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Morfologia comparada do sistema do hormônio concentrador de melanina

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RESUMO

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O sistema do hormônio concentrador de melanina (MCH) consiste em três genes: *Pmch, Mchr1, e Mchr2*, e nas proteínas originadas desses três genes: MCH, Neuropeptídeo E-I (NEI), Neuropeptídeo G-E, e os dois subtipos de receptores de MCH, MCHR1 e MCHR2. O sistema neuropeptidérgico do MCH tem sido implicado em diversos papéis fisiológicos, tais como a integração e promoção de comportamentos motivados, incluindo reprodução e comportamento maternal, e sono. Uma melhor compreensão dos elementos que compõem o sistema do MCH pode nos ajudar a entender melhor como ele executa essas funções. Um possível método para se obter isto é comparar diferentes espécies, identificando elementos em comum e divergências que podem nos informar sobre as correlações morfofuncionais conservadas, e sobre a evolução de neuromoduladores como um todo. No Capítulo 1, uma introdução mais extensiva sobre o MCH é providenciada. No Capítulo 2, nós conectamos o racional por trás de cada trabalho apresentado nesta tese, com um foco nas perspectivas deste campo de investigação. No Capítulo 3, empregamos imuno-histoquímica para investigar a distribuição de MCH e NEI no sistema nervoso central de três espécies diferentes de muroides: ratos (*Rattus novergicus*), camundongos (*Mus musculus*), e camundongos vulcânicos Mexicanos (*Neotomodon alstoni*). Também empregamos camundongos fêmeas em diferentes estágios do ciclo reprodutivo para identificar correlações entre a distribuição de MCH e sua função em fêmeas. No Capítulo 4, identificamos um anticorpo comercial que seletivamente marca MCHR1. Esse anticorpo foi então utilizado para mapear a distribuição de MCHR1 no prosencéfalo de ratos (machos) e camundongos (machos e fêmeas em todos os estágios do ciclo estral). Algumas áreas onde o MCHR1 foi encontrado foram utilizadas para caracterização neuroquímica. No Capítulo 5, propusemos uma normatização para a nomenclatura do MCH, e revisamos os dados disponíveis para o sistema do MCH em um grande número de espécies. Esta análise nos permitiu traçar paralelos entre a evolução do MCH e eventos genômicos de larga-escala que ocorreram na linhagem dos vertebrados. No Capítulo 6, sumarizamos os principais pontos encontrados neste trabalho. Em resumo, nossos resultados mostram que, apesar das tímidas diferenças entre machos e fêmeas, há substancial diferença entre espécies, mesmo entre espécies próximas, impossibilitando a extrapolação de dados obtidos em animais-modelo sem que haja verificação experimental prévia. Nossos resultados também sugerem que mecanismos de transmissão por volume – incluindo a liberação de MCH livre no líquor e no espaço extracelular – podem desempenhar um importante papel na função normal do sistema do MCH. Acreditamos que os dados obtidos neste trabalho avançam nossa compreensão sobre o MCH e podem ser usados como guia para outros projetos de pesquisa.

Palavras-chave: Neurociência. Neuroanatomia. Evolução comparada. Hipotálamo. MCH.

ABSTRACT

Diniz GB. Comparative morphology of the melanin-concentrating hormone system. [Thesis (Ph.D. thesis in Systems Biology)] – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo; 2019.

The melanin-concentrating hormone (MCH) system consists of three genes: *Pmch, Mchr1* and *Mchr2*, and the proteins originated from these three genes, namely: MCH, Neuropeptide E-I (NEI), Neuropetide G-E, and the two subtypes of MCH receptors, MCHR1 and MCHR2. The MCH neuropeptidergic system has been involved in several physiological roles, such as the integration and promotion of motivated behaviors, including reproduction and maternal behavior, and sleep. A better comprehension of the elements that comprise the MCH system may help us understand how it executes the aforementioned roles. One possible approach to achieve this is to compare different species, identifying commonalities and divergences that may inform us about conserved morphofunctional correlates, and about the evolution of neuromodulators as a whole. In Chapter 1, a more extensive introduction about the MCH system is provided. In Chapter 2, we connect the reasoning behind each work presented in this thesis, with a focus on the perspectives of the field. In Chapter 3, we employed immunohistochemistry to investigate the distribution of MCH and NEI in the central nervous system of three different species of muroids: rats (*Rattus novergicus*), mice (*Mus musculus*), and Mexican volcano mice (*Neotomodon alstoni*). We also employed female mice in different stages of the reproductive cycle to identify correlates between MCH distribution and function in females. In Chapter 4, we identified a commercial antibody that selectively labels MCHR1. This antibody was then used to map the distribution of MCHR1 in the prosencephalon of rats (male) and mice (males and females in all stages of the estrous cycle). Some areas where MCHR1 was detected were selected for further neurochemical characterization. In Chapter 5, we proposed a normalization for the nomenclature of MCH and reviewed the available data on the MCH system for a large number of species. This analysis allowed us to draw parallels between the evolution of MCH and large-scale genomic events that occurred in the vertebrate lineage. In Chapter 6, we summarize the main points of this work. In summary, our results show that, despite timid differences between males and females, there are substantial interspecies differences, even between closely related species, precluding the extrapolation of data obtained in animal models without experimental verification. Our results also suggest that volume transmission mechanisms - including the release of free MCH in the cerebrospinal fluid and extracellular space – may play an important role for the normal function of the MCH system. We believe the data obtained in this work furthers our comprehension of MCH and may be used to guide other research projects.

Keywords: Neuroscience. Neuroanatomy. Comparative evolution. Hypothalamus. MCH.

1 INTRODUCTION

As a cornerstone of vertebrate evolution, a centralized nervous system has been a constant after the emergence of *phylum Chordata*. Among the most basal structures of this central nervous system (CNS) is the hypothalamus, as its homologous structures are found even in early-diverging chordates, such as cephalochordates and tunicates (1). The hypothalamus has been fundamentally conserved for its role in the maintenance of body homeostasis. This cardinal role cannot be executed without an enormous associated complexity since each organism finds diverse environmental challenges during their lifetime and must respond appropriately to each one of them. It is natural, therefore, that animals with a more robust hypothalamus, capable of a broader spectrum of responses, will have a fitness advantage against their peers, what resulted in a remarkable development in hypothalamic complexity regarding its spatial organization, connectivity, and chemical composition.

In the current scheme of parcellation employed by most atlas, approximately all the hypothalamus comprehended between the fornix and the internal capsule and between the optic chiasm and the mammillary bodies is considered part of the lateral hypothalamic area (LHA) (2-4), closely following the definition of lateral hypothalamus provided by Gurdjian (5). The LHA is also known as the bed nucleus of the medial forebrain bundle (mfb), the largest rostrocaudal tract of fibers in the prosencephalon (6, 7), as mfb fibers are found intermingled with LHA neurons (8, 9). This confers a remarkable connectivity to the LHA, which can send and receive projections from virtually all areas within the CNS. Although a great effort has been made to comprehend the LHA, this region still stands as one of the least understood aspects of the hypothalamus (10).

In addition to its complex hodology, the LHA contains a large number of neuronal populations characterized by different neurochemical markers (11). Various neuromodulators are synthesized by neurons within the LHA, such as melanin-concentrating hormone (MCH) (12), orexins (13), cocaine- and amphetamine-regulated transcript (14), neurokinin B (15), and galanin (16). These different neuronal populations are found intermingled with a complex population of GABAergic and glutamatergic interneurons (17). Although all these neuromodulators play essential roles in maintaining the normal function of the organism, MCH stands out for the full range of roles it has been associated with in vertebrate physiology.

1.1 Melanin-concentrating hormone

The MCH system consists of three genes: *Pmch, Mchr1* and *Mchr2*, and the proteins originated from these three genes (as reviewed in Bittencourt and Diniz (18)). The *Pmch* gene encodes the MCH precursor, which is 165 amino acids (aa)-long in rodents. This precursor contains a signal peptide of 21 aa, necessary for protein targeting, followed by a structural chain of 144 aa, and three peptides: neuropeptide G-E (NGE), a linear peptide with 19 aa; neuropeptide E-I (NEI), a linear peptide with 13 aa; and MCH, with 19 aa and cyclic structure (19, 20). The generation of the mature forms of these peptides depends on the actions of endopeptidases that act on dibasic sites, in a mechanism that is not entirely understood (21). While the synthesis of MCH and NEI has been thoroughly demonstrated (21), NGE remains as a predicted peptide, without any available evidence of its actual synthesis by neurons or biological activities. Therefore, for the purposes of this work, the term "MCH peptidergic system" refers mainly to MCH and its receptors and NEI.

The rat (*Rattus novergicus*) has been the preferred animal model for morphological studies on MCH and NEI. In this species, MCH neurons are found predominantly in the diencephalon, including the medial and lateral LHA, the incerto-hypothalamic area, the dorsomedial and posterior hypothalamic areas, and the *zona incerta*. Smaller groups are found outside the diencephalon, such as in the olfactory tubercle, pontine reticular formation, and laterodorsal tegmental nucleus (12, 18, 22). An additional group of MCH cells is observed exclusively in the preoptic hypothalamus of lactating females (23-27). Dissimilar to the exceptionally restricted pattern of MCH synthesis, MCH-ir fibers are found widespread throughout the whole CNS. Regions that contain fibers include olfactory areas, the septal nuclei, the basal nuclei, the hippocampal formation, the neocortex, several diencephalic nuclei, the mesencephalic and pontine reticular formation, the periaqueductal gray matter, and all levels of the spinal cord (12, 28-33).

While the rat has been the focus of morphological and hodological studies, mice (*Mus musculus*) have been extensively used to probe the function of MCH neurons. The most well-described roles of MCH include the integration of information to coordinate motivated behaviors (reviewed in Diniz and Bittencourt (34)) and sleep (reviewed in Ferreira et al. (35) and Gao (36)). However, MCH has also been implicated in several other functions, including emotion and stress response (37), sexual physiology (38), learning and memory consolidation (39, 40), and ventricular homeostasis (41). Despite the extensive knowledge obtained about MCH, attempts to leverage the MCH system as a pharmacological target have largely failed (42, 43). Some of the difficulties in turning the MCH system into a pharmacological option

may result from the disconnection between animal models used to obtain morphological and functional data.

An additional function has been associated with MCH: the control of skin color in vertebrates that display adaptive color change (44). Melanin-concentrating hormone released into the circulation acts as a pallor-promoting agent at melanophores in the skin (45). The development of MCH as a neurohormone in teleosts occurred concomitantly with the appearance of MCH-immunoreactive neurons in the lateral hypothalamus of those animals (46). Few attempts have been made, however, to conciliate the evolution of MCH in teleosts with other clades and, in particular, mammals. It is also unclear how MCH has originated in the vertebrate lineage.

1.2 The melanin-concentrating hormone receptors

Melanin-concentrating hormone binds to two different receptors, commonly termed MCHR1 and MCHR2 in the literature. Both receptors show typical characteristics of G protein-coupled receptors (GPCRs), such as seven transmembrane domains, a DRY motif between transmembrane domain 3 and the second intracellular loop and an Asp-linked glycosylation region near the N-terminus. Rat MCHR1 is a 353 aa-long protein, with 91% sequence identity between rats and humans (47, 48). The actions of MCHR1 are selective to $G_{i/o}$ and G_q subunits, with a predominantly inhibitory effect (49, 50). Significantly less is known about MCHR2, because this receptor was lost in the *Glires* clade (including lagomorphs – rabbits and pikas - and rodents), limiting the number of animal models available for its study (51). Through the use of MCHR1 antagonists and knockout animals, essential roles for this receptor in feeding behavior and energy expenditure have been revealed (52-54). Other works have also implicated MCHR1 in mood regulation (55) and the control of ciliary beating (41).

While the distribution of *Mchr1* expression has been investigated in both rats and mice (56-59), a single work has attempted to map immunoreactivity to MCHR1 in the CNS of male rats (56). Expression of *Mchr1* is found in several areas of the murine CNS, including olfactory areas, the cerebral cortex, striatum, hippocampal formation, amygdala, several thalamic and hypothalamic nuclei, and discreet regions of the midbrain and hindbrain, but no consensus exists among published works (56-59). Contrasting to the wealth of information obtained about *Mchr1* expression, the single mapping available for MCHR1 immunoreactivity is hard to interpret, in particular in light of recent developments in our knowledge about the subcellular localization of MCHR1.

Hervieu et al. (56) report that labeling to MCHR1 is localized to the cellular membrane. Gao and van den Pol (17) suggest that MCHR1 is found in the presynaptic membrane, based on the property of MCH to decrease the frequency of miniature glutamate-mediated postsynaptic currents in the presence of tetrodotoxin. Berbari et al. (60), on the other hand, were the first to find MCHR1 in the neuronal primary *cilia* of transfected cultured cells and slices of mice brains, observation replicated in rats using the same antibody (61) . The neuronal primary *cilium* is a sensory structure, a single non-motile *cilium* that extends from the membrane towards the extracellular space (ECS) and is often coated in receptors (62). It is believed that neuronal primary *cilia* play a major role in volume transmission (VT), a mode of communication employed by some neurons where neurochemical messengers are released in the cerebrospinal fluid (CSF) or the ECS, rather than at the synaptic cleft (63). Since MCH has been suggested to employ VT to modulate feeding behavior (64), it is important to understand what role is played by the receptor in this VT paradigm.

1.3 Aims and outline of this thesis

Given the full range of functions played by MCH in maintaining the correct function of the organism, the disconnection between animal models used to study its morphology and its physiology, the lack of a complete mapping on the distribution and subcellular localization of MCHR1, and the few attempts to conciliate the data obtained in different clades of vertebrates, we proposed in this work to study the morphology and hodology of the MCH peptidergic system in mice, in addition to the distribution of MCHR1 in both rats and mice.

In **Chapter 2**, we describe the relationship between the articles that make up this thesis, integrating the knowledge obtained in each work in a unified framework of interpretation, and applying such framework on our current knowledge about the MCH system.

In **Chapter 3**, we employed immunohistochemical methods to investigate the distribution of MCH immunoreactivity in three muroid species: *R. novergicus*, *M. musculus*, and the Mexican volcano mouse (*Neotomodon alstoni*). By identifying differences in the synthesis of MCH between rats and mice, we may better understand the functional discrepancies reported in the literature. Furthermore, the comparison between murines and a distant relative, the Mexican volcano mouse (Cricetidae), can provide us with insights on the plasticity of neuromodulatory systems, and to what extent we can extrapolate data between species.

In **Chapter 4**, we identified a commercial antibody that can be used to locate MCHR1 immunoreactivity with high specificity. Employing that antibody, we verified that MCHR1 is widely colocalized with a neuronal primary *cilium* marker in the murine CNS, and that several areas related to previously indicated functions of MCH contain ciliary MCHR1, including several distinct neurochemical populations within some of those areas. These results suggest that VT may play an important role in normal MCH function. Although no sex-linked differences were observed, we identified some differences between rats and mice, indicating that there is a certain degree of plasticity in the MCH receptors between species.

In **Chapter 5**, we systematically compare the MCH peptidergic system among vertebrates, including the genetic composition of the MCH system, amino acid sequence changes, and the morphological distribution of MCH-synthesizing neurons and their fibers, in an attempt to identify critical events in the evolution of the MCH system. We also propose a standardization for the nomenclature of elements belonging to the MCH system. By overlaying the evolution of MCH with broader genetic events, we can better understand how hypothalamic neuromodulatory systems evolved, and how they may have contributed to the increasing complexity of the nervous system in extant species.

In **Chapter 6**, we discuss the main conclusions obtained in the aforementioned works, with a focus on the perspectives of the field.

6 CONCLUSION

By comparing the MCH system between multiple species, both through the generation of new data and through the analysis of gene databases and the literature, we were able to draw meaningful conclusions about this system. Here, we proposed a model for its origin in vertebrates; we identified changes in the MCH system linked to whole-genome alterations that occurred in the vertebrate lineage; we proposed a model to explain the two major events of lateral migration of MCH neurons that occurred during evolution; we showed significant plasticity in the distribution of MCH neurons, even between closely related species; we demonstrated the conservation of cellular characteristics; we identified a new antibody for the localization of MCHR1 and employed it to map the distribution of ciliary MCHR1 in the murine nervous system; and we provided overwhelming evidence towards the existence of an anatomical basis for volume transmission in the MCH system. We believe the data produced in this work will be of value to the scientific literature, as we hope the questions raised in this work may encourage others to pursue the fascinating MCH system.

A better understanding of the MCH system is paramount to our knowledge of the human brain, not only due to the actions of MCH in mammalian physiology, but because it serves as a model for different neuromodulatory systems. While its phylogenetic history tells us about the events that shaped the brain of extant species as we know them, the conserved/diverged aspects of MCH cells between different species serves as a cautionary tale about the limitations of extrapolating data obtained in one model to other animal species, even closely related ones. More than an interesting topic, comparative morphology is an essential aspect of translational research, equipping us with the knowledge necessary to make appropriate interspecies considerations.

Finally, our discovery of a strong basis for volume transmission in the MCH system of multiple rodent species is a starting point for numerous other works necessary to fully comprehend how MCH operates. It is vital that we understand how released MCH travels within the brain, and how does it operate outside the synapse. It is insufficient for an MCHR1 agonist or antagonist to bind to the receptor in the same way MCH does. To fully mimic or block MCH actions, these substances must have a half-life and a transport system that imitates that of volume transmission MCH. By screening substances that have this broader range of similarities with MCH, we may be able to identify compounds with practical use for the treatment of human pathologies.

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