Characterization of melanin-concentrating hormone cells coexpressing cocaine- and amphetamine-regulated transcript

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A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in partial fulfillment of the requirements for the degree of

Master of Science
in
Neuroscience

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Ottawa, Ontario

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ABSTRACT

Melanin-concentrating hormone (MCH) is a neuropeptide produced almost exclusively in the lateral hypothalamus. MCH cells regulate many behaviours including feeding, sleep, stress, cognition, and maternal behaviour. There are also subpopulations of MCH cells that are neurochemically distinct, which could underlie the functional diversity of this cell type. Notably, about half of MCH cells coexpress cocaine- and amphetamine-regulated transcript (CART). CART influences many of the same behaviours as MCH, but these two peptides often act in opposition. For example, MCH promotes feeding and positive energy balance, but CART can be either orexigenic or anorexigenic. The unique roles of MCH/CART+ and MCH/CART− cells are not known, but these neurochemically distinct subpopulations could contribute to distinct behaviours. To help define their roles in MCH-related neuronal networks, we defined the neuroanatomical, electrical, and morphological properties of MCH/CART+ and MCH/CART− cells in male and female mice. First, we made spatial maps of the distribution of CART in MCH cells and found that MCH/CART+ and MCH/CART− cells were anatomically divided. Second, we analyzed the electrical activity and structure of each cell type and found differences in their excitability and response to stimulation. Third, we traced their dendritic structure and found that male MCH/CART+ were highly branched.

These results highlight variations in the regulation of each MCH cell subtype and suggest that they could be implicated in different cellular networks. This work will help resolve the complexity of the MCH system by characterizing MCH/CART+ and MCH/CART− cells that could underpin the diverse functions of MCH neurons.
Acknowledgements

Thank you to my committee Dr. Zachary Patterson and Dr. Hongyu Sun, and to my external examiner, Dr. Masha Prager-Khoutorsky. Thank you for your time, insights, and counsel which have helped me grow as a researcher and have pushed this project beyond where I thought it could go.

I would also like to thank my supervisor, Dr. Melissa Chee, for her unwavering support and guidance during my thesis. Thank you, Melissa, for always helping me achieve my highest potential. You continually inspire me, and I am so grateful for everything you have taught me.

A big thank you to the wonderful members of the Chee Lab. In particular, thank you to Jesukhogie Williams-Ikhenoba for his tireless help with the neuroanatomical mapping. Thank you to Aditi Sankhe, for many long days helping me patch cells, and thank you to Brendan Hoffe who taught me how to use Neurolucida.

Thank you to my synaptic sisters – Nikita, Aditi, Bianca, and Yasmina – for filling my lab days with laughter.

Finally, thank you Mom, Dad, Max, and Pat. Your love and support have guided me through every good and bad day. I am so lucky to have such a wonderful family and chosen family, I love you all so very much.
Dedication

This thesis is dedicated to Benson, my sweet pup who stayed awake all night with me when I was writing and who sings every time I come home from school.
### List of abbreviations

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<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tbody>
<tr>
<td>ACB</td>
<td>Nucleus accumbens</td>
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<td>ACSF</td>
<td>Artificial cerebrospinal fluid</td>
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<tr>
<td>AHNp</td>
<td>Anterior hypothalamic nucleus, posterior part</td>
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<tr>
<td>ARA</td>
<td>Allen Reference Atlas</td>
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<tr>
<td>ARH</td>
<td>Arcuate nucleus</td>
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<tr>
<td>CART</td>
<td>Cocaine- and amphetamine-regulated transcript</td>
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<tr>
<td>Cartpt</td>
<td>Cocaine- and amphetamine-regulated transcript prepropeptide</td>
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<tr>
<td>Cy3</td>
<td>Cyanine 3</td>
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<tr>
<td>DAB</td>
<td>3,3'-diaminobenzidine</td>
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<tr>
<td>DapB</td>
<td>Bacillus dihydricolinate reductase</td>
</tr>
<tr>
<td>DMHa</td>
<td>Dorsomedial nucleus of the hypothalamus, anterior part</td>
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<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
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<tr>
<td>fISH</td>
<td>Fluorescence in situ hybridization</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>ip</td>
<td>Intraperitoneal</td>
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<td>ir</td>
<td>Immunoreactive</td>
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<tr>
<td>L</td>
<td>Level</td>
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<tr>
<td>LHA</td>
<td>Lateral hypothalamic area</td>
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<td>MCH</td>
<td>Melanin-concentrating hormone</td>
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<tr>
<td>MCHR1</td>
<td>Melanin-concentrating hormone receptor 1</td>
</tr>
<tr>
<td>MPO</td>
<td>Medial preoptic area</td>
</tr>
<tr>
<td>NDS</td>
<td>Normal donkey serum</td>
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<tr>
<td>NK3R</td>
<td>Neurokinin 3 receptor</td>
</tr>
<tr>
<td>NKB</td>
<td>Neurokinin B</td>
</tr>
<tr>
<td>NMDG</td>
<td>N-methyl-D-glucamine</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PH</td>
<td>Posterior hypothalamic nucleus</td>
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<tr>
<td>Pmch</td>
<td>Pro-melanin concentrating hormone</td>
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<tr>
<td>Ppib</td>
<td>Peptidylprolyl isomerase B</td>
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<tr>
<td>PST</td>
<td>Preparasubthalamic nucleus</td>
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<tr>
<td>PSTN</td>
<td>Parasubthalamic nucleus</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
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<tr>
<td>sEPSC</td>
<td>Spontaneous excitatory post-synaptic current</td>
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<tr>
<td>STN</td>
<td>Subthalamic nucleus</td>
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<td>Tacr3</td>
<td>Tachykinin receptor 3</td>
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<td>Abbreviation</td>
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<tr>
<td>TSA</td>
<td>Tyramine signal amplification</td>
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<tr>
<td>VMH</td>
<td>Ventromedial hypothalamus</td>
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<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>ZI</td>
<td>Zona incerta</td>
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1. INTRODUCTION

1.1 Melanin-concentrating hormone

1.1.1 MCH: A protein of many functions

Melanin-concentrating hormone (MCH) is an evolutionarily conserved neuropeptide produced primarily in the lateral hypothalamic area (LHA) (Nahon et al., 1989), though it may also be produced in extrahypothalamic regions like the lateral septum and medial preoptic nucleus in female mice during lactation (Rondini et al., 2010; Benedetto et al., 2014; Beekly et al., 2020). In rodents, this peptide is primarily inhibitory and acts via its G\textsubscript{i/o} or G\textsubscript{q} coupled receptor, MCH receptor-1 (MCHR1) (Gao, 2009; MacNeil, 2013). Functionally, MCH has been best characterized for its role in energy homeostasis. Intraventricular administration of MCH in rats (Qu et al., 1996; Rossi et al., 1997; Clegg et al., 2003) or mice (Gomori et al., 2003; Glick et al., 2009) increases chow consumption. Similarly, MCH- or MCHR1-knock out mice are leaner than controls, due to decreased chow consumption and increased energy expenditure (Shimada et al., 1998; Marsh et al., 2002). MCH also contributes to the hedonic aspect of feeding, as MCH injection in the nucleus accumbens (ACB) shell promotes chow consumption (Georgescu et al., 2005).

However, MCH is involved in many functions and has many widespread projections throughout the brain (Bittencourt, 1992). These fibers may innervate the olfactory bulbs rostrally to the brain stem caudally, and MCH fibers can contact many regions including the basal ganglia, hippocampus, and several diencephalic and mesencephalic nuclei (Bittencourt, 1992). Via these brain-wide projections, MCH cells have also been implicated in the regulation of many other behaviours including sleep.
(Verret et al., 2003; Hassani et al., 2009; Monti et al., 2013), learning and memory (Monzon et al., 1999; Adamantidis and de Lecea, 2009; Concetti and Burdakov, 2021), anxiety and depression (Borowsky et al., 2002; Georgescu et al., 2005; Sankhe et al., 2022), and reproduction (Gonzalez et al., 1997; Williamson-Hughes et al., 2005; Wu et al., 2009). As such, MCH is a multifaceted peptide, capable of modulating many diverse physiological and psychological behaviours, that acts at multiple downstream targets to achieve its effects.

1.1.2 Inputs to MCH cells

In line with their many functions, several neuronal and peripheral systems interact with MCH cells (Guyon et al., 2009). Within the hypothalamus, MCH cells are regulated by other cell populations in LHA and the arcuate nucleus (ARH) (Elias et al., 1998). Notably, neighboring orexin neurons in the LHA can have a direct stimulatory (Van Den Pol et al., 2004), or indirect inhibitory (Apergis-Schoute et al., 2015) effect on MCH neurons. In the ARH, NPY/AgRP neurons can inhibit MCH cells (Van Den Pol et al., 2004), and injections of MCH in the ARH promotes food consumption (Abbott et al., 2003). From the paraventricular nucleus, both oxytocin and vasopressin can depolarize MCH cells (Yao et al., 2012).

MCH cells also respond to peripheral factors. Leptin indirectly inhibits MCH cells, as $Lep^{ob/ob}$ mice express twice the level of $Pmch$ as wildtype controls (Qu et al., 1996), but MCH cells do not express the leptin receptor (Louis et al., 2010). Interestingly, both insulin (Hausen et al., 2016a) and glucose (Burdakov et al., 2005) can directly depolarize MCH cells, thus these cells are a component of the central regulation of glucose
homeostasis. Finally, changes in diet can also impact the function of MCH neurons. Acute exposure to high-fat diet suppresses the excitatory contacts to MCH cells (Linehan et al., 2020). In contrast, both fasting and long-term exposure to high-fat diet exacerbated the excitatory inputs to these cells (Linehan et al., 2020; Linehan and Hirasawa, 2022).

Beyond feeding, MCH cells interact with endogenous opioids. Nearly 90% of MCH neurons express the transcript for the nociception/orphanin FQ receptor (Parks et al., 2014b), which has been implicated in stress (Köster et al., 1999), feeding (Stratford et al., 1997), and reducing reward (Sakoori and Murphy, 2004). In another example, around 50% of MCH cells express the kisspeptin receptor (Parks et al., 2014b). As kisspeptin is a mediator of pituitary gonadotropin release (Han, 2005), this implicates MCH in the control of reproductive circuitry. Overall, as key modulators of behaviour MCH cells are regulated by multiple neuronal and hormonal systems to effectuate their outputs.

1.1.3 Outputs of MCH cells

In turn, MCH cells modulate behaviour through their vast connections (Bittencourt, 1992). Notable projections from these cells include those to the hippocampus, amygdala, lateral septum, brain stem, and ventral tegmental area. Intra-hippocampal infusions of MCH improved memory retention in rats during a foot shock memory task (Monzon et al., 1999; Varas et al., 2002), while intra-amygdala MCH injections improved memory in response to emotion-dependent tasks (Monzon et al., 1999; Varas et al., 2002). In the lateral septum, MCH neurons contributed to the formation of spatial memories by modulating the response of septum cells to excitation from hippocampal place cells (Liu et al., 2022). Furthermore, one of the regions with the highest MCHR1 expression is the
ACB shell (Hervieu et al., 2000; Saito et al., 2001; Chee et al., 2013). MCH injection in this region promoted chow consumption and blocking MCHR1 had the opposite effect (Georgescu et al., 2005). Similarly, deletion of MCHR1 from GABAergic neurons in the ACB lead to lower body weight and increased locomotion (Chee et al., 2019). Finally, MCH cells can indirectly modulate energy homeostasis through at least two brainstem pathways. MCH cells can contribute to salivation and mastication by indirectly modulating the masseter muscles and salivary glands via the brainstem (Pérez et al., 2011). Secondly, using a polysynaptic viral tracer, Oldfield et al. (2002) found connections to MCH cells from the brown adipose tissue in rats. This is in line with the discovery of Pereira-Da-Silva et al. (2003) that MCH is upregulated by almost 60% in cold-exposed animals. Thus, this suggests that MCH cells can modulate thermoregulation through the brainstem. Overall, congruent with their functional diversity, MCH cells are implicated in many different neuronal and peripheral systems, which allows them to regulate homeostasis in response to internal and external cues.

1.1.4 Heterogenous firing patterns of MCH cells

Without external stimulation, MCH cells have a resting membrane potential between -50 to -60 mV (Guyon et al., 2009) but an action potential threshold of about -30 mV (Gao et al., 2003; Huang et al., 2007) and are thus silent at rest. Notably, when depolarized, MCH cells can display multiple firing phenotypes. These cells can respond with either a tonic (Eggermann et al., 2003; Van Den Pol et al., 2004) or accommodating (Jo et al., 2005; Fujita et al., 2021) stream of action potentials. The firing pattern of a neuron is important as this can differentially impact the activity of its target. For example,
studies in dorsal root ganglion neurons found that longer bursts of action potentials downregulated sodium channel (Klein et al., 2003; Iacobas et al., 2019) and growth factor transcripts in the receiving cell (Lee et al., 2017). Similarly, the frequency of firing was critical, as more c-fos expression was induced by a shorter interval between action potentials (Fields et al., 1997). As such, the spike pattern of a neuron can uniquely impact the genetic activity of its target (Iacobas et al., 2019). Furthermore, different firing patterns can release different chemical messengers. Notably, bursts of action potentials more effectively stimulate neuropeptide release than chronic firing (Poulain and Wakerley, 1982). Likewise, the amount of neuropeptide released per action potential increases with a faster firing frequency (Dreifuss et al., 1972; Gainer et al., 1986). MCH cells express neuropeptides, like MCH, but have also been shown to release glutamate (Chee et al., 2015) and express the vesicular GABA transporter (Jego et al., 2013). As such, distinct firing patterns could release either neuropeptides or fast-acting neurotransmitters at the downstream cell. Thus, MCH cells display unique action potential characteristics, and these firing patterns can differentially modulate the activity of their targets.

1.1.5 The impact of cell morphology on electrical activity

MCH neurons are multipolar cells, with around 3-4 dendrites projecting from the soma (Eggermann et al., 2003). In the present study, we will analyze both the morphology and electrical activity of MCH cells. The morphological features of a neuron can influence both the integration of its inputs and the pattern of its outputs (Connors and Regehr, 1996). When inputs arrive at a cell, the ability of the receiving dendrite to convey this information to the soma depends on the geometry, active membrane properties, and
resistance of the dendrite (Poirazi and Papoutsi, 2020). For example, excitatory inputs arriving further from the soma attenuate as they travel down the dendrite and are thus less likely to trigger an action potential (Rall and Rinzel, 1973; Stuart and Spruston, 1998). However, this effect can be reversed if the dendrite contains active conductances, as these can trigger dendritic spikes and further depolarize the soma (Williams and Stuart, 2000; Komendantov and Ascoli, 2009; Cook and Johnston, 2023). In turn, the branching pattern of a cell can also influence its firing pattern. For example, computer simulation studies have reported that CA3 pyramidal neurons with large dendritic trees were less likely to display burst firing compared to neurons with smaller arborizations. Similarly, computational models of the prefrontal cortex found that regular-spiking and burst-spiking cells can be discriminated based on dendritic length, volume, and number (Psarrou et al., 2014). In sum, dendritic morphology has a critical impact on electrical activity, by modulating both the integration of inputs and generation of outputs from a cell.

1.1.6 Sex-dependent morphological heterogeneity of the hypothalamus

To date, the morphology of MCH cells has not been investigated in depth. However, other hypothalamic nuclei are structurally heterogenous, and these differences can impact behaviour (Lenz and Mccarthy, 2010). Notably, studies have found sex-dependent differences in the cell structure of the ARH, medial preoptic area (MPO), and ventromedial hypothalamus (VMH) (Lenz and Mccarthy, 2010). In the ARH, males have more axosomatic synapses, while females have more axodendritic synapses (Matsumoto and Arai, 1980; Mong et al., 1999). These differences are regulated by estradiol, as neonatal castration of males (Matsumoto and Arai, 1980; Mong et al., 2001), or steroid
treatment of females (Mong and McCarthy, 1999; Mong et al., 1999), reversed the synaptic phenotype in the ARH. In the MPO, male neurons have over twice the number of dendritic spines as females (Amateau and McCarthy, 2002, 2004). This difference is behaviourally relevant, as masculinization of the MPO resulted in females displaying masculine copulatory behaviour, while feminization of this region significantly reduced these behaviours in males (Amateau and McCarthy, 2004; Wright and McCarthy, 2009). Finally, in the VMH, male neurons have more synapses on their dendrites and a more highly branched morphology (Matsumoto and Arai, 1980; Mong et al., 1999). This phenotype was due to glutamate signalling, as estradiol indirectly increased glutamate release, to promote spine formation (Shwarz and McCarthy, 2008). Overall, the hypothalamus is a structurally diverse area between male and female rodents. Although the morphological features of MCH cells are currently unknown, differences in dendritic patterns could potentially underlie the heterogeneity of MCH firing phenotypes and could be distinct between the sexes.

1.1.7 The MCH system is heterogenous between the sexes

Adding to the complexity of the MCH system, MCH and its effects are not identical between males and females. Only females present MCH cells in the laterodorsal tegmental nucleus (Rondini et al., 2007), where they could act to regulate REM sleep (Ferreira et al., 2017). Furthermore, several MCH clusters only appear in lactating dams. Notably, in the medial and anterodorsal preoptic nuclei, the anterior portion of the paraventricular nucleus, the ventral lateral septum, and in the anterodorsal nucleus of the thalamus (Knollema et al., 1992; Rondini et al., 2010; Beekly et al., 2020). In these dams,
the MCH system is a key regulator of maternal behaviour, and its impairment leads to decreased lactation, nesting, and pup retrieval and increased pup cannibalization and maternal aggression (Knollema et al., 1992; Adams et al., 2011; Benedetto et al., 2014; Alachkar et al., 2016).

In males, MCH administration is widely recognized to increase feeding (Qu et al., 1996; Rossi et al., 1997; Tritos et al., 1998; Della-Zuana et al., 2002; Clegg et al., 2003; Gomori et al., 2003; Glick et al., 2009), but it has little effect on food intake in females (Mogi et al., 2005; Messina et al., 2006; Santollo and Eckel, 2007; Terrill et al., 2020). Indeed, the orexigenic effect of MCH appears sensitive to the presence of estrogen as chronic, but not acute (Tritos et al., 2004), estrogen treatment blocked fasting-induced increases in Pmch gene expression (Murray et al., 2000; Mystkowski et al., 2000; Morton et al., 2004). In sum, the MCH system is heterogenous both functionally and between the sexes.

1.2 CART: An opposite yet overlapping protein

The broad functional contributions of MCH cells may also be attributed to their neurochemical diversity. As previously mentioned, (section 1.1.4), MCH cells release glutamate (Chee et al., 2015) and express the vesicular GABA transporter (Jego et al., 2013), along with other neuropeptides such as nesfastin and neuropeptide-glutamic acid-isoleucine (Bittencourt, 1992; Harthoorn et al., 2005; Fort et al., 2008; Mickelsen et al., 2017, 2019). Most notably, around half of MCH cells coexpress CART (Broberger, 1999; Vrang et al., 1999; Elias et al., 2001; Cvetkovic et al., 2004; Croizier et al., 2010; Mickelsen et al., 2017, 2019), a messenger RNA found abundantly in the central and
peripheral nervous systems, the gut, adrenal gland, and pancreas (Koylu et al., 1997, 1998; Kuhar and Yoho, 1999; Vrang, 2006; Subhedar et al., 2014). In rodents, the processing of this transcript results in two biologically active peptides, CART 55-102 and CART 62-102 (Thim et al., 1999), which act through their newly identified receptor, GPR160 (Yosten et al., 2020).

CART is robustly expressed in the hypothalamus (Koylu et al., 1997), and CART cells have been implicated in many functions that overlap with the MCH system. These include the regulation of food intake (Kristensen et al., 1998; Thim et al., 1998), sleep-wake behavior (Keating et al., 2010), reproduction (Lebrethon et al., 2000; Parent et al., 2000; True et al., 2013), and stress (Smith et al., 2004; Vicentic et al., 2004; Koylu et al., 2006). Curiously, the effects of MCH and CART often act in opposition. For instance, MCH neurons are activated during sleep (Verret et al., 2003; Hassani et al., 2009), and MCH infusion can increase REM sleep episodes (Verret et al., 2003), but CART mediates wake-promoting effects (Keating et al., 2010). In another example, MCH and CART act as opposing signals of nutritional stores on fertility status. Gonadotropin-releasing hormone neurons, a key player in the hypothalamic reproductive axis, are inhibited by MCH but activated by CART.

Like MCH cells, CART cells have also been extensively implicated in the control of energy homeostasis. However, while MCH promotes positive energy balance (Qu et al., 1996; Shimada et al., 1998), CART has shown both anorexigenic and orexigenic effects (Lau and Herzog, 2014; Subhedar et al., 2014). Intracerebroventricular injections of CART inhibited food intake (Kristensen et al., 1998; Aja et al., 2001) and reduced body weight (Larsen et al., 2000; Rohner-Jeanrenaud et al., 2002; Nakhate et al., 2010).
Similarly, antibodies directed against the CART peptides increased feeding (Kristensen et al., 1998; Nakhate et al., 2010, 2013). CART is also extensively coexpressed with proopiomelanocortin in the ARH (Elmquist, 2001), a potent inhibitor of feeding (Yaswen et al., 1999). On the other hand, directed CART overexpression in specific hypothalamic areas, including the lateral, paraventricular, and ventromedial hypothalamic nuclei, increased food intake (Abbott et al., 2001; Kong et al., 2003; Smith et al., 2008; Farzi et al., 2018; Lau et al., 2018). Ultimately, CART is another complicated regulator of behaviour, that could contribute to multiple discrete neurocircuits to exert its functions.

1.3 MCH/CART+ cells: A unique MCH subpopulation

In many ways, MCH/CART+ cells are distinct from the MCH/CART– population. Several studies have noted that these populations are anatomically divided, as MCH/CART+ cells are found in the medial portion of the LHA, while MCH/CART– cells are found predominantly in the lateral LHA (Broberger, 1999; Vrang et al., 1999; Brischoux et al., 2001; Elias et al., 2001; Croizier et al., 2010). MCH/CART+ cells also emerge later in development than MCH/CART– cells. MCH/CART+ cells are produced around embryonic day 12-13 but MCH/CART– cells arise at embryonic day 11 (Brischoux et al., 2001; Cvetkovic et al., 2004; Risold et al., 2009; Croizier et al., 2010). As well, MCH/CART+ cells uniquely coexpress other neuropeptides (Mickelsen et al., 2017). Transcriptomic evidence has revealed that between half to two-thirds of Pmch+ cells coexpress both Cartpt and Tacr3, the gene encoding the neurokinin-3 receptor (NK3R) (Mickelsen et al., 2019; Fujita et al., 2021). This receptor preferentially binds to neurokinin
B (Steinhoff et al., 2014), which has been implicated in reproductive physiology and behaviour (Rance et al., 2010, 2013).

To date, an in-depth analysis of the target regions of MCH/CART+ cells does not yet exist but, broadly, these cells have been found to project towards the cerebral cortex, while MCH/CART– cells project towards the spinal cord (Brischoux et al., 2001; Cvetkovic et al., 2004; Risold et al., 2009; Croizier et al., 2010). Of note, MCH/CART+ projections have been found in the ventral tegmental area (VTA) and ACB (Dallvechia-Adams et al., 2002; Philpot et al., 2005; Ekstrand et al., 2014). The mesolimbic dopamine pathway, including the VTA and ACB, has been implicated in the hedonic drive to eat (Liu and Borgland, 2015). Both MCH and CART can modulate energy balance through this circuity (Liu and Borgland, 2015; Diniz and Bittencourt, 2017; Chee et al., 2019). The ACB shell and VTA express MCHR1 (Saito et al., 2001; Borowsky et al., 2002), and anatomical studies have found both MCH and CART fibers in these areas (Bittencourt, 1992; Koylu et al., 1998). However, acting again in opposition, MCH injection into the ACB shell of rats increased chow consumption (Georgescu et al., 2005) but intra-ACB shell injections of CART peptides decreased food intake (Yang et al., 2005). Interestingly, co-injection of MCH and CART in the ACB prevented CART-induced dopamine release in this region (Yang and Shieh, 2005), thus it is possible that MCH and CART could self-regulate to modulate hedonic feeding. Overall, while the significance of MCH and CART coexpression is unknown, the MCH/CART+ population could exert a dual mechanism to regulate homeostasis that is distinct from MCH-only or CART-only cells. The MCH/CART+ population marks the intersection of two dynamic neuropeptides, whose co-contributions to behaviour remain to be elucidated.
1.4 Rationale, aims, and hypothesis

MCH cells are a hypothalamic population that modulate a wide variety of behaviours. About half of MCH cells coexpress CART, which is also associated with many functions in the brain. We hypothesize that MCH/CART+ and MCH/CART− cells are neurochemically distinct subpopulations that contribute to unique cellular networks to modify behaviour. As the cellular properties of these neurons could elucidate their functional relevance, we have characterized MCH/CART+ and MCH/CART− cells anatomically, electrically, and morphologically in male and female mice.

Aim 1: Map the spatial distribution of MCH/CART+ and MCH/CART− cells

We quantified and mapped the colocalization of MCH cells, as marked by their native EGFP expression in Mch-cre;L10-Egfp transgenic mice, with CART and/or NK3R immunoreactivity.

Aim 2: Determine and compare the electrophysiological properties of MCH/CART+ and MCH/CART− cells

We used whole-cell patch-clamp recordings from Mch-cre;L10-Egfp mice to elucidate the passive and active membrane properties of MCH/CART+ and MCH/CART− cells. The chemical identity of each recorded cell was defined by post hoc immunolabeling for CART-immunoreactivity. Additionally, we also compared the frequency, amplitude, and kinetic properties of excitatory afferent input to each cell type.
Aim 3: Define and compare the morphological properties of MCH/CART+ and MCH/CART− cells

Individual biocytin-filled MCH/CART+ or MCH/CART− cells were visualized by 3,3'-diaminobenzidine staining to analyze the complexity of their dendritic arborization. We analyzed the length of dendrites, the number of nodes and the proximity of nodes to the soma.
2. MATERIALS & METHODS

2.1 Animals

All animal procedures were completed in accordance with guidelines and approval of the Animal Care Committee at Carleton University. Mice were group-housed at 21–22 °C with a 12:12 h light-dark cycle and provided with ad libitum access to water and standard mouse chow (Teklad Global Diets 2014, Envigo, Mississauga, Canada).

To visualize MCH neurons, Pmch-cre mice (Kong et al., 2010) were crossed to R26-lox-STOP-lox-L10-Egfp reporter mice (Krashes et al., 2014), kindly provided by Dr. B. Lowell (Beth Israel Deaconess Medical Center, Boston, MA), to generate Mch-cre;L10-Egfp mice expressing enhanced green fluorescent protein (EGFP) under the Mch promoter.

2.2 Antibody characterization

Table 1. List and details of primary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Immunogen</th>
<th>Clonality, Isotype</th>
<th>Source, Product no., Lot no.</th>
<th>RRID</th>
<th>Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-MCH</td>
<td>Full length MCH peptide</td>
<td>Polyclonal, IgG</td>
<td>Dr. E. Maratos-Flier</td>
<td>AB_2314774</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Rabbit anti-NK3R</td>
<td>Amino acids 375–452 at C-terminus of rat NK3R</td>
<td>Polyclonal, IgG</td>
<td>Novus Biologicals, NB300-102, F-4</td>
<td>AB_1000327</td>
<td>1:150,00</td>
</tr>
<tr>
<td>Rabbit anti-CART</td>
<td>Amino acids 61–102 of CART</td>
<td>Polyclonal, IgG</td>
<td>Phoenix Pharmaceuticals, H-003-61, 1625-3</td>
<td>AB_2922768</td>
<td>1:2,000</td>
</tr>
</tbody>
</table>
Table 1 lists the details of the following primary antibodies and how they were used in immunohistochemistry (IHC) or dual IHC and in situ hybridization experiments.

**Rabbit anti-MCH antibody** was made and generously provided by Dr. E. Maratos-Flier (Beth Israel Deaconess Medical Center, Boston, MA). Antibody specificity was demonstrated by a lack of MCH-immunoreactivity in brain tissue from MCH knockout mice (Chee et al., 2013) and following MCH peptide absorption (Elias et al., 1998).

**Rabbit anti-NK3R antibody** was raised against amino acids 375–452 in the C-terminus of the rat NK3R protein. This antibody recognized a 50–52 kDa band from an immunoblot of rat brain homogenate (Le Brun et al., 2008). Our NK3R immunoreactivity pattern matched that reported in the rat hypothalamus (Ding et al., 1996; Burke et al., 2006), and there was a lack of staining following preabsorption with the NK3R peptide (Novus Biological, product datasheet).

**Rabbit anti-CART antibody** was raised against amino acids 61–102 of the CART protein. Antibody specificity was established by the lack of CART immunoreactivity when 1 ml of diluted antibody was preabsorbed with 10–100 μg of CART antigen (Arciszewski et al., 2009).

Secondary antibodies used were raised in donkey against the rabbit or were streptavidin-conjugated (Table 2).
Table 2. List and details of secondary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Source, Product no., Lot no.</th>
<th>RRID</th>
<th>Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donkey anti-rabbit Alexa Fluor 647</td>
<td>IgG (H+L)</td>
<td>Thermo Fisher Scientific, A-31573, 2420695</td>
<td>AB_2536183</td>
<td>1:500</td>
</tr>
<tr>
<td>Biotin-SP AffiniPure donkey anti-rabbit</td>
<td>IgG (H+L)</td>
<td>Jackson ImmunoResearch, 711-065-152, 134054</td>
<td>AB_2340593</td>
<td>1:500</td>
</tr>
<tr>
<td>Cy3 Streptavidin</td>
<td></td>
<td>Jackson ImmunoResearch, 016-160-084, 146014</td>
<td>AB_2337244</td>
<td>1:1,000\ 1:5,000</td>
</tr>
</tbody>
</table>

2.3 Tissue preparation

*Mch-cre;L10-Egfp* mice (6–13 weeks) were anesthetized with an intraperitoneal (ip) injection of 7% chloral hydrate (700 mg/kg; Sigma-Aldrich, St-Louis, MO), and brain tissue was collected as previously described (Negishi et al., 2020). In brief, the mice were transcardially perfused with cold 0.9% saline and 10% formalin, and their brains were post-fixed in formalin for 12–24 h (4 °C) and cryoprotected in 20% sucrose for 12–24 h. Each brain was sliced along the coronal plane into five series of 30 μm-thick sections using a freezing stage microtome (Spencer Lens Co., Buffalo, NY) and collected in phosphate-buffered saline (PBS).

Free-floating tissue sections were stored in antifreeze solution at −20 °C for immunohistochemistry (IHC) experiments or were immediately mounted onto Superfrost Plus microscope slides (Fisher Scientific, Hampton, NH), air dried at room temperature (RT; 21–22 °C, 20 min) and −20 °C (30 min), then stored at −80 °C, as previously
described (Bono et al., 2022) for Nissl staining. For fluorescence in situ hybridization (fISH) experiments, free-floating tissue sections were used immediately.

2.4 Immunohistochemistry (IHC) and fluorescence in situ hybridization (fISH)

2.4.2 Single-label IHC

As previously described (Chee et al., 2013; Negishi et al., 2020), free-floating slices were washed six times PBS for 5 min each (RT), blocked in 3% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories, West Grove, PA; RRID: AB_2337258) prepared in PBS containing 0.05% sodium azide and 0.25% TritonX-100 (PBT-azide) for 2 h, and then incubated in rabbit anti-MCH antibody (1:2,000) overnight. After six 5-minute PBS washes (RT), the sections were incubated (2 h, RT) in donkey anti-rabbit Alexa Fluor 647 (1:500) and then rinsed with three 10-minute PBS washes (RT). The brain sections were mounted onto Superfrost Plus glass microscope slides (Fisher Scientific) and coverslipped with ProLong Diamond Antifade Mountant (Thermo Fisher Scientific) and 1.5 thickness glass (22-266-882P, Fisher Scientific).

2.4.2 Dual-label IHC

Primary antibodies for anti-NK3R and anti-CART were both made in the rabbit (Table 1), thus, to minimize cross-reactivity between NK3R- and CART-immunoreactive (-ir) signals from rabbit-specific secondary antibodies, we labeled each antigen in series where labeling for NK3R immunoreactivity preceded that for CART.

Brain sections were rinsed via six exchanges of PBS (5 min each, RT), treated with 0.3% hydrogen peroxide (20 min), and rinsed with three PBS washes (10 min each,
RT). The sections were blocked in 3% NDS (2 h) and immediately incubated (24 h, RT) with a rabbit anti-NK3R primary antibody (1:150,000), which was prepared in blocking solution. This anti-NK3R titer was established by serial dilution (Hoffman et al., 2016) and revealed that NK3R-ir signals were not detectable without tyramine signal amplification (TSA) (Hunyady et al., 1996; von Wasielewski et al., 1997; Tóth and Mezey, 2007).

After primary incubation, the sections were rinsed with six 5-minute PBS washes, then incubated in a biotinylated donkey anti-rabbit secondary antibody (1:500) for 1 h (RT) before rinsing again with three 10-minute PBS exchanges. The sections were then treated with an avidin-biotin-horseradish peroxidase solution (1:1:833; PK-6100, Vector Laboratories, Burlingame, CA; RRID: AB_2336819) for 30 min (RT), rinsed thrice with PBS (10 min each), and incubated (20 min) in a PBT solution comprising 0.005% hydrogen peroxide and 0.5% biotinylated (EZ-Link sulfo-NHS-LC-biotin; ThermoFisher Scientific) tyramine (Sigma-Aldrich, St. Louis, MO), which was made in-house (Diniz et al., 2020). The sections were rinsed with three 10-min PBS exchanges and treated with Cyanine 3 (Cy3)-conjugated streptavidin (1:1,000) in 3% NDS without azide for 2 h (RT).

To prepare for anti-CART immunolabeling, the sections were washed in three 10-minute PBS washes and incubated in the rabbit anti-CART antibody (1:2,000) for 24 h (RT). They were then rinsed six times in PBS (5 min each) before treatment with donkey anti-rabbit Alexa Fluor 647 antibody (1:500) for 2 h (RT). Following three final PBS washes (10 min each), the slices were promptly mounted and coverslipped with ProLong Diamond Antifade Mountant (ThermoFisher Scientific).
2.4.3 Dual IHC and fISH

Immediately after tissue sectioning, free-floating tissues were rinsed with six 5-minute PBS washes (RT), blocked in 3% NDS for 1 h, and then incubated with a rabbit anti-MCH primary antibody (1:2,000) for 2 h. Subsequently, the sections were washed in three 5-minute PBS exchanges (RT), treated with donkey anti-rabbit Alexa Fluor 647 secondary antibody (1:500) for 2 h, washed again in three 5-minute PBS exchanges, and then mounted onto Superfrost Plus microscope slides (Fisher Scientific). The slides were air dried at RT (X min) and −20 °C (30 min), and then sealed in a microscope slides box (HS15994GF, Fisher Scientific) for storage at −80 °C to promote tissue adherence.

One week later, the slides were warmed in a HybEZ II oven (Advanced Cell Diagnostics (ACD), Newark, CA) at 37 °C for 45 min, dehydrated in ascending ethanol concentrations (50%, 70%, and 100%) at RT for 5 min each, and air-dried at RT for 15 min before implementing RNAscope-mediated fISH as previously described (Bono et al., 2022). Three parallel sets of slides were established for RNAscope fISH to determine the coexpression of Pmch and Egfp mRNA; hybridization for mouse peptidylprolyl isomerase B (Ppib) to verify RNA and tissue quality; and hybridization for Bacillus dihydrodipicolinate reductase (dapB) to assess background staining. All RNAscope probes were acquired from ACD and detailed in Table 3.
Table 3. List and details of RNAscope probes

<table>
<thead>
<tr>
<th>Probe</th>
<th>Gene target</th>
<th>Target base pair</th>
<th>Z pairs</th>
<th>Product no., Lot no.</th>
<th>Accession ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mm-Ppib</td>
<td>Peptidylprolyl isomerase B</td>
<td>98–856</td>
<td>15</td>
<td>313911, 21057A</td>
<td>NM_011149.2</td>
</tr>
<tr>
<td>dapB</td>
<td>Dihydridipicolinate reductase</td>
<td>414–862</td>
<td>10</td>
<td>320871, 21106A</td>
<td>EF191515</td>
</tr>
<tr>
<td>Gfp-O2</td>
<td>Green fluorescent protein</td>
<td>12–686</td>
<td>17</td>
<td>409011-C2, 22084D</td>
<td>AF275953.1</td>
</tr>
<tr>
<td>Mm-Pmch</td>
<td>Pro-melanin-concentrating hormone</td>
<td>4–652</td>
<td>11</td>
<td>478721, 21154E</td>
<td>NM_029971.2</td>
</tr>
</tbody>
</table>

Briefly, the sections were treated with hydrogen peroxide (322335, ACD) for 10 min (RT) to quench endogenous peroxidase activity and rinsed twice for 3 min each in distilled water (RT). The slides were then briefly (30 s) acclimated in distilled water at 99 °C before incubated with Target Retrieval Reagent (322000, ACD) for 5 min (99 °C). The slides were again rinsed in distilled water (15 s, RT), submerged in 100% ethanol (3 min, RT), and allowed to air-dry for 15 min (RT). A hydrophobic barrier was drawn around the tissue sections to prevent subsequent reagents from running off the slide.

The tissue was treated with Protease III (322337, ACD) at 40 °C for 30 min and washed with distilled water (3 min each, RT). RNAscope probes for Pmch and Egfp, Ppib, and dapB were applied to their respective slides and to hybridize for 2 h at 40 °C before washing three times (5 min each, RT) with Wash Buffer (3100931, ACD). Probe signals were amplified by the following incubations at 40 °C: AMP 1 (30 min; 323101, ACD), AMP 2 (30 min; 323102, ACD), and AMP 3 (15 min; 323103, ACD) that alternated with two exchanges in fresh Wash Buffer (5 min each, RT).
All tissues were treated with HRP-C1 (15 min, 40 °C; 323104, ACD), washed twice in Wash Buffer (5 min each, RT), and incubated with TSA Plus Cyanine 3 (1:750; 30 min, 40 °C; NEL744E001KT, PerkinElmer, Waltham, MA) prepared in TSA Buffer (322809, ACD). After two washes in Wash Buffer (5 min each, RT), the tissues were incubated in HRP Blocker (15 min, 40 °C; 323107, ACD). HRP-C2 (15 min, 40 °C; 323105, ACD) was then applied to the tissue followed by Opal 520 (1:500, 30 min, 40 °C; FP1487001KT, Akoya Biosciences, Marlborough, MA) prepared in TSA Buffer. The tissue was rinsed twice with Wash Buffer (5 min each, RT), incubated with HRP Blocker (15 min, 40 °C), and again rinsed in Wash Buffer (5 min each, RT). All slides were coverslipped with ProLong Diamond Antifade Mountant (ThermoFisher Scientific).

2.5 Nissl stain

Nissl stains (Simmons and Swanson, 1993) were performed on tissues adjacent to our experimental tissue to determine the levels and cytoarchitectural boundaries within each slice. As previously described (Negishi et al., 2020), the mounted tissues were dehydrated in serial washes of 50%, 70%, 95%, and three exchanges of 100% ethanol (3 min each, RT) and then delipidated in two incubations with fresh xylene for 10 min and 15 min, respectively. The tissues were then rehydrated by six ethanol washes (3 min each) repeated in reverse order and then soaked in distilled water for 3 min.

The tissues were stained with a 0.25% w/v thionine dye solution (pH 4.5) at RT for about 5 s. If the staining appeared too dark, the slides were rinsed in 4% glacial acetic acid to remove excess dye (or in distilled water if dye is excessive), and if the staining was too faint, the slides were dipped again in thionine solution until the grey and white
matter were clearly distinguishable by eye. The reaction was quenched in distilled water, and the slides were dehydrated in increasing concentrations of ethanol (3 min each), cleared in in two xylene incubations (15 min), and then coverslipped using Richard-Allan Scientific Mounting Media (Fisher Scientific).

2.6 Microscopy

All images were captured using an Eclipse Ti2 inverted microscope (Nikon Instruments Inc., Mississauga, Canada) equipped with a motorized stage, DS-Ri2 color camera (Nikon), Prime 95B CMOS camera (Photometrics, Tucson, AZ), and Plan Achromat 10× (0.45 numerical aperture), 20× (0.75 numerical aperture), or 40× (0.95 numerical aperture) objective lenses. Captured images were stitched and/or adjusted for brightness using NIS Elements software (Nikon), exported as TIFF files, and then imported into Adobe Illustrator 26.2.1 (Adobe Systems Inc., San Jose, CA) for analysis and figure assembly.

2.6.1 Epifluorescence imaging

Large overview images of the entire brain slice in fluorescence were acquired with a Prime 95B CMOS camera using the 10× objective lens. Tissue structures were illuminated by a 561-nm wavelength light via a SPECTRA X engine (Lumencor, Beaverton, OR). Tiled images were stitched into a single overview image of the entire slice using NIS Elements software.
2.6.2 Confocal imaging

High resolution images (2,048 × 2,048 pixels) of the hypothalamus were captured with a C2 confocal system (Nikon) using either a 10× or 20× objective lens. Regions of interest were selected from overview images (section 2.7.1) and acquired with a 488-, 561-, or 640-nm wavelength lasers to visualize native EGFP or Opal 520, Cy3 or Alexa Fluor 594, and Alexa Fluor 647, respectively. Resulting images were stitched to simultaneously display individual cells and an overview of the entire hypothalamus, where applicable, and pseudo-colored green, orange, or magenta, respectively.

In RNAscope experiments, the laser and image settings were adjusted to eliminate visible fluorescence in the dapB negative control sections and then applied to capture and process images from experimental tissue. All images were exported as TIFF files to Adobe Illustrator for assembly and quantification.

2.6.3 Brightfield imaging

Large field-of-view images of the entire brain slice from Nissl-stained tissues were acquired with the DS-Ri2 color camera using a 10× objective lens and were stitched using NIS Elements (Nikon) to resolve white and gray matter distinctions within each slice. These were exported as TIFF files and imported into Adobe Illustrator for plane-of-section analysis and alignment with epifluorescence or confocal images.
2.7 Image analysis

2.7.1 Plane-of-section analysis and Nissl-based parcellation

Stitched overview brightfield and epifluorescence images of each entire brain slice were imported into Adobe Illustrator and were overlayed and aligned to one another to determine its corresponding Allen Reference Atlas (ARA) level (Dong, 2008). Nissl-based parcellations were drawn using the Pen tool to distinguish the boundaries of each brain region or white matter. Parcellations were defined based on cell morphology, density, and directionality, and they were superimposed onto the matching epifluorescence overview image of the brain slice. Additionally, higher magnification confocal images were aligned to the epifluorescence overview image thus effectively transferring parcellations onto confocal images also.

Using the regions defined in each slice by their corresponding parcellations, we recreated the pattern of cell expression on templates from the ARA. If a slice was uneven dorsoventrally, it was subdivided into three horizontal segments; from the mamillary bodies dorsally to the ventral tip of the globus pallidus; then to the fornix dorsally; and then to the ventral edge of the hypothalamus slice. A slice was only included for cell quantification if: i) the same level was assigned to the top two horizontal segments of a slice, as these horizontal segments comprised the majority of MCH cells, and ii) if a matching level was found for the bottommost horizontal segment in the same brain series.

To quantify the cellular distribution mediolaterally, each ARA map was divided into four 0.5 mm wide vertical sections, stretching from the 3rd ventricle to the lateral border of
the hypothalamus. We quantified the proportion of each cell type expressed within each vertical segment to determine their mediolateral distribution.

### 2.7.2 Cell quantification and atlas-based mapping

An epifluorescence overview image of the entire brain slice and a high-magnification confocal image comprising preselected regions of interest were assembled in an Adobe Illustrator file to be aligned so that both images together would simultaneously display the entire brain slice and the distribution of cells within our region of interest. Each cell type was marked with a filled circle using the Ellipse tool and then counted by determining the number of circles selected in the Document Info menu of Adobe Illustrator. The relative positions of filled circles were mapped onto ARA brain templates (Dong, 2008). Mean cell counts per level was reported unilaterally. Total cell counts per brain was reported bilaterally.

Number of cells reported was corrected for oversampling, as previously described (Chee et al., 2013; Negishi et al., 2020; Bono et al., 2022), using the Abercrombie formula (Abercrombie, 1946): 

\[
    P = A \left( \frac{M}{L+M} \right)
\]

where \( P \) is the corrected count, \( A \) is the original count, \( M \) is the mean tissue thickness (17.42 μm) determined from five brain slices, and \( L \) is the mean cell diameter (14.60 μm) determined from 107 MCH-ir cells.

### 2.8 Electrophysiology

#### 2.8.1 Slice preparation

Male and female \textit{Mch-cre;L10-Egfp} mice (4–23 weeks) were anesthetized with 7% chloral hydrate (700 mg/kg, ip) and then were transcardially perfused with ice-cold N-
methyl-D-glucamine based (NMDG)-slice artificial cerebrospinal fluid (ACSF) containing (in mM): 50 NMDG, 1.25 KCl, 10 HEPES, 10 MgSO\(_4\), 12.5 glucose, 15 NaHCO\(_3\), 0.25 CaCl\(_2\), 1 thiourea, 2.5 L-ascorbic acid, 1.5 sodium pyruvate (300 mOsm/L, pH 7.4). The brains were rapidly removed from the skull and were sliced with a vibratome (VT1000S, Leica, Wetzlar, Germany) into 250 μm-thick coronal sections. Slices were incubated in NMDG-slice ACSF at 37 °C for 5 min and then transferred to bath ACSF comprising (in mM): 124 NaCl, 3 KCl, 1.3 MgSO\(_4\), 1.4 NaH\(_2\)PO\(_4\), 10 glucose, 26 NaHCO\(_3\), 2.5 CaCl\(_2\) (300 mOsm/L) for 5 min (37 °C). Slices were allowed to recover at RT for at least one hour prior to slice recording. All slice and bath ACSF solutions used were constantly carbogenated (95% O\(_2\), 5% CO\(_2\)).

2.8.2 Whole-cell patch-clamp recordings

Slices were equilibrated in a recording chamber and maintained by a continuous flow (1–1.5 ml/min) of carbonated bath ACSF at 31–32 °C. MCH cells were identified by epifluorescence illumination of native EGFP and visualized by infrared differential interference contrast at 40× magnification on either an Eclipse FN1 microscope (Nikon) equipped with a mercury lamp (C-SHG1, Nikon), pco.panda 4.2 sCMOS camera (Excelsitas PCO GmbH, Kelheim, Germany), and NIS Elements imaging software (Nikon) or an Examiner.A1 microscope (Carl Zeiss Inc, Oberkochen, Germany) equipped with a mercury lamp (HXP 120, Zeiss), an AxioCam Mrm camera (Zeiss), and AxioVision 4.8 software (Zeiss).

Glass micropipettes used for recording were pulled using a Flaming/Brown Micropipette puller (P-1000, Sutter Instruments, Novato, CA) with a pipette resistance of
7–10 MΩ when backfilled with an internal solution containing (in mM): 120 K-gluconate, 10 KCl, 10 HEPES, 1 MgCl₂, 1 EGTA, 4 MgATP, 0.5 NaGTP, 10 phosphocreatine, and 0.2% biocytin (pH 7.24, 280–290 mOsm/L). All recordings were acquired using a MultiClamp 700B amplifier (Molecular Devices, San Jose, CA) and digitized by either a Digidata 1440A or 1550B (Molecular Devices) running pClamp 10.3 or 11.2 software (Molecular Devices).

2.8.3 Electrophysiological parameters

All recordings were filtered at 1 kHz, and all reported values were corrected for a +15 mV liquid junction potential. In between recordings, the cells were held at a holding potential $V_h = -60$ mV. Electrophysiological data was collected using Clampfit 10.3 or 11.2 software (Molecular Devices), unless indicated otherwise.

**Resting membrane potential (RMP)** was averaged over two minutes in current-clamp mode with no current injection immediately after establishing whole-cell configuration.

**Cell excitability** was determined by rheobase, and the number of action potentials elicited from $V_h = -60$ mV by +10 pA depolarizing current steps (3 s) evoked from 0 pA to 90 pA. Action potentials were considered if their amplitudes exceeded 0 mV.

**Input resistance** was determined by measuring the voltage output from a −30 pA hyperpolarizing current step (3 s).

**Current–voltage (I–V) relationship.** Current change was determined in voltage-clamp at $V_h = -60$ mV in response to −10 mV voltage steps (250 ms) elicited from −40 mV to −110 mV.
Excitatory synaptic input was measured as the spontaneous excitatory post-synaptic current (sEPSC) events recorded at $V_h = -60$ mV. sEPSC frequency, interevent intervals, and amplitude were detected using MiniAnalysis (Synaptosoft, Fort Lee, NJ). sEPSC kinetics were determined by the rise time, which was calculated as the slope at 10–90% of each sEPSC rising phase, and decay time, which was calculated as τ based on a single-exponential fit from the peak to the end of the sEPSC decay phase. Amount of charge carried by each sEPSC was determined by the area under the curve of each event. Cells were excluded if noise threshold ≥ 9 or if the cell was identified as an outlier, in at least 2 parameters, by ROUT test when $Q = 0.1\%$.

2.9 Post hoc labeling of Mch-cre;L10-Egfp cells

All recordings were maintained in whole-cell configuration for at least 30 min to ensure full penetration of biocytin fill throughout the recorded cell. At the end of the recording, the pipette was gently extracted by slowly gliding the pipette in a diagonal along the XZ plane, as described by Swietek et al. (2016).

2.9.1 Thick tissue IHC

The whole slice was immediately submerged in 10% formalin for 15–18 h and then rinsed in six 5-min PBS washes (RT). The slices were permeabilized by incubation in PBS comprising 2% TritonX-100 for 45 min and blocked in 3% NDS prepared in PBT-azide composed of 0.25% TritonX-100 and 0.02% sodium azide for 2 h. Next, slices were incubated with rabbit anti-CART primary antibody (1:2,000) for 48 h at 4 °C.
The slices were rinsed in six 5-min exchanges of PBS (RT) and then incubated in donkey anti-rabbit Alexa Fluor 647 (1:1,000) and Cy3-conjugated streptavidin (1:5,000; Jackson ImmunoResearch; AB_2337244) for 2 h (RT) prepared in 3% NDS. Slices were wet-mounted onto Superfrost Plus microscope slides for confocal imaging with a 20× objective lens to determine native EGFP and CART-ir signals in biocytin-filled cells. Only biocytin-filled cells that expressed EGFP were included in our analyses.

2.9.2 3,3′-diaminobenzidine (DAB) labeling of biocytin-filled cells

Following imaging analysis, the slides were soaked in PBS at RT and gently slipped off the microscope slide. The free-floating brain slices were prepared for morphological analysis. They were treated with 1% hydrogen peroxide in PBT for 30 min (RT) to quench endogenous peroxidase activity, rinsed in three 10-min PBS exchanges (RT), and treated to form the avidin-biotin complex in PBS (1:1:500; Vector Laboratories; AB_2336819) for 72 h (4 °C). After three PBS washes (10 min each), the slices were incubated in a nickel-enhanced DAB solution, which was prepared according to manufacturer instructions, until the cells were dark black, and the cell outline was clearly visible (~5 min). The thick slices were then mounted onto Superfrost Plus microscope slides, dehydrated in increasing ethanol concentrations (50%, 70%, 95%, and 100%) for 6 min each, delipidated in 100% xylene for 2 h, and coverslipped with Richard-Allan Scientific Mounting Media. Morphological features of the biocytin-filled DAB-stained cell was matched via brightfield imaging using a 20× objective lens.
2.10 Morphological reconstruction and analysis

For cell reconstruction, the thick tissue slice was visualized in brightfield on a BX51 (Olympus, Tokyo, Japan) upright microscope that was equipped with a CX9000 camera (MBF Bioscience, Williston, VT) and fully motorized stage. Cells were analyzed live using an oil immersion Olympus Plan N 100× objective lens (0.95 numerical aperture). The soma, dendrites, and axon of each cell were traced using Neurolucida 2020.1.3 software (MBF Bioscience). A single line with varying widths was traced through the center of each process to match the thickness of each axon and/or dendrite.

Each reconstructed cell was exported into Neurolucida Explorer 2019.2.1 (MBF Bioscience). The number of nodes was determined by branched structure analysis, and the total length of each process and distance of each node from the soma were determined by Sholl analysis (Sholl, 1953), with each concentric ring increasing by a 10 μm radius. Primary dendrites were defined as those originating at the soma; secondary dendrites branched from primary dendrites, and tertiary dendrites branched from secondary dendrites. Similarly, primary nodes originated on primary dendrites, while secondary nodes originated on secondary dendrites, and henceforth. Cell tracings were exported as vectors and imported into Adobe Illustrator.

2.11 Statistics

Data are represented as mean ± SEM, and the sample size, shown in parenthesis within the figures, is represented as n for number of cells and N for number of mice. Means compared across time, voltage, distance, or node order were compared using two-way, where †, p < 0.05; ††, p < 0.01; †††, p < 0.001 or mixed-design (if some data points
were missing) ANOVA, where $^\wedge$, $p < 0.05$ with the Geisser-Greenhouse correction for lack of sphericity. If there were significant main effects or interaction, a Bonferroni multiple comparison post hoc test was also implemented, where significance was determined as: $^*, p < 0.05; ^{**}, p < 0.01; ^{***}, p < 0.001$. Three-way ANOVA was used to test for sex differences between cell types across time, current injection, voltage range, or node order. Tests were chosen based on normality and homogeneity of variance. Simple linear regression was used to estimate the relationship between morphological and electrophysiological data, with significance reported as: $^*$, $p < 0.05$. All statistical analyses were performed in Prism 9.3.1 (GraphPad Software Inc., San Diego, CA). Graphs and sample electrophysiological recordings traces were obtained using Prism and OriginPro (OriginLab, Northampton, MA), respectively.
3. RESULTS

3.1 Neuroanatomy

3.1.1 Validity and specificity of $\text{EGFP}^\text{Mch}$ expression in $\text{Mch-cre}$ mice

We selected MCH neurons by native EGFP fluorescence ($\text{EGFP}^\text{Mch}$) in $\text{Mch-cre;L10-Egfp}$ mice. EGFP$^\text{Mch}$ was detected only within the hypothalamus (Figure 1a, b), and nearly all MCH-ir neurons ($92 \pm 2\%$, $N = 5$) expressed EGFP$^\text{Mch}$ (Figure 1c). Importantly, we also determined the specificity of EGFP$^\text{Mch}$ labeling for MCH neurons and found that $81 \pm 4\%$ ($N = 5$) of EGFP$^\text{Mch}$ cells coexpressed MCH immunoreactivity (Figure 1d). However, by analyzing the distribution of MCH-ir and EGFP$^\text{Mch}$ signals across the MCH field rostrocaudally, we found that MCH-negative EGFP$^\text{Mch}$ cells were largely restricted to ARA level (L) 73 (Figure 1e; $N = 4$).

In the anterior MCH field (L63–66), we observed scattered MCH-ir cells that did not express EGFP (EGFP$^\text{Mch}$−/MCH+) and EGFP-labeled cells that did not express MCH immunoreactivity (EGFP$^\text{Mch}$+/MCH−) in the medial zona incerta (ZI) and the posterior part of the anterior hypothalamic nucleus (AHNp; Figure 2a–c). Towards the middle of the MCH field (L67–72), we saw robust colocalization between EGFP$^\text{Mch}$ and MCH immunoreactivity (EGFP$^\text{Mch}$+/MCH+), with only few non-colocalized cells in the ZI, LHA, and anterior part of the dorsomedial hypothalamic nucleus (DMHa), paraventricular nucleus (PSTN) and subthalamic nucleus (STN; Figure 2d–i). However, at L73, we consistently observed an EGFP$^\text{Mch}$+/MCH− cluster that appeared immediately dorsal to the fornix (Figure 2j), as described by Beekly et al. (2020). In the posterior hypothalamic MCH field (L74–77), we found consistent EGFP$^\text{Mch}$+/MCH+ colocalization, with only a
few EGFP<sup>Mch</sup> −/MCH+ cells and a few EGFP<sup>Mch</sup>+/MCH− cells in the medial LHA (Figure 2k, l).

Figure 1. Validation of hypothalamic EGFP<sup>Mch</sup> expression in Mch-cre mice. Representative merged channel confocal photomicrographs from level 72 of the Allen Reference Atlas (ARA; Dong, 2008) at −1.755 mm relative to Bregma β within the hypothalamus (a). Merged channel confocal photomicrograph from outlined area in a (bi) of native EGFP fluorescence (EGFP<sup>Mch</sup>; bii) and MCH immunoreactivity (biii) from the brain of a Mch-cre;L10-Egfp mouse. Representative sample of EGFP<sup>Mch</sup> cells that coexpressed MCH immunoreactivity (white arrowheads), EGFP<sup>Mch</sup> cells that were MCH-negative (open arrowheads), and MCH-immunoreactive (MCH-ir) cells that did not express EGFP<sup>Mch</sup> (asterisk; b). Percentage of MCH-ir cells marked by the presence (dark teal) or absence (magenta) of EGFP<sup>Mch</sup> (c). Percentage of EGFP<sup>Mch</sup> labeled cells marked by the presence (dark teal) or absence (light blue) of MCH-ir signals (d). Anteroposterior distribution of the mean number of EGFP<sup>Mch</sup>−/MCH+ cells (magenta), EGFP<sup>Mch</sup>+/MCH− cells (light blue), and dual-labeled EGFP<sup>Mch</sup>+/MCH+ cells (dark teal) at each level of the hypothalamus assigned in accordance with the ARA. Scale bar: 200 μm (a); 50 μm (b). fx, fornix; int, internal capsule; mtt, mammillothalamic tract; V3, third ventricle.

Overall, we found that EGFP<sup>Mch</sup> cells robustly identified MCH neurons in Mch-cre;L10-Egfp mice throughout the MCH field, with the exception of a distinctive cluster of EGFP<sup>Mch</sup>+/MCH− cells situated within or around the fornix (Figure 3a, b). These
EGFP<sup>Mch</sup>+/MCH− cells also appeared smaller and fainter than surrounding MCH-ir cells. We performed dual in situ hybridization for Gfp mRNA and Pmch mRNA, but nearly all EGFP<sup>Mch</sup>+/MCH− cells did not express either transcript (Figure 3c) and may represent dormant MCH cells. As well, though rare, EGFP<sup>Mch</sup> cells that are not MCH-ir can express Pmch (Figure 3ciii, asterisk).

### 3.1.2 Spatial distribution of CART and/or NK3R coexpression at MCH neurons

As EGFP<sup>Mch</sup> expression robustly represented MCH neurons (Figure 1, 2), we proceeded to determine the colocalization of CART and NK3R immunoreactivity in EGFP<sup>Mch</sup> cells (Figure 4a). The number and proportion of each EGFP<sup>Mch</sup> cell type was similar between female and male mice. EGFP<sup>Mch</sup>-only cells comprised over half of all EGFP<sup>Mch</sup> cells counted from the hypothalamus of female (53 ± 7%, N = 4; Figure 4b) and male (55 ± 2%, N = 2; Figure 4c) Mch-cre;L10-Egfp mice. There were relatively equal proportions of EGFP<sup>Mch</sup> cells that coexpressed both CART and NK3R (EGFP<sup>Mch</sup>/CART/NK3R; female: 21 ± 3%; male: 23 ± 3%) or that coexpressed CART only (EGFP<sup>Mch</sup>/CART; female: 22 ± 4%; male: 20 ± 2%), thus nearly half of all EGFP<sup>Mch</sup> cells were CART-positive. The remaining EGFP<sup>Mch</sup> cells included a small proportion of EGFP<sup>Mch</sup> cells that coexpressed NK3R only (EGFP<sup>Mch</sup>/NK3R; female: 4 ± 1%; male: 2 ± 0%). All four subpopulations of EGFP<sup>Mch</sup> cells could be seen throughout the rostrocaudal axis of the hypothalamus (L63–L77), but the availability of EGFP<sup>Mch</sup> cells gradually peaked at L71 then decreased posteriorly in a normally distributed pattern (Figure 4d).
Figure 2. Spatial distribution of EGFP\textsuperscript{Mch} expression in \textit{Mch-cre} mice. Representative coronal maps using \textit{Allen Reference Atlas} (ARA; Dong, 2008) brain templates arranged from rostral to caudal (a–l) showing the relative distribution of EGFP\textsuperscript{Mch−/MCH+} cells (magenta circles), EGFP\textsuperscript{Mch+/MCH−} cells (light blue circles), and dual-labeled EGFP\textsuperscript{Mch+/MCH+} cells (dark teal circles) from the hypothalamus of \textit{Mch-cre;L10-Egfp} mice. Each panel includes the corresponding ARA level (top right), stereotaxic coordinate inferred from Bregma (β; bottom right), and brain region labels according to ARA nomenclature.
As CART-expressing MCH cells are expectedly more abundant in the medial hypothalamus (Broberger, 1999; Vrang et al., 1999; Elias et al., 2001; Croizier et al., 2010), we expressed the proportion of each subpopulation across the mediolateral axis of the hypothalamus in 0.5 mm increments away from the third ventricle. All defined EGFP<sup>Mch</sup> subtypes could be identified across the mediolateral axis of the hypothalamus, but overall, CART-positive EGFP<sup>Mch</sup> cells were more abundant medially then gradually
diminished laterally (Figure 4e). Interestingly, CART-positive cells that also expressed NK3R were evenly distributed mediolaterally (Figure 4e). By contrast, CART-negative EGFP\textsuperscript{Mch} only and EGFP\textsuperscript{Mch}/NK3R cells gradually increased in abundance laterally (Figure 4e).

Figure 4. Diverse MCH cell types defined by CART and NK3R coexpression. Merged channel (ai) confocal photomicrographs of native EGFP fluorescence (EGFP\textsuperscript{Mch}; aii), CART immunoreactivity (aiii), and NK3R immunoreactivity (aiiv) within the hypothalamus of Mch-cre;L10-Egfp mice. Representative sample of EGFP\textsuperscript{Mch}-only cells (open arrowheads), EGFP\textsuperscript{Mch} cells that coexpressed both CART and NK3R (EGFP\textsuperscript{Mch}/CART/NK3R; filled arrowheads), EGFP\textsuperscript{Mch} cells that coexpressed CART but not NK3R (EGFP\textsuperscript{Mch}/CART; * asterisk), and EGFP\textsuperscript{Mch} cells that were CART-negative but coexpressed NK3R (EGFP\textsuperscript{Mch}/NK3R; † dagger). Percentage of EGFP\textsuperscript{Mch}/CART (blue), EGFP\textsuperscript{Mch}/CART/NK3R (orange), EGFP\textsuperscript{Mch}/NK3R (purple), and EGFP\textsuperscript{Mch}-only cells (turquoise) determined in female (b) and male (c) Mch-cre;L10-Egfp mice. Anteroposterior distribution of all EGFP\textsuperscript{Mch} cell types at each level of the hypothalamus assigned in accordance with the Allen Reference Atlas (Dong, 2008; d). Mediolateral distribution of all EGFP\textsuperscript{Mch} cell types relative to the third ventricle (V3) in accordance with stereotaxic coordinate from the Allen Reference Atlas (e). Scale: 100 μm (a).

To determine the relative distribution of each EGFP\textsuperscript{Mch} cell type, we used Nissl-based parcellations to delineate hypothalamic subnuclei and generate high-resolution...
spatial maps of all EGFP<sub>Mch</sub> cells. There were few EGFP<sub>Mch</sub> cells anteriorly (L63–L65), where they were loosely scattered medially and not confined to a specific hypothalamic nucleus (Figure 5a, b). The number of EGFP<sub>Mch</sub>/CART and EGFP<sub>Mch</sub>/CART/NK3R cells increased in L66–L69 and were found laterally within the AHNp, the DMHa, and the ZI (Figure 5c–f). CART-negative EGFP<sub>Mch</sub>-only cells emerged and became more prominent posteriorly (L68–L73), where they were distributed lateral to the fornix in the LHA, in the region below the ZI and ventral border of the internal capsule and cerebral peduncle (Figure 5e–j). A few EGFP<sub>Mch</sub>/NK3R cells were scattered within this cluster of EGFP<sub>Mch</sub>-only cells (i.e., Figure 5h). Towards the posterior field (L72–74), EGFP<sub>Mch</sub> cells started to separate into two clusters. The medial cluster comprised more CART-positive cells, both EGFP<sub>Mch</sub>/CART and EGFP<sub>Mch</sub>/CART/NK3R, and they were distributed between the fornix and the DMH (Figure 5i, j) and along the ventral border of the posterior hypothalamic nucleus (PH; Figure 5k). A second cluster was lateral to the fornix and largely comprised EGFP<sub>Mch</sub>-only cells in the prepiramidal nucleus (PST; Figure 5i), ventral edge of the subthalamic nucleus (STN; Figure 5i), and parasubthalamic nucleus (PSTN; Figure 5k). The availability of EGFP<sub>Mch</sub> cells was sparse in posterior hypothalamic regions (L75–L77; Figure 5l).

As expected, our spatial maps revealed neuroanatomical division between medial CART-positive and lateral CART-negative MCH cells. However, there was no mediolateral separation for NK3R expression in CART-positive MCH cells. This topographical division suggested that heterogenous MCH cells, especially based on CART coexpression, could contribute to unique neuronal networks.
Figure 5. Spatial distribution of CART and/or NK3R coexpression in heterogeneous MCH cells. Representative coronal maps using Allen Reference Atlas (ARA; Dong, 2008) brain templates ordered from rostral to caudal (a–l) showing the relative distribution of EGFP<sup>Mch</sup>-only (turquoise circles), EGFP<sup>Mch</sup>/CART (blue circles), EGFP<sup>Mch</sup>/NK3R (purple circles), and EGFP<sup>Mch</sup>/CART/NK3R cells (orange circles) from the hypothalamus of Mch-cre;L10-Egfp mice. Each panel includes the corresponding ARA level (top right), stereotaxic coordinate inferred from Bregma (β; bottom right), and brain region labels according to ARA nomenclature.
3.2  Electrophysiology

3.2.1 Female MCH cells differed in passive membrane properties

To determine if differences in electrophysiological characteristics, including passive and active membrane properties, also contribute to the overall heterogeneity of MCH cells, we performed whole-cell patch-clamp recordings from CART-positive (MCH/CART+) and CART-negative (MCH/CART−) MCH cells from Mch-cre;L10-Egfp male and female mice (Figure 6a). We recorded and biocytin-filled cells identified by EGFP<sup>Mch</sup> and processed them for post hoc immunostaining to determine the presence (Figure 6b) or absence of CART immunoreactivity (Figure 6c) in MCH/CART+ and MCH/CART− cells, respectively.

There was a strong effect of cell type (i.e., based on CART coexpression; F(1, 96) = 8.8, p = 0.004) on input resistance. The effect of sex was less pronounced (F(1, 96) = 3.9, p = 0.051) but interacted with cell type to effect input resistance (F(1, 96) = 4.6, p = 0.034). We found that the mean input resistance of MCH/CART+ cells was lower than MCH/CART− cells in female (t = 3.9, df = 96, p = 0.003) but not male mice (t = 0.53, df = 96, p = 0.999; Figure 6d). Accordingly, CART expression also impacted the current–voltage relationship between female MCH cells (F(1, 47) = 11.99, p = 0.001) and interacted with voltage (F(7, 329) = 8.639, p < 0.0001), as a larger inward current was elicited at negative voltage potentials in MCH/CART+ cells (Figure 6e). In male MCH cells, there was no effect of cell type on current output (F(1, 39) = 2.2, p = 0.141), but CART expression also interacted with voltage (F(7, 273) = 3.0, p = 0.004) as the current elicited tended to be greater at negative voltage steps (Figure 6f). To determine if there is a sex difference in the current–voltage relationship between MCH cells, we ran a three-
way ANOVA with sex included as a factor with cell type and voltage. We found significant effects of voltage (F(1.110, 95.45) = 172.4, p < 0.0001) and cell type (F(1, 86) = 11.63, p = 0.001) on current output, but there was no overall effect of sex (F(1, 86) = 0.51, p = 0.4767), and there was no significant interaction between sex and voltage (F(7, 602) = 1.2, p = 0.280) or cell type (F(1, 86) = 1.19, p = 0.2787).

Figure 6. Lower input resistance and larger inward current elicited at female MCH/CART+ cells. Low (ai) and high magnification (aii, from outlined region in ai) photomicrograph captured by infrared differential interference contrast imaging of a coronal brain slice from Mch-cre;L10-Egfp mice. Merged channel (i) confocal photomicrograph of native EGFP fluorescence (EGFP<sub>Mch</sub>, ii), biocytin-labeling (iii), and CART immunoreactivity (iv) in MCH/CART+ (b; filled arrowhead) and MCH/CART− cell (c; open arrowhead). Comparison of input resistance (d) and current–voltage relationship (e, f). Representative sample traces of voltage steps (top) and current elicited (bottom) in female (ei) and male (fi) MCH/CART+ and MCH/CART− cells. Current-voltage relationship elicited in female (eii) and male (fii) MCH/CART+ and MCH/CART− cells. Scale: 200 μm (a), 40 μm (b, c), 200 pA, 50 ms (ei, fi). Two-way ANOVA: †, p < 0.05; ††, p < 0.01; ††††, p < 0.0001 with Bonferroni multiple comparisons posttest: **, p < 0.01; †††††, p < 0.0001.
3.2.2 Male MCH cells differed in active membrane properties

There was no effect of cell type (F(1, 110) = 0.21, p = 0.646) or sex (F(1, 110) = 1.24, p = 0.267) on the resting membrane potential of MCH cells, and there was also no interaction between cell type and sex (F(1, 110) = 0.17, p = 0.678). There were no resting membrane potential differences between the MCH/CART+ and MCH/CART− cells in female (t = 0.03, df = 62, p = 0.974) or male mice (t = 0.7, df = 47.9, p = 0.490; Figure 7a).

While there were no differences in resting membrane potential, we determined if there were differences in the electrical fingerprint or excitability of MCH/CART+ and MCH/CART− cells. We first determined if there were differences in the rheobase of MCH cell types by comparing the minimum amount of current required to elicit action potential firing. We found no difference in the rheobase of MCH/CART+ and MCH/CART− cells in female (t = 0.82, df = 57, p = 0.41) or male mice (t = 0.7, df = 43.37, p = 0.47), and there was no effect of cell type (F(1, 106) = 0.0002, p = 0.987), sex (F(1, 106) = 0.08, p = 0.779), or cell type by sex interaction (F(1, 106) = 1.2, p = 0.283; Figure 7b).

All MCH cells increased the number of action potentials elicited with increasing current steps from the rheobase (female: F(8, 309) = 80.7, p < 0.0001; male: F(8, 274) = 123.1, p < 0.0001). Interestingly, while there was also no effect of cell type at female (F(1, 53) = 0.1, p = 0.783; Figure 7c) or male cells (F(1, 43) = 2.9, p = 0.097; Figure 7d), we found that cell firing depended on the amount of current injected at each cell type. There was a significant interaction between current injection and cell type at male (F(8, 274) = 2.5, p = 0.013; Figure 7d) but not female MCH cells (F(8, 309) = 1.4, p = 0.213; Figure 7c). To determine if there was a sex difference in the excitability of
MCH cells, we included sex as a factor in an overall model with cell type and the amount of current injected. There was no main effect of sex (F(1, 95) = 0.4, p = 0.547), but sex was an important factor in a three-way interaction with cell type and current injection to impact action potential firing (F(8, 583) = 3.4, p = 0.001).

As previously shown (Burdakov et al., 2005), all MCH cells exhibited spike rate adaptation. We evaluated firing frequency in three 1-second bins (Figure 7ei, fi) to determine differences in firing at the start and end of the depolarizing current step (3 s), and we found a strong effect of time in female (F(1.5, 61.1) = 53.0, p < 0.0001) and male cells (F(1.2, 42.8) = 88.8, p < 0.0001). Interestingly, there was no main effect of cell type (female: F(1, 42) = 0.5, p = 0.480; male: F(1, 35) = 0.02, p = 0.899), but there was a significant interaction between the firing frequency of each cell type and time in female (F(2, 84) = 3.9, p = 0.024; Figure 7eii) and male cells (F(2, 70) = 33.6, p < 0.0001; Figure 7fii) that produced differences in spike rate adaptation between MCH/CART+ and MCH/CART− cells. In male MCH cells, MCH/CART+ cells had a higher firing rate at the start of the current step (i.e., within the first 1-second current bin; t = 2.9, df = 31.4, p = 0.018) but had much lower firing by the end of the current step (i.e., in the third 1-second current bin; t = 3.1, df = 29.5, p = 0.011; Figure 7fii). This was consistent with a unique electrical fingerprint in male MCH/CART+ cells that comprised an initial burst of action potentials followed by a sharp drop in firing frequency (Figure 7fii). To determine if there was a sex difference in the strength of spike rate adaptation, we included sex as a factor in a three-way ANOVA but found no main effect of sex (F(1, 77) = 2.7, p = 0.103). Interestingly, there was a strong interaction between cell type and time (F(2, 154) = 22.7, p < 0.0001) that persisted regardless of sex (F(2, 154) = 2.8, p = 0.064). Taken together,
our results indicated that, regardless of sex, all MCH cells underwent spike rate adaptation upon sustained depolarization. This firing rate accommodation was most pronounced in male CART+ cells.

Figure 7. Elevated spike rate adaptation at male MCH/CART+ cells. Comparison of resting membrane potential (RMP; a), rheobase (b), and the number of action potentials elicited with increments of +10 pA current steps above rheobase between MCH/CART+ and MCH/CART− cells in female (c) and male Mch-cre;L10-Egfp mice (d). Representative sample current traces of action potential firing (t; top) elicited by a current step (3 s) at +50 pA above rheobase for its respective cell (bottom, black trace) from female (e) and male cells (f). Comparison of instantaneous frequency of action potentials elicited by the current step was determined over three 1-second bins (fii). Repeated measures: †, p < 0.05; ††††, p ≤ 0.0001 or mixed-effect two-way ANOVA: ^, p < 0.05 with Bonferroni multiple comparisons posttest: *, p < 0.05. Scale: 20 mV, 500 ms (ei, fi).
3.2.3 Differences in rise time kinetics of excitatory events at MCH cells

The differences in spatial distribution and electrical properties of MCH/CART+ and MCH/CART− cells suggested that MCH cells may be differentially regulated by afferent input. In particular, excitatory inputs to MCH cells are regulated by feeding (Li and Van Den Pol, 2009; Linehan et al., 2020) and sleep (Briggs et al., 2018), thus we analyzed the spontaneous excitatory postsynaptic currents (sEPSC) arriving at MCH/CART+ and MCH/CART− cells in female (Figure 8a) and male mice (Figure 8b).

We found no difference in the cumulative distribution of interevent intervals (p = 0.489; Figure 8c), amplitude (p = 0.081; Figure 8f), or area (p = 0.174; Figure 8i) of sEPSCs arriving at female MCH cells. However, we found differences in the distribution of sEPSC events collected from male MCH cells. There was a rightward shift in the cumulative probability of interevent intervals (p = 0.004; Figure 8d) and leftward shift in the cumulative probability of amplitude (p < 0.0001; Figure 8g) and area of sEPSC events (p = 0.0002; Figure 8j) thus implicating that a subset of sEPSC events at male MCH/CART+ cells were smaller and occurred less frequently. However, we found no significant effect of cell type on the mean sEPSC frequency (F(1, 57) = 1.3, p = 0.263; Figure 8e), amplitude (F(1, 57) = 3.4, p = 0.071; Figure 8h), or area (F(1, 57) = 0.3, p = 0.573; Figure 8k). There was also no sex difference on the mean sEPSC frequency (F(1, 57) = 0.005, p = 0.943; Figure 8e), amplitude (F(1, 57) = 0.8, p = 0.388; Figure 8h), or area (F(1, 57) = 0.6, p = 0.434; Figure 8k), and no significant interactions between cell type and sex on these sEPSC parameters.

Interestingly, we found differences in sEPSC kinetics at female and male MCH cells, as a rightward shift in the cumulative distribution of sEPSC rise time rate (i.e.,
amount of time it takes for a sEPSC event to cover 10–90% of its amplitude) at female (p < 0.0001; Figure 8l) and male MCH/CART+ cells (p < 0.0001; Figure 8m) indicated that a subset of sEPSC events at MCH/CART+ cells had slower rise rate than MCH/CART− cells. Cell type was a significant factor in rise time kinetics (F(1, 57) = 10.5, p = 0.002), but there was no sex difference (F(1, 57) = 2.2, p = 0.146) or interaction with cell type to influence the rising rate of sEPSC events (F(1, 57) = 0.006, p = 0.937). In effect, pairwise comparisons of the mean sEPSC rise rate at MCH/CART+ cells in female (t = 2.3, df = 58, p = 0.049) and male mice (t = 2.3, df = 58, p = 0.046) tend to be slower than at MCH/CART− cells (Figure 8n).

By contrast, differences in decay time of sEPSC events were less prominent. There was a slight rightward shift in the cumulative distribution of sEPSC decay times at female MCH/CART+ cells (p = 0.009; Figure 8o), thus suggesting that a small subset of sEPSC events had longer or slower decay times. No differences were observed between male MCH cells (p = 0.111; Figure 8p). There was no effect of cell type (F(1, 57) = 0.0, p = 0.996), sex (F(1, 57) = 0.2, p = 0.668), or interactions between cell type and sex (F(1, 57) = 1.0, p = 0.320; Figure 8q).

Overall, our findings indicated that there were no gross differences in the frequency, amplitude, or area of sEPSC events at either subtype or sex of MCH cells, though a subset of events at male CART+ cells may occur less frequently and have smaller amplitudes. Interestingly, we found cell type-dependent differences in the kinetics of sEPSC events, as those arriving at CART+ cells had slower rise rates in both sexes, and a subset of events may also have slower decay times.
Figure 8. Slower sEPSC event kinetics at MCH/CART+ cells. Representative current trace (i) and sample of individual spontaneous excitatory postsynaptic current (sEPSC) events superimposed, with average trace shown in bold (ii), when recorded at a holding potential of $V_h = -60$ mV from female (a) and male (b) MCH/CART+ and CART− cells. Cumulative probability plot and mean of sEPSC event properties in female and male cells, including interevent intervals and frequency (c–e), amplitude (f–h), area (i–k), rise time, measured as the sEPSC slope or sEPSC amplitude change between 10–90% of its peak over time (l–n), and decay time (o–q). Two-way ANOVA: ††, $p < 0.01$. Kolmogorov-Smirnov test: **, $p < 0.01$; †††, $p < 0.001$; ††††, $p < 0.0001$. Scale: 5 s, 10 pA (ai, bi); 5 s, 5 pA (aii, bii).
3.3 Morphology

3.3.1 Reduced branching at male CART+ cells

To determine if the properties of sEPSC events were a function of cell morphology, we first determined CART immunoreactivity in biocytin-filled EGFP<sup>Mch</sup> cells (Figure 9a, b) from whole-cell recordings (Figure 6a–c). Next, we used nickel-enhanced DAB staining to label the soma, axons, and dendrites of each cell (Figure 9c) so they may be reconstructed to evaluate dendritic branching patterns using the Sholl analysis (Figure 9d–f). Cell type had no effect on the total dendritic length of female MCH cells (F(1, 13) = 0.4, p = 0.545; Figure 9g) but influenced that of male MCH cells (F(1, 17) = 6.9, p = 0.018; Figure 9h). To determine if there was an overall sex difference, we included sex in our model with cell type and distance, but we did not see an effect of sex (F(1, 28) = 0.04, p = 0.845), cell type (F(1, 28) = 0.06, p = 0.816), or any interactions involving sex or cell type on dendritic length.

We also used cell reconstructions to determine the number of nodes (i.e., branch points) at each node order (e.g., primary, secondary, tertiary, quaternary branch). Female cells did not show an effect of cell type on the number of nodes at each increasing node order (F(1, 13) = 2.7, p = 0.123; Figure 9i), but we found an effect of cell type at male cells (F(1, 17) = 5.0, p = 0.040), as male MCH/CART+ cells tended to have fewer nodes at each branch order (Figure 9j). However, there was no overall sex difference in the number of nodes (F(1, 30) = 1.0, p = 0.316), but in a three-way ANOVA that included sex, cell type, and node order, we found that CART expression significantly interacted with sex (F(1, 30) = 7.3, p = 0.011) and also with node order (F(3, 90) = 3.6, p = 0.016).
Therefore, while male CART+ cells tended to have less nodes at each node order compared to CART− cells, female cells may show the opposite or no pattern.

Given that there may be a difference in the number of branch points in male cells, we also determined if the number of nodes varied as a function of distance from the soma. There were no differences in the number of nodes at any distance away between CART+ and CART− female (F(1, 13) = 1.2, p = 0.284; Figure 9k) or male cells (F(1, 312) = 3.1, p = 0.080; Figure 9l). There was no sex difference (F(1, 30) = 0.05, p = 0.629), but the dendrites of MCH cells can have different node distributions depending on sex (F(1, 28) = 4.4, p = 0.044).

Taken together, as male MCH/CART+ cells had less dendritic length and fewer branch points, they may have a less complex morphological structure. Meanwhile, female MCH cells did not appear to be morphologically distinct from each other.

3.3.2 Increased dendritic branching predicted larger evoked current in CART+ cells

To determine if electrophysiological features were related to the dendritic morphology of MCH cells, we performed correlational analyses between the passive and active properties of MCH cells compared to their dendritic length or branching.

Current evoked from a −100 mV hyperpolarizing voltage step was correlated with total dendritic length of female ($R^2 = 0.65$, p = 0.016; Figure 10a) and male ($R^2 = 0.43$, p = 0.027; Figure 10c) CART+ cells. The number of nodes was also predictive of current flow in female ($R^2 = 0.51$, p = 0.045; Figure 10b) and male ($R^2 = 0.35$, p = 0.056; Figure 10d) CART+ cells. However, none of these relationships were
significant in CART− cells (Figure 10a-d). In sum, increased branching is associated with greater current flow in CART+ cells.

Figure 9. Reduced branching and dendritic coverage at male MCH/CART+ cells. Schematic of a whole-cell recording at an EGFP<sup>Mch</sup> cell in Mch-cre;L10-Egfp brain tissue that was biocytin-filled during the recording (a). Biocytin was first labelled in fluorescence (b) to determine the presence or absence of CART coexpression (not shown) and then nickel-enhanced with DAB staining (c) to trace and reconstruct the cell for Sholl analysis (d). Representative reconstructed female (e) and male (f) MCH/CART+ (i) and MCH/CART− (ii) cells with axons shown in gray. Dashed circles outline the field analyzed within a 200 μm radius from the soma. Comparison of total dendritic length coverage (g, h), number of nodes per node order (i, j), or distance of nodes from the soma (k, l). Two-way ANOVA with Bonferroni multiple comparisons posttest: *, p < 0.05. Scale: 100 μm (b, c).
The main difference in the electrical fingerprint between CART-positive and CART-negative MCH cells was the presence of high frequency firing that accommodated with sustained depolarization in male MCH/CART+ cells (Figure 7fi, fii). Since burst firing is associated with cells with smaller dendritic trees (Krichmar et al., 2002; Van Elburg and Van Ooyen, 2010; Psarrou et al., 2014), we calculated the average firing rate during the first second of a depolarizing pulse and determined if it was related to dendritic branching or length.

There was no relationship between branch length and action potential frequency in either female CART+ ($R^2 = 0.03, p = 0.799$) or CART− ($R^2 = 0.47, p = 0.316$; Figure 10e) cells. Likewise, the number of branch points did not correlate with firing frequency in female CART+ ($R^2 = 0.57, p = 0.14$) or CART− ($R^2 = 0.67, p = 0.18$; Figure 10f) cells.

In male cells, the dendritic length was not associated with firing frequency in CART+ ($R^2 = 0.38, p = 0.10$) or CART− ($R^2 = 0.06, p = 0.64$; Figure 10g) cells. Similarly, the quantity of nodes was not predictive of firing pattern in male CART+ ($R^2 = 0.29, p = 0.17$) or CART− ($R^2 = 0.054, p = 0.66$; Figure 10h) cells. However, when the data of both MCH/CART+ and MCH/CART− was combined, we found that both dendritic length and branching were significantly predictive of action potential frequency in males ($R^2 = 0.33, r = -0.57, p = 0.033$). As such, it appears that morphology is predictive of firing phenotype in male cells, but this relationship is independent of CART expression.

Overall, dendritic branching and length were predictive of current flow at MCH/CART+ cells. Increased branching correlated with slower firing frequency in male cells, but this association was not cell type specific.
Figure 10. Increased dendritic branching is associated with increased current flow in MCH/CART+ cells. Correlational relationship between current flow evoked from a −100 mV voltage step (a–d) or instantaneous frequency of action potentials elicited during a 1 s depolarizing pulse (e–h) with the dendritic length or quantity of nodes in MCH/CART+ or MCH/CART− cells. Lines on plots represent Pearson correlation (r) with significance determined by simple linear regression: *, p < 0.05.

3.3.3 Increased dendritic branching predicted larger charge but lower frequency excitatory input at male CART− cells

As synaptic input arrived at dendrites, we determined if properties of synaptic events were related to dendritic morphology of MCH cells.

In CART+ female cells, we found a significant correlation between the total dendritic length and the frequency of sEPSC events ($R^2 = 0.84$, $p = 0.030$ Figure 11a) and the slope ($R^2 = 0.78$, $p = 0.046$; Figure 11i) but no relationship with the area ($R^2 = 0.37$, $p = 0.280$; Figure 11e) or decay ($R^2 = 0.55$, $p = 0.149$; Figure 11m). In CART− female cells, we found no relationship between the total dendritic length and the frequency ($R^2 = 0.66$, $p = 0.094$; Figure 11a), area ($R^2 = 0.18$, $p = 0.479$; Figure 11e),
slope ($R^2 = 0.42, p = 0.238$; Figure 11i), or decay ($R^2 = 0.53, p = 0.163$; Figure 11m) of excitatory currents. Similarly, there was no correlation between the number of nodes and the frequency ($R^2 = 0.26, p = 0.380$; Figure 11b), area ($R^2 = 0.62, p = 0.115$; Figure 11f), slope ($R^2 = 0.48, p = 0.197$; Figure 11j) or decay ($R^2 = 0.27, p = 0.371$; Figure 11n) of sEPSC events in female CART− cells.

However, in male mice, we found that the number of dendritic nodes, though not dendritic length, at MCH/CART− cells was related to the properties of sEPSC events. In male MCH/CART− cells, the availability of dendritic nodes was inversely related with sEPSC event frequency ($R^2 = 0.63, p = 0.018$; Figure 11d) but positively related with greater sEPSC area ($R^2 = 0.52, p = 0.045$; Figure 11h) or decay time ($R^2 = 0.64, p = 0.018$; Figure 11p). sEPSC rise time was not related to the number of nodes in male cells ($R^2 = 0.10, p = 0.442$; Figure 11l).

In sum, the dendritic length was positively correlated with the sEPSCs frequency and slope at female MCH/CART+ cells, while the number of dendritic nodes was significantly related to the frequency, area, and decay kinetics of sEPSC events arriving at male MCH/CART− cells.
Figure 11. Dendritic branching predicted lower frequency sEPSC events with larger area at male MCH/CART− cells. Correlational plot of relationship between sEPSC frequency (a–d), area (e–h), slope (i–l), and decay (m–n) with dendritic length and number of nodes in MCH/CART+ or MCH/CART− cells. Lines representing Pearson correlation are denoted by r, with significance determined by simple linear regression: *, p < 0.05.
4. DISCUSSION

This study aimed to characterize the neuroanatomical distribution, passive and active electrophysiological properties, and dendritic morphology of MCH/CART+ and MCH/CART− cells in male and female mice. There were no gross sex differences in the distribution or properties of MCH cells, however sex interacted with CART coexpression to influence the electrical fingerprint and dendritic branching of MCH cells. Furthermore, MCH/CART+ and MCH/CART− cells also differed based on their cellular conductance and on the kinetic properties of incoming synaptic events. CART coexpression thus delineated a distinct subpopulation of MCH cells, and our findings suggested that each MCH cell type could form distinct neural circuits and elicit functionally distinct roles in the expression of MCH-mediated behaviours.

4.1 Neuroanatomy: Mapping CART and NK3R expression in hypothalamic MCH cells

About half of the hypothalamic MCH cell population expressed CART. The prominence of CART coexpression in half of MCH cells has also been independently reported by others (Broberger, 1999; Elias et al., 2001; Cvetkovic et al., 2004; Vrang, 2006; Croizier et al., 2010; Mickelsen et al., 2017) while Cartpt mRNA coexpression may be even higher (Mickelsen et al., 2019; Fujita et al., 2021). CART-positive MCH cells can also be spatially differentiated from CART-negative cells. Consistent with previous reports (Broberger, 1999; Vrang et al., 1999; Brischoux et al., 2001; Wang et al., 2021), CART-positive MCH cells appeared more anteriorly and were largely distributed medial to the fornix, within the anterior DMH, the medial ZI, and medial regions of the LHA. Meanwhile,
CART-negative MCH cells were predominantly located in the lateral ZI and lateral regions of the LHA, near the internal capsule, the substantia innominata, and the cerebral peduncle.

Interestingly, NK3R expression defined another subset of MCH cells, as NK3R was almost exclusively seen in MCH/CART+ cells, and only a few MCH cells expressed NK3R in the absence of CART. CART and NK3R coexpression in MCH cells is known (Broberger, 1999; Vrang et al., 1999; Brischoux et al., 2001, 2002; Elias et al., 2001; Cvetkovic et al., 2004; Croizier et al., 2012; Wang et al., 2021) and might be even more prominent as the coexpression of Cartpt and Tacr3, the genes for CART and NK3R respectively, can comprise over 70% of MCH cells (Mickelsen et al., 2019). Interestingly, MCH/CART/NK3R+ cells were equally distributed along the medio-lateral axis of the hypothalamus. However, these NK3R-expressing MCH cells may mediate different functions depending on their spatial distribution because the medial and lateral regions of the hypothalamus can receive unique neurokinin B (NKB), the main ligand at NK3R, projections (Cvetkovic et al., 2003). The medial hypothalamus was mostly innervated by NKB axons from the lateral septal complex, multiple hypothalamic nuclei, and periaqueductal grey. In contrast, the lateral regions received the densest NKB innervation from the lateral hypothalamus and the pallidum, especially the substantia innominata and the diagonal band nucleus. In addition, Fujita et al. (2021) also recently identified the bed nucleus of the stria terminalis and central amygdala as major sources of NKB innervation, and these projections were densest at the lateral border of the hypothalamus. Therefore, while MCH/CART/NK3R+ cells appear equally distributed along the medio-lateral axis, they may be differentially innervated.
As CART-positive and CART-negative MCH cells had distinct spatial distributions, they may also have different efferent projection targets and recruit unique behavioural networks. Specific tracing experiments have highlighted targets of CART-positive MCH cells within the mesolimbic pathway, including the accumbens (Ekstrand et al., 2014) and ventral tegmental area (Dallvechia-Adams et al., 2002; Philpot et al., 2005), where half of MCH-ir varicosities colocalized with CART (Dallvechia-Adams et al., 2002). Both MCH and CART can modulate energy balance through the mesolimbic pathway, but these peptides have opposing effects. MCH injection into the accumbens shell of rats increased chow consumption (Georgescu et al., 2005), but similar injections of CART peptides decreased feeding (Yang et al., 2005). Consistently, co-injection of MCH and CART in the accumbens prevented CART-induced dopamine release in this region (Yang and Shieh, 2005), thus it is possible that coincident MCH and CART release in the accumbens could self-regulate to modulate feeding outcomes (Diniz and Bittencourt, 2017).

4.2 Electrophysiology: Electrical fingerprint of MCH cells varied with sex

MCH cells had unique electrical behaviours depending on sex and CART coexpression. The input resistance was lower in CART-positive MCH cells from female mice, so they may require a stronger current input to be stimulated or the same current input would elicit a lower voltage response in these female cells. MCH cells receive direct inputs from diverse brain regions at varying intensities (González et al., 2016), thus differences in input resistance may complement the strength of afferent inputs that innervate MCH cells. For instance, weak inputs at CART-negative MCH cells that have higher input resistances may be sufficient for functional activation.
CART-positive MCH cells, especially in female mice, displayed significantly greater inward current flow at negative voltage potentials. The current-voltage relationship at all MCH cells revealed greater current flow at voltages negative to the reversal potential that suggested the expression of G-protein coupled inwardly-rectifying potassium channels (GIRK). GIRK activation permits outward K+ flow that hyperpolarizes and dampens membrane excitability (Lüscher and Slesinger, 2010), and both nociceptin/orphanin FQ (Parsons and Hirasawa, 2011) and histamine (Parks et al., 2014a) can inhibit MCH cells through GIRK channel activation to suppress anxiogenic and sleep-promoting effects of MCH cells, respectively. As female CART-positive MCH cells elicited greater GIRK conductances, they may be particularly implicated for regulating MCH-mediated anxiety-like and sleep behaviours.

MCH cells in male mice largely differed based on their capacity for excitation. Male CART-positive MCH cells exhibited burst firing that dissipated with sustained depolarization, and this was seen by their pronounced spike rate adaptation. Our findings extended those by Fujita and colleagues (2021) who have also noted multiple firing phenotypes in MCH cells, including a greater proportion of Pmch/Cartpt+ cells displaying burst firing, by demonstrating that this effect was stronger in males. The firing pattern of a neuron can influence both the target postsynaptic cell and the originating presynaptic cell. For example, different stimulation frequencies can influence gene expression in the target cell (Klein et al., 2003; Lee et al., 2017; Iacobas et al., 2019). At the presynaptic terminal, select firing patterns may differentiate the release of coexpressed chemical messengers. MCH neurons can utilize fast-acting neurotransmitters (Jego et al., 2013; Chee et al., 2015; Mickelsen et al., 2019; Sankhe et al., 2022), but they also coexpress
multiple neuropeptides. Bursts of action potentials more effectively stimulate neuropeptide release than chronic firing (Poulain and Wakerley, 1982), and the amount of neuropeptide released per action potential is directly related to the firing frequency (Dreifuss et al., 1972; Gainer et al., 1986). Therefore, we predict that cell firing is imperative for their function. As both short, high frequency stimulation (Jiang et al., 2020) and sustained depolarization may be required for MCH release (Hausen et al., 2016b; Noble et al., 2018), burst firing at CART-positive MCH cells may initiate neuropeptide release that is sustained with high frequency firing to permit maximal neuropeptide signaling (Jiang et al., 2020).

4.3 **Morphology: Differential dendritic structure at MCH cells varied with sex**

To date, there is little data on the dendritic branching pattern of MCH cells, which have been reported to be large and multipolar in hypothalamic cultures (Eggermann et al., 2003). Differences in the morphology between CART-positive and CART-negative MCH cells were most prominent in males, where CART-positive cells had fewer branch points, and these dendritic nodes were farther from the soma. In effect, we can infer that male CART-positive cells would have a smaller dendritic tree. Computer modelling studies have reported that the morphology of a neuron can directly impact the generation of outputs. In cortical and hippocampal neuron models, firing phenotype could be predicted by dendritic branching, where larger dendritic trees were less likely to display burst firing (Krichmar et al., 2002; Van Elburg and Van Ooyen, 2010; Psarrou et al., 2014). Consistent with these cortical models, our male MCH/CART⁺ cells, which had larger dendritic arborizations, were also less likely to exhibit burst firing when depolarized.
Indeed, high frequency firing in response to current stimulation was overall related to increased dendritic branching and length at male MCH cells, however this relationship did not depend on CART expression.

Glutamatergic signalling is a key factor in dendritic development (Rajan and Cline, 1998; Sin et al., 2002; Richards et al., 2005). In our cells, there were no significant differences in the average frequency or size of excitatory events to MCH cells in both sexes. However, we did detect a sample of glutamatergic events in male CART-negative cells that occur with greater frequency and size. As sEPSC area positively correlated with an increased number of nodes, we could also speculate that this would promote increased dendritic growth (Wong and Ghosh, 2002; Cline and Haas, 2008). MCH cells receive higher glutamatergic input during development (Li and Van Den Pol, 2009), following sleep deprivation (Briggs et al., 2018), or high fat feeding (Linehan et al., 2020). Therefore, as cells in the lateral hypothalamus can increase dendritic branching following restraint stress (Grafe et al., 2019), behavioural experiences may also shape the branching pattern and contribute to the plasticity of MCH cells even in adulthood.

Estrogen is another key modulator of dendritic structure. Male cells in the ventromedial hypothalamic nucleus are more branched than female cells, and this effect was due to an estradiol-mediated increase in glutamatergic signalling (Mong and McCarthy, 1999; Shwarz and McCarthy, 2008). MCH cells are sensitive to the effects of chronic, but not acute (Tritos et al., 2004), estrogen treatment that blocked fasting-induced increases in Pmch gene expression (Murray et al., 2000; Mystkowski et al., 2000; Morton et al., 2004). As CART-negative MCH cells in the male brain were more branched, this suggested that they may be more sensitive to the influence of estrogen. Furthermore,
as differences in branching were most prominent in the male brain, it is also possible that these differences were hardwired by estradiol during the sexual differentiation and masculinization periods of the developing brain (McCarthy, 2008).

4.4 Intersection of form and function in MCH cells

The structure of a neuron can have a substantial impact on its electrical behaviour and can help define the role it plays in a larger synaptic network. In both sexes, we found that increased branching was correlated with greater passive current flow in CART-positive MCH cells. In male CART-negative cells, increased branching modulated the frequency, size, and decay kinetics of sEPSCs.

Foremost, we found that dendritic structure was predictive of passive current flow in CART-positive MCH cells so that the amplitude of current evoked increased with the number of nodes and branch length. The current-voltage relationship of MCH cells, especially at negative voltage potentials, was consistent with the availability of GIRK channels, which were more prevalent in highly-branched MCH/CART+ cells. This relationship may form the basis for peptidergic regulation at MCH/CART+ cells, especially by neuropeptides that signal via G-protein coupled receptors. Consistently, NK3R is a Gq-coupled receptor (De Tassigny and Colledge, 2010) and is preferentially expressed at MCH/CART+ cells. NK3R activation has been shown to suppress GIRK activation leading to cell depolarization (Boyle et al., 2022), and NK3R activation was predicted to excite MCH cells (Fujita et al., 2021).

In male CART-negative cells, we found that dendritic branching positively correlated with sEPSC area but negatively correlated with sEPSC frequency. Nodes or
branch points act as a point of synaptic integration, thus two synaptic events arriving at separate dendritic branches can converge and summate at a node to elicit synergistic effects and greater potential change than their independent events (Kamijo et al., 2014). The summation of synaptic events may reflect the direct positive relationship between sEPSC area or decay time and the availability of nodes at MCH/CART− cells, which may also receive a higher distribution of larger amplitude sEPSC events. Furthermore, the prevalence of sEPSC event summation may reflect a lower frequency of sEPSC events detected at highly branched cells. By contrast, branch nodes can also be points of synaptic attenuation especially as synaptic events travel from smaller branches with low intracellular resistance to larger diameter branches at proximal dendrites where current can be attenuated by charging larger membrane capacitances (Spruston et al., 2016). Therefore, synaptic events at distal dendrites of MCH/CART− cells that are highly branched may exhibit increased decay times as current dissipates along the dendrite. Paradoxically, while cells with greater dendritic branching may present more synaptic sites, there was an inverse relationship between sEPSC events detected at MCH/CART− cells with more dendritic nodes. This may be attributed to significant attenuation of sEPSC events arriving at distal dendrites of highly branched cells that fail to be detected at the soma.

4.5 Limitations

Majority of MCH cells (> 90%) could be identified by EGFP\textsuperscript{Mch} in the hypothalamus of Mch-cre;L10-Egfp mice, but as previously shown (Beekly et al., 2020), some EGFP\textsuperscript{Mch} cells, though infrequent and sporadically distributed, did not express MCH
immunoreactivity. There was a unique perifornical cluster of MCH-negative EGFP$^{Mch}$ cells that appeared smaller and fainter than neighboring MCH-ir cells and were often grouped in a circular pattern immediately dorsal to the fornix at ARA L73. Our work expanded upon the initial analysis by Beekly and colleagues by defining the level that these MCH-negative EGFP$^{Mch}$ cells occur relative to Bregma. Interestingly, we found that this MCH-negative cluster also did not express $Pmch$ or $Egfp$ mRNA, so they were not actively producing EGFP. As EGFP production in the $Mch$-cre;L10-Egfp mouse arose from a fate-mapping strategy to mark the genetic lineage of a cell (Zinyk et al., 1998; Padilla et al., 2010), EGFP fluorescence would permanently mark cells that expressed $Pmch$ at any point during gestation or development, even if $Pmch$ transcription has since been turned off in the cell. We did not record from EGFP-labelled cells immediately surrounding the fornix, but it is possible that a MCH-negative EGFP-labeled cell may have been inadvertently included in our dataset.

In order to draw stronger associations between electrophysiological and morphological properties, we reconstructed the dendritic structure of MCH cells that we recorded from. The addition of dendritic spine analysis would help integrate and interpret differences in excitatory input between MCH cell types. However, we were unable to analyze the type or density of dendritic spines on our reconstructed cells because spines were not always visible with biocytin-labelling. Our reconstructed cells underwent extensive post hoc thick-tissue processing that may be prohibitive for maintaining the integrity of dendritic spines. However, sex-dependent differences in spine properties within other hypothalamic nuclei can relay functional outcomes on male and female behaviour (Matsumoto and Arai, 1980; Mong and McCarthy, 1999; Amateau and
McCarthy, 2002, 2004) and would be relevant in future studies to further elucidate the heterogeneity among MCH cells.

To further characterize the heterogeneity of the MCH system in both sexes, it could be important to consider fluctuations in circulating estrogen, as this may impact the cellular and behavioral effects of MCH. Estrogen is a known modulator of MCH, as it prevents fasting-induced increases in Pmch expression (Mystkowski et al., 2000; Morton et al., 2004) and reduces the orexigenic effects of MCH administration in male (Mystkowski et al., 2000; Terrill et al., 2020) or ovariectomized female (Murray et al., 2000) rats. Similarly, females are more susceptible to the actions of MCH during diestrus when estrogen levels are low (Santollo and Eckel, 2007), thus the stage of the reproductive cycle can impact the MCH system, including the electrical or morphological features of MCH cells. While we did not monitor the estrous cycle of female mice used in this study, our recordings have included group-housed females performed across the same week to include sampling throughout the estrus cycle.

### 4.6 Conclusion

There were no overt sex differences in the neuroanatomical makeup of MCH cells. However, MCH cell type can affect electrophysiological properties depending on sex. Differences in electrophysiological and morphological outcomes help elucidate the neural circuit involving CART-positive or CART-negative MCH cells. For instance, CART-positive cells may be innervated by strong afferent input and encode behaviours with short bursts of high frequency stimulation. By contrast, CART-negative cells may be sensitive to even weak or subthreshold afferent input but provide sustained cell firing to
mediate long-lasting behavioural output. Interestingly, in males, CART-negative cells have more complex dendritic structure and are more likely to receive higher frequency and larger amplitude excitatory events. As such, it is possible that these events can facilitate the activation of male CART-negative MCH cells. Overall, this study expanded on the cellular heterogeneity of the MCH system to help guide future work unpacking the neurochemical subtypes that underpin the diverse functions of MCH neurons.
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