containing the MCH neurons (Wellman 2000). NE was applied to the recorded neuron through a flow pipe aimed at the neuron. NE (50 μM) hyperpolarized the membrane potential in all MCH neurons tested (n = 7; Fig. 4F) from −56.3 ± 1.6 to −61.3 ± 1.5 mV; neurons recovered to −54.5 ± 2.3 mV after washout of NE (ANOVA; P < 0.05). To determine if NE exerted a direct effect on membrane potential in MCH neurons, we studied the effect of NE in TTX (1 μM in all solutions). In the presence of TTX, the membrane potential was significantly hyperpolarized from −60.7 ± 2.0 to −66.2 ± 2.3 mV by NE and returned to −60.8 ± 2.5 mV after the washout of NE in all six MCH neurons tested (P < 0.05, ANOVA test).

NPY and MCH inhibit voltage-dependent calcium currents in MCH neurons

MCH and NPY are both involved in energy homeostasis (Schwartz et al. 2000). MCH (1 μM) did not cause any change in membrane potential or input resistance in MCH neurons in the presence of TTX. Since there is an intensive innervation of MCH fibers in the LH area and MCH receptors were expressed in the LH area (Bittencourt et al. 1992; Hervieu et al. 2000), we postulated that MCH might inhibit voltage-dependent calcium currents in MCH neurons, parallel to the response of other LH neurons (Gao and van den Pol 2002). Voltage-dependent cal-
Basic membrane properties of MCH neurons and response to GABA, glutamate, and norepinephrine. A: current injections were applied to MCH neurons under current clamp. Raw traces of voltage responses are shown in A (top traces). B: spontaneous action potentials recorded from MCH neurons. C: when MCH neurons were injected with a long depolarizing pulse, spike frequency adaptation (C, bottom trace) and termination of firing (C, top trace) were observed. D–I: response of MCH neurons to neurotransmitters. Glutamate depolarized (D) and GABA hyperpolarized (E) MCH neurons held at resting membrane potential under current clamp. Norepinephrine hyperpolarized MCH neurons in the absence and presence of TTX (1 μM) and GABA hyperpolarized (F) MCH neurons. Glutamate depolarized (D) and GABA hyperpolarized (E) MCH neurons held at resting membrane potential under current clamp. Norepinephrine hyperpolarized MCH neurons in the absence and presence of TTX (1 μM). Raw traces are presented in F and H. Pooled data from all tested MCH neurons are plotted in G and I.

Calcium currents (319.8 ± 58.2 pA, n = 8, Fig. 5A) were induced by a test pulse from −80 to 0 mV (100-ms duration) in MCH neurons held at −80 mV (Gao and van den Pol 2002). After a stable baseline recording was obtained, MCH was applied to the recorded neuron through a flow pipe (Fig. 5B). Two concentrations of MCH applications were tested. Voltage-dependent calcium currents were reduced to 84.8 ± 5.3% of the control by MCH (100 nM) and returned to 100.8 ± 2.2% of control after the withdrawal of MCH (n = 4; Fig. 4C). ANOVA, P < 0.05). In the presence of 500 nM MCH, voltage-dependent calcium currents were significantly decreased to 67.5 ± 4.9% of control and returned to 102.9 ± 4.8% of control after removal of MCH (n = 4, Fig. 5C; ANOVA, P < 0.05). Our results indicate the presence of MCH receptors on MCH neurons.

NPY was one of the most widespread peptides in the brain and participates in a number of hypothalamic functions, including energy homeostasis (Schwartz et al. 2000). When NPY was applied to MCH neurons in the presence of TTX, we found no change in membrane potential or input resistance. Voltage-dependent calcium channels are important modulators of neuronal functions and can be modulated by NPY. We therefore tested if NPY had any effect on voltage-dependent calcium currents in MCH neurons using the same stimulus parameters described in the paragraph above. In the presence of NPY (100 nM), calcium currents (304.2 ± 77.1 pA, n = 6) were depressed to 64.6 ± 5.8% of control (range, 80–42% of control; n = 6; Fig. 5, D and E). The inhibition of calcium currents by NPY was significant (ANOVA test, P < 0.001; Fig. 5F) and desensitized over time, indicating the presence of NPY receptors on MCH neurons. After NPY washout, calcium currents recovered to 103.8 ± 5.3% of control (n = 6).

Action potential firing properties can be modulated by neurotransmitters. We tested whether MCH- or NPY-mediated inhibition in calcium currents led to change in spike properties; because spontaneous spikes were infrequent, a long depolarizing current (25–55 pA, 1-s duration) was injected to the MCH-GFP positive neurons, and a train of action potentials was observed. In addition, a brief depolarizing current (120 pA, 8-ms duration) was injected into MCH-GFP positive neurons, and a train of action potentials was observed. Two parameters, action potential width and AHP, were examined. The action potential width was measured at half-amplitude of the evoked action potential. The AHP was measured from the resting membrane potential. The action potential width was 3.9 ± 0.4 ms (n = 6) before MCH application, 4.1 ± 0.5 ms (n = 6) during MCH application, and 4.0 ± 0.5 ms (n = 6) after MCH application (P > 0.05, ANOVA test). The AHP was 5.6 ± 1.6 mV (n = 5) before, 6.0 ± 1.5 mV (n = 5) during, and 6.3 ± 1.6 mV (n = 5) after MCH application (P > 0.05, ANOVA test). In parallel experiments with NPY, the action potential width was 4.2 ± 0.4 ms (n = 6) before, 4.2 ± 0.4 ms (n = 6) during, and 4.0 ± 0.4 ms
The AHP was 6.5 ± 1.7 mV (n = 5) before, 5.6 ± 1.4 mV during, and 5.6 ± 1.4 mV after the application of NPY (P > 0.05, ANOVA test). Thus neither MCH nor NPY altered the width or AHP of evoked spikes in MCH neurons.

**Discussion**

In this paper, we describe a means of selectively identifying a peptidergic transmitter phenotype in culture by using specific constructs with the neuropeptide promoter driving expression of dsRed or GFP selectively in live neurons for electrophysiological study. Using this approach, we examined the functional transmitter responses of identified MCH neurons in culture. Our data demonstrate that MCH neurons are directly excited by glutamate and inhibited by GABA, NE, MCH, and NPY through receptors expressed by MCH neurons.

**Identified neurons in culture**

Using either GFP or dsRed as reporter genes driven by the MCH promoter, we found expression only in hypothalamic cultures and not in neurons from cortex, hippocampus, thalamus, or striatum. Furthermore, immunocytochemistry revealed that only cells containing MCH peptide expressed the reporter gene construct. Together, these data suggest that expression of a reporter gene under control of a peptide promoter is highly selective after transfection of a general cellular population including glia and neurons and can therefore be used as a novel means to identify the transmitter phenotype of live cells in culture for subsequent whole cell recording and other types of cellular analysis. Although many cell types were transfected with the plasmid, only MCH neurons showed reporter gene expression. Since MCH neurons are only a small percentage of neurons in the LH, identifying live MCH neurons is a critical step toward studying the physiological properties of these cells.

In addition, the transfection procedure should work for neurons from a wide variety of species. A parallel approach of identifying GFP-expressing neurons in cultures or slices for recording purposes can be achieved with transgenic animals (van den Pol et al. 2002), but this is generally restricted to mice. Previous work has shown that neurons and glia could be visualized following biolistic application of GFP constructs driven by a preprotachykinin promoter in chronic slices (Walker et al. 2000) and that a calcium-sensitive GFP variant could be used to track calcium changes in transfected cultured neurons (Tsuchiya et al. 2002).

A large number of studies have shown that many specific properties of neurons are maintained in culture. For instance, hypothalamic circadian clock neurons of the suprachiasmatic nucleus maintain circadian firing rhythms in culture (Welsh et al. 1995). Long-term potentiation and depression common in hippocampal slices similarly are detected in cultured hippocampal neurons (Goda and Stevens 1998; Malgaroli and Tsien 1992). In experiments where cultured hypothalamic cells and hypothalamic slices were compared, NPY actions were similar in each (Chen and van den Pol 1996; van den Pol et al. 1996). Differential mechanisms of transmitter release can be elucidated with synaptically coupled cultured neurons (Jo and Role 2002). Neurons also maintain their transmitter identity in culture (Bekkers and Stevens 1991; Petit et al. 2002) and many of their morphological attributes. For instance, in this study, we found that MCH neurons had axons that were many times longer than cerebellar granule cells, consistent with the normal...
long axons of MCH neurons (Bittencourt et al. 1992) and short axons of granule cells in vivo. Finally, our data show that MCH neurons retain their phenotypic transmitter identity long after removal from the embryonic hypothalamus. Some electrophysiological features of MCH neurons identified by immunostaining in hypothalamic slices have been briefly reported recently (Eggermann et al. 2003). In slices, MCH neurons showed a very low spike frequency and no sag during hyperpolarizing current injections, suggesting no \( I_h \) current. Our observations in MCH neurons in culture are consistent with those findings, suggesting that MCH neurons maintain many of their electrophysiological properties in vitro.

Neurotransmitter actions can be studied both in cultured cells or in brain slices. Each approach has important attributes, particularly for the study of neuromodulators that may have either slow or fast onset and long-lasting effects. Where glutamate antagonists AP5/CNQX act quickly and recover immediately on washout in cultures, in slices, the antagonists may take several minutes to exert an effect and can take more than 1 h to achieve full recovery to baseline after the initiation of washout. Therefore slow onset or long-lasting effects of peptide actions in culture may be indicative of real effects rather than artifacts of slow perfusion that may be a concern in slices. In cultured suprachiasmatic nucleus neurons, many cells showed slow (>30 min) recovery after NPY application (van den Pol et al. 1996); in contrast, MCH neurons in this study showed a very rapid (15–60 s) recovery after washout, suggesting some difference in second messenger signaling between the two cell types. Whereas cultures have some substantial advantages for certain mechanistic questions of transmitter actions and release, ideally, findings from cultured neurons would be confirmed in whole animals or slices where synaptic connections remain to some degree normal. Transgenic mice with reporter genes identifying MCH neurons are not currently available, and the transfection procedure used here would not work for identification of neurons in acute hypothalamic slices but might work in chronic slices maintained in vitro for extended periods. Neurons continued to survive and grow for many weeks after transfection with the GFP sequence, supporting the view that transfection and expression of GFP is not deleterious to neuronal health. We have previously shown that detectable levels of GFP expression do not appear to alter neuron input resistance, spike threshold or frequency, current-voltage relations, or responses to peptides (van den Pol et al. 2002), suggesting that GFP expression here after transfection is not neurotoxic and should not substantially alter cellular physiology.

Transmitter responses of MCH neurons

As found in all other mature hypothalamic neurons tested, glutamate excited MCH neurons, and GABA inhibited them. NE is found in axons in the area of MCH neurons in the hypothalamus; previous work has shown that NE can be excitatory or inhibitory in specific hypothalamic neurons depending on which NE receptor is expressed (Daftary et al. 1998; Wellman 2000). NE was consistently inhibitory and exerted a direct hyperpolarizing action on MCH neurons, similar to its effect on nearby hypocretin/orexin neurons (Li et al. 2002).

Although neither MCH nor NPY influenced membrane potential or current-voltage relations, both depressed calcium currents in the presence of TTX. Calcium channels play an important role in neuronal behavior, neurotransmitter release (Chen and van den Pol 1998; Hirning et al. 1988) and modulation of expression of transcription factors such as CREB (Rajadhyaksha et al. 1999). Complete blockade of calcium currents by specific calcium channel blockers can lead to broadened action potentials and reduced AHP (Cloues and Sather 2003). Although MCH and NPY inhibited voltage-dependent calcium currents in MCH neurons here, no effect of MCH or NPY on evoked spike width or AHP was observed. This might be due to fact that calcium currents were only partially inhibited by MCH or NPY. This view is consistent with observations that partial blockade of calcium currents by selective channel blockers may have little effect on spike characteristics (Cloues and Sather 2003).

Because the depression of calcium currents by MCH can lead to a reduction in transmitter release (Gao and van den Pol 2001), these data suggest that MCH neurons express autoreceptors for MCH depression, potentially leading to an inhibitory feedback of the peptide on the parent cell. If MCH neurons also express autoreceptors on their axons, MCH may act at presynaptic sites to inhibit further release of MCH. Immunocytochemical studies have shown both NPY and MCH axons terminate on or near MCH cells (Broberger et al. 1998; Elias et al. 1998). NPY has been suggested as a robust orexigenic peptide that may act by stimulating MCH neurons (Mystkowski et al. 2000). However, present data suggest that MCH neurons show an inhibitory response to NPY, suggesting if MCH is involved in the final feeding behavior induced by NPY, it is probably not due to direct NPY mediated excitation of MCH neurons, but rather through other mechanisms that may best be addressed in hypothalamic slices.

DISCLOSURES

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