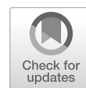
Optogenetic Control of the Melanin-Concentrating Hormone Expressing Neurons



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Abstract Since the discovery of the neuropeptide melanin-concentrating hormone (MCH) more than half a century ago, MCH neurons have spawned hundreds of scientific studies. MCH neurons are a phylogenetically well-conserved group of hypothalamic neurons. The MCH neurons project diffusely throughout the CNS but only reside within a restricted area encompassing the incerto-lateral and perifornical hypothalamus. Here we review optogenetic studies focused on understanding the functions of the MCH neurons, particularly their role as sleep modulators. We attempted to put optogenetic findings in context with other studies focused on neuronal/behavioral modulation by MCH or its receptors. We also laid a theoretical framework to understand better the data on MCH neuronal activity in relation to the activity of the orexin neurons. For readers not familiar with optogenetics, we also went over major developments in this new field.

1 Introduction

The implementation of optogenetic technology to the study of brain functions has made possible a sophisticated control over one of its chief biological units; the neurons. This leap forward in technology has been applied to numerous studies of the brain functions including the understanding of one of its most basic functions, the wake–sleep behavior. Here we are reviewing optogenetic findings from our own laboratory as well as from others that have used it to understand the activity of one important phenotype among hypothalamic neurons, neurons expressing the melanin-

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concentrating hormone (MCH). Ahead of the main topic, we introduced the reader not acquainted with optogenetics with a chronological account of its major developments. Readers already familiar with optogenetics may certainly skip this section. We divide the review optogenetic studies in those experiments that stimulated and those that inhibited the activity of MCH neurons. At the end, we put forward a model for interpretation of all sort of data, i.e., sleep and other behaviors. Our model proposes a counterbalance between MCH and orexin neuronal activity.

2 Development of the Optogenetic Tools

Before reviewing the studies that used optogenetic tools to control the excitability of MCH neurons, it is helpful to follow its major developments. Optogenetics only has been around for 12 years. Yet its development has been breathtaking. This biotechnology could not have fully developed without the parallel development of molecular biology tools and photonics (optics + electronics). Laser invention, and particularly laser application to imaging, opened the door for using laser pulses as surrogate of the electrical stimulator. In early years medical laser applications like tumor laser heating gave incentive to study the optical properties of intact brain tissue, i.e., absorbance, diffraction, etc. Optical measurements indicated that, for instance, blue light from lasers penetrates 0.5 mm into white matter and 1.84 mm into the gray matter making a light-neuron interface theoretically possible (Yaroslavsky et al. 2002). Two decades after laser was invented, Farber and Grinvald "photostimulated" neurons in the mollusk *Aplysia* by synthetizing a photoexcitable dye (Farber and Grinvald 1983). Alas, Grinvald's Lab later focused heavily on the development of fluorescent voltage sensors and stopped pursuing the development of photostimulation tools. This application had to wait another decade until Callaway and Katz used laser to generate the photochemical release of the excitatory neurotransmitter glutamate, i.e., "uncaging." Using either one- or two-photon laser scanning microscopy, it became possible to activate individual neuronal synapsis in vitro by photolysis of caged neurotransmitters (Callaway and Katz 1993; Denk 1994). Photon-driven uncaging of neurotransmitters requires the caged compounds to be loaded into all neurons, hence lacked cell specificity. Also, caged neurotransmitters are loaded by bath applications making this approach only amenable to in vitro preparations.

Advances toward activity control of specific neurons were first made by transfecting nematode-derived genes of ligand-gated chloride ion channels into mammalian neurons, i.e., ivermectin inhibition. This strategy of genetic inhibition of neurons swiftly moved up from in vitro constitutive (Johns et al. 1999) to in vitro reversible (Lechner et al. 2002; Slimko et al. 2002), and ultimately it was applied to freely behaving mice (Lerchner et al. 2007). Still this approach is dependent on the slow pharmacokinetics of the drug binding to the ion channel. This problem was partially solved by coupling genetic control of excitability with photo-uncaging.

Ectopic ionotropic excitatory receptors were transfected into specific neurons, while its ligands were caged and bath-loaded. Thus successfully transfected neurons were forced to fire when light pulses shone over them (Zemelman et al. 2003). A subsequent refinement of photolysis uncaging was the creation of a mutant potassium channel that could be chemically gated depending on light wavelength (Banghart et al. 2004). This technology is the forbearer of what few years later will be the "step function bidirectional control of excitability." Time resolution of neuronal response by photolysis uncaging is in the order of seconds, better than minutes-hours taken by drugs but still far slower than traditional electrical stimulation, i.e., milliseconds.

Gero Miesenböck's research group (currently in Oxford University) provided the first proof of principle behind the modern optogenetic approach. A chimeric protein made up of arrestin-2, rhodopsin and the subunit of a G-coupled excitatory protein "chARGe" was transfected into hippocampal neurons. White light pulses triggered action potentials (AP) only in the chARGe transfected neurons (Zemelman et al. 2002). Same research group took this approach one step further evoking behavioral responses in fruit flies that had chARGe receptors expressed in specific neurons. Caged ligands for the ectopic ion channel receptors were microinjected into the fruit flies. Then 150 ms duration white light pulses uncaged the ligands stimulating the transgenic neurons (Zemelman et al. 2003). The photochemical strategy had nonetheless two limitations. First, either the cofactor retinol or the uncaged ligands were still required to be applied prior to the experiment. Also the kinetic of the neuronal response lagged behind the precise time resolution of traditional electrical stimulation. An ectopic photo-driven cation channel with ultra-rapid response and no requirement of cofactors loads was needed to advance the field.

The discovery of microbial ion channels responsive to light (opsins) kindled the field of optogenetics. The proton pump channelrhodopsin-1 (crChR1) and the mono/divalent cation channel channelrhodopsin-2 (crChR2) were the first microbial opsins cloned from the unicellular freshwater green algae *Chlamydomonas reinhardtii* (Nagel et al. 2002, 2003). A couple of years after its cloning, Karl Deisseroth's Lab (Stanford University) used for the first time crChR2 to activate neurons by giving millisecond (ms)-duration pulses of blue light (Boyden et al. 2005). Cultured hippocampal neurons transfected with lentivirus containing the mammalian codon-optimized crChR2 gene showed fast and phase-locked spiking in response to 5–30 Hz frequency light pulses. Light-evoked spikes were indistinguishable from those produced by intracellular depolarizing current. Light pulses at low intensity were also able to elicit subthreshold responses. Electrophysiological and cell viability markers were minimally affected by crChR2 transfection or its photoactivation. Despite crChR2 requires retinal, no cofactor was needed to add to the culture media. It seems that mammalian stores of retinal are large enough to fuel the chromophore. Two independent labs corroborated the stimulatory effects of crChR2 (Li et al. 2005; Ishizuka et al. 2006). These labs reported that in mice brain slices, photostimulation mediated by crChR2 elicited depolarization/repolarization events occurring at a faster rate than the neuron membrane time constant. The fast response of crChR2 made the neuronal spiking get phase-locked to the light pulses up to a frequency of

20 Hz. The turning on phase of crChR2 photocurrent was light intensity dependent with a time constant of only 2.2 ms. By contrast, the turning off phase was independent of light intensity having a slightly slower time constant than the chChR2 opening (Ishizuka et al. 2006). Soon after cloning of ChR2, the first gain-of-function ChR2 mutant was engineered, i.e., $ChR2_{H134R}$. Photostimulation of $ChR2_{H134R}$ evoked larger and faster photocurrents (Nagel et al. 2005). $ChR2_{H134R}$ was then ectopically expressed in muscle and mechanosensory neurons of the round worm *C. elegans* eliciting appropriate behavioral responses. Unlike transfection of ChR2 into rat hippocampal neurons, positive photoactivation of ChR2 in chick embryonic neurons or in the round worm required the presence of retinal. These seminal experiments were also the first to use fast optical switching coupled to a laser/arc lamps/LED as light stimulator.

A crucial milestone in optogenetics came with the development of the first light-neuron interface capable of eliciting a predictable behavioral response in freely behaving rodents (Aravanis et al. 2007). The αCaMKII promoter was selected for targeting expression of crChR2 in excitatory cortical motoneurons controlling whisker movements. Using an optic fiber (200 microns O.D.) and blue light laser (473 nm), photostimulation pulses significantly elicited whisker deflections with a latency of few seconds. Also for the first time, it was described the optical properties of the rat/mouse cerebral cortex. It was estimated that with a laser power of roughly 38 mW/mm^2, a brain volume of roughly 0.5 mm^3 below the tip of the optic fiber would receive enough energy (>1 mW/mm^2) to activate all neurons transfected with crChR2. Considering the mouse neuronal cortical density is 920,000/mm^3 (Schuz and Palm 1989), this energy would activate more than 400,000 neurons. Thus 137 years after Hitzig and Fritsch (Thomas and Young 1993) elicited a predicted muscular contraction by electrical stimulation of the motor cortex, selective optogenetic stimulation of cortical motoneurons exquisitely reproduced this paradigm.

Since 2005, channelrhodopsin-based technology has gone through a vigorous development; for a recent review of this topic, see Wietek and Prigge (2016). Thus other channelrhodopsins from different species of freshwater algae have been both characterized and tested as optogenetic tools. The cation channel opsin from *Volvox carteri* (vChR1) also evokes depolarizing photocurrents in response to light. vChR1 shows red-shifted (520 nm) photosensitivity as compared to crChR2 or $ChR2_{H134R}$ (470 nm) (Zhang et al. 2008). Channelrhodopsin from *Mesostigma viride* (mChR) is another red-shifted (534 nm) opsin with better channel kinetic properties, including broader pH range, that undergoes minimal inactivation upon sustained illumination (Govorunova et al. 2011). Other two channelrhodopsins from two different species of the *Chlamydomonas* genus show similar red-shifted (520 nm) spectral photosensitivity as well as lack of fast desensitization (Hou et al. 2012). These other channelrhodopsins are named CaChR1 (*C. augustae*) and CyChR1 (*C. yellowstonensis*). Red-shifted optimal photosensitivity has two main advantages over blue photosensitivity. First it is well established that longer-wavelength photons penetrate deeper into intact mammalian brain tissue (Yaroslavsky et al. 2002; Al-Juboori et al. 2013); henceforth red-shifted ChRs can excite more neurons with the same intensity of illumination. Likewise,

neuronal photostimulation with red-shifted ChRs is compatible with blue light excitable calcium indicators like GCAMP6.

In addition to find more WT ChRs, other research groups used protein chimeric technology (or site-directed mutagenesis) to engineer improved channelrhodopsins. Swapping the six-helix domains between ChR1 and ChR2, it yielded the red-shifted opsin ChRGR (Lin et al. 2009; Wen et al. 2010). ChRGR transfected cells show light-induced photocurrents featuring faster channel kinetics as well as less desensitization upon constant illumination. Site-directed mutagenesis of crChR2 significantly improved the channel kinetics measured during fast frequency illumination (Gunaydin et al. 2010). The resultant mutant called $ChETA_A$ showed ultrafast on response (1 ms), faster of time constant, lack of doublets or missing spikes, and no current plateau when stimulated at frequencies as high as 200 Hz. $ChETA_A$ can be activated with blue-greenish light pulses as brief as 2 ms and still evoke 100% of neuronal spiking. Another mutant $ChR2_{TC}$ can swiftly excite neurons at illumination levels as low as 1.9 mW/mm^2 (Berndt et al. 2011). Readers wishing to know more about the particular performance for each ChRs variant, please consult Mattis et al. (2011).

One of the most interesting developments in ChR biotechnology was the bioengineering of “step function opsins” (SFO). A step function opsin is a photo-induced cation channel with slow closing kinetics allowing long (several seconds) and steady depolarization. SFO undergo rapid channel closing with a brief pulse of yellow light (Gunaydin et al. 2010). Since the original SFO ($ChR2_{E123T}$) was slow for bidirectional control of fast-spiking neurons, a faster double mutant SFO called $ChR2_{E123T/T159C}$ was engineered to evoke step function bidirectional control up to 60 Hz (Berndt et al. 2011). At the same time, vChR1 was fused with crChR1 and subjected to point mutations in order to come up with a fast bistable SFO (Yizhar et al. 2011). The resulting $ChR2_{C128S/D156A}$ double mutant was tested in vivo eliciting significant behavioral changes in mice (Yizhar et al. 2011). Same year Boyden’s research group (MIT) also engineered bistable optogenetic expressing vectors fusing different varieties of ChRs (Kleinlogel et al. 2011).

As important as the application of ChR2 to photostimulate neurons, it was the successful application of the photosensitive inhibitory channel halorhodopsin (HR). HR evolved in archaea halophiles, i.e., salt-thriving primitive bacteria. In nature, HR uses the energy from photons to drive an electrogenic chloride pump to keep osmotic balance amidst an hyperosmotic environment (Lanyi 1990). The photosensitive pump cycle of HR only takes a few milliseconds (Tittor et al. 1987). Furthermore, HR shows red-shifted (560–590 nm, yellow) optimal photosensitivity (Bamberg et al. 1993) and hence can be used coupled with ChR2. All these features make HR a good candidate as a photo-gated inhibitory chloride channel. The extracellular concentration of chloride ions is many times higher than the intracellular, and the chloride equilibrium potential is close to the neuron’s membrane potential. Thus, chloride ion influx follows the opening of a chloride-selective channel (i.e., $GABA_A$ receptors) bringing the membrane potential toward the resting level counteracting depolarization currents.

Karl Deisseroth's Lab in collaboration with German scientists tested a couple of HRs derived from two species of archaea: *Halobacterium salinarum* (HsHR) and *Natronomonas pharaonis* (NpHR) (Zhang et al. 2007). In vertebrate cells, NpHR showed higher affinity for chloride and was therefore chosen for transfecting mammalian neurons. Mice neurons transfected with mammalian codon-optimized NpHR-YFP showed yellow light-elicited hyperpolarizing currents (−40 mV) with rise/decay time constants of roughly 6 ms. Yellow light pulses silenced neurons being activated either by depolarization currents or by optogenetic activation of ChR2. NpHR-YFP transfected neurons did not show electrophysiological/vital alterations either at baseline or after light-induced inhibition. NpHR inhibited neuronal firing in a light intensity manner (threshold = 7 mW/mm^2; ceiling = 22 mW/mm^2), over a continuous illumination (1–10 min) or at a single spike level over frequencies ranging up to 30 Hz. NpHR inhibition occurred without interfering with other inhibitory currents mediated by $GABA_A$ receptors. In mice Selective expression of NpHR caused complete inhibition of APs and intracellular calcium transients. Similar to ChR2, the mammalian neurons transfected with NpHR did not require the cofactor retinol to evoke photocurrents. Yet retinol was necessary to observe behavioral responses in *C. elegans*. In these invertebrates, NpHR produced reliable photoinhibition of muscles in charge of swimming and cholinergic motoneurons as well. NpHR was also effective in inhibiting swimming behavior elicited by ChR2 activation (Zhang et al. 2007). Boyden's Lab produced similar results as Zhang et al. transfecting cortical projecting neurons both with NpHR and ChR2 (Han and Boyden 2007). Remarkably a Gaussian mode of alternating yellow/blue light pulses was able to alter neuronal spike timing without altering the neuron firing rate. In a further refinement, NpHR was engineered to show increased mammalian neuronal expression and reduced toxicity by removing the motif signals that caused its buildup inside the endoplasmic reticulum. The modified NpHR, called eNpHR2.0, displayed increased peak photocurrent in the absence of aggregations or toxicity and potent optical inhibition both in vitro and in vivo (Gradinaru et al. 2008). Two years later an improved version of NpHR2.0, dubbed NpHR3.0, was engineered (Gradinaru et al. 2010). NpHR3.0 was made modifying the C-terminus of NpHR2.0 with a trafficking signal motif borrowed from the rectifying potassium channel gene Kir2.1. Thanks to this addition, NpHR3.0 mostly expressed along the plasma membrane. As a result, the photocurrents measured in neurons transfected with NpHR3.0 showed a threefold increase, and the ensuing hyperpolarization increased by 50% (−100 mV). The potent hyperpolarization of NpHR3.0 was also observed under orange/reddish light (630 nm) and even at nearly infrared wavelengths (680 nm) (Gradinaru et al. 2010). The large red-shifted photosensitivity of NpHR3.0 allows to be used simultaneously with ChR in the same neuron. This application was made possible creating a bicistronic lentivirus vector containing both ChR2-EYFP and NpHR-mCherry genes, i.e., eNPAC. eNPAC enabled bidirectional (excitation/inhibition) control with blue (excitation) and orange (inhibition) light pulses of the same transfected neuron (Gradinaru et al. 2010).

Around the same time NpHR was being developed, Boyden's group screened several other photosensitive HRs derived from both fungi and archaea. In mice archaerhodopsin-3 (Arch) from *Halorubrum sodomense* showed stronger neuronal inhibition as compared to NpHR (Chow et al. 2010). Data strongly suggested that Arch hyperpolarizes neurons by a protonic outward current. Unlike NpHR which shows long-lasting deactivation after continuous yellow light illumination, Arch recovered its photosensitivity spontaneously in matter of seconds. Inhibitory currents elicited by Arch were larger (hundreds of pA) than those caused by NpHR (100 pA) but with similar on/off time scales. Arch was also more photosensitive (0.3 mW/mm^2) and showed lack of saturation at high intensity illumination (36 mW/mm^2). Inhibitory yellow photocurrents mediated by Arch occurred regardless of the presence of potassium or chloride ions. Arch has shown to be a better inhibitory optogenetic tool than chloride channel eNpHR3.0 in GABAergic synapsis where the photoactivation of chloride pumps changed the reversal potential eliciting after light spiking (Raimondo et al. 2012). Lentivirus having the mammalian codon-optimized Arch gene produced robust plasma membrane neuronal expression. In awake mice, Arch transfected cortical neurons were completely silenced for several seconds. One month after Arch transfection, neurons expressing EYFP had similar passive and active electrophysiological/vital properties as the non-expressing neurons. Boyden's group also developed a blue light-shifted photosensitive proton pump derived from the fungus *Leptosphaeria maculans* (Mac). Mac allowed simultaneous inhibition of two different neuronal clusters using blue (470 nm; −20 mV) and red (630 nm; −30 mV) light pulses. They also developed a more photosensitive inhibitory opsin from the archaea *Halorubrum* strain TP009 (ArchT) (Han et al. 2011). Similar to Arch, ArchT expresses along plasma membrane at somata and terminals of mice and in non-human primates. ArchT showed the same level of photoinhibitory currents as the WT Arch, but it had a threefold increase in photosensitivity (1–10 mW/mm^2) resulting in doubling of the volume of tissue being inhibited. Recently a fusion protein made between Arch and ChR has yielded another SFO (Kleinlogel et al. 2011).

A significant development of the optogenetic field is the creation of transgenic (Tg) lines of mice constitutively expressing ChR2-YFP in different phenotypes of neurons. The first Tg ChR2 mice line was developed to photostimulate projecting neurons (Arenkiel et al. 2007; Wang et al. 2007). On these neurons blue light pulses evoked phase-locked spiking similar to those reported using viral transfection of ChR2. The in vitro studies indicated light-elicited spiking was both dependent on light intensity (threshold $= 0.2$ mW/mm^2; ceiling ≈ 9 mW/mm^2) and frequency of stimulation (5–30 Hz). Despite ChR2-YFP was expressed in both perikarya and processes, APs were only evoked by the illumination of the perikarya. Illumination focused on proximal dendrites only produced subthreshold photocurrents. The optogenetic stimulation of neurons also evoked postsynaptic currents in downstream neurons confirming that light-induced APs cause release of neurotransmitters. The capacity of optogenetics to stimulate neurons whose projections are simultaneously recorded opens the door for the creation of high-resolution circuit brain maps.

The repertoire of Tg mice line expressing ChR2 has grown ever since to include VGAT-ChR2$_{H134R}$-EYFP, ChAT-ChR2$_{H134R}$-EYFP, Tph2-ChR2$_{H134R}$-EYFP, and Pvalb$_{H134R}$-ChR2-EYFP (Zhao et al. 2011). Slice recordings from these Tg mice lines confirmed that blue light pulses reliably stimulated GABAergic, cholinergic, serotonergic, or parvalbumin-expressing neurons. Transgenic lines of mice selectively expressing ChR2-YFP in glutamatergic (Hagglund et al. 2010), sensory olfactory (Dhawale et al. 2010), and striatal medium spiny neurons (Chuhma et al. 2011) have been also engineered.

Another major development in optogenetics has been the bioengineering of multiple Tg lines of mice expressing the Cre-Lox system in different neuronal phenotypes. Currently there are dozens of commercially available Tg Cre mice specific for genes relevant for neurotransmission like TH-Cre, vGABAT-Cre, MCH-Cre, etc. Cre-Lox is a conditional gene expression system requiring the presence of two DNA components. One is the Cre gene inserted downstream of a specific promoter sequence: TH promoter, MCH promoter, etc. The other component is the gene of interest, in this case ChR2, NpHR3.0, or ArchT. Located upstream of the opsin genes, there is a transcription STOP codon flanked by a pair of specific DNA sequences called Lox sites. Without Cre the STOP signal will prevent the opsin to be transcribed. Yet once both sets of genes are present within the same cell, Cre will bind to the Lox sites, and depending on its orientation, it will either cut it (same) or invert it (opposite). Researchers at the Allan Institute for Brain Science bioengineered four Tg lines of mice harboring the Lox flanked STOP codon upstream of ChR2$_{H134R}$-dTomato (Ai27), ChR2$_{H134R}$-EYFP (Ai32), Arch-EGFP (Ai35), or eNpHR3.0-EYFP (Ai39) (Madisen et al. 2012). The progeny of the crossing between a Cre Tg line mouse and a mouse harboring the floxed-stop/opsins will show selective expression of the opsin inside Cre-expressing neurons. As a proof of principle, the Tg opsin mice were first crossed with parvalbumin-Cre mice corroborating that the Cre-dependent opsins performed as expected (Madisen et al. 2012). Alternatively, the Cre-lox system can be used to virally transfect the microbial opsins into a specific subpopulation of Cre-expressing neurons. This system is called double-floxed inverse ORF or DIO for short. DIO vectors contain the gene of interest (ChR2, Arch, NpHR) oriented as antisense and flanked by two different lox sites also in reverse orientation. Once the DIO virus infects the Cre-expressing neuron, Cre will cut both pairs and place the gene in the sense orientation for transcription. After recombination a mismatch Lox pair will remain preventing further Cre binding (Sohal et al. 2009). DIO viruses containing the genes for all optogenetic opsins are commercially available from virus vector cores at Stanford University, University of Pennsylvania, and the University of North Carolina at Chapel Hill.

Now that the reader had acquired a firm foot onto optogenetics, it follows focus on melanin-concentrating hormone (MCH) neurons.

3 Brief Evolutionary Perspective of the MCH Neurons

It is necessary to mention that the name given to MCH does not suit well its various roles in the brain of many vertebrates expressing it. The name of this neuropeptide derived from the discovery that, in bony fishes, MCH functions as depigmentation hormone, i.e., "melanin-concentrating." It can be said this endocrine role evolved as a secondary specialization in jawed fishes, i.e., gnathostomes. Comparative anatomy studies reveal that the original small periventricular neurons expressing MCH in agnathans (e.g., lampreys, hagfish) later evolved in jawed fishes into large neuroendocrine-type cells whose axons mainly project to the neurohypophysis (Baker and Bird 2002). The skin light tone effect of endocrine MCH has been replaced later on in some tetrapod (e.g., frogs, lizards) by the most potent action of the pituitary gland-secreted hormone melatonin (Filadelfi and Castrucci 1994). In most reptiles, and all birds and mammals, the magnocellular incerto-latero hypothalamic MCH neurons send its axons throughout the neuroaxis staying away of the neurohypophysis (Cardot et al. 1994; Vallarino et al. 2009). Thus in more recent vertebrates, the MCH neurons evolved away from endocrine regulation into paracrine (cell to cell) regulation of a wide variety of intra- and extra-hypothalamic neurons. Noteworthy in mammals MCH still plays a role regulating the beat frequency of ependymal cilia and ventricular volume (Conductier et al. 2013) perhaps in the same way the parvocellular periventricular MCH neurons did it. In any case in all mammals hitherto studied, MCH-expressing neurons only reside within a region covering the zona incerta (ZI), the lateral hypothalamus (LH), and the perifornical area (PeF) (Bittencourt 2011). In mammals, the total number of MCH neurons has significantly increased along with brain size and brain complexity. It is estimated that the mouse has roughly 5000 (Toossi et al. 2016; McGregor et al. 2017), whereas the rat has 10,000 (Mikrouli et al. 2011), and humans may have from 90,000 up to 140,000 MCH neurons (Thannickal et al. 2007; Aziz et al. 2008). The number of cognate receptors for MCH has also increased. Rodents only have one MCH receptor (Chambers et al. 1999; Saito et al. 1999), while primates have two types of MCH receptors (Sailer et al. 2001). Nonetheless the primary amino acid sequence of MCH has not evolved, and there is also a high degree of conservation (90%) to the level of its base pair gene sequence (Nahon 1994). In hominids a second MCH precursor gene has evolved (Courseaux and Nahon 2001). In summary, the evolutionary trends in mammals highlight the increasing importance for the brain of having more synapsis involving MCH neurons.

4 Electrical Stimulation of Brain Areas Containing MCH Neurons: Findings and Caveats

Long before optogenetic tools were used to activate hypothalamic neurons, clues of its functions emerged out of electrical stimulation studies; for a review see Berthoud and Munzberg (2011). More than a century ago, it was discovered that discrete electrical currents applied to the brain can evoke discrete and specific movements/ behaviors (Thomas and Young 1993). Ever since electrical stimulation has produced direct evidence that activation of specific brain regions is responsible for all sort of brain functions. Nowadays elegant microsimulation experiments still produce meaningful data; for review see Clark et al. (2011). By the middle of the twenty-first century, Nobel Prize awardee Weiss R. Hess discovered that electrical stimulation of the LH elicited biologically crucial emotional responses such as flight or fight, voracious eating, or mating (Hess and Akert 1955). In particular the LH was recognized as the main feeding center (Anand and Brobeck 1951), and later on it was discovered feeding and reward (self-stimulation) were both integrated there (Hoebel and Teitelbaum 1962). Electrical stimulation of the LH also produced changes in motor output (Sinnamon 1993) and emotionally triggered changes in cardiovascular function (Smith et al. 1990). Electrical stimulation of the ZI also has an effect on motor outputs like locomotion/stepping (Sinnamon 1984), cardiovascular function (van der Plas et al. 1995), and thermoregulation (Kelly and Bielajew 1996). Recent studies, still using electrical stimulation, confirm that activation of the ZI is crucial to modulate skeletal muscle tone and the pace of movements. Thus electrical activation of the ZI is very effective for control of tremors, bradykinesia, and rigidity in patients with Parkinson disease (Plaha et al. 2006). Using nonspecific gene transfer of the arousal peptide orexin, we found that orexin release from all kinds of incerto-lateral hypothalamic neurons prevents sudden bouts of muscle paralysis known as cataplexy (Liu et al. 2011). Cataplexy and sleep attacks are cardinal symptoms of narcolepsy. In contrast, we observed that transferring the orexin gene specifically into MCH neurons exacerbated cataplexy and sleep attacks suggesting MCH neurons exert negative control over the skeletal muscle tone and levels of arousal.

Our contrasting results using gene transfer vividly underscore the need for attaining cell specificity during experiments of complex networks. Electrical currents are limited in this regard. Electrophysiological studies have been done almost always using ex vivo preparations, i.e., neurons in slices or in culture. Glass pipette microelectrodes in whole-cell patch clamp are exquisite tools for controlling the excitability of individual neurons. Neurons in these preparations can also be identified post hoc either by intracellular injection of dyes or a priori through genetic tagging. Alas behaviors like sleep/wake engaging multiple neurons and being displayed only in vivo settings are an unsurmountable challenge for using the whole-cell patch-clamp approach. To achieve intracellular stimulation in vivo, the animal must be anesthetized and even though it is still a very challenging study (Brecht et al. 2004). Even the enthusiasm to study the role of discrete brain regions

using very fine microelectrodes (5–30 microns) has significantly waned. By the end of the twenty-first century, it became unavoidable the realization that electrical fields produce activation of the neural tissue by a myriad of factors making extremely hard to interpret the results using a cellular framework. Type of neural tissue (gray vs. white matter), type of electrode configuration (monopolar vs. bipolar), type of current (anodal, cathodal, biphasic), size of electrode tip, distance to the excitable unit, orientation of electrical field with respect to axons or cell bodies, whether the neuron has or not myelinated axons, and the chronoaxie signature all influence whether neurons or axons [or both] will be activated by electrical currents (Ranck 1975; Iggo 1978; Brocker and Grill 2013). Artificial electrical currents can even activate axons instead of neuronal perikarya (Nowak and Bullier 1998a, b). Even small electrical currents are able to stimulate multiple neurons and often activate thousands of them (Tehovnik 1996). In the case of chronic electrical stimulation, its efficacy diminishes with time, based on how brain tissue reacts to the electrode material (Biran et al. 2005). These many drawbacks motivated researchers to develop new approaches so as to unravel the role of specific neurons on behavior or its electrophysiological correlates. These novel approaches should overcome the caveats of electrical stimulation but still keep its advantages, i.e., small size and precise time resolution. The solution came combining molecular biology tools with photonics, i.e., optogenetics. Now it follows reviewing the optogenetic experiments done to control the excitability of MCH neurons.

5 Optogenetic Control of MCH Neurons and Sleep Regulation

5.1 Stimulation of MCH Neurons

Findings from numerous previous studies led to the proposition that activity of MCH neurons plays a role regulating the sleep states. Yet any of those studies directly tested this hypothesis. Therefore we reasoned that if MCH neurons are sleep active (Hassani et al. 2009), its stimulation should push the awake brain to transit into NREM sleep and when in NREM sleep to transit into REM sleep. Thus we were the first research group to apply optogenetics to selectively stimulate MCH neurons while measuring its effects on sleep/wake stages (Konadhode et al. 2013). Because of the genetic advantages that afford studying mice, we chose mice as our first animal model, i.e., WT C57/BL. In the past we used a very specific and efficient MCH promoter to target the expression of heterologous proteins solely in MCH neurons (Liu et al. 2011). Our MCH promoter was donated and validated by one of our collaborators (van den Pol et al. 2004). Using the same gene expression cassette, we inserted the ChR2$_{H134R}$-YFP gene (donated by K. Deisseroth) replacing the orexin gene. The re-engineered plasmid was packed into recombinant adeno-associated virus (stereotype 5) at a titer of 5 × 10^{12GC} /μl (University of North

Carolina, Chapel Hill, NC). As a control we used our previous rAAV-MCH promoter-GFP vector (Liu et al. 2011). To transfect a large number of MCH neuron vectors, we injected the vectors at two different loci within the LH. Likewise the injected volume was large (0.75 μl/hemisphere). Histological analysis confirmed that our MCH promoter produced a eutopic expression of YFP solely in MCH neurons. For instance, no orexin neurons expressed YFP even though orexin neurons are closely intermingled with MCH neurons. The YFP expression was strong and restricted to the plasma membrane particularly in perikarya but it was less dense in proximal dendrites. Roughly 53% of MCH neurons expressed EYFP.

Whole-cell patch-clamp studies indicated that our vector efficiently drove the expression of the ChR2 gene. In current clamp mode, the YFP-positive-labeled neurons showed APs in responses to 10 ms blue light pulses delivered up to 30 Hz. As already reported by others using $ChR2_{H134R}$ (Gunaydin et al. 2010), light pulses sometimes evoked spikes in doublet or even triplets. Under constant illumination transfected neurons responded with depolarization current of 30 mV. Photocurrents were evoked in a light intensity manner. At subthreshold light intensity, transfected neurons only showed depolarization currents. Transfected neurons were also able to follow the light pulses when stimulated chronically at 10 Hz for 1 min repeated every other minute.

The in vitro findings gave us the confidence to test optogenetic stimulation in vivo. To test the hypnotic effect of MCH, we chose to stimulate MCH neurons over a 24 h period starting when lights turned off, i.e., mouse active period. Instead of stimulating MCH neurons within a particular vigilance state, we chose an unbiased approach stimulating neurons for 1 min every 4 min so stimulation would fall in all vigilance states over the course of the experiment. A LED blue light produced 10 ms duration pulses with at an output power of 1 mW (at the tip of fiber optic) that shone the transfected neurons via bilateral 200 μm O.D. optic fiber probes. We stimulated MCH neurons at three different frequencies, 5, 10, and 30 Hz, keeping 72 h apart between experiments. During the first 12 h (night), stimulation at 5 Hz was ineffective to change sleep amounts; however, stimulation at 10 and 30 Hz significantly increased both NREM sleep and REM sleep. The hypnotic effects started immediately and lasted for the next 6 h. Stimulation at 10 Hz was the most effective increasing NREMS by 40% and REMS by 70%. This finding was expected since in vitro recordings indicated 10 Hz stimulation evoked APs with the highest amplitude and even elicited double or triple spiking. Higher sleep amounts were caused by a significant reduction in the number of long-duration wake bouts. As such during stimulation, mice could not stay awake longer than 8 min. Yet sleep bout average duration remained unchanged. During stimulation mice fell more often asleep as well as transited more often into REM sleep. Transitions out of sleep stages also increased suggesting that activation of MCH neurons facilitates all sorts of state transitions. Increase in sleep drive was also observed measuring delta power during NREM sleep.

When stimulation continued over the next 12 h (day), sleep amounts remained unchanged likely due to the ceiling effects. Remarkably NREM sleep delta power analysis revealed a paradoxical increase during daytime stimulation. Sleep amounts remained unchanged over the 24 h of stimulation in mice infected with the control vector. To assess whether ceiling effects observed during the day time could have been caused by exhaustion of MCH neurons, MCH neurons were stimulated for 6 h starting at noon. Protocol of stimulation was the same as before, that is, 10 ms of pulse duration, at 10 Hz for 1 min every 4 min. Unlike stimulation started at night, stimulation started at noon failed to increase sleep amounts or change the sleep architecture. Our conclusion was then that the hypnotic effects of optogenetic stimulation of MCH neurons depend on the time of day manifesting very strongly during the mouse's active period.

To corroborate the hypnotic effect of stimulation of MCH neurons, we repeated the study in rats (Blanco-Centurion et al. 2016). We followed closely the same protocol of transfection and stimulation except we delivered more viruses (3 μl/hemisphere) as well as we used a wider optic fiber (rat = 400 μm O.D. vs. mouse = 200 μm O.D.). The larger number of MCH neurons and brain size in rats made these adjustments compulsory. First we set out to confirm the effects of optogenetic stimulation in vitro. As reported earlier for mouse MCH neurons, rat MCH neurons did not show spontaneous firing activity and had almost identical membrane resting potential (≈ -60 mV). Rat transfected MCH neurons responded with APs when illuminated with blue light pulses of 10 ms of duration. However unlike mouse MCH neurons, rat MCH neurons could not fire in synchrony with light pulses delivered faster than 10 Hz. Voltage-clamp recordings showed that fast-frequency stimulation evoked a single spike first but then only evoked subthreshold depolarization currents. Histological analysis in rats indicated that our vector specifically transfected MCH neurons as we observed in mice. We observed very intense YFP labeling within the LH, ZI, and PeF. YFP was also exclusively present along neuronal plasma membranes, and it was particularly dense along dendrites unlike what occurred in mice. Similar to what was measured in mice, 52% of MCH neurons were transfected. We then tested stimulation of MCH neurons in vivo at three different frequencies: 5, 10, and 30 Hz. Again stimulation lasted for 24 h starting at lights off. Similar to previous findings in mice during the first 12 h of stimulation at 10 Hz, NREM sleep and REM sleep were significantly increased. The magnitude of NREM sleep increase was similar to that in mice ($\approx$ 50%); however, in rats REM sleep was further augmented ($\approx$ 200%). Another difference from the observations in mice is that stimulation at 5 Hz significantly increased REM sleep, whereas at 30 Hz, it did not. Optogenetic stimulation of MCH neurons in rats also had an immediate hypnotic effect that lasted for the next 9 h. During the peak of effects, REM sleep was augmented by 300% and NREM sleep by 100%. During the next 12 h of stimulation (day), all frequencies of stimulation significantly increased REM sleep amounts. Particularly stimulation at 5 Hz enhanced REM sleep amounts by 50% breaking the ceiling effects seen before in mice. Analysis of the sleep architecture confirmed that the hypnotic effect was mainly due to a dramatic reduction of long-duration wake bouts. Stimulation also increased the number of short duration NREM and REM sleep

bouts instead of lengthening them. It corroborates our prior hypothesis that activation of MCH neurons is meant to facilitate brain transitions from waking into NREM sleep and from NREM sleep into REM sleep. This function was clearly revealed when we analyzed NREM EEG delta power over the 24 h of the stimulation. Normally NREM sleep delta power waxes and wanes across the 24 h, i.e., waxing during the active period and waning during the rest period. However during stimulation delta power daily oscillation became flat. The simplest explanation of it is that in order to discharge delta power during the resting phase, NREM sleep bouts should remain consolidated, i.e., plenty of long-duration bouts. Because during the day stimulation forced the brain to switch regularly into REM sleep, it caused a reduction of the long-duration bouts of NREM sleep blunting the delta power discharge. At night, constant intrusion of NREM sleep into the waking also produced a noticeable trend to elevate delta power so it too failed to wax. EEG spectral analysis in REM sleep revealed that theta power was also significantly higher for almost the entire 24 h of the stimulation. It indicates that activation of MCH neurons significantly builds up REM sleep pressure as well. In conclusion, optogenetic stimulation of MCH neurons had a potent hypnotic effect that was capable of counteracting circadian wake drive and, in the case of REM sleep, breaking the ceiling effect during daytime.

Other two research groups have also used optogenetic tools to investigate the role of activity of MCH neurons on sleep stages. Just a few months after we published our study in mice, Jego et al. published another study using a Tg line of mice expressing Cre in MCH neurons (Jego et al. 2013). A Cre-dependent DIO virus was microinjected into the hypothalamus of the MCH-Cre mice for targeting $ChETA_A$-YFP expression in MCH neurons. Around 87% of MCH neurons expressed YFP which was almost double from what we achieved in mice and rats. Whole-cell patch-clamp recordings from transfected neurons indicated blue light pulses also elicited spiking. Similar to transfected neurons in rats, transfected neurons in MCH-Cre mice showed 100% fidelity on their response to light pulses delivered between 1 and 20 Hz. However when stimulated at faster frequencies, it failed to evoke spiking. The fact that MCH neurons in the Cre mice could not be driven to fire faster than 20 Hz, even with $ChETA_A$, indicates that rodent MCH's neurons feature strong spike adaptation. Transfected neurons did not show spontaneous firing either.

Unlike our stimulation paradigm of unbiased timing, Jego et al. stimulated MCH neurons across sleep states during the second half of the resting phase (afternoon). They stimulated at the two opposite fringes of the response curve: 1 and 20 Hz. Unlike us, they used a blue laser light as source of illumination. Since lasers emit photons in a coherent way, the photon energy in laser light is higher than the LED energy (30–40 mW vs. 1 mW) and is also non-divergent. Stimulation at either frequency across NREM sleep episodes did not lengthen the episodes but significantly increased the number of transition into REM sleep. When stimulation was given at 20 Hz across the REM sleep episodes, it significantly prolonged the bout average duration. Yet when stimulated at 1 Hz, it failed to modify any of the sleep states. Stimulation at any rate during REM sleep did not change the EEG power spectra. Subsequently, they transfected MCH neurons with a ChR2 SFO confirming that 50 ms blue light pulses delivered every 10 s evoked steady depolarization

currents until it was stopped by a 50 ms yellow pulse. Spiking was also produced under those conditions upon activation of postsynaptic excitatory inputs. They used the SFO to stimulate MCH neurons in vivo. Similar to previous findings with $ChETA_A$, stimulation across NREM sleep did not change this stage, whereas stimulation across REM sleep episodes prolonged its duration. Jego et al.'s experiments both confirmed and contradicted ours. Both studies found activation of MCH neurons significantly increased REM sleep by increasing transitions from NREM sleep. However, Jego et al. found that it also prolonged REM sleep bout duration. We found no evidence of MCH neurons activation prolonged bout duration.

It is possible to explain the discrepancies. Jego et al. observed effects on REM sleep during daytime stimulation, whereas we did not simply because they transfected the double of neurons than us. Also, blue light energy used to activate ChR2 was more powerful and concentrated in Jego et al.'s study than in ours (Laser vs. LED). When we used a thicker optic fiber and produced very dense expression of ChR2 on dendrites in the rat study, REM sleep amount was potently increased across a spectrum of stimulation frequencies. The fact that we did not observe a lengthening of the REM sleep bout might have to do with the duration of the stimulation. Whereas we stimulated for 1 min, Jego et al. stimulated along the entire REM sleep episode either intermittently (20 Hz) or continuously (SFO). Our analysis of the sleep architecture revealed that REM sleep bouts distribution was phase-locked to the duration of the stimulation; i.e. 1 min. Since 1 Hz stimulation did not evoke longer REM sleep episodes, it suggests this effect only occurs when the MCH neurons are forced to stay highly active across the REM sleep episode. Jego et al. found that MCH neuronal activation did not increase NREM sleep. When we stimulated during daytime, we did not observed this effect either. We only observed it during the nighttime stimulation. Since Jego et al. did not stimulate MCH neurons at that time, the controversy could not be resolved then.

The following year another study from Tsunematsu et al. (2014) attempted to tackle it. These researchers engineered a double Tg line of mice to conditionally express the SFO $ChR2_{E123T/T159C}$ in MCH neurons. For that purpose they used the tetracycline-controlled transcriptional activation system. Crossing a mouse endowed with the tetracycline transactivator protein (tTA) downstream of the MCH promoter gene with another mouse endowed with the tetracycline operator (TetO) upstream of the $ChR2_{E123T/T159C}$-YFP gene resulted in offspring having MCH-tTA/TetO. Histology of these MCH-tTA/TetO revealed that almost 90% of the MCH neurons expressed the reporter gene YFP. Its expression was exclusive to MCH neurons and was observed in somata and dendrites. It is worthy to note that antibodies against GFP were needed to see YFP; hence it is inferred that the endogenous gene expression had to be weak. Voltage-clamp mode recordings showed the YFP-expressing neurons responded with depolarizing currents. The response was elicited by either sustained blue light, or stronger yet, by 10 ms duration light pulses given at 10 Hz. Blue light pulses at 10 Hz for 1 min every 5 min also produced consistent spiking with 100% of fidelity at each stimulation bout. Tsunematsu et al. stimulated the MCH-tTA/TetO mice both during daytime and nighttime. During the first experiment, the daytime stimulation lasted for 3 h at 10 Hz (pulse = 10 ms, light

power = 26 mW). Their findings closely replicated our results in the sense that REM sleep amount doubled. This effect was caused by a higher number of REM sleep transitions associated with fragmentation of NREM sleep and waking as well as increased NREM sleep delta power. The average duration of REM sleep remained unchanged despite mice had constant stimulation for 3 h. When 10 Hz stimulation was given across the night for 1 min every 5 min, transitions into REM sleep were also significantly augmented. The authors stated that REM sleep transitions always occurred from NREM sleep, never from waking, i.e., no SOREMPs. Thus it can be surely inferred that the number of NREM sleep bouts had to increase as well. These researchers did not measure the amounts of vigilance states because they were only interested in transitions into REM sleep. The hypnogram presented on the paper to illustrate this experiment clearly shows more transitions into NREM sleep during the stimulation. Then in a second set of experiments, stimulation at 10 Hz for 1 min was given at the beginning of either wake or NREM sleep episodes. Experiments were done during night and day. Authors claimed that only stimulation at the beginning of NREM sleep caused changes in vigilance states. However they only quantified the state transitions that occurred during the minute of stimulation and the next minute of post-stimulation. These conditions did not reproduce our experiments, and their conclusion should be taken with caution due to sampling bias. Even their state transition plots show a clear trend to increase the number of animals who had NREM sleep as stimulation time went on. We claimed that activation of MCH neurons during waking and during the active period will not put the animal to sleep unless it is given repetitively. At this time the circadian drive to stay awake is too strong to be counteracted by only 1 min of stimulation.

Findings from a recent study using DREADDs to activate MCH neurons highlight our point that effects on the two main sleep states will depend on strength and pattern of stimulation. MCH-Cre mice were transfected with DIO vector containing the excitatory DREADD hM3-DGq-mCherry (Vetrivelan et al. 2016). More than 90% of the mCherry-positive neurons co-labeled with MCH. When recorded in vitro mCherry-labeled neurons responded with continuous spiking following a bath application of the DREADD ligand CNO. c-Fos activation was also observed in mCherry-labeled neurons after CNO administration. c-Fos usually signals the continuous activation of neurons for at least 90 min. In vivo between 24% and 56% of MCH neurons were activated by CNO. DREADD-mediated activation of MCH neurons caused a selective increase of REM sleep both during day and night. A higher number of REM sleep bouts caused this effect. REM sleep average bout duration remained unchanged again confirming that MCH neurons normally do not play a role in REM sleep maintenance. It can be said that continuous activation of a small number of MCH neurons seems to be insufficient to significantly change the full spectrum of sleep stages. In our study in mice, the activation of 50% of those neurons did not yield meaningful changes in any of the sleep states during the day likely because we could not break the ceiling effect recruiting only half of the population. Likewise, the Vetrivelan et al.'s study where only one third of the total MCH neuronal population was steadily activated, it did not observe effects in NREM sleep because it did not have the cadence and strength to counteract the

strong circadian drive to stay awake. Whereas CNO activates MCH neurons for several hours, our protocol of intermittent stimulation only activated these neurons for 2% of the total time. As mentioned earlier MCH neurons show prominent spike adaptation when stimulated continuously (Gao et al. 2003). Tsunematsu et al. also noticed how, when MCH neurons are stimulated at 10 Hz, the evoked depolarizing currents were larger than when stimulated continuously for 1 s (Tsunematsu et al. 2014). Because of this intrinsic property of the MCH neurons, we chose to stimulate them intermittently. Neurons encode their messages to other neurons by timing its APs. In vivo MCH neurons have both tonic and phasic firing, and both modes are quite dissimilar (Hassani et al. 2009). Tonic firing is only 1 Hz, whereas instantaneous firing is 22 Hz more or less 8 Hz. In lieu of stimulating MCH neurons at its tonic rhythm, we assumed that the message of MCH neurons is mainly encoded through their phasic firing. Hence we tested stimulation frequencies from 5 to 30 Hz but delivered intermittently. Thus in our rat's study combining the appropriate phasic stimulation with enough recruitment of stimulated neurons (more light + more ChR2), we then were able to evoke strong effects on both types of sleep at any time of the day counteracting the circadian constraints. Other researchers have also suggested that depending on the mode of optogenetic stimulation, stimulated neurons will release either amino acid (like GABA, glutamate), neurotransmitters, or neuropeptides (like MCH or orexin). They proposed that tonic mild stimulation releases fast amino acid neurotransmitters, whereas neuropeptides may require sustained strong stimulation (Arrigoni and Saper 2014). This concept perfectly would explain why our protocol of stimulation likely resulted in strong MCH release and effects on both types of sleep.

Now it follows to review experiments where optogenetic tools were used to inhibit the activity of MCH neurons.

5.2 *Inhibition of MCH Neurons*

Both Jego et al. (2013) and Tsunematsu et al. (2014) used optogenetic tools to silence MCH neurons and study its effect on sleep. Jego et al. transfected MCH-cre mice with a DIO viral vector containing either the eNpHR3.0-YFP or Arch HR gene. In vitro slice recordings of those transfected neurons corroborated that yellow light illumination for 30 s caused a complete silencing of spikes as well as an outward current when those neurons were voltage clamped. Yellow light 30 s duration pulses were then used to test the in vivo effect on REM sleep and theta power. Inhibition either by eNpHR3.0 or Arch significantly decreased the 6–9 Hz EEG theta power bandwidth at the expense of increasing the 3–5 Hz bandwidth. These results corroborate ours when we found stimulation of MCH neurons significantly increased EEG theta power during REM sleep.

Remarkably yellow light inhibition of MCH neurons neither decreased the average bout duration nor the amount of REM sleep. This result contradicts their hypothesis claiming activity of the MCH neurons is necessary for REM sleep

initiation or maintenance. Since these researchers did not publish any histology for those particular experiments, it is not possible to determine whether the lack of effect had to do with technical issues. We claim that it is not sufficient to rely on the in vitro data to assure the data in vivo had the same degree of validity. Placement of the optic fiber with respect to the targeted cells, the extent of transfection (or selective expression), and strength of transgene expression judged by the reporter gene fluorescent signal all are critical factors that must be reported to interpret correctly the data from optogenetic experiments. It is important that researchers produce maps depicting optic probe placement in relation to transfected cells. The reader must keep in mind that optogenetic tools demand that great number of photons reach the opsins as well as a robust expression of the opsins among a relatively large population of transfected neurons. One important question is how to assess which of the total transfected neurons are actually being stimulated. One way of answering this should be using a marker of activity during the stimulation, like c-Fos, but could be another one more sensitive like GCAMP6. In any case it can be said that the strong results we measured in rats can be explained because rats have more MCH neurons in the first place. However in addition to this species intrinsic difference, the remarkable expression of ChR2 we observed and correct placement of a thicker optic probe that transmitted more photons all likely caused enough neuronal recruitment resulting in strong effects on both types of sleep.

Tsunematsu et al. also inhibited MCH neurons and studied its effects on REM sleep. They again used the TtA/TtO system to express ArchT in MCH neurons. Unlike Jego et al., Tsunematsu et al. published their histology analysis corroborating ArchT only expressed in MCH neurons. Again the reporter signal must have been weak since IF was needed to visualize the transfected neurons. In any case almost all MCH neurons (97.2%) showed colocalization with YFP. In vitro recordings indicated that green light illumination produced 100% of inhibition of APs induced by current injection. Despite the outstanding expression of ArchT and complete inhibition of APs recorded in vitro, 3 h of inhibition failed to change any of the vigilance states. Total amount, average bout duration, or number of episodes of all three states remained unchanged. The conclusion derived from both studies indicates that acute silencing MCH neuronal activity does not affect any of the sleep states.

Tsunematsu et al. also made selective ablations of MCH neurons with the diphtheria toxin A gene under the control of the TtA/TtO gene expression system. One week after doxycycline was removed from the diet, MCH neurons started to die. By the fourth week, only 2.4% of MCH neurons remained. Contrary to the findings obtained with acute silencing of MCH neurons, its ablation had significant impacts on both wake and NREM sleep daily amounts. Thus wake amounts started to significantly increase by the second week and continued along that trend all the way to the fourth week. The increase in wake amounts was due to the expense of a significant decrease of NREM sleep. Reduction in NREM sleep was measured both during the active and resting phases although it was more remarkable during daytime. The reduction of NREM sleep during daytime was mainly caused by decrease in the number of episodes, whereas during nighttime NREM sleep bout duration significantly decreased. No changes in the EEG spectral power were

observed after the loss of MCH neurons. REM sleep amounts were not altered by the complete loss of MCH neurons.

Thus the results obtained after a total loss of MCH neurons corroborate our hypothesis concerning the role of these neurons in regulating daily amounts of NREM sleep. Also they negate the hypothesis that these neurons are critical to generate REM sleep. MCH neurons seem to execute the NREM sleep regulation differently depending on the time of day. This hypothesis is supported by our results where stimulation had different effects depending on the phase of the diurnal cycle. Previously we measured the highest level of MCH in the CSF of rats around noon, a timing associated with high amounts of NREM sleep and REM sleep (Pelluru et al. 2013). Likewise in rats the ICV infusion of MCH at night onset was followed by a significant increase of NREM sleep (+70%) and REM sleep (+200%) (Verret et al. 2003). At night onset MCH levels in the CSF is waning and stay low during the active period (Pelluru et al. 2013). In humans the release of MCH was highest after sleep onset which is also normally associated with higher amounts of delta sleep (Blouin et al. 2013). Thus forced stimulation of MCH neurons, or ICV infusion of MCH after night onset, yielded higher levels of MCH receptor activation recapitulating the sleep facilitation role of MCH occurred otherwise at the onset of the resting phase.

Pharmacological and gene knockout studies provide additional strong support for the role of MCH neurons as modulators of both NREM and REM sleep. In rats selective pharmacological blocking of the MCH1 receptors is followed by a remarkable decrease of both delta sleep and REM sleep (Ahnaou et al. 2008). This effect is dose-dependent, not followed by a sleep rebound and caused by a reduction of bouts duration. MCHR1 KO mice also show significantly less daily amounts of NREM sleep and higher core temperature at rest (Ahnaou et al. 2011). Similar effects were reported by Jego et al. who measured sleep in double MCH-Cre/MCH1R KO mice (Jego et al. 2013). These double transgenic mice had significant reductions in both NREM and REM sleep during daytime.

Recent studies making selective ablations of MCH neurons have put forward an interesting twist on the role of MCH neurons regulating REM sleep during the resting phase. One study replicated the Tsunematsu et al. lesion approach by using diphtheria toxin (DT) to kill selectively MCH neurons (Vetrivelan et al. 2016). MCH-Cre mice were crossed with floxed-P Tg mice harboring the DT receptor. MCH neurons were killed in the offspring with systemic administrations of DT. During the active period, lesioned mice had higher core body temperature, hyperactivity, as well as a significant loss of body mass but no changes in the amounts of sleep states. Remarkably during the resting period, the number of REM sleep bouts paradoxically increased. Interestingly this effect was linked to a shortening of wake episodes. On the second study, researchers made partial lesions of the MCH neurons inserting the Ataxin-3 lethal gene upstream of the MCH promoter (Varin et al. 2016). Adult mice showed ≈30% less MCH neurons. The surviving MCH neurons had clear signs of degeneration such as severe reduction in projection terminals as well as 80% reduction in hypothalamic MCH mRNAs. Similar to the previous lesion study during daytime, MCH Ataxin-3 mice had higher number and longer duration of the REM sleep bouts.

In addition MCH Ataxin-3 mice showed sleep fragmentation. After 12 h of total sleep deprivation, MCH Ataxin-3 mice also had a significant deficit to consolidate NREM sleep rebound including a decrease in delta power. Declining and recovering slopes for REM sleep amounts were also significantly decreased in lesioned mice. Another study in MCHR1 KO mice also found higher amounts of REM sleep during the day (Adamantidis et al. 2008).

It is obvious from these three studies that MCH neuronal activity cannot be critical for generation of REM sleep because it is hard to explain the increase of REM sleep without MCH neurons or MCH receptors. Since the pioneering studies of brain transections, it is known that REM sleep is generated by the brainstem. However it is also evident that absence of MCH neurons does impact how the brain shifts among vigilance states, a function that is particularly important to achieve during sleep homeostasis. Deficit in sleep homeostasis is tightly linked to disturbances of metabolism/body temperature. As pointed out extensively already, loss of MCH neurons or MCH receptors is followed by nighttime hyperactivity, loss of lean body mass, and high core body temperature in addition to loss of appetite. It is conceivable that all these changes have led to abnormal sleepiness during the resting period. The persistent higher core temperature can lead to increase of entries into REM sleep because higher core/brain temperature is known to occur during the transition from NREM to REM sleep (Obal et al. 1985; Alfoldi et al. 1990; Gao et al. 1995). Higher basal/brain metabolism during the rest period also leads to sleep fragmentation (Nofzinger et al. 2004).

6 Conclusion: Ying and Yang Interplay Between MCH and Orexin Neurons

Next, we propose a model where orexin and MCH neuronal activity counterbalance each other. The main assumption of our model demands that both populations of neurons show firing activity in close synchrony. Under the prevalent view, this is unlikely because MCH neurons are considered strictly as sleep active, whereas orexin is considered strictly as wake active. A recent study, however, has debunked the dogma that MCH neurons are completely silent during waking (Hassani et al. 2009). Using fiber photometry and GCAMP6 as calcium sensor in MCH-Cre mice, this study found that MCH neurons showed strong population bursts during novelty exploration (Gonzalez et al. 2016). MCH neuronal activation bursts were inversely correlated with activity bursts from orexin neurons. During a novel object presentation, orexin neurons became very active, while MCH neurons did not, and then MCH neurons became active, while orexin neurons quieted down. During the entry into the exploration area, MCH neurons also turned suddenly active, while orexin neurons remained inactive. The calcium proxy of neuronal activity between orexin and MCH corroborates what in vivo juxtacellular recordings reported earlier, that is, orexin and MCH neurons fire in opposite phases (Hassani et al. 2009). However, in

this case, counterbalancing is occurring during waking. It is conceivable that activity of MCH neurons during waking is geared toward balancing the activity of orexin neurons. In vitro studies have shown MCH blocks the increase of firing in orexin neurons driven by positive feedback (Rao et al. 2008). Without MCH receptors, the glutamatergic inputs into orexin neurons evoke larger excitatory postsynaptic potentials and stronger firing. During novelty exploration, orexin release within the LH is the highest (Kiyashchenko et al. 2002) and so is orexin firing (Mileykovskiy et al. 2005). Thus it is conceivable that MCH neurons become active to offset the positive feedback of orexin neurons preventing a state of hyperarousal, hyperactivity, and anxiety. Overexpression of orexin leads to this abnormal state (Willie et al. 2011). Highest level of activity of orexin neurons is also associated with behaviors characterized by strong and positive emotions like playing or exploration (Mileykovskiy et al. 2005). These behaviors feature high levels of arousal, memory retrieval, and attention. By contrast, orexin neurons are moderately active during repetitive motor behaviors like treadmill running, ingesting, or grooming which are less emotionally charged. Perhaps this is so because MCH tone is counterbalancing the orexin tone. When we stimulated repetitively MCH neurons during waking, we putatively set up an intermittent restrain over the burst of activity in orexin neurons. In other words, we facilitated NREM sleep during the active period by creating a relaxed state of mind conducive for sleep onset.

MCH neuronal activity may be assisting other inhibitory pathways like GABA in setting up a restraining tone for stable vigilance state switching. During NREM, orexin neurons are already under restrain by GABAergic sleep-active neurons (Alam et al. 2005) and fire very occasionally. Orexin neurons have multiple GABAergic synapses contacting them (Henny and Jones 2006). When GABA receptors on orexin neurons are removed, there is a decrease in sensitivity of orexin neurons to both excitatory and inhibitory inputs. As a result these mice show sleep fragmentation switching frequently among vigilance states (Matsuki et al. 2009). Frequent switching among vigilance states is what we also produced by stimulating MCH neurons. We propose that, during NREM sleep, extra inhibition from MCH neurons to orexin neurons would create the right conditions for REM sleep to initiate. However, when MCH tone is abnormally high, orexin neurons become desensitized creating instability among vigilance states. This hypothesis would explain squarely why optogenetic activation of MCH neurons during NREM sleep is so effective facilitating transition into REM sleep but also why we observed frequent transitions among all vigilance states.

Narcolepsy with cataplexy can also be understood as an imbalance between orexin and MCH neurons. Narcolepsy features disturbance of all vigilances states. Narcoleptics show deficits staying awake, staying asleep, and maintaining proper muscle tone during waking and sleep. In narcolepsy with cataplexy, orexin neurons have died, but MCH neurons are still present in normal numbers creating an imbalance (Thannickal et al. 2000). Normally orexin inhibits voltage-dependent calcium channels on MCH neurons (Gao et al. 2003). Furthermore optogenetic stimulation of orexin neurons inhibits MCH firing burst by exciting GABAergic synapsis onto MCH neurons (Apergis-Schoute et al. 2015). Hence in narcoleptics

the lack of orexin inhibition onto MCH neurons may trigger abnormal activity bursts across all vigilance states. This burst of activity in MCH neurons will, in turn, break down arousal (i.e., sleep attacks), and sometimes, when the emotional tone becomes very high, it will bring down muscle tone resulting in cataplexy attacks or direct transitions into REM sleep, i.e., SOREMPs. It also creates sleep fragmentation manifested as constant switches among waking, NREM, and REM sleep, particularly transitions into REM sleep.

Narcolepsy also illustrates how deficits to stay fully alert and maintain skeletal muscle tone are tightly connected to disturbances regulating emotional tone (Bayard and Dauvilliers 2013). We claim that the connection of cataplexy with unrestrained emotional tone can be also explained by an imbalance between orexin and MCH neuronal activity. In narcoleptic rodents or dogs, cataplexy attacks are usually preceded by emotionally laden motor repetitive behaviors such as eating palatable food (Gerashchenko et al. 2001; Clark et al. 2009) play, and copulation (Nishino and Mignot 1997) or other less emotive like grooming or running (Chemelli et al. 1999; Beuckmann et al. 2004; Espana et al. 2007). Infusion of orexin (Mieda et al. 2004) or orexin gene therapy within the dorsolateral pons, a REM sleep generator (Blanco-Centurion et al. 2013); or within the central nucleus of the amygdala, a strong emotional center (Liu et al. 2016); or in the ZI/LH (Liu et al. 2011), where MCH neurons are located, all blocks cataplexy. However we found that when orexin is expressed in MCH neurons only, cataplexy and sleep fragmentation actually worsen. Thus when the timing of orexin and MCH release becomes simultaneous, emotionally driven cataplexy and hyperarousal during sleep occur more often than when MCH is released without any counterbalance from orexin tone. Repetitive pattern motor behaviors like eating are precisely the behaviors that we know kindle MCH release in the amygdala of humans (Blouin et al. 2013). In the amygdala every behavior where orexin tone turns high is negatively mirrored by low MCH tone and vice versa. We propose this counterbalance scenario within the main center of emotional regulation is not epiphenomena, but it reflects an essential counterbalance modulation.

Burst of MCH activity is critical to integrate caloric value, ingesting behavior and level of locomotor activity. It is hard to understand this function if MCH neurons would only fire during sleep. Thus MCH neurons were optogenetically stimulated during a drinking choice preference test where animals had to choose between drinking an artificial sweetener or sucrose (Domingos et al. 2013). In the control group, animals preferred sucrose because of its true caloric content and rewarding effect assessed by dopamine release. Stimulation of MCH neurons at 20 Hz, or continuously for 1 s, given during the choice test completely reversed the preference for sucrose. Stimulation at 5 Hz was ineffective. Effective stimulation also caused a peak of dopamine release, whereas the ineffective stimulation did not. Selective ablation of MCH neurons abolished the sucrose preference as well as the peak in dopamine release elicited by sucrose intake. An antagonist of the MCH receptor also blocks the preference for ingesting highly palatable food (Morens et al. 2005). Remarkably in humans the second high level of MCH release occurs after eating (Blouin et al. 2013). In this context years ago direct evidence emerged that activation of MCH receptors is key to regulate caloric intake. MCH receptor KO mice are lean,

eat less, and show high basal metabolism (Shimada et al. 1998). When MCH receptor KO mice are fed with high caloric diet, animals also become hyperactive (Kokkotou et al. 2005). Hyperactivity in MCH receptor KO mice fed with fat diet is especially prominent during the active period and also occurs in MCH KO mice (Zhou et al. 2005) and mice with selective genetic ablation of MCH neurons (Alon and Friedman 2006). By contrast, overexpression of MCH receptors produced a phenotype of obesity and insulin resistance (Ludwig et al. 2001). Same results occurred in mice that had continuous ICV infusions of MCH (Gomori et al. 2003). MCH neurons are indeed strongly activated by glucose (Burdakov et al. 2005) or by cannabinoids which are well known for its orexinergic effect (Huang et al. 2007).

Glucose has the opposite effect on orexin neurons causing strong inhibition of its firing (Burdakov et al. 2005). In contrast to the lean mice whose MCH neurons were ablated, mice without orexin neurons are obese despite eating less. These mice are obese because they have significantly low locomotor activity during the active period so calories are not burned (Hara et al. 2001). On top of that, sleepiness lower even further both the metabolism and the energy expenditure. The effects on large body weight with low activity and metabolism are not surprising because the selective activation of orexin neurons causes an increase of eating, drinking, locomotor activity, and metabolism (Inutsuka et al. 2014).

As we already mentioned, electrical stimulation experiments indicated that the LH is a brain hub for integration of feeding and reward behaviors. The link between MCH neuronal activation and relaxed emotions is rooted in several studies that firmly established the anxiolytic effects of MCH (Monzon et al. 2001; Borowsky et al. 2002; Carlini et al. 2006). Unlike the calming effects of MCH neuronal activation, optogenetic activation of orexin increases anxiety in mice (Heydendael et al. 2014). The orexin pathway regulating anxiety and fear involves connection from the amygdala and the locus coeruleus (Sears et al. 2013). By contrast, GABAergic neurons from the amygdala make monosynaptic connections with MCH neurons inhibiting them (Gonzalez et al. 2016). Altogether the picture that emerges depicts the MCH neurons becoming active in response to biological critically goal-directed behaviors like eating. Ingestion is primarily driven by high orexinergic tone that if unchecked could become excessive. Therefore it is conceivable MCH neurons would activate, counterbalancing the orexin drive and generating a calming/pleasant sensation that leads to the end of the appetitive behavior. The satisfaction of the appetitive drive may eventually lead to sleep onset. This balance/counterbalance model of orexin/MCH may also apply to understand other essential appetitive behavior like drinking or mating.

Stress and anxiety inhibit activity of MCH neurons (Gonzalez et al. 2016) through an orexin and amygdala jointed pathway. This effect of stress would explain why during the juxtacellular recordings of these neurons firing activity during waking was barely detected, i.e., only during transitions to NREM sleep (Hassani et al. 2009). Juxtacellular recordings demand the rat to be head fixed and held lifted onto a hammock. Even though daily habituation makes the rat to eventually fall asleep during the recording, it is obvious that the animal must be somewhat stressed while awake. Stress will prevent the MCH neurons to fire giving a bias toward sleep activity.

The counterbalance between orexin and MCH neurons also can take place during REM sleep. Contrary to the prevalent view of orexin neurons being silent during REM sleep, a microdialysis study found that orexin release, within the LH and basal forebrain, is highest during REM sleep (Kiyashchenko et al. 2002). This finding is supported by the fact that, in unrestrained animals, orexin neurons produce burst of activity linked to the phasic movements occurring during REM sleep (Mileykovskiy et al. 2005). Jego et al. observed that 20 Hz optogenetic stimulation of the MCH projections to the posterior hypothalamus, LH, and medial septum significantly increased the duration of REM sleep episodes (Jego et al. 2013). All these regions receive strong projections from orexin neurons as well (Peyron et al. 1998). We claim that MCH projections to those areas are such to counterbalance the burst of phasic activity from orexin neurons during the phasic events of REM sleep. Recent unpublished data from our laboratory indicate that the pathway connecting MCH neurons to noradrenergic neurons in the locus coeruleus also mediate the increase in REM sleep produced by the optogenetic stimulation. A previous study found projections from MCH to the REM sleep generator zone in the pons. Local infusion of MCH into this pontine area increased REM sleep (Torterolo et al. 2009). Needless to add that orexin neurons also project to the locus coeruleus and the pontine reticular formation. Orexin input into both areas is critically involved in the control of muscle tone during REM sleep and cataplexy (Wu et al. 1999; Kiyashchenko et al. 2001). Thus our hypothesis neatly explains why burst of activity of MCH neurons occurs phase-locked to the phasic motor events of REM sleep (Hassani et al. 2009). Whether the optogenetic stimulation of MCH neurons would lengthen REM sleep, as Jego et al. observed, must depend on how effective counteracting the burst of activity of orexin neurons is. Yet if the latter is mild, optogenetic activation of MCH neurons could be ineffective prolonging REM sleep episode as we and other researchers have observed. As we sustain the timing between orexin and MCH burst of neuronal activity is what will determine the outcome of behavior. An out-of-phase interplay between these two hypothalamic types of neurons is what ultimately governs the expression of the vigilance states.

7 Future Directions

Our hypothesis can be empirically tested in two ways. One is to monitor the in vivo activity of both MCH and orexin neurons using state-of-the-art imaging techniques. Nowadays it is possible to observe intracellular calcium transients in specific neuronal phenotypes occurring in real time and to single-cell spatial resolution level. Hundreds of neurons can be imaged in this way. Less than a second time-resolution scale observations are possible. For instance, a recent imaging study focused on observing how GABAergic neurons within the LH get activated during either the consummatory or appetitive phases of the feeding behavior (Jennings et al. 2015). Calcium imaging in vivo also has been applied to study neuronal activity of both GABAergic and glutamatergic dorsolateral pontine neurons occurring during

the sleep states (Cox et al. 2016). If our counterbalance hypothesis is correct and the out-of-phase interplay regulates the states of vigilance, it should be observed as distinctive patterns of activity burst in orexin and MCH neurons. Furthermore, using optogenetics coupled to in vivo micro-endoscopic imaging, we can ask hypothesis-driven experimental questions. Either stimulating or inhibiting orexin and MCH neurons and at the same time imaging those neurons, we should be able to predict changes in the neuronal activity patterns and sleep behavior.

Another aspect that is critical to explore is how different projections of both orexin and MCH are integrating sleep states. Using clarity to make the brain transparent, we discovered MCH neurons are organized in three different clusters according to the direction of its projections (Shiromani and Peever 2017). One cluster projects mostly to the forebrain and the other toward the brainstem, and a third one seems to link both clusters within the LH. We believe this arrangement may reflect the way MCH neurons counterbalance orexin neurons along different aspects of sleep–wake behavior. Perhaps forebrain projections counterbalance orexin signal in terms of memory, attention, and emotion and may regulate the transition from wake into NREM sleep. Projections to the brainstem may integrate the counterbalance of orexin tone to restrain sympathetic and muscle tone that may underlie the transitions into REM sleep. In any case what is needed is to stimulate or inhibit in a specific projection manner and observe its impact on neuronal activity and behavior. Using retrograde virus carrying either opsins or GCAMP6 Cre-dependent gene, we could address this type of important questions.

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