The Genetic and Neuronal Substrates of Melatonin Signaling in Zebrafish Sleep

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ABSTRACT

Sleep is hypothesized to be regulated by two processes: a circadian drive, which communicates time of day to ensure that sleep is timed to the appropriate day/night phase, and a homeostatic drive, by which the propensity for sleep becomes stronger over the course of prolonged wakefulness. While studies suggest that adenosine and serotonin signaling in part mediate the homeostatic sleep drive, factors that act downstream of the circadian clock to promote sleep were unidentified until recently. Previous work in the Prober lab has shown that the nocturnal hormone melatonin acts downstream of the circadian rhythm to promote sleep in zebrafish. The downstream processes by which melatonin promotes sleep is poorly understood across all animal models. This is likely because melatonin research has been primarily conducted using nocturnal laboratory rodent models, in whom melatonin does not seem to play a role in sleep, and because of the widely held view that melatonin informs the circadian clock and does not promote sleep directly. In Chapter 1 of this thesis, I review some of the research conducted over the last 50 years that has informed our current understanding of melatonin and its role in sleep. In Chapter 2, I describe our efforts to use the zebrafish, in which melatonin is both potently sedating and essential for nightly sleep, to uncover some of the mechanisms by which melatonin might promote sleep. We found that melatonin acts through a particular melatonin receptor family called MT1, whereas melatonin receptors belonging to other families were dispensable for sleep. We show that MT1 receptors are expressed broadly throughout the zebrafish brain and are enriched in brain regions involved in sensory processing, particularly in those related to vision. We tested the hypothesis that melatonin promotes sleep, at least in part, by dampening visual responsiveness at night. We show that, separable from sleep, exogenous melatonin suppresses behavioral responses to light stimuli, and loss of endogenous melatonin results in day-like behavioral responses to light stimuli during the night. We are using whole brain imaging in live zebrafish to corroborate our behavioral results with neuronal GCaMP recordings. We hope that the findings presented here contribute to a greater understanding of melatonin's role in sleep, which may help enhance its value as a natural therapeutic aid.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER 1

A REVIEW OF MELATONIN'S SLEEP-PROMOTING ROLE ACROSS ANIMAL MODELS

1.1 Introduction

In the early 1900s it was discovered that by feeding or injecting tadpoles with crude pineal gland extracts, the tadpoles' skin became remarkably lighter (McCord & Allen, 1917). Forty years later, in 1958, a dermatologist named Aaron Lerner purified the relevant pineal hormone and showed that it blocked the darkening of frogs' skin by melanocyte-stimulating hormone with 100 or more times greater efficacy than other neurotransmitters (Lerner et al., 1958). He called this molecule melatonin. Dr. Lerner had hoped that melatonin was "the long sought for factor" of vitiligo, a skin condition that he studied, but injections of melatonin into adult men did not result in de-pigmentation like it did in frogs. One of Dr. Lerner's patients instead reported sedation after melatonin injection (Lerner & Case, 1960).

Around the same time, several laboratories had experimentally established a relationship between the pineal gland, environmental lighting, and gonadal maturation, but the unifying substance, housed in the pineal gland, was not yet identified (Wurtman, 1985). By 1965, it became clear that the pineal gland indeed converts photic information into a hormonal message, melatonin, that regulates certain aspects of animal physiology (Wurtman, 1985). This basic light-pineal-melatonin pathway has been exhaustively studied and is well-accepted among scientists who study melatonin and pineal-related processes, but those *certain aspects* of animal physiology—the *outputs* of melatonin—remain controversial.

The role of melatonin in sleep is one such debated function. Since the unexpected sleep-inducing effect reported by Dr. Lerner's patient, several studies have endeavored to replicate this finding. Some have succeeded, while others have failed to find a soporific effect of melatonin. Even some recent scientific reviews claim that melatonin is *not* a sleep

hormone (Arendt, 2019; Foster, 2021). The purpose of this review is (1) to highlight important studies that use model organisms to test the hypothesis that melatonin promotes sleep, (2) to point out the complications and sources of confusion that have led to alternative claims about the function of melatonin vis-à-vis sleep, and (3) to discuss gaps in knowledge about the mechanism by which melatonin promotes sleep and how to fill in those gaps.

1.2 Synthesis and regulation of melatonin

Melatonin is synthesized from the neurotransmitter serotonin in two enzymatic steps (Figure 1.1). First, serotonin is acetylated by *aryl alkylamine N-acetyltransferase* (AANAT) to form N-acetylserotonin. Then, N-acetylserotonin is methylated by *hydroxyindole-O-methyltransferase* (HIOMT) to form melatonin (Reiter, 1991). Melatonin is highly lipophilic, and it therefore freely crosses lipid bilayers and cannot be contained in vesicles like its precursor, serotonin (Yu et al., 2016). As a result, melatonin behaves like a hormone and not as a neurotransmitter, and it is expected to reach all tissues of the body in animals with a cardiovascular system. It also means that the main regulation of melatonin signaling happens at the level of its synthesis: specifically, by control of the AANAT enzyme (Schomerus & Korf, 2005).

Melatonin is synthesized primarily in two organs: the eye and the pineal gland (Wiechmann & Sherry, 2013; Ostrin, 2019). Interestingly, the pineal gland is considered to be a 'vestigial eye' because in non-mammalian vertebrates it contains photoreceptors and can therefore 'sense' light, even though it does not play a role in vision (Mano & Fukada, 2007, Wiechmann & Sherry, 2013). Thus, melatonin is poised to communicate photic information from tissues that receive such information to every other tissue in the animal. However, melatonin synthesis is not a *direct* output of light status. Instead, it is an output of the circadian clock, which is entrained by cycles of light and dark (or, more naturally, day and night), and by other circadian cues (Liu & Borjigin, 2005). The circadian clock and clock-controlled behaviors persist even in the absence of normal day/night cues. Thus, an entrained animal that is subsequently confined to constant darkness will still produce melatonin at the time that corresponds to night ('subjective night').

In birds and fish, cells of the pineal gland express photoreceptors and have self-sustaining circadian rhythms that are directly entrainable by light (Cahill, 1996; Doyle & Menaker, 2007). Thus, when pineal glands of birds and fish are cultured *ex vivo*, the circadian release of melatonin persists and can be re-entrained by light (Cahill, 1996; Zatz et al., 1988). By contrast, in mammals, cells of the pineal gland are not directly photosensitive, and circadian rhythms are entrained elsewhere, in the suprachiasmatic nucleus (SCN) of the hypothalamus, which is sometimes called the master pacemaker. This difference is explained by the anatomical location of the pineal gland: in birds and fish the pineal gland is located on top of the head, and thus is directly exposed to light, whereas in mammals the pineal gland is buried deep within the brain.

The photo-neuro-endocrine transduction pathway of mammals is depicted in Figure 1.2. Non-image forming light information (i.e., irradiance) is primarily detected in the retina by intrinsically photosensitive retinal ganglion cells (ipRGCs). ipRGCs express melanopsin, a type of photoreceptor that is distinct from those used by rods and cones for image-forming vision. Whereas rods and cones activate retinal ganglion cells via bipolar cell interneurons, ipRGCs themselves are directly responsive to light. ipRGCs deliver light-dark information from the retina to the SCN via the retinohypothalamic tract, and this light-dark information 'entrains' the SCN (for a review, see Coomans et al., 2015). Neurons in the SCN exhibit a circadian rhythm of activity, with high firing rates during the day and lower firing rates at night, and this rhythm persists in constant darkness for several circadian cycles. SCN outputs are thought to synchronize the circadian rhythms of cells in peripheral tissues, which is why the SCN is thought to serve as a master pacemaker (reviewed in Mohawk et al., 2012).

The SCN is connected to the pineal gland via a multi-synaptic pathway, starting with one inhibitory connection from the SCN to the paraventricular nucleus (PVN) of the

hypothalamus. Neurons of the PVN innervate preganglionic sympathetic neurons in the spinal cord, known as the intermediolateral nucleus (IML), and these sympathetic neurons innervate the superior cervical ganglia (SCG). Finally, noradrenergic SCG axons terminate at the pineal. At night, when SCN firing is low, inhibitory input to the PVN is reduced, and noradrenergic input to the pineal is increased (Borjigin et al., 2012). Binding of norepinephrine to beta-adrenergic receptors on pinealocytes stimulates the accumulation of cyclic AMP (cAMP), which positively regulates the AANAT enzyme in (at least) two ways.

First, cAMP stabilizes AANAT protein by promoting protein kinase A (PKA)-mediated phosphorylation, which prevents the otherwise rapid degradation of AANAT by the proteasome (Gastel et al., 1998). Second, cAMP promotes transcription of the *aanat* gene by PKA-mediated phosphorylation of the cAMP response element-binding protein (CREB). This mode of AANAT regulation is typical in most rodents, but appears not to be the case in ungulates, such as sheep and cow. In ungulates, *aanat* transcripts do not oscillate in a circadian manner; regulation of AANAT instead occurs only on a post-translational basis (Schomerus & Korf, 2005).

The protection of AANAT from degradation explains an *acute* inhibitory effect of light on AANAT activity that has been observed in many organisms, including rodents (Deguchi & Axelrod, 1972), birds (Binkley et al., 1975; Hamm et al., 1983), and fish (Falcón et al., 2001). Separate from its role in entraining the circadian clock, light stimulation—either via the SCN or by direct action on a photoreceptive pineal gland—leads to a loss of cAMP, which results in the rapid destabilization and degradation of AANAT. This acute effect of light on AANAT fine-tunes the rhythm of melatonin synthesis.

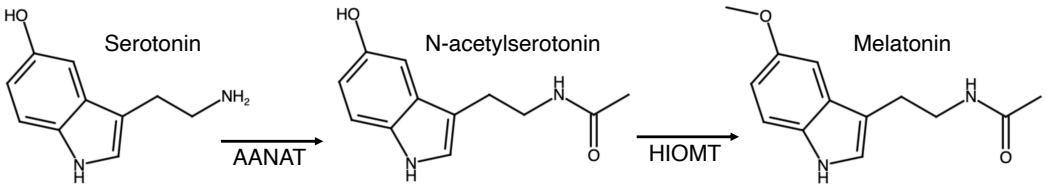

Figure 1.1. Melatonin synthesis pathway. Serotonin is acetylated by the enzyme AANAT to form N-acetylserotonin. N-acetylserotonin is then methylated by HIOMT to form melatonin.

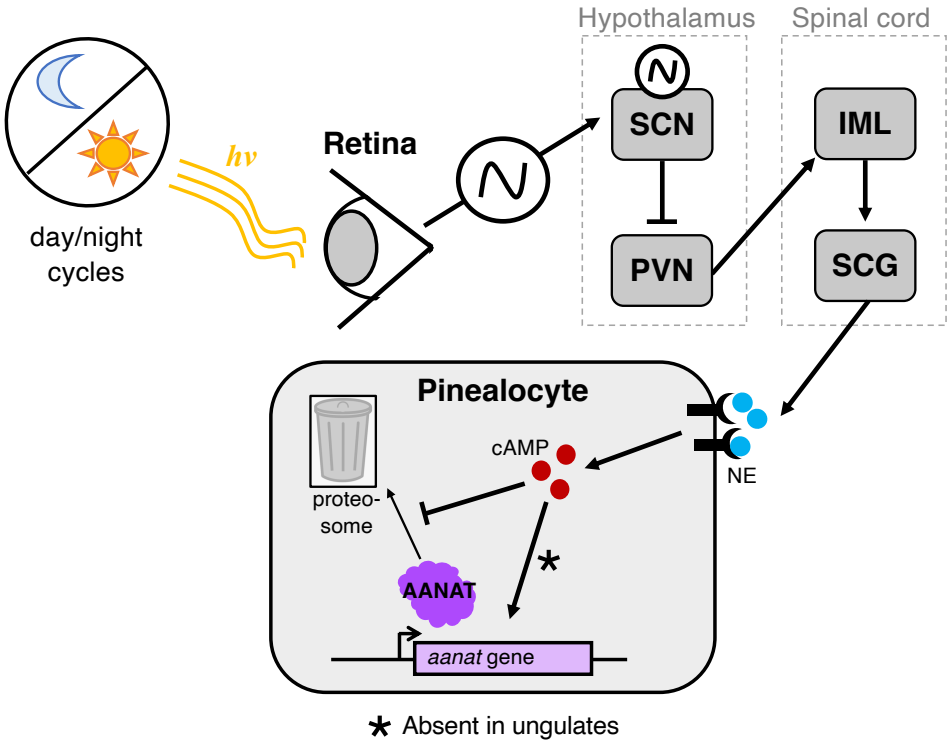

Figure 1.2. Circadian regulation of AANAT in the mammalian pineal gland. Cycles of day (light) and night (darkness) entrain the circadian rhythm of the suprachiasmatic nucleus (SCN) via intrinsically photosensitive retinal ganglion cells of the retina that project to SCN. Entrained SCN neurons, which maintain a circadian rhythm of electrical activity, send inhibitory projections to the paraventricular nucleus (PVN). PVN neurons innervate the intermediolateral nucleus of the spinal cord (IML), which in turn innervates the superior cervical ganglion (SCG). Noradrenergic SCG neurons terminate at the pineal and activate beta-adrenergic receptors of *aanat*-expressing pinealocytes. Activation of beta-adrenergic receptors causes an increase in cyclic AMP, which has two effects on AANAT. First, it up-regulates expression of *Aanat* transcripts, and second, it prevents the rapid degradation of AANAT protein by the proteosome.

1.3 Functions of melatonin

Free-radical scavenger. Melatonin has been identified in nearly every organism studied, even in primitive bacteria (Hardeland, 1999; Tan et al., 2016). Melatonin and its metabolites are highly effective antioxidants, and it has been hypothesized that melatonin's "oldest" function was to counter oxidative stress from free radicals that are generated by metabolic processes (Manchester et al., 2015; Zhao et al., 2019). Many of melatonin's effects on the physiology of more complex organisms have been ascribed to this antioxidant capacity. Whether or not these effects, which are based solely on administration of exogenous melatonin, represent a *normal* physiological function of melatonin has yet to be substantiated by loss-of-function experiments. A recent review by Zhao et al. summarizes this function of melatonin (Zhao et al., 2019).

Seasonal control of reproduction. The nighttime release of melatonin enables tissues that are receptive to its message to know not only *when* it is night (or when it is supposed to be night, more specifically), but also the *duration* of night. This latter metric is useful for directing seasonal changes in physiology and behavior, most notably those associated with reproduction. In the winter months of the year, the longer night is translated into a longer melatonin signal, and this increased signal duration is sufficient to evoke winter-related reproductive changes in seasonally breeding mammals. The effect of melatonin on the maturation of rat gonads (Wurtman, 1985) was one of the first identified functions of melatonin aside from its skin-lightening property. The melatonin receptor-enriched pars tuberalis of the pituitary gland was later shown to be the recipient tissue for this function of melatonin signaling (Reppert & Weaver, 1995; Wood et al., 2020). The loss of melatonin synthesis in most laboratory mouse strains (see below), and thus

the loss of seasonal control of reproduction by melatonin, is likely a result of selective pressures for mice to breed throughout the year.

Metabolism. A considerable amount of recent research has demonstrated a role for melatonin in regulating various aspects of metabolism. Loss-of-function experiments (by pinealectomy or genetic deletion of melatonin receptors) have shown that loss of melatonin signaling exacerbates weight gain and hyperglycemia in a diet-induced obesity model, impairs leptin signaling and eventually leads to leptin resistance, and disrupts glucose metabolism in certain tissues. Owino et al. recently reviewed animal studies that support this role of melatonin (Owino et al., 2019). This function of melatonin appears to be conserved in humans, as common variants in the MT2 melatonin receptor are associated with fasting glucose levels and risk of type 2 diabetes (Prokopenko et al., 2009; Lyssenko et al., 2009).

Circadian rhythms and sleep. Perhaps the most widely studied, yet controversial, role for melatonin involves the regulation of circadian rhythms and/or sleep. Melatonin treatment has been shown to be sedating in a wide range of diurnal species, including nematodes (Tanaka et al., 2007; Niu et al., 2020), zebrafish (Zhdanova et al., 2001; Gandhi et al., 2015), birds (Hishikawa et al., 1969; Paredes et al., 2007), non-human primates (Zhdanova et al., 2002), and humans (Brzezinski et al., 2005), and can affect circadian rhythms in some contexts (Lewy et al., 1992; Lewy et al., 1996; Arendt, 2005). However, melatonin treatment is generally not sedating in nocturnal species (Murakami et al., 2001; Huber et al., 1998; Langebartels et al., 2001), which is inconsistent with a general role for melatonin in sleep. In addition, treatment with exogenous melatonin is a gain-of-function perturbation that may induce phenotypes that do not reflect the normal function of melatonin. Most melatonin loss-of-function studies have used surgical removal of the pineal gland (pinealectomy, Px). This is a relatively crude procedure whose efficacy

likely varies in different species and in experiments performed in different labs, which may account for the variety of reported results. More importantly, Px removes not only cells that synthesize melatonin, but also other cells in the pineal gland whose loss may confound conclusions about the function of melatonin. Another caveat with Px experiments is that in most cases the results observed were interpreted as effects on circadian rhythms, when in fact the assays used did not distinguish between effects on the circadian clock itself vs. outputs of the circadian clock such as sleep. The more recent use of genetic techniques to abolish melatonin synthesis, combined with assays that distinguish between effects on the circadian clock and on sleep, suggest that melatonin normally functions downstream of the circadian clock to promote sleep, at least in diurnal animals. Below I discuss the evidence for a role for melatonin in regulating circadian rhythms and/or sleep in several model organisms and in humans.

1.4 Melatonin studies using invertebrate model systems

Studying sleep using simpler model organisms that are not readily amenable to EEG/EMG recordings—considered to be the 'gold standard' for measuring sleep—has been made possible by the development of a set of behavioral criteria that define a sleep state. Sleep can be behaviorally defined as periods of reduced locomotor activity that are rapidly reversible, are associated with an increased arousal threshold, and are under homeostatic regulation (Borbély, 2022). Indeed, behavioral measures can identify sleep and wake states with ~95% accuracy compared to EEG/EMG recordings in mammals (Pack et al., 2007; Fisher et al., 2012). As a result, behavioral measures of sleep can be used not only for animals where EEG/EMG recordings are challenging to perform, such as nematodes, flies, and fish, but also for studies of sleep in mammals.

C. elegans is one such simple invertebrate that has recently emerged as a model system for the study of sleep. Using the above criteria, *C. elegans* are reported to display sleep-like states during periods of molting (known as 'lethargus'; Raizen et al., 2008), when sated (You et al., 2008), and following exposures to cellular stressors (Hill et al., 2014). Signaling pathways that regulate at least some of these sleep-like states in the worm appear to be conserved regulators of sleep in other invertebrate and vertebrate models (Kushikata et al., 1998; Lenz et al., 2015; Lee et al., 2019). Despite its recent utility in understanding the mechanisms governing sleep, using *C. elegans* to study the role of melatonin in sleep has been challenging.

Tanaka et al. (2007) provided the first evidence that melatonin might regulate sleep in *C. elegans.* They observed a rapid and dose-dependent decrease in locomotor activity upon treatment with melatonin. The authors did not posit that melatonin's influence on locomotion involved a sleep-like state, which is appropriate, since they did not test whether

melatonin-treated worms fulfilled the behavioral criteria for a sleep state. The effect of melatonin on locomotion was attributed to the MT1-type melatonin receptor based on drug experiments: the effect was blocked by pre-treatment with an MT1/MT2 antagonist but not with an MT2-selective or an MT3-selective antagonist. However, it is unknown whether different melatonin receptor subtypes are present in the *C. elegans* genome, and it is unclear whether the specificity of mammalian melatonin receptor subtype-selective drugs is preserved in nematodes.

Consistent with a function for melatonin in nematodes, Tanaka et al. (2007) identified endogenous melatonin in *C. elegans* using HPLC. Surprisingly, the *C. elegans* genome appears to lack an *aanat* ortholog (Ganguly et al., 2001; Tanaka et al., 2007; Migliori et al., 2012), despite the presence of abundant N-acetylserotonin, which suggests that serotonin is N-acetylated by a different enzyme. A putative HIOMT ortholog (called *homt-1*) and a single melatonin receptor ortholog (called *pcdr-1*) have been identified, and using mutations in these genes, Niu et al. (2020) provided compelling evidence that melatonin functions through *pcdr-1* to inhibit neuronal activity, which is likely associated with sleep in this organism. They demonstrated that treatment with melatonin reduced excitatory post-synaptic current (ePSC) amplitude and decreased the frequency of miniature ePSCs ('minis'), together indicating a reduction in neuronal activity. They also showed that worms treated with melatonin remained in lethargus, a developmentally regulated sleep state, for longer. More interestingly, loss of *homt-1* or *pcdr-1* increased ePSC amplitudes and mini frequencies, reduced time spent quiescent during lethargus, and increased the frequency of 'active events' during lethargus, all pointing to a role for endogenously-produced melatonin in nematode sleep. *homt-1; pcdr-1* double mutant animals did not show additive phenotypes, implying that these two genes function in the same pathway. Finally, the authors showed that melatonin treatment restored the wild-

type duration of lethargus in *homt-1* mutants but had no effect on the shortened lethargus of *pcdr-1* mutants. These findings offer a potentially powerful window into the mechanism by which melatonin promotes sleep. *C. elegans* has a simple nervous system and is highly genetically tractable; thus, identifying a melatonin pathway that promotes sleep in *C. elegans* would enable opportunities to study melatonin's effect at the neuronal and molecular levels. Indeed, Niu et al. (2020) showed that the putative melatonin receptor PCDR-1 is complexed with the BK channel SLO-1 to regulate neurotransmitter release.

Despite these observations, there are caveats to the notion that melatonin promotes sleep in *C. elegans*. First, it remains to be determined if melatonin is synthesized by the putative HIOMT homolog *homt-1*. This can be clarified by assaying for melatonin using HPLC/MS measurements in wild types and *homt-1* mutants. Until it is shown that *homt-1* animals are defective for melatonin synthesis, it will remain unclear if the mutant phenotypes are attributable to a loss of melatonin, especially given the weak homology of *homt-1* to mammalian HIOMT.

Second, it remains unclear if *pcdr-1* encodes a melatonin receptor. Niu et al. (2020) demonstrate that PCDR-1 shows sequence homology to human melatonin receptors, but sequence analysis by BLASTP suggests that PCDR-1 is just as, if not more, homologous to several *other* human GPCRs, including a serotonin receptor and neuropeptide Y receptor (unpublished observation). A previous study by Keating et al. (2003) also predicts that *pcdr-1* encodes a melatonin-binding GPCR based on phylogenetic analysis, but when Keating et al. knocked down *pcdr-1* using RNA interference, worms exhibited a sluggish phenotype, contrary to the expected effect of melatonin receptor knockdown and contrary to the study by Niu et al. (Keating et al., 2003; Niu et al., 2020). As mentioned above, it is difficult to predict how melatonin receptor-specific drugs will affect a putative worm ortholog such as PCDR-1. Nevertheless, treatment with different agonists and antagonists

yield confusing results: a non-selective antagonist gives agonist-like effects, and while an MT2-selective antagonist appears to block the effect of melatonin treatment on behavior, an MT2-selective agonist has no effect on its own (Niu et al., 2020). These findings also do not reconcile with the drug experiments reported in Tanaka et al. (2007). Niu et al. (2020) conclude that PCDR-1 has pharmacological properties distinct from mammalian MT1 and MT2.

Finally, whereas melatonin is tightly linked to circadian rhythms in other organisms, its relationship with the clock in *C. elegans* is not clear. *C. elegans* has been reported to display circadian rhythms in melatonin synthesis (Migliori et al., 2012), N-acetylation activity (by a presumptive worm AANAT) (Migliori et al., 2012), gene expression (van der Linden et al., 2010; Goya et al., 2016), and certain behaviors (Simonetta et al., 2009; Winbush et al., 2015; Saigusa et al., 2002), but no sleep-like state in *C. elegans* occurs with circadian rhythmicity (Raizen et al., 2008; You et al., 2008; Hill et al., 2014). Furthermore, the expression of nematode homologs of core circadian clock genes do not oscillate in a circadian manner in *C. elegans* (Chan et al., 2003; Tennessen et al., 2006); instead, these clock genes, such as the PERIOD homolog LIN-42, regulate the timing of developmental cycles, including lethargus (Monsalve et al., 2011). That the worm PERIOD homolog regulates lethargus, a sleep-like state shown to require melatonin signaling (Niu et al., 2020), might suggest an ancient link between genes that mediate the circadian clock and melatonin in an animal whose sleep is regulated by developmental transitions instead of circadian rhythms.

Drosophila melanogaster is another invertebrate species that is widely used for circadian and sleep research. Melatonin is synthesized in *Drosophila* by the same enzymatic pathway as in other species, and a single clear ortholog of AANAT called *aanat1*, *speck*, or *Dat*, has been identified (Finocchiario et al., 1988; Hintermann et al.,

1996; Brodbeck et al., 1998; Spana et al., 2020). However, unlike in vertebrate species, neither *Drosophila aanat1* transcripts nor AANAT1 protein levels oscillates with a 24-hour rhythm (Hintermann et al.,1996; Brodbeck et al., 1998, Amherd et al., 2000). This lack of circadian *aanat* expression is also true in prawns (Withyachumnarnkul et al., 1992) and may reflect a feature of arthropod AANAT regulation. Further, *Drosophila aanat1* mutants have normal baseline sleep levels, although sleep rebound is enhanced (Shaw et al., 2000). The likely explanation for this behavior is melatonin-independent and instead involves AANAT's capacity to convert serotonin into N-acetylserotonin, as serotonin has been shown to play a role in sleep rebound (Oikonomou et al., 2019; Davla et al., 2020). Thus, while few published studies have investigated a role for melatonin in *Drosophila* circadian rhythms or sleep, there is currently no compelling evidence that melatonin regulates these processes in this species.

1.5 Melatonin studies using zebrafish

The zebrafish (*Danio rerio*) has recently emerged as a useful model system to study mechanisms that regulate both circadian rhythms and sleep. Key advantages of this model include a vertebrate brain that is anatomically and molecularly homologous to the mammalian brain, rapid development with complex behaviors evident at only 5 days post-fertilization, optical transparency during embryonic and larval development, and mechanisms that regulate circadian rhythms and sleep that are well-conserved with those of mammals. Most important for studies of melatonin, zebrafish are diurnal and produce melatonin, like humans, and unlike the nocturnal rodents used by most research laboratories. Thus, zebrafish have distinct advantages over nocturnal rodents in efforts to model the role of melatonin in circadian rhythms and sleep in humans. However, a key difference between zebrafish and mammals is that there is no evidence that zebrafish have a brain structure analogous to the suprachiasmatic nucleus (SCN), which serves as a circadian pacemaker in mammals. Like in other species of fish and in birds, the zebrafish pineal gland is located above the dorsal surface of the brain and is thus directly exposed to environmental light. In these species, many cells of the pineal gland express photoreceptors, which enables the circadian rhythm of these cells to be directly entrained by light, as shown in *ex vivo* experiments (Cahill, 1996). In fact, zebrafish circadian organization is likely comprised of a decentralized network of cell-autonomous clocks, as the circadian clocks of peripheral tissues can be directly entrained by light (Whitmore et al., 2000), and the zebrafish pineal is not required for the entrainment of peripheral circadian rhythms (Livne et al., 2016). Thus, due to these anatomical differences between fish and mammals, the role of melatonin in circadian rhythms and sleep may not be exactly the same.

In 2001, Zhdanova et al. (2001) established the use of larval zebrafish as a model to study sleep. Using behavioral criteria to define sleep (see above), Zhdanova et al. showed that the locomotor activity of larval zebrafish is significantly reduced at night, and that this reduced activity is associated with reduced responses to stimuli and is under homeostatic regulation, consistent with a sleep state. They also demonstrated that several sedating drugs commonly used in humans induce sleep in larval zebrafish. This included melatonin, whose sleep-inducing effect was blocked by pre-treatment with the melatonin receptor antagonist luzindole, suggesting that the sleep induced by melatonin was not an off-target effect of melatonin or the consequence of a supraphysiological dose, but rather the natural response of endogenously-expressed melatonin receptors (Zhdanova et al., 2001). The observation that melatonin is sedating in zebrafish has been corroborated by several other studies (Appelbaum et al., 2009; Rihel et al., 2010; Gandhi et al., 2015).

More definitive evidence for melatonin's role in circadian rhythms and sleep in zebrafish was provided by a study from Gandhi et al. (2015), which took a genetic approach to abolish melatonin synthesis by introducing a null mutation in *aanat2*, the sole zebrafish *aanat* paralog expressed in the pineal gland. At night, *aanat2* mutant animals slept about half as much as their wild-type control siblings, indicating that endogenous melatonin plays an important role in promoting sleep at night (Gandhi et al., 2015). No behavioral phenotypes were observed during the day, consistent with melatonin's nocturnal production, and the night-time mutant phenotype was rescued by exogenously administered melatonin, suggesting that the mutant phenotypes were indeed due to loss of melatonin and not a pleiotropic effect of the *aanat2* mutation. The *aanat2* mutant effect was phenocopied by a chemogenetic method that specifically ablated melatonin-producing pinealocytes. Notably, the persistence of as few as 10% of pinealocytes was sufficient to maintain normal sleep levels at night, suggesting that pinealectomy studies

that have been widely used in other species would need to remove almost all pinealocytes to observe a melatonin loss-of-function phenotype.

Gandhi et al. also provided compelling evidence that melatonin is not required for a normal circadian rhythm but instead functions as the sleep-promoting output of the circadian clock. They showed this in three key experiments. First, they compared the amount of sleep of *aanat2* mutants to that of their wild-type siblings after being raised in constant darkness, which prevents entrainment of a circadian rhythm. The *aanat2* mutants still slept much less than sibling controls without an entrained rhythm, suggesting that the sleep-promoting role of melatonin is not *via* effects on the circadian clock.

Second, Gandhi et al. showed that circadian rhythms are intact in *aanat2* mutants. Specifically, they measured both behavioral and molecular circadian rhythms in free-running conditions (i.e., animals that were entrained in a light-dark cycle, then moved to constant darkness). In neither case was there a difference in circadian period length, phase, or amplitude between *aanat2* mutants and controls, suggesting that, at least in fish, melatonin is not required for a normal circadian rhythm.

Last, if melatonin acts downstream of the circadian clock to promote sleep, then *aanat2* mutants should have no difference in sleep amount during subjective day versus subjective night in free-running conditions. Gandhi et al. found that this is indeed the case: after entrainment, *aanat2* mutants showed constant levels of sleep across several subjective days and nights in constant darkness, while wild-type control siblings maintained rhythmic sleep, with increased sleep during subjective night. Notably, while *aanat2* mutants have reduced sleep at night compared to wild-type siblings under normal light-dark conditions, they do sleep more at night than during the day. This observation indicates that the direct effects of light and dark on behavior (a phenomenon known as ‘masking’; Hubbard et al., 2013) remain intact in melatonin-deficient zebrafish.

A subsequent study by Livne et al. (2016) tested the importance of the pineal gland in controlling circadian behaviors; in doing so, they provided corroborating insight into the relationship of melatonin, circadian rhythms, and sleep. To disrupt circadian rhythms specifically in the pineal gland, they expressed a dominant-negative Clock variant (ΔCLK) under control of the *aanat2* promoter, which is specific to melatonin-producing pinealocytes. In the pineal glands of these transgenic fish, the rhythmic expression of clock-controlled genes was disrupted, including that of *aanat2*. Importantly, these animals were not melatonin-deficient, but rather maintained steady production of melatonin across the circadian period in free-running conditions. As a result, melatonin was present at atypically high levels during subjective day. Echoing the findings of Gandhi et al. (2015), under free-running conditions, ΔCLK animals maintained wild-type circadian period lengths of locomotor activity, and molecular circadian rhythms were normal in peripheral tissues (Livne et al., 2016). However, in constant darkness or constant dim light, when melatonin synthesis is not inhibited by light, the amplitude of locomotor activity cycles was significantly dampened during subjective day in ΔCLK animals, consistent with higher levels of melatonin during the day in the transgenic fish. Conversely, in constant light, where melatonin synthesis is inhibited, ΔCLK and control animals had similar amplitudes of locomotor activity rhythms. These observations support the notion that melatonin does not normally regulate circadian rhythms, but rather acts downstream of the circadian clock to promote sleep.

1.6 Melatonin studies using avian species

Studies using both EEG/EMG recordings and behavioral measurements found that melatonin treatment induces sleep in several species of diurnal birds, including those belonging to the passerine (Hendel & Turek, 1978; Murakami et al., 2001), columbine (Phillips & Berger, 1992; Mintz et al., 1998; Paredes et al., 2007), and gallinaceous (Hishikawa et al., 1969; Bermudez et al., 1983; Zeman et al., 1993; Murakami et al., 2001) orders. In contrast, melatonin treatment did not induce sleep in nocturnal owls, although it did lower their body temperature (Murakami et al., 2001). This finding suggests that melatonin may lack its sleep-promoting role in nocturnal animals, consistent with similar observations made in nocturnal rodents. It also suggests, in owls at least, that melatonin's effect on sleep and body temperature might be separable; that is, melatonin may not lower body temperature solely by promoting sleep.

To my knowledge, the only loss-of-function experiments, which are essential to evaluate the role of melatonin in regulating sleep, that have been carried out in birds involve pinealectomy (Px); i.e., surgical removal of the pineal gland. Seminal work from the lab of Dr. Michael Menaker illustrated the necessity of the pineal gland for the circadian control of locomotor (Gaston & Menaker, 1968) and body temperature (Binkley et al., 1971) rhythms in sparrows.

Menaker's studies showed that free-running sparrows became arrhythmic in both perching (a proxy for locomotor behavior) and body temperature soon after pinealectomy. In sham-operated controls, the free-running rhythm resumed after surgery. Pinealectomized birds synchronized their activity and temperatures to cycles of light-dark, demonstrating that the masking effect of light on behavior was maintained, but rapidly lost rhythmicity in constant darkness. In a series of impressive follow-up experiments,

Zimmerman and Menaker (1975; 1979) transplanted the pineal glands of normal, entrained sparrows into the anterior chamber of the eye in pinealectomized, arrhythmic birds. They found that the pineal-recipient birds displayed free-running locomotor rhythms after transplantation, and that the phase of the rhythm matched the phase of the donor bird (Zimmerman & Menaker, 1975; Zimmerman & Menaker, 1979). These experiments confirmed a number of characteristics of the avian pineal gland. First, it showed that the circadian rhythm of the pineal gland in birds is self-contained and does not require input from other parts of the brain; although, it is not clear how much contribution the pineal receives from other photoreceptive tissues, such as the eyes and putative SCN, for circadian entrainment (for reviews, see: Gwinner & Brandstätter, 2001; Cassone, 2014). This is in contrast with the mammalian pineal gland, which depends entirely on the SCN for its circadian rhythm (Moore & Klein, 1974; Kalsbeek et al., 2000). Second, it confirmed that the pineal gland conveys circadian information via a diffusible messenger and not by direct innervation.

These studies sought to define the role of the pineal gland in regulating circadian rhythms by using locomotor behavior and body temperature as read-outs of the circadian clock. They demonstrate a crucial role for the pineal gland in regulating these outputs; however, whether the observed effects of Px are due to a loss of circadian rhythm itself, or to a loss of an output of the circadian clock, were not distinguished. The conventional interpretation of these studies is that Px (when coincident with loss of light cues) abolishes the circadian rhythm, and as the major hormonal output of the pineal gland, it is reasonable to assume that melatonin is therefore orchestrating the circadian rhythm. However, a more parsimonious explanation that is consistent with observations based on treatment with melatonin, and with zebrafish genetic loss-of-function studies, is that melatonin is an

output of the circadian clock that regulates sleep and, either directly or indirectly, body temperature.

Another caveat is that Px is a relatively crude and imprecise procedure, and it is impossible to know if the manipulation uniquely affects melatonin production in the animal. First, tissues near the pineal gland may be damaged by the procedure, which could result in confounding behavioral effects. Second, the pineal gland is likely involved in melatonin-independent aspects of animal physiology, and perturbation of these functions of the pineal would obscure interpretation of the effects of Px. For example, in addition to the pinealocytes that produce melatonin, the pineal gland of frogs, fish, and mammals contains neurons that project into the brain (Ekström & Meissl, 1990; Erkström & Meissl, 1997; Korf et al., 1986), which presumably have melatonin-independent functions. Third, it is apparent from zebrafish experiments that chemogenetic ablation of ~90% of *aanat2*-expressing pinealocytes is not sufficient to phenocopy the complete loss of melatonin in *aanat2* mutants. This indicates that a small number of pineal cells is sufficient to maintain normal melatonin-mediated behaviors. Inconsistencies in the observed effects of Px across different species of birds, and among different laboratories (see Rutledge & Angle, 1977; Gwinner, 1978), could be due to incomplete Px.

In summary, while loss-of-function experiments in avian species demonstrate a requirement for the pineal in driving circadian behaviors, the common interpretation from these experiments—that the pineal (via melatonin) controls the circadian clock—has not been directly tested. One issue is the use of certain behaviors as measurements of circadian rhythm. If melatonin acts as a direct output of the circadian clock to promote sleep, then using an assay that measures behaviors related to sleep (e.g., locomotor activity, perching, feeding, or body temperature) is inherently problematic. Thus, rather than assaying outputs of the circadian clock, such as circadian controlled behaviors,

effects of Px on the circadian clock itself should be directly tested. Another significant issue is that the fidelity and completeness of Px can only be estimated visually, and both damage to surrounding tissues and incomplete removal of all pineal cells complicates interpretation of these experiments. These caveats can be avoided by using animals harboring mutations in the genes required for the synthesis of melatonin or in the receptors through which it acts.

1.7 Melatonin studies using laboratory rodents.

Experiments using laboratory rodents have been very illuminating in efforts to understand many aspects of human physiology. Most physiological processes shared among rodents and humans evolved only once, long before the two groups were split by different evolutionary trajectories, and those processes are expected to be regulated by common mechanisms. Thus, among the widely used animal models, studies in rodents are typically considered to be the most immediately applicable to humans. The role of melatonin, however, may be a rare exception.

An important difference between rodents and most other species in which melatonin has been shown to promote sleep is that most rodents—and almost all commonly used laboratory rodent strains—are nocturnal. Moreover, in every species examined, the peak of melatonin synthesis occurs at night, regardless of whether the species shows a diurnal or nocturnal pattern of behavior (Borjigin et al., 2012). Another important difference is that while wild nocturnal rodents synthesize melatonin, most strains of laboratory mice have acquired mutations in *AANAT* or *HIOMT* and therefore do not synthesize melatonin, possibly an outcome of artificial selection against melatonin's effect on breeding seasonality (Kasahara et al., 2010; Roseboom et al., 1998). The fact that these melatonin-deficient mice are still able to sleep and have apparently normal circadian rhythms suggests that melatonin is not essential for these processes in nocturnal rodents.

The peak of melatonin synthesis at night in nocturnal animals is not consistent with a sleep-promoting function for the hormone and has led to the prevailing hypothesis that melatonin promotes night-specific aspects of behavior and physiology in both diurnal and nocturnal rodents. According to this view, one would expect melatonin treatment at night to promote sleep in diurnal animals and wakefulness in nocturnal animals. However, while

one of the first studies that examined the acute pharmacological effects of melatonin treatment found that it promoted sleep in diurnal chicks, no gross behavioral effects were observed in nocturnal mice (Barchas et al., 1967). Since then, reports using exogenous melatonin have largely corroborated the observation that melatonin does not affect sleep in nocturnal rodents (Tobler et al., 1994; Huber et al., 1998; Langebartels et al., 2001; Mailliet et al., 2001), although a few studies described a sleep-promoting effect of melatonin treatment (Holmes & Sugden, 1982; Mirmiran & Pévet, 1986; Wang et al., 2003), and one report observed a wake- or activity-promoting effect (Mendelson et al., 1980). These observations suggest that melatonin does not simply act as a hormone that promotes night-specific behaviors, at least in the context of sleep, but rather acts to promote sleep at night in diurnal but not nocturnal animals. While there have been studies that examine the effect of melatonin on *diurnal* rodents, these studies did not measure sleep directly, but instead focused on circadian rhythms of wheel-running behavior (*Funambulus pennantii* (palm squirrel): Rajaratnam & Redman, 1997; *Articanthis ansorgei*: Slotten et al., 2002; *Octodon degus*: Vivanco et al., 2007; *Funambulus pennantii*: Soni et al., 2020). From the actograms in these studies, it seems that melatonin does not obviously promote sleep in diurnal rodents, but a closer examination is required.

A consistently observed effect of melatonin treatment is its ability to entrain the circadian rhythm of locomotor activity in free-running nocturnal rodents and diurnal birds. When melatonin is delivered at a consistent time of day to free-running animals kept in constant dark or dim light, entrainment occurs such that the start of the behavioral phase normally associated with ‘night’ becomes gradually aligned to the time of melatonin administration. Thus, in diurnal birds, entrainment occurs such that the *inactive* phase follows the time of melatonin administration, and by contrast, nocturnal rodents entrain to melatonin delivery such that the *active* phase follows the time of melatonin administration.

In birds, entrainment by melatonin can be explained by the sleep-promoting effect of melatonin directly driving sleep, and a gradual attunement of the animal's daily behavioral rhythm to consolidate sleep over the 24-hour day. However, since melatonin has only rarely been shown to promote wake behaviors in nocturnal rodents, this effect of scheduled melatonin on rhythmic behavior cannot be interpreted as such in these animals. Evidence suggests that melatonin-induced circadian entrainment in nocturnal rodents is likely due to melatonin binding its receptors in the SCN. Indeed, melatonin receptors are expressed in the SCN and are required for an inhibitory effect of melatonin on the firing of SCN neurons (Liu et al., 1997), and the ability of melatonin to entrain behavioral rhythms is abrogated in mice with lesioned SCNs (Cassone et al., 1986). However, experiments demonstrate that neither melatonin nor the pineal gland are required for circadian rhythms in nocturnal rodents (Cassone, 1990), so whether this effect of melatonin on circadian entrainment represents a *normal* physiological function of melatonin is unclear.

Melatonin loss-of-function studies using rodents can be broadly categorized into three groups: (1) effects of pinealectomy, (2) comparisons of strains that are melatonin-proficient or -deficient, and (3) effects of mutation or pharmacological inhibition of melatonin receptors.

Pinealectomy. For reasons described above, the use of pinealectomized animals to determine the role of melatonin is far from ideal. With this in mind, pinealectomy studies in rodents have not consistently demonstrated a role for melatonin in affecting sleep (much like the gain-of-function studies). Among studies that measured baseline sleep in pinealectomized nocturnal rats and sham-operated control rats (which are melatonin-proficient), Rechtschaffen et al. (1969) reported no difference in time asleep or time in REM sleep, and Fisher and Sugden (2010) similarly concluded that pineal-derived melatonin had no effect on total amount or diurnal distribution of locomotor activity, wake,

NREM sleep, or REM sleep (Rechtschaffen et al., 1969; Fisher & Sugden, 2010). Mendelson and Bergmann (2001) report a slight (2.5%) increase in NREM in pinealectomized rats compared to sham controls, consistent with the same author's gain-of-function report that melatonin promotes wakefulness (Mendelson et al., 1980; Mendelson & Bergmann, 2001), but in contrast with the other pinealectomy studies. Together, these reports support the notion that melatonin does not regulate sleep or circadian rhythms in nocturnal rodents.

Melatonin-proficient vs. -deficient strains. Using laboratory rodents to study melatonin can be problematic due to the loss of endogenously produced melatonin in many common strains. For example, C57BL/6J mice harbor mutations in both *aanat* and *hiomt* genes and produce no detectable melatonin (Goto et al., 1989; Rosebloom, 1998; Kasahara et al., 2010). As a result, these strains cannot be used to assay for loss of melatonin phenotypes since there are no wild-type controls for comparison. Some lab strains, such as C3H and MSM/Ms, retain the ability to synthesize melatonin (Goto et al., 1989; Kasahara et al., 2010), but loss of melatonin synthesis using genetics has not been described for these strains. Studies that compare melatonin-proficient to melatonin-deficient strains of mice are problematic for determining the functions of melatonin since different strains have many genetic differences, resulting in many differences in physiology and behavior (Kopp et al., 1998; Huber et al., 2000). For example, a close comparison via automated video analysis of C57BL/6 and C3H mice over several behaviors related to activity, rest, exploration, and diet revealed significant differences between the two strains, including, notably, rest-related behaviors (Adamah-Biassi et al., 2013). Importantly, melatonin treatment did not affect the behavior of C57BL/6 mice compared to untreated controls (Adamah-Biassi et al., 2013); thus, the melatonin deficiency of C57BL/6 mice cannot explain these differences in behaviors.

In a recent study, Zhang et al. (2021) restored melatonin synthesis to melatonin-deficient C57BL/6J mice in order to compare melatonin-proficient and -deficient animals with similar genetic backgrounds. They did this by crossing C57BL/6J to MSM/Ms, and then back-crossing the progeny to C57BL/6J for at least 10 generations while selecting for MSM/Ms-derived wild-type *aanat* and *hiomt* genes. Melatonin-proficient (*aanat+/+; hiomt+/+)* and -deficient (*aanat-/-; hiomt-/-)* siblings were then compared to test whether melatonin is required for a variety of behaviors. While sleep was not quantified, data from wheel-running assays and quantification of home cage behaviors suggest that melatonin is not necessary for sleep or circadian regulation of locomotor activity in these nocturnal mice (Zhang et al., 2021). In addition, there was no difference among genotypes in re-entrainment to a complete light schedule reversal. However, in response to a 6-hour advance in the light schedule, melatonin-proficient mice entrained *faster*, by about 2 days, than melatonin-deficient mice. The ability of melatonin to play a role in circadian entrainment is thought to be mediated by its actions on the SCN (Cassone et al., 1986). If melatonin facilitates re-entrainment by acting on the circadian clock in the SCN, then PER1 levels in the SCN should differ among melatonin-proficient and -deficient animals at a given time point until entrainment is complete. However, at day 4 after the light shift, when neither genotype had fully entrained to the new schedule, there was no difference in levels of PER1 protein in the SCN at the same time of day (ZT10), pre- versus post-light shift (Zhang et al., 2021). Admittedly, this assay may not be sensitive enough to detect small adjustments in PER1, but assays such as these, which measure the circadian rhythm by molecular markers and not solely by overt behavior, are important for accurately assessing the role of melatonin in circadian behaviors.

Thus, in studies that attempt to restore melatonin to melatonin-deficient strains of nocturnal mice, melatonin seems to be largely dispensable for regulating sleep or

circadian rhythms. In Zhang et al. (2021), melatonin-proficient nocturnal mice entrained faster to a 6-hour shift in the light schedule, which may demonstrate some role for melatonin in facilitating circadian re-entrainment. However, that the melatonin-proficient mice entrained to a complete reversal in light schedule at the same pace as melatonin-deficient mice raises some confusion about this particular function of melatonin. A role for melatonin in rodent sleep should be clarified using similar approaches (i.e., comparing melatonin-deficient and -proficient siblings in the same genetic background) with direct measurements of sleep using EEG and EMG recordings in both nocturnal and diurnal rodents.

Mutation and pharmacological inhibition of MT receptors. In mammals, melatonin is thought to act via two G-protein coupled receptors (GPCRs), MT1 and MT2, that bind melatonin with high affinity *in vitro*. This notion is supported by knockout (KO) studies that have demonstrated that these receptors are necessary for the effects of melatonin treatment on the firing of cultured SCN neurons (Liu et al., 1997). *In vivo*, treatment with melatonin receptor antagonists or MT1 receptor KO each blocks the ability of melatonin to phase shift behavioral rhythms in mice (Dubocovich et al., 1998; Dubocovich et al., 2005).

To test the role of melatonin receptors in behavior, the laboratories of Steven Reppert and David Weaver generated MT1 and MT2 KO mice (Liu et al., 1997; Jin et al., 2003). The original mouse strains used to generate the MT1 and MT2 KO mutant lines were two melatonin-deficient strains: a 129/Sv/C57BL/6 hybrid strain and 129/Sv, respectively. Both mutant lines were subsequently bred to melatonin-proficient C3H/He strains for multiple generations.

In the inaugural publications for these two lines, the authors reported no circadian defects in either single mutant line based on wheel-running behavior: amplitude, phase,

and period length of free-running mutant mice were no different from their wild-type siblings (Liu et al., 1997; Jin et al, 2003). Moreover, Jin et al. (2003) reported that they were unable to entrain even wild-type mice to a timed melatonin injection, as had been reported by others (Dubocovich et al., 1998), and thus could not test the dependency of this effect on MT1 or MT2 *in vivo*. The authors instead turned to an *in vitro* assay, in which they examined the effect of melatonin treatment on the electrical activity of cultured SCN neurons collected from WT and KO mice. The authors described two effects of melatonin treatment in their *in vitro* assay. First, melatonin acutely inhibited SCN firing, and this effect was completely blocked in MT1, but not MT2 KO cells (Liu et al., 1997; Jin et al., 2003). Second, in their report describing the MT1 KO line (Liu et al., 1997), melatonin treatment phase shifted the circadian rhythm of SCN firing, which was attenuated, but not abolished, in MT1 KO cells. Treatment with pertussis toxin completely blocked this effect (Liu et al., 1997), suggesting the role of another GPCR, possibly MT2 receptors, which had already been implicated using receptor-selective small molecule antagonists (Dubocovich et al., 1998). However, in their later report using the MT2 KO line (Jin et al., 2003), the authors were unable to reliably record electrical activity of even their wild-type SCN preps, and therefore could not test whether the phase shifting effect of MT was dependent on MT2 receptors.

A limitation of these initial studies is that sleep was not quantified in MT1 or MT2 KO mice. However, sleep studies were subsequently performed by several labs (reviewed in Gobbi & Comai, 2019). One study observed normal amounts of NREM and REM sleep in MT1/MT2 double KO mice (Comai et al., 2013), indicating that melatonin receptors are not required for normal sleep in these melatonin-proficient nocturnal mice. This lack of a sleep phenotype is consistent with Px loss-of-function studies, but it is confounded by the surprising phenotypes observed in MT1 and MT2 single KO mice. Loss of MT1 caused a

significant decrease in REM sleep, while loss of MT2 caused a significant decrease in NREM sleep, leading the authors to suggest that MT1 signaling promotes REM sleep and MT2 promotes NREM sleep. The latter conclusion is supported by experiments using MT2-specific agonists, which enhanced NREM sleep only (Ochoa-Sanchez et al., 2011). MT1-specific agonists have not been developed, so it has not been possible to corroborate the MT1 KO phenotype using pharmacology. Based on these observations, it was hypothesized that each receptor type affects a particular sleep stage and perhaps has opposing effects on the other stage. In this model, simultaneous activation or inhibition of both receptors (e.g., by melatonin or non-selective agonists) would not yield a sleep phenotype. However, this model also predicts that loss of either receptor would lead to an increase in the sleep state that that receptor opposes, which has not been shown to be the case. MT2 KO mice did not show increased REM sleep, and MT1 KO mice only showed increased NREM sleep when sleep was scored in 10-second bins but not in 4-second bins (Ochoa-Sanchez et al., 2011; Comai et al., 2013). Furthermore, an MT2-selective agonist did not inhibit REM sleep (Ochoa-Sanchez et al., 2011). The role of each receptor, and their dependence on melatonin, could be clarified by evaluating the effect of melatonin treatment on REM and NREM sleep in each single receptor KO line, but these experiments have not been reported.

A major caveat in interpreting these findings is that the effects observed were primarily restricted to the light phase, the main sleep phase of these nocturnal animals, when melatonin levels are at their minimum. As a result, the relevance of the MT1/MT2 KO experiments to a role for MT in sleep is unclear. It is possible, at least in mouse, that these receptors act constitutively and are either unresponsive to or inhibited by melatonin. Constitutive activity has been inferred from experiments using MT1-specific antagonists in rat artery (Ersahin et al., 2002), and for both MT1 and MT2 receptors transfected into

Chinese hamster ovary and Neuro2A cells (Devavry et al., 2012). However, this interpretation does not reconcile with the MT1 KO experiments using cultured SCN cells described above, since the phase and amplitude of SCN firing were comparable in wild type and MT1 KO mice in the absence of MT (Liu et al., 1997).

In summary, the role of melatonin in sleep in nocturnal rodents remains unclear. Administration of exogenous melatonin is often reported to have no effect, but some studies have found that it can promote sleep. Many commonly used strains of laboratory rodents do not synthesize melatonin, yet they have apparently normal circadian rhythms and sleep; however, it is impossible to accurately judge the role of MT in these strains since MT-proficient controls in the appropriate genetic background do not exist. In nocturnal rodents that *do* synthesize melatonin naturally, melatonin levels peak at night, which is their active phase. Thus, if melatonin does play a role in sleep in these animals, it is either very minor or complex. The lack of an apparent sleep phenotype in nocturnal rodents (when compared to the very strong effects of melatonin administration or loss of melatonin in birds and fish) has led to the alternate hypothesis that melatonin promotes night-specific behaviors. In this model, melatonin is expected to promote wake in nocturnal rodents. However, administration of melatonin has only been reported to promote wakefulness in a single study, and loss of the pineal has been reported to increase NREM in only one study, as well (from the same laboratory). Much work is still needed to parse out the potentially complicated functions of melatonin in nocturnal rodents' circadian behaviors.

1.8 Human melatonin studies

There is abundant data from numerous human sleep studies since the 1970s, as well as modern-day meta-analyses, which show that exogenous melatonin is sedating in humans (Antón-Tay et al., 1971; Cramer et al., 1974; Waldhauser et al., 1990; Brzezinski et al., 2005, Moon et al., 2022). However, there have also been reports of melatonin *not* improving sleep in humans, and a debate surrounding these discrepant reports has ensued (van den Heuvel et al., 2005). Arguments in support of a role for melatonin in promoting sleep in humans point out that the effects of melatonin are subtle—particularly when compared to other well-known sedatives—and that its efficacy in inducing sleep is likely sensitive to the varying methodologies and preparations employed across studies from different laboratories (Zhdanova, 2005). Opposing arguments favor a model where melatonin's sleep-promoting role—if any—occurs via its actions on the circadian clock (Arendt, 2019; Foster, 2021). This stance is supported by the observed effects of melatonin treatment on the SCN in nocturnal rodents, as well as some human studies that show that exogenous melatonin can be useful for circadian rhythm entrainment and for jet lag recovery (Arendt, 2019).

There is limited loss-of-function data to test the role of endogenous melatonin in humans, and the few published studies are typically comprised of small sample sizes, are retrospective (and therefore correlative), and the 'loss-of-function' manipulations are not specific to melatonin—that is, they involve surgeries or injuries that likely affect other circuits or physiological processes, making it difficult to attribute any observable differences to a loss of melatonin. In this section, I describe three notable loss-of-function studies that examine sleep behavior in patients with disrupted melatonin signaling.

A report by Slawik et al. (2016) is, to my knowledge, the only *prospective* study that examines the role of melatonin in human sleep. The authors measured melatonin levels and several subjective sleep parameters in patients before and after pinealectomy, which was performed due to the presence of a pinealoma. They also compared this data to a retrospective cohort of pinealectomized patients. They found that, despite a depletion of salivary melatonin following pinealectomy, there were no statistically significant changes in any sleep parameter before versus after pinealectomy (Slawik et al., 2016). These data suggest that pineal-derived melatonin, and the pineal itself, are dispensable for sleep. In fact, loss of the pineal seemed to improve subjective sleep quality, although still not significantly. This improvement is difficult to interpret since on average the cohort scored above threshold for 'impaired sleep quality' pre-operatively, perhaps due to effects of the pinealoma itself, and remained slightly above that threshold post-operatively. The study itself mentions this limitation: sleep quality in these patients could not be measured prior to tumor growth. The effect of personal concerns about the tumor or upcoming surgery also was not controlled and may have contributed to the poor pre-operative sleep quality.

When comparing retrospective pinealectomized patients against pre-operative prospective patients, the pinealectomized group had a marked (but not significant) increase in time spent awake after sleep onset and in frequency of night awakenings, consistent with a role of pineal-derived melatonin in maintaining the sleep state (Slawik et al., 2016). However, the validity of this comparison is questionable due to a low sample size. Finally, in none of the patients were daily activity-sleep rhythms shifted or irregular after pinealectomy compared to before. While these measurements were qualitative only and subject to the aforementioned caveats, this finding suggests that pineal-derived melatonin is not required for normal circadian rhythms in humans.

In mammals, melatonin synthesis in the pineal is regulated by the SCN via a multi-synaptic pathway that includes the superior cervical ganglion (SCG). As a result, melatonin secretion is absent in individuals with compete cervical spinal cord injury (SCI) (Zeitzer et al., 2000). In a study by Scheer et al. (2006), melatonin rhythms and sleep patterns were measured in three patients with complete cervical SCI. Two patients with complete thoracic SCI, who have normal melatonin rhythms, were studied as a control group. The melatonin-proficient thoracic SCI group was also compared to melatonin-proficient uninjured controls to gauge the effect of SCI alone. They found that melatonin-deficient individuals had significantly decreased total sleep duration and sleep efficiency compared to both control groups, suggesting a role for melatonin in promoting human sleep (Scheer et al., 2006). Interestingly, both in this study and in Slawik et al. (2016), no difference in sleep onset latency was reported, which may indicate that the timing of sleep in humans is not solely regulated by melatonin—indeed, given the importance of sleep, one would expect it to be regulated by multiple redundant pathways—and that melatonin's function in humans might be more important for the maintenance of sleep.

While the loss-of-function observations reported by Scheer et al. (2006) suggest a role for melatonin in human sleep that is consistent human gain-of-function studies and with studies using other diurnal models, they are nevertheless correlative, as this study was retrospective. While the study does include a thoracic SCI cohort, whose melatonin production remains intact and whose sleep patterns do not differ from uninjured controls, it is still impossible to say that the lack of melatonin itself is the cause of the sleep defects in the cervical SCI cohort. Instead, the differences in sleep could be due to another factor that is similarly affected by cervical, but not thoracic, SCI. One way to help show that loss of melatonin was responsible for the sleep defects would be to show 'rescue' of normal sleep patterns by administering melatonin to these patients. Indeed, the authors

suggested that melatonin might be an effective treatment for insomnia in cervical SCI patients, but this was not tested.

Neurons of the SCG project not only to the pineal gland but also to the heart, and in humans with heart disease, sleep-wake cycles are often also perturbed (Thosar et al., 2018) and melatonin secretion is impaired (Brugger et al., 1995). A report by Ziegler et al. (2023) identifies a link between heart disease and loss of melatonin in both humans with heart disease and in a mouse model for heart disease. In pineal glands harvested post-mortem from humans with heart disease, as well as from mice subjected to transverse aortic constriction (TAC), innervation of the pineal gland by sympathetic neuronal axons was significantly reduced relative to heart-healthy or sham-operated controls (Ziegler et al., 2023). The authors trace the pineal denervation to the SCG, which, in both heart disease patients and TAC mice, is hypertrophied and fibrotic, with a loss of presumptively pineal-projecting neurons. The loss of pineal-projecting neurons is likely macrophage-induced, suggesting that inflammatory responses in the SCG in patients with heart disease underlie the loss of pineal innervation and loss of melatonin release.

In the mouse model, TAC resulted in a loss of melatonin and in the diurnal rhythm of oxygen consumption, energy expenditure, and locomotor activity. This is noteworthy, as it demonstrates a role for the pineal in regulating rhythmic behaviors in a nocturnal rodent, which has not been observed in pinealectomy studies (Rechtschaffen et al., 1969; Fisher & Sugden, 2010); however, it may be explained by additional effects of TAC that are not necessarily pineal- or melatonin-related. The authors also report a dampening of day-night rhythms in oxygen consumption and locomotor activity after surgical removal of the SCG (SCGx). In SCGx mice, a single dose of peritoneally delivered melatonin at night restores activity rhythms over the subsequent 24-hr period, suggesting that the loss of melatonin in SCGx mice is the cause of the reduced rhythm. The authors report activity

as simply the ratio of locomotor activity during the light versus dark phase, in lieu of a typical actogram, and as a result, it is unclear what specific effect melatonin had on activity rhythms. Additionally, neither a vehicle nor handling control was reported, which leaves open the possibility that the restoration of activity rhythms by melatonin in SCGx mice was not an effect of melatonin, specifically. If the changes in activity rhythms in the TAC or SCGx mice are indeed due to a loss of melatonin, then this model may offer a useful new avenue to explore the role of endogenous melatonin in regulating circadian rhythms and sleep in a laboratory rodent.

In summary, studies that examine the effects of exogenous melatonin on human sleep are abundant and clearly demonstrate a sedating effect of melatonin. Melatonin's ability to promote sleep in humans is less robust than that of commonly prescribed hypnotics, such as diazepam (Valium) and zolpidem (Ambien). The relatively subtle effect of melatonin is expected, as melatonin is a naturally-produced sleep hormone, and, given the vulnerability of an animal during sleep, a potently sedating endogenous somnogen would likely be maladaptive. Meanwhile, loss-of-function studies that test the role of melatonin in human sleep are scarce and difficult to interpret. Sample sizes are typically small, and the loss-of-function manipulation in each case is accompanied by extraneous perturbations that likely confound interpretation of the data. Thus, human studies tend to be less informative than those using animal models for understanding melatonin's role in sleep. Nevertheless, both the gain- and loss-of-function reports are generally supportive of a role for melatonin in human sleep.

1.9 Mechanisms by which melatonin promotes sleep

Since the role of melatonin in sleep has remained controversial, especially in mammalian systems, it is not surprising that the mechanism by which melatonin promotes sleep is poorly understood. While two groups of receptors that bind melatonin with high affinity have been identified, and the expression patterns of these receptors have been gradually revealed via different labeling techniques, the specific receptor subtypes that mediate melatonin's sleep-promoting function, as well as their specific site of action in the brain, remain unclear.

Melatonin's earliest known function was its ability to aggregate melanin in the skin of tadpoles, and thus, the first melatonin receptor was cloned using a cDNA library from an immortalized *Xenopus* dermal melanophore cell line (Ebisawa et al 1994). After the sequence of this receptor was revealed, degenerate primers were used to identify a putative melatonin receptor in mammals. Over the next few years, three groups of melatonin-binding receptors were discovered across various species, initially called *Mel1a, Mel1b,* and *Mel1c*. Today, in mammals, *Mel1a* and *Mel1b* are referred to as MT1 and MT2, while the mammalian homolog of *Mel1c* appears to not bind melatonin and is known as GPR50. Nearly all efforts to elucidate the melatonin receptors involved in transducing melatonin's effects on sleep have been carried out in mammals; accordingly, I will refer to the two mammalian receptors as MT1 and MT2 in this section.

The MT1 and MT2 receptors represent their own group within the GPCR superfamily, with similar structural motifs that make them unique from other GPCR families (Dubocovich et al., 2010). Both receptor types demonstrate a high affinity for melatonin—in the picomolar range—and exhibit similar but slightly different rankings in affinities for related molecules (Dubocovich et al., 2010). Both receptors inhibit cAMP formation via

inhibition of adenylate cyclase in a pertussis toxin (PTX)-sensitive fashion, suggesting that MT1 and MT2 couple to the G_i/G_o family of G proteins. This intracellular response to MT1 and MT2 activation was the first reported and is perhaps the most well-known (Reppert et al., 1994), but further studies have implicated numerous other signaling pathways downstream of MT1 or MT2 activation. In different cell types, activation of MT1 has been shown to increase intracellular calcium by PTX-insensitive G proteins (Brydon et al., 1999) and inhibit calcium influx via PTX-sensitive G proteins (Slanar et al., 2000). MT1 activation has also been shown to both increase (Nelson & Quayle, 1995; Geary et al., 1998; Masana et al., 2002) and decrease (Steffens et al., 2003) BK_{Ca} channel activity, as well as activate inwardly rectifying potassium channels (Jiang et al., 1995). MT2 receptor activation has been reported to inhibit cyclic GMP formation (Petit et al., 1999) and increase protein kinase C activity (Hunt et al., 2001). The varied downstream effectors of MT1 and MT2 activation are possibly cell-type specific, and, except for the putative *C. elegans* melatonin receptor PCDR-1, which was shown to promote quiescence through the BK channel SLO-1 (Niu et al., 2020), it remains unclear which intracellular pathways are important for melatonin's sleep-promoting function.

Before the sub-cellular mechanisms by which melatonin promotes sleep can be elucidated, a role for either or both melatonin receptors in sleep should first be established. An overview of the loss-of-function experiments that seek to identify the sleep-relevant melatonin receptor(s) using nocturnal rodents is provided in section 1.7. The consensus from these reports suggests that MT1 promotes REM sleep while MT2 promotes NREM sleep; however, due to a number of caveats (also described in section 1.7) and lack of corroborating reports, the roles of MT1 and MT2 in regulating sleep are not yet clear.

This model is supported by drug experiments using two MT2-selective agonists, IIK7 and UCM765. In one study, IIK7 delivered by intraperitoneal injection to rats during

the dark phase caused a decreased latency to NREM onset and increased time spent in NREM sleep over a 1-hour window compared to vehicle-injected controls (Fisher & Sugden, 2009). In contrast, IIK7 had no significant effect on REM sleep. Because IIK7 affected sleep in a very similar manner as the non-selective melatonin receptor agonist ramelteon (Fisher et al., 2008), the authors concluded that MT2 likely mediates the hypnotic effect of ramelteon. The significance of this conclusion in unclear, as the natural ligand for this receptor, melatonin, does not reliably promote sleep in this species. The MT2-selective agonist UCM765 was reported to have effects similar to those of IIK7 on NREM sleep in rats and mice (Ochoa-Sanchez et al., 2011). These effects were abrogated in MT2 KO mice (but not in MT1 KO mice) and by pre-treatment with the MT2-selective antagonist, 4-P-PDOT, which confirmed the specificity of the drug. MT1-selective agonists have not been described, so whether or not specific activation of MT1 promotes REM sleep has yet to be tested.

Identifying the neuronal populations that express melatonin receptors—and are therefore expected to be directly modulated by melatonin—is an important first step in determining the neuronal circuits through which melatonin may affect sleep. Early attempts to identify the expression pattern of MT receptors used autoradiography to detect sites of radio-labeled melatonin binding; this technique is highly sensitive but cannot discriminate between MT1 and MT2. In these early reports, strong melatonin binding was observed in the SCN and the pars tuberalis of the pituitary gland (Reppert et al., 1994). Once the sequences of the receptors were identified, *in situ* hybridization- and antibody-based methods revealed a much more expansive expression pattern (Reppert et al., 1995; Lacoste et al., 2015). In a recent review that summarizes work by multiple labs, MT1 and MT2 receptors were found to be widely distributed throughout the brain (Ng et al., 2017). The general distribution of melatonin receptors in the brain, summarized by Ng et al.

(2017) using data from humans, rats, and mice, is reprinted in Table 1.1. More specific regions that were found to have the highest expression in the rat brain are listed in Table 1.2 (from Lacoste et al., 2015). While melatonin may be acting through any of these brain regions to promote sleep, here I discuss areas that may be particularly relevant to sleep.

Retina. While the precise functions of melatonin in the retina are unclear, its role as a modulator of retinal physiology is relatively well-studied. Melatonin synthesis enzymes are exclusively expressed in the photoreceptor layer, but melatonin receptors are expressed in the photoreceptor, inner nuclear, and retinal ganglion cell layers, in a pattern of distribution that is reported to vary in different species (Wiechmann & Sherry, 2013). Melatonin synthesis in the retina is under circadian control and is thought to play a local role in retinal physiology (Wiechmann & Sherry, 2013). It is unclear whether retina-derived melatonin plays a role in non-ocular physiology, as high acetylase activity within the eye is suggested to rapidly metabolize melatonin, possibly preventing its diffusion beyond the retina (Grace et al., 1991). Retinal melatonin may play a role in the daily shedding of photoreceptor outer segments and in the dark adaption of the retina, primarily by opposing dopamine signaling (Wiechmann & Sherry, 2013). To my knowledge, a relationship between melatonin's direct actions on eye physiology and its ability to promote sleep—if such a connection exists—has not been described.

Superior and inferior colliculi. Neither the superior nor inferior colliculi are reported to play a role in sleep regulation (outside of masking, see below); however, I mention it here for a handful of reasons. First, in Chapter 2, I find that melatonin receptors in the zebrafish larvae are relatively highly expressed in the optic tectum, a structure homologous to the superior colliculus (SC) of mammals.

Second, the SC receives direct input from retinal ganglion cells, which suggests a trend of melatonin receptor expression in vision-related brain structures. The SC plays a

role in oculomotor control and in processing of visual information as well as other sensory modalities. Meanwhile, the inferior colliculus receives inputs from the auditory cortex and from somatosensory nuclei. In the zebrafish, the optic tectum has been shown to process visual, auditory, and water flow sensory information (Thompson et al., 2016). A well-known feature of sleep is the attenuation of sensory input; that melatonin receptors are expressed in brain regions dedicated to such tasks perhaps suggests a specific role for melatonin in dampening sensory responses to stimuli.

Third, a role for the SC in masking (i.e., the direct effect of light on sleep and wake) has been reported (Miller et al., 1998; Zhang et al., 2019). In mice exposed to three hours of darkness during the light phase, sleep is suppressed and wakefulness is induced. Conversely, when exposed to three hours of light during the dark phase, sleep is induced and wakefulness is suppressed. In mice with caspase-mediated ablation of GABAergic SC neurons (or their synaptic partners in the ventral tegmental area), the wakefulness induced by dark exposure is completely absent (Zhang et al., 2019). Sleep induced by light during the dark phase was intact, suggesting that this form of masking is either mediated by a different brain region or by a different subpopulation of SC neurons. An earlier report shows defective light induction of NREM in mice whose SC had been completely removed, which suggests that the SC still mediates this type of masking (Miller et al., 1998).

Reticular thalamic nucleus. MT2 receptors are highly expressed in the reticular thalamic nucleus (Rt), whereas MT1 receptors are absent in this region (Ochoa-Sanchez et al., 2011; Lacoste et al., 2015). The Rt is suggested to regulate the “flow” of information between thalamus and cortex depending on attentional state and, relatedly, plays a role in generating the EEG rhythms observed during NREM sleep (McAlonan & Brown, 2002; Vantomme et al., 2019). In the same study that reported a role for MT2 receptors in

regulating NREM sleep using the MT2-selective agonist UCM765, the authors determined that the site-of-action of the agonist were MT2 receptors in the Rt (Ochoa-Sanchez et al., 2011). To show site-of-action, they recorded electrical activity from Rt neurons while systemically administering UCM765, with and without focal delivery of an MT2 antagonist to the Rt. They saw that antagonist delivered to Rt suppressed UCM765-induced firing of Rt neurons.

Hypothalamus. The mammalian hypothalamus, which includes the SCN, is perhaps the most well-studied domain of melatonin receptor expression. *In vitro* studies have demonstrated a clear role for melatonin in suppressing SCN firing, and this effect is abolished in MT1 receptor mutants. The ability of melatonin to phase shift circadian rhythms *in vivo* and *in vitro* is also dependent on the MT1 (and possibly MT2) receptor and the SCN. These findings have led to the speculation that melatonin's sleep-promoting role is *through* its actions on the circadian rhythm by modulating SCN firing. However, data from zebrafish show that melatonin acts downstream of the clock, and in every animal model tested, loss of the pineal, melatonin, or melatonin receptors has either failed to show a circadian phenotype or was not rigorously tested. Thus, melatonin's actions on the SCN may play a minor feedback role in regulating circadian rhythm (indeed, by suppressing SCN firing it would 'reinforce' the lower firing rate of SCN neurons at night), but there is currently inadequate evidence that melatonin promotes sleep through the SCN.

Besides the SCN, melatonin receptors (primarily MT2) were also detected in the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) of the hypothalamus (Lacoste et al., 2015). A role for the PVN in sleep-wake regulation has been described (Ono et al., 2020; Chen at al., 2021); it also acts downstream of the SCN to regulate pineal

melatonin production. Whether or not melatonin influences sleep via receptors in this region of hypothalamus remains to be determined.

Brain region	Type of melatonin receptor	Species
Retina	MT1, MT2	Rat, guinea pig, human
Cerebral cortex	MT1, MT2	Mouse, rat, human
Reticular thalamic nucleus	MT2	Rat
Habenula	MT1	Mouse, rat
Hypothalamus	MT1, MT2	Rat, human
Pituitary gland	MT1	Rat, human
Periaqueductal grey	MT1, MT2	Mouse, rat
Dorsal raphe nucleus	MT1	Rat
Midbrain	MT1, MT2	Rat, human
Cerebellum	MT1, MT2	Mouse, rat, human

Table 1.1. Brain regions where melatonin receptors are reported to be expressed. Table reproduced from Ng et al., 2017, who compiled the data from multiple references.

Selected brain regions (with highest MT1 or MT2 expression reported by Lacoste et al., 2015)	**MT1 density**	**MT2 density**
Cerebral cortex: retrosplenial	+ + +	+
Basal forebrain: Islands of Calleja	+ + +	+
Hippocampus: CA3	+ (+)	+ + +
Epithalamus: medial habenula	+ + +	-
Thalamus: reticular nucleus	-	+ + +
Hypothalamus: supraoptic nucleus	-	+ + +
Hypothalamus: suprachiasmatic nucleus	+ + (+)	-
Dorsal mesencephalon: superior colliculi	+ + +	+
Dorsal mesencephalon: inferior colliculi	+ + (+)	+ + (+)
Ventral mesencephalon: substantia nigra *pars compacta*	+ + (+)	+
Ventral mesencephalon: substantia nigra *pars reticulata*	+	+ + (+)
Pituitary gland: pars tuberalis	+ + +	-

Table 1.2. Selected brain regions where melatonin receptors were found to be highly expressed in the rat. Table adapted from Lacoste et al., 2015; specifically, only the brain regions where expression of MT1 and/or MT2 were reported to be highest are included. Authors performed immunostaining of adult rat brains with antibodies specific for the MT1 or MT2 receptor. For each brain region, the authors assessed the level of receptor density accordingly: -, no immunostaining; +, weak; ++, moderate; or +++, strong. Intermediate levels indicated with a parenthesized (+).

1.10 Conclusions and future directions

Melatonin's first reported effect in humans is also its most controversial. In this review, I have outlined the experimental evidence across a variety of model systems that test the relationship between melatonin and sleep regulation, with a primary focus on loss-of-function experiments where available. I broadly summarize the findings as follows:

1. **Exogenous melatonin is sedating in many diurnal species, including zebrafish, birds, and humans.** Melatonin seems to have no sedating effect on nocturnal animals, including rodents and owls. Actograms from melatonin-treated *diurnal* rodents suggest that melatonin does not affect sleep in these animals; however, direct measurements of sleep have not been reported and are necessary to determine whether melatonin's role in sleep is taxa-specific or diurnal-specific.
2. The sedative effects of melatonin in humans are not as potent as those of commonly-prescribed benzodiazepine (e.g., Valium) and non-benzodiazepine (e.g., Ambien) hypnotics, as expected for an endogenous somnogen.
3. **Endogenous melatonin is required for sleep in zebrafish and likely in birds.** Genetic loss-of-function studies support a role for endogenous melatonin in promoting sleep in zebrafish and possibly nematodes. Zebrafish studies showed that melatonin is specifically required for the circadian regulation of sleep. Pinealectomy (Px) studies may support a role for endogenous melatonin in promoting sleep in birds, as well, but published experiments do not distinguish between Px effects on sleep versus circadian rhythm. Loss-of-function data from human studies is consistent with a role for melatonin in sleep, but conclusive evidence is lacking due to small sample sizes and confounding factors associated with each loss-of-function condition.

4. **Exogenous melatonin *can* entrain and phase shift circadian rhythms in birds, rodents, and humans**, and may function through melatonin receptors in the SCN, which in turn regulates melatonin synthesis in the pineal gland. However, **in zebrafish, rodents, and humans, there is virtually no loss-of-function evidence to support a role for endogenous melatonin in regulating circadian rhythms**. Studies in birds have been interpreted as showing a requirement for melatonin for normal circadian rhythms, but experiments that (a) distinguish between effects on sleep versus circadian rhythm and (b) examine the specific loss of melatonin rather than the pineal gland are still needed.

Taken together, published studies suggest that melatonin acts downstream of the circadian clock to promote sleep in diurnal animals.

The role of melatonin in sleep seems to become less apparent (and/or more nuanced) in 'higher' organisms. This is evident when comparing the relatively strong effects of gain- or loss-of-function manipulations in zebrafish or birds with the relatively weak or non-existent effects in rodents or humans. What anatomical or physiological differences between these groups might explain the differences in melatonin's effects? Perhaps the simplest dividing factor is the complexity of their nervous systems. In an animal with a more complex nervous system, the requirement for sleep may be greater, and accordingly, the regulation of sleep might be under control of multiple redundantly acting mechanisms. In this view, melatonin's role in sleep may be more 'important' in birds and fish and less so in humans or diurnal rodents, where other sleep-regulating pathways or circuits are present.

A second (non-mutually exclusive) possibility is that both the human and rodent lineages likely share a common nocturnal ancestor. The 'nocturnal bottleneck' hypothesis

suggests that the mammalian ancestor occupied nocturnal niches to avoid predation by diurnal dinosaurs, and not until after the extinction of dinosaurs were diurnal mammals able to flourish (Gerkema et al., 2013). Many aspects of physiology in modern mammals—even diurnal ones—are reflective of this evolutionary period. One example may be a decreased dependence on melatonin for regulating sleep, as nocturnally secreted melatonin would be maladaptive if it promoted sleep in a nocturnal animal.

Third, as noted above, both zebrafish and birds have pineal glands that are positioned near the surface of their heads and are directly exposed to and entrained by light. In species whose circadian rhythms are so readily and directly attuned to environmental lighting, perhaps the melatonin signal is more reliable and therefore more potent, as there was no need to develop redundant or compensatory sleep-regulating circuits. Conversely, in animals whose pineal glands are buried deep within the brain (and thus not exposed to light), circadian information is received indirectly via a multi-synaptic pathway. This 'outsourcing' of circadian information might have led to a less reliable melatonin signal and a subsequent development of alternative, redundant mechanisms of circadian regulation of sleep.

Understanding the role of melatonin in sleep is important for evaluating and optimizing its use as a therapeutic agent. Since melatonin is a naturally-occurring sleep hormone in humans, its value in remedying sleep disorders is potentially very high. The most well-known pharmaceutical options for treating sleep disorders act by enhancing inhibitory GABAergic signaling in the brain, and their effects, while potently sedating, do not fully recapitulate natural sleep. Designing therapies around melatonin signaling, which has a key function in natural sleep regulation, could result in more natural and restorative sleep for those suffering from insomnia. Agonists or antagonists that target specific melatonin receptors with high affinity or that act on parts of the brain that mediate the

sleep-promoting role melatonin could prove useful as therapeutic agents. Optimizing melatonin's utility as a sleep aid hinges on a greater understanding of its mode(s) of action with regards to sleep. While plenty of research has been done to uncover some of the subcellular events elicited by melatonin receptor activation, very little work has been done to identify sites of action in the brain or downstream effectors of melatonin signaling.

To advance our understanding of melatonin's role in sleep, it seems crucial to expand melatonin research to include animal models beyond nocturnal rodents. While invertebrate research has been useful for the study of sleep, even leading to the discovery of sleep-promoting neuropeptides that play a role in vertebrate sleep (Nelson et al., 2014; Nath et al., 2016; Lee et al., 2017), the function of melatonin in these species is questionable, and the lack of brain homology with vertebrates limits the potential for modeling melatonin's role in human sleep. Instead, zebrafish, diurnal birds, and diurnal rodents hold great promise as model organisms to understand the functions of melatonin in diurnal vertebrates, including humans.

Zebrafish are a useful model for sleep research for the reasons described in section 1.5, and melatonin has been shown to be required for circadian regulation of sleep in this species. The genetic tractability of the zebrafish and its amenability to non-invasive whole-brain neuronal imaging makes it particularly suitable to uncover the genetic and neuronal mechanisms through which melatonin regulates sleep.

Studies using diurnal birds have been instrumental in understanding the role of the pineal gland in regulating behavior. Diurnal birds are strongly dependent on the pineal gland for normal sleep-wake cycling, but this relationship has been interpreted as an action of the pineal on the circadian rhythm, not on sleep directly. In order to determine the role of melatonin in these processes, loss-of-function manipulations that specifically target melatonin and not the entire pineal gland, perhaps using transgenesis or mutations

(Scott et al., 2010), and assays that distinguish between effects on the circadian clock versus outputs of the clock such as sleep, are needed.

Finally, a close examination of sleep (i.e., using EEG/EMG recordings) in diurnal rodents in the context of melatonin gain- and loss-of-function manipulations are necessary to determine the role of melatonin in sleep in these species. If the lack of an apparent role for melatonin in sleep in nocturnal rodents is simply a consequence of their nocturnality, then diurnal rodents could be used to validate current and future findings in nocturnal rodents—such as the role of MT2 in the reticular thalamic nucleus—and build confidence that the mechanisms uncovered in 'lower' organisms like birds, fish, and even worms are conserved in mammals. Studies of melatonin's function in diurnal rodents would also aid in the development of melatonin-based therapies for human sleep disorders.

Next, in Chapter 2, I describe my efforts using zebrafish to uncover some of the mechanisms by which melatonin promotes sleep.

CHAPTER 2

THE GENETIC AND NEURONAL SUBSTRATES OF MELATONIN SIGNALING IN ZEBRAFISH SLEEP

2.1 Loss of any single melatonin receptor-encoding gene has no overt effects on activity or sleep

Vertebrates melatonin receptors are classified into four subtypes: *mtnr1a, mtnr1d* (or *mtnr1a-like/1al*)*, mtnr1b, and mtnr1c.* These subtypes are believed to have arisen from two genome duplication events in animal evolution, starting from the ancestral *mtnr1*, which was duplicated to yield *mtnr1ad* and *mtnr1bc*, which were each then duplicated to yield the four subtypes (Maugars et al., 2020). In the mammalian lineage, *mtnr1d* was lost, and the *mtnr1c* homolog—known as *GPR50*—lost its ability to bind melatonin (Maugars et al., 2020; Denker et al., 2019; Dufourny et al., 2008). Thus, in mammals, there are two functional melatonin receptors, *mtnr1a* and *mtnr1b*, commonly known as MT1 and MT2, respectively. The role that each receptor plays in mediating melatonin's effects on physiology is an area of active investigation, and the receptor(s) that mediates sleep, specifically, has yet to be identified (discussed in Chapter 1).

In the teleost infraclass, to which zebrafish belong, a third genome duplication event yielded, hypothetically, eight receptors: *mtnr1aa, mtnr1ab, mtnr1da, mtnr1db, mtnr1ba, mtnr1bb, mtnr1ca, mtnr1cb.* The *mtnr1db* gene was rapidly lost, and later, in the zebrafish lineage, *mtnr1cb* was also lost (Maugars et al., 2020). (The eel lineage retains the 7 melatonin receptors after loss of *1db*.) Thus, six melatonin receptors are encoded in the zebrafish genome: *mtnr1aa, mtnr1ab, mtnr1d/mtnr1al, mtnr1ba, mtnr1ba,* and *mtnr1c*. We refer to *mtnr1d/mtnr1al* as simply *mtnr1al* because (a) it is designated as *mtnr1al* by the ZFIN database and other databases, (b) it is positioned more closely to *mtnr1aa* and *mtnr1ab* on a phylogenetic tree (in contrast to *mtnr1c,* which does not cluster near *mtnr1ba* and *mtnr1bb*) (Maugars et al., 2020), and (c) it appears to be related to *mtnr1aa* and *mtnr1ab* based on its phenotypes (below).

To test which, if any, of these melatonin receptors are required for melatonin's role in promoting sleep, we used CRISPR/Cas9 to create insertion and/or deletion (indel) mutations in each of the six melatonin receptors (Liu et al., 2019). In generating these alleles, we designed guide RNAs (gRNAs) to target a region of the receptor-encoding gene that was 'downstream' of (3' to) the sequence encoding the second transmembrane domain, which would ensure that the mutation is retained in any alternate transcripts that are transcribed from an alternate promoter, but 'upstream' of (5' to) the fourth transmembrane domain to ensure that the majority of the transcript—including regions that are important for melatonin binding and G-protein signaling—is affected by the shift in the translational reading frame and early STOP codon caused by the indel mutation.

After verifying the mutagenicity of each gRNA, wild-type embryos at the 1-cell stage were injected, into the cell, with 1-2 μL of a solution containing Cas9-gRNA complexes. To identify 'founder' fish that carried heritable mutations, injected embryos were raised to adulthood and outcrossed to parental wild-type fish, and their progenies were screened by PCR and T7 endonuclease for mutations. Identified founder fish were outcrossed again, their progenies were raised to adulthood, and these adult F1 fish were genotyped by fin-clip. F1 heterozygotes were kept, and the specific genetic lesion in each mutant line was determined by sequencing. To mitigate any effects of potential off-target mutations, mutant lines were outcrossed two more times before testing, such that the progeny of F3 fish were used in the first experiments for every mutant line.

Using this strategy, we were able to recover two independent mutant alleles, each resulting in a frameshift and early stop codon (and thus a likely null allele), for every melatonin receptor gene except one. The exception was *mtnr1c*, for which we recovered only one allele, a 99-bp deletion that does not result in a frameshift but removes an entire transmembrane domain and is predicted to invert the extracellular and cytoplasmic

domains for a large swath of the receptor. Table 2.1 lists the melatonin receptor sequences targeted and describes the nature of each mutant allele.

We expected that larvae carrying mutations in the relevant melatonin receptors would show the following phenotypes: First, based on the lab's previous work demonstrating that melatonin-deficient *aanat2-/-* animals are strongly impaired for nightly sleep (Gandhi et al., 2015), we expected that the relevant melatonin receptor mutants would also be impaired for sleep at night compared to their wild-type siblings. Second, we expected the relevant mutants to be resistant to sleep induced by treatment with exogenous melatonin (Zhdanova et al., 2001; Gandhi et al., 2015).

We used a video-tracking system to record the behavior of up to 96 larvae at a time for 2-3 days and nights, spanning the larval zebrafish ages of 5 days post-fertilization (dpf) to 6 or 7 dpf, when robust sleep/wake rhythms are present (Prober et al., 2006). As many as 96 larvae are distributed in wells among a 96-well plate at the age of 4 dpf, then the 96-well plate is loaded into a video-tracker, which is programmed to alternate between light and dark at 9:00 am and 11:00 pm, respectively; these are the same lighting conditions that adult fish experience in the fish facility and that larvae experience in the incubator in which they are raised prior to the experiment. Recording starts later in the day on 4 dpf, but we only use data from the morning of 5 dpf onwards.

Data from the video-trackers report the locomotor activity for each larva over a window of time defined by the experimenter. For normal sleep/wake assays, we integrate activity over 1-min bins. From these activity data, sleep is quantified as the number of 1-min bins during which there was no detectable movement of the larva for the entire 1-min period. This method of defining sleep is based on the observation that larvae that have been immobile for 1 minute or longer exhibit a higher arousal threshold, consistent with a ≥1-min inactive bout representing a sleep state. Accordingly, sleep is reported as 'mins/10

mins' or 'mins/hour.' From the video-tracker output, we can also calculate various other sleep and wake parameters, such as the number of sleep bouts per hour, the average duration of each sleep bout, and the latency to first sleep bout at night.

We compared the sleep-wake profiles among the wild-type, heterozygous, and homozygous mutant progeny yielded from adult heterozygote in-crosses (Figures 2.1-2.6). For every receptor mutant, we observed similar levels of locomotor activity and sleep among all three genotypes (A, B). We also quantified the average hourly activity (C) and sleep (E) during the day, average hourly activity (D) and sleep (F) during the night, average sleep bout length at night (G), average number of sleep bouts per hour at night (H), and latency to first sleep bout at night (I). For each receptor, we found no significant differences among the three genotypes for most of these measurements. We noticed a slight but non-significant decrease in locomotor activity during the day in *mtnr1aa-/-* mutants (Fig. 2.1 A, C), as well as a slight but non-significant increase in locomotor activity during the day in *mtnr1ba-/-* mutants (Fig. 2.4 A, C). There was a significant decrease in the daytime locomotor activity of *mtnr1bb-/-* mutants compared to their wild-type siblings (Fig. 2.5 C). This *mtnr1bb-/-* phenotype is not accompanied by any changes in locomotor activity or sleep during the night, when melatonin is present, and is therefore difficult to interpret. The finding that no significant sleep deficit was observed in any single melatonin receptor mutant suggests that melatonin, which is essential for normal amounts of sleep at night, does not act through any one melatonin receptor paralog in zebrafish.

Target MT receptor gene	Target sequence (5' ⟶ 3') (PAM sequence underlined.)	Allele ID (Allele used in this study in red.)	Lesion	Predicted change
mtnr1aa	GGCCATTTACCCATATCCGCTGG	d20	20 b.p. deletion	Frameshift @ 80th AA & early STOP codon; truncated 350 ⟶ 116 AAs.
"	"	i19	19 b.p. insertion	Frameshift and early STOP codon.
mtnr1ab	CCGTAGCTGACCTTGTTGTGGCC	d11a	11 b.p. deletion	Frameshift @ 74th AA & early STOP codon; truncated 352 ⟶ 114 AAs.
"	"	d10	10 b.p. deletion	Frameshift and early STOP codon.
mtnr1al	CCTACCCGCTTGTCCTGACCGCC	i5	5 b.p. insertion	Frameshift @ 110th AA & early STOP codon; truncated 344 ⟶ 113 AAs.
"	"	i8	8 b.p. insertion	Frameshift and early STOP codon.
mtnr1ba	AATAGAAAACTCAGAAACGCGGG	d5	5 b.p. deletion	Frameshift @ 65th AA & early STOP codon; truncated 355 ⟶ 173 AAs.
"	"	i4	4 b.p. insertion	Frameshift and early STOP codon.
mtnr1bb	CCACAGCTTTGCCTACGGACGTC	d11b	11 b.p. deletion	Frameshift @ 135th AA & early STOP codon; truncated 347 ⟶ 286 AAs.
"	"	d5i12	5 b.p. deletion & 12 b.p. insertion	Frameshift and early STOP codon.
mtnr1c	CCACAGTCTTCGCTACGACCGAC	d99	99 b.p. deletion	33-amino acid del. @ 4th TM domain [135:167]; inversion of some intracellular and extracellular domains.
mtnr1aa	TACCGAAGACGACCGCAGGGGGG CCACACTGCACATTCCAGTGCCA	Ex1-KO	846 b.p. deletion	Removal of 1st exon.

Table 2.1. Melatonin receptor mutant alleles generated by CRISPR/Cas9. For every melatonin receptor gene, guides were developed to target the coding region between the 2nd and 4th transmembrane domains. Sequences are shown as 5' → 3' on the 'sense' DNA strand, with the PAM sequence underlined. For some genes, the guide RNA is targeted to the opposite strand, and the PAM sequences in those cases are written as 'CCN' and positioned 5' to the target. We recovered two independent alleles (different indels, but same target) for each gene, except for *mtnr1c*, for which we only recovered one allele. The allele for each gene that is represented in this study is shown in red text. The specific DNA lesion, and how the modification is predicted to disrupt the peptide, is described for each allele.

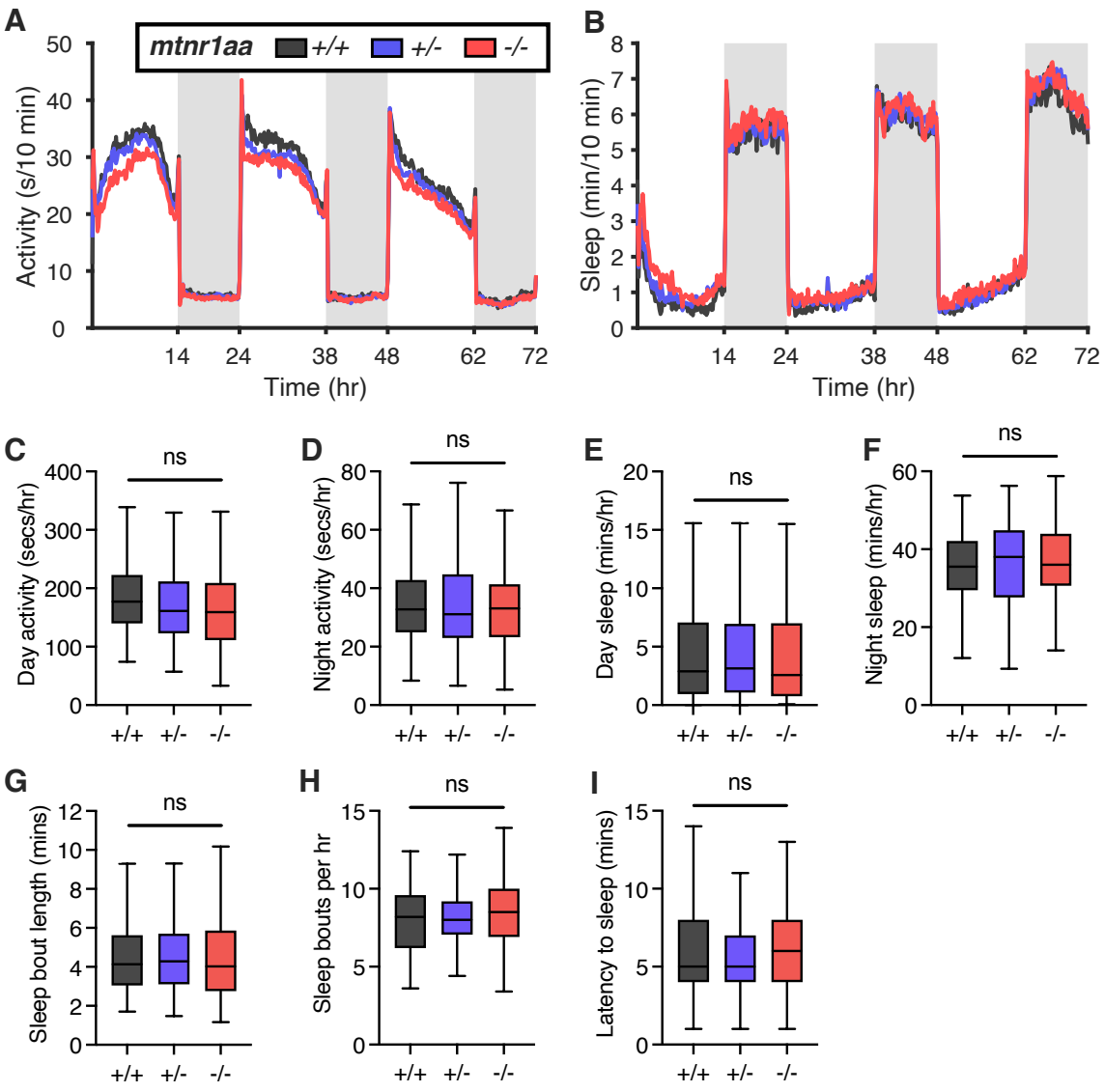

Figure 2.1. Mutation of *mtnr1aa* has no significant effect on locomotor activity or sleep. (A) Average locomotor activity over three days (unshaded segments) and nights (shaded segments) of 5- to 7-dpf *mtnr1aa+/+* (black), *mtnr1aa+/-* (blue), and *mtnr1aa-/-* (red) siblings. **(B)** Same as in (A) but measuring average sleep (average number of consolidated ≥1-minute inactive bouts per 10 minutes). **(C-D)** Box plot of locomotor activity measurements during day 6 (C) and night 6 (D) of *mtnr1aa+/+* (black), *mtnr1aa+/-* (blue), and *mtnr1aa-/-* (red) siblings. **(E-F)** Box plot of sleep amounts (minutes per hour) during day 6 (E) and night 6 (F) for each genotype. **(G-I)** Box plots of average sleep bout lengths (in minutes) (G), average number of sleep bouts per hour (H), and latencies to first sleep bout (in minutes) (I) during night 6 for each genotype. Data are pooled from 4 video-tracker experiments, n = 80 for *mtnr1aa+/+*, 145 for *1aa+/-*, 93 for *1aa-/-*. Box plots use Tukey method for determining whiskers and outliers; outliers are not shown. ns = not significant ($P > 0.05$); Kruskal-Wallis test.

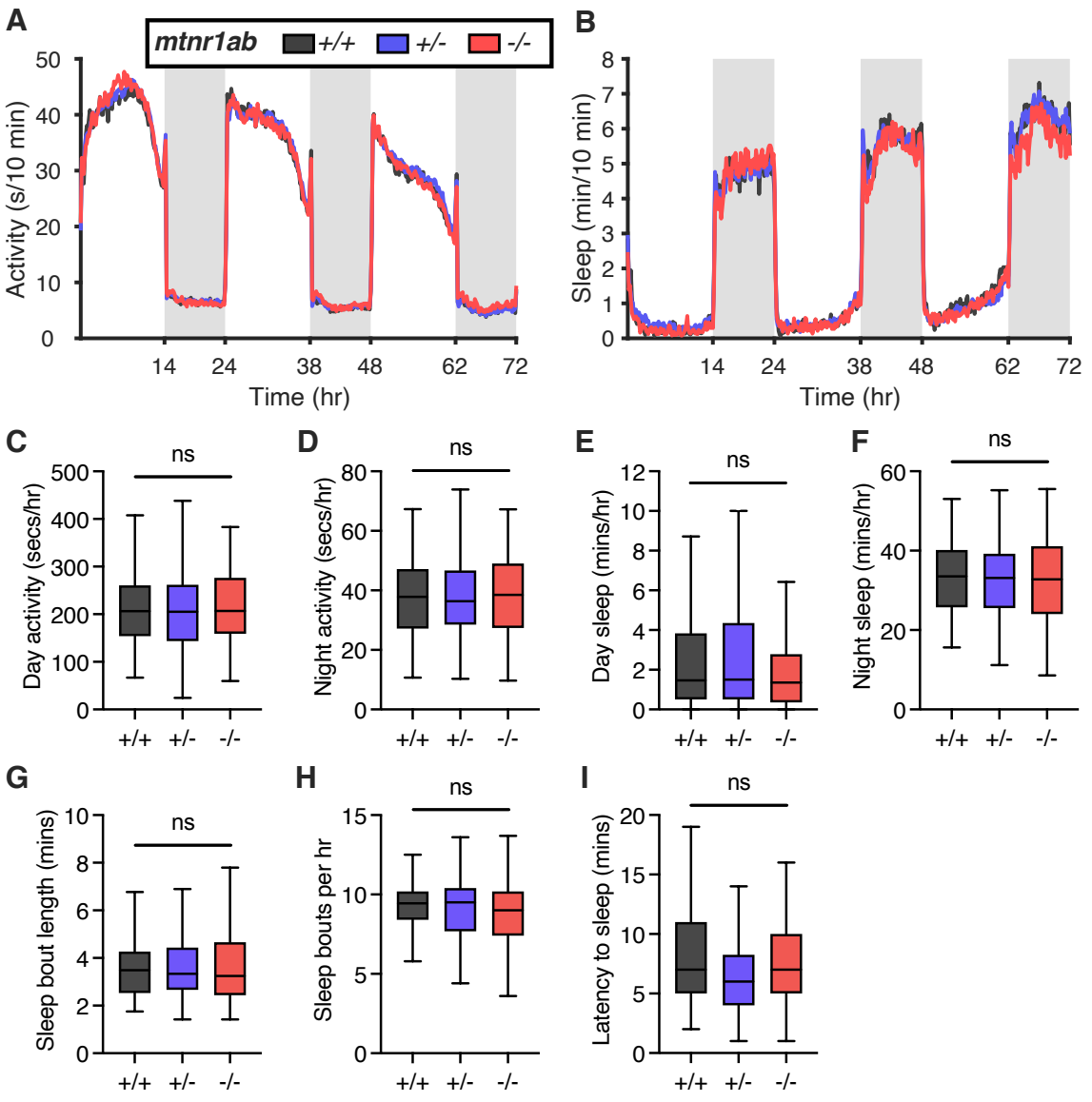

Figure 2.2. Mutation of *mtnr1ab* has no significant effect on locomotor activity or sleep. (A) Average locomotor activity) over three days (unshaded segments) and nights (shaded segments) of 5- to 7-dpf *mtnr1ab+/+* (black), *mtnr1ab+/-* (blue), and *mtnr1ab-/-* (red) siblings. **(B)** Same as in (A) but measuring average sleep (average number of consolidated ≥1-minute inactive bouts per 10 minutes). **(C-D)** Box plot of locomotor activity measurements during day 6 (C) and night 6 (D) of *mtnr1ab+/+* (black), *mtnr1ab+/-* (blue), and *mtnr1ab-/-* (red) siblings. **(E-F)** Box plot of sleep amounts (minutes per hour) during day 6 (E) and night 6 (F) for each genotype. **(G-I)** Box plots of average sleep bout lengths (in minutes) (G), average number of sleep bouts per hour (H), and latencies to first sleep bout (in minutes) (I) during night 6 for each genotype. Data are pooled from 4 video-tracker experiments, n = 76 for *mtnr1ab+/+*, 170 for *1ab+/-*, 75 for *1ab-/-*. Box plots use Tukey method for determining whiskers and outliers; outliers are not shown. ns = not significant ($P > 0.05$); Kruskal-Wallis test.

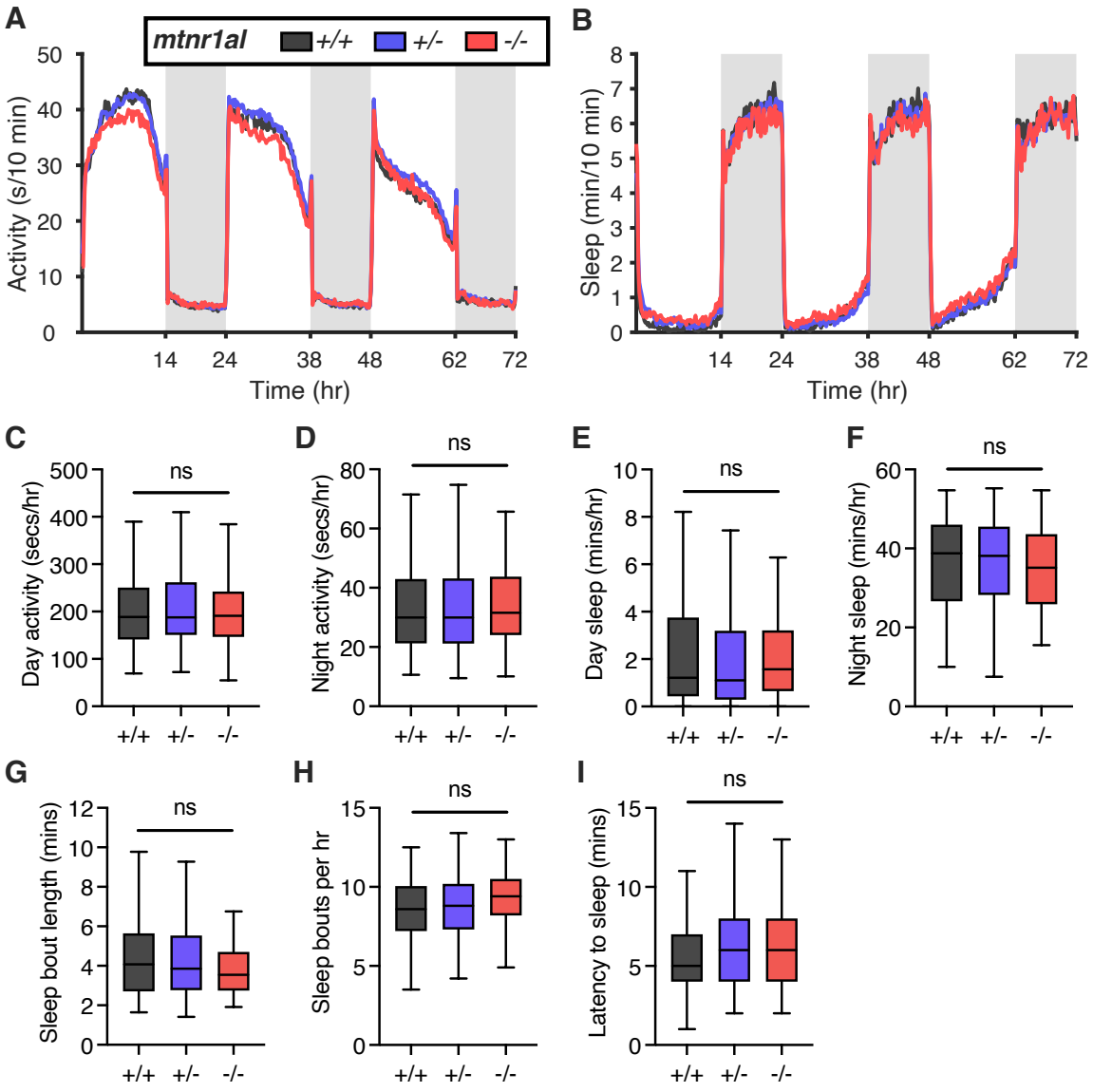

Figure 2.3. Mutation of *mtnr1al* has no significant effect on locomotor activity or sleep. (A) Average locomotor activity over three days (unshaded segments) and nights (shaded segments) of 5- to 7-dpf *mtnr1al+/+* (black), *mtnr1al+/-* (blue), and *mtnr1al-/-* (red) siblings. **(B)** Same as in (A) but measuring average sleep (average number of consolidated ≥1-minute inactive bouts per 10 minutes). **(C-D)** Box plot of locomotor activity measurements during day 6 (C) and night 6 (D) of *mtnr1al+/+* (black), *mtnr1al+/-* (blue), and *mtnr1al-/-* (red) siblings. **(E-F)** Box plot of sleep amounts (minutes per hour) during day 6 (E) and night 6 (F) for each genotype. **(G-I)** Box plots of average sleep bout lengths (in minutes) (G), average number of sleep bouts per hour (H), and latencies to first sleep bout (in minutes) (I) during night 6 for each genotype. Data are pooled from 4 video-tracker experiments, n = 81 for *mtnr1al+/+*, 168 for *1al+/-*, 75 for *1al-/-*. Box plots use Tukey method for determining whiskers and outliers; outliers are not shown. ns = not significant ($P > 0.05$); Kruskal-Wallis test.

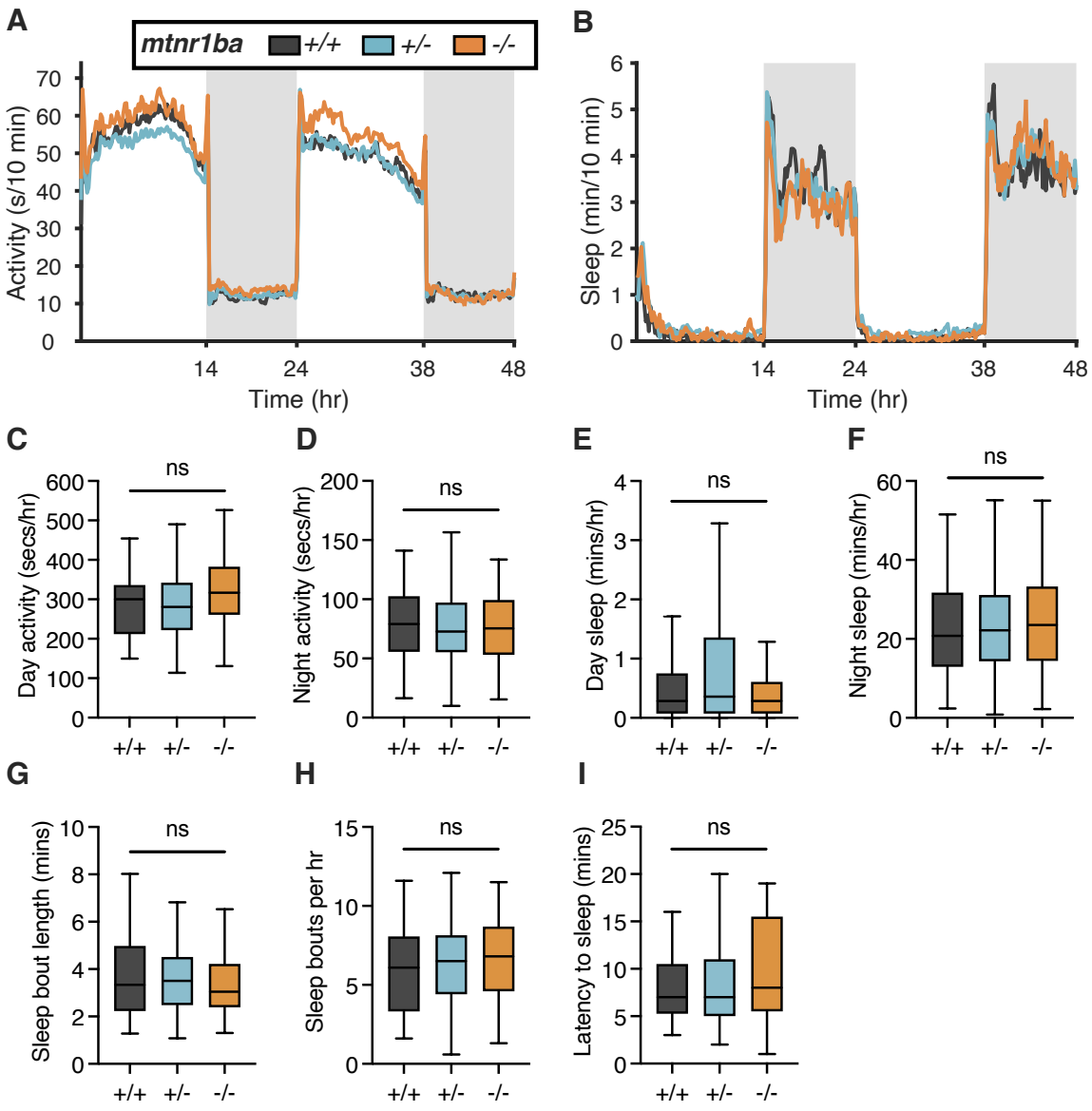

Figure 2.4. Mutation of *mtnr1ba* has no significant effect on locomotor activity or sleep. (A) Average locomotor activity over two days (unshaded segments) and nights (shaded segments) of 5- to 6-dpf *mtnr1ba+/+* (black), *mtnr1ba+/-* (light blue), and *mtnr1ba-/-* (orange) siblings. **(B)** Same as in (A) but measuring average sleep (average number of consolidated ≥1-minute inactive bouts per 10 minutes). **(C-D)** Box plot of locomotor activity measurements during day 6 (C) and night 6 (D) of *mtnr1ba+/+* (black), *mtnr1ba+/-* (light blue), and *mtnr1ba-/-* (orange) siblings. **(E-F)** Box plot of sleep amounts (minutes per hour) during day 6 (E) and night 6 (F) for each genotype. **(G-I)** Box plots of average sleep bout lengths (in minutes) (G), average number of sleep bouts per hour (H), and latencies to first sleep bout (in minutes) (I) during night 6 for each genotype. Data are pooled from 3 video-tracker experiments, n = 52 for *mtnr1ba+/+*, 133 for *1ba+/-*, 57 for *1ba-/-*. Box plots use Tukey method for determining whiskers and outliers; outliers are not shown. ns = not significant ($P > 0.05$); Kruskal-Wallis test.

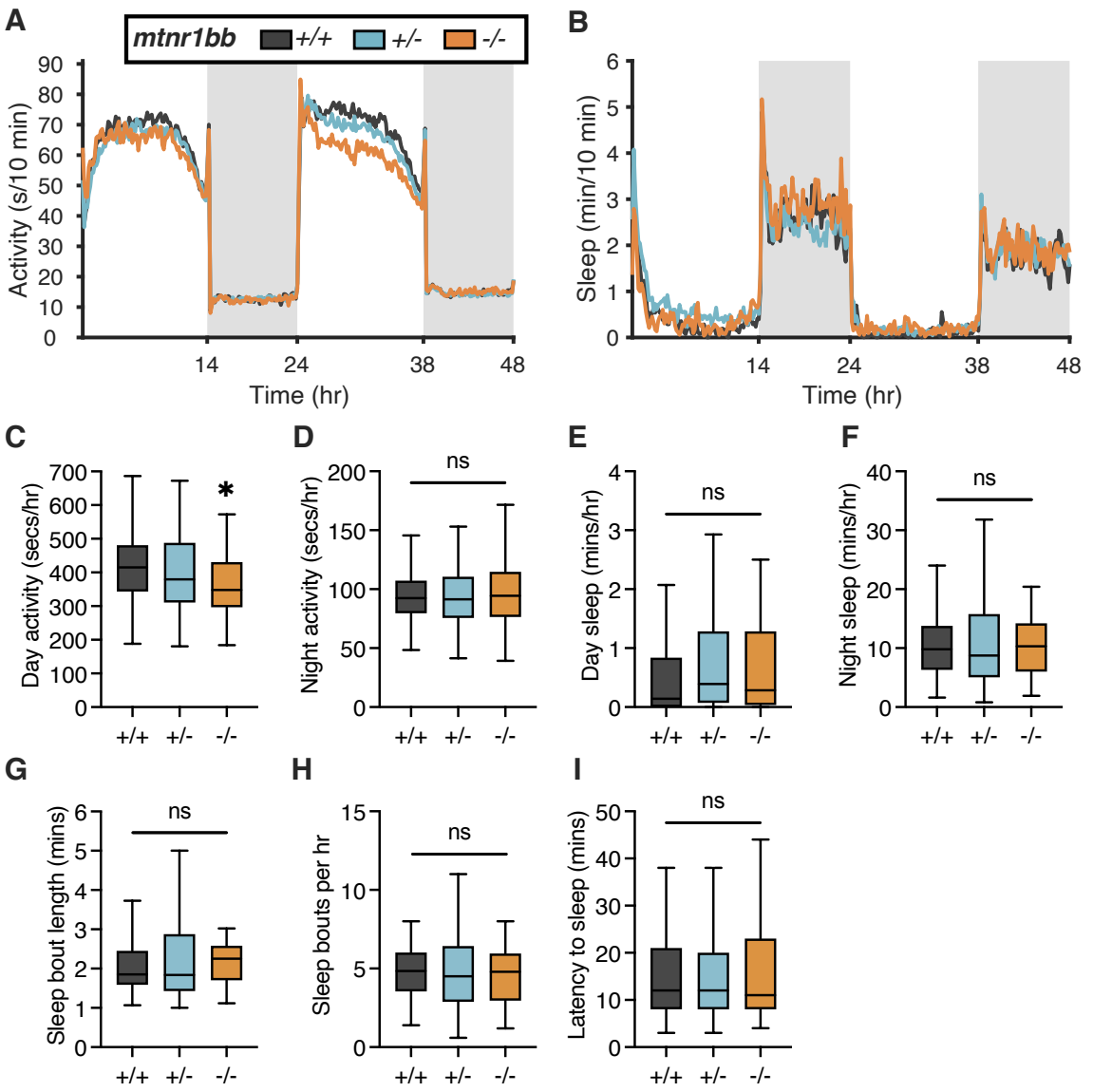

Figure 2.5. *mtnr1bb* mutants exhibit decreased locomotor activity during the day but have wild-type levels of sleep. (A) Average locomotor activity over two days (unshaded) and nights (shaded) of 5- to 6-dpf *mtnr1bb+/+* (black), *mtnr1bb+/-* (light blue), and *mtnr1bb-/-* (orange) siblings. **(B)** Same as in (A) but measuring average sleep (average number of consolidated ≥1-min inactive bouts per 10 mins). **(C-D)** Box plot of locomotor activity measurements during day 6 (C) and night 6 (D) of *mtnr1bb+/+* (black), *mtnr1bb+/-* (light blue), and *mtnr1bb-/-* (orange) siblings. *mtnr1bb-/-* larvae are significantly less active than wild-type siblings on day 6. **(E-F)** Box plot of sleep amounts (minutes per hour) during day 6 (E) and night 6 (F) for each genotype. **(G-I)** Box plots of average sleep bout lengths (in minutes) (G), average number of sleep bouts per hour (H), and latencies to first sleep bout (in minutes) (I) during night 6 for each genotype. Data are pooled from 3 video-tracker experiments, n = 56 for *mtnr1bb+/+*, 114 for *1bb+/-*, 53 for *1bb-/-*. Box plots use Tukey method for determining whiskers and outliers; outliers are not shown. ∗ = $P <$ 0.05 vs. *mtnr1bb+/+*, ns = not significant ($P >$ 0.05); Kruskal-Wallis test with Dunn's multiple comparisons test.

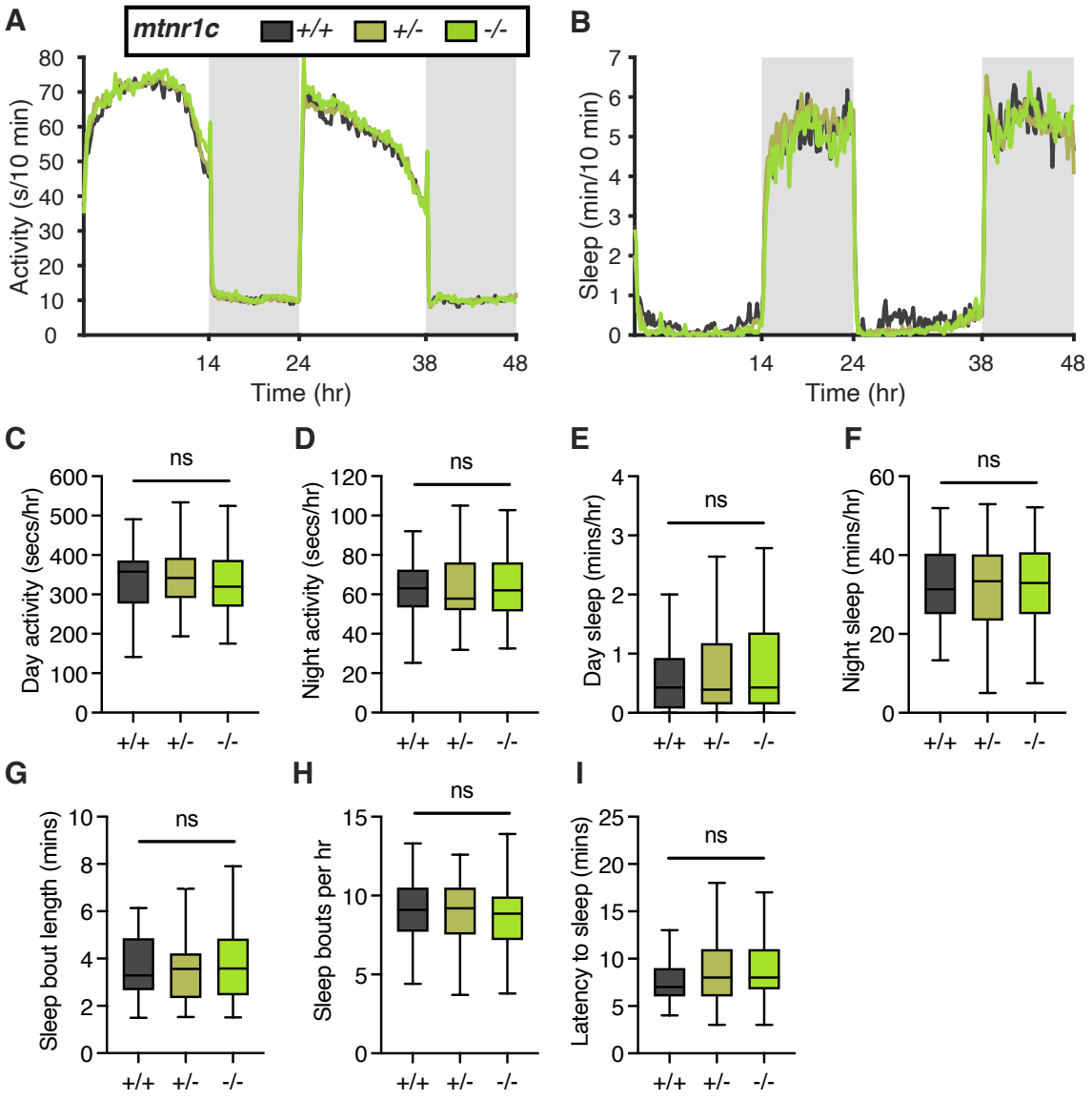

Figure 2.6. Mutation of *mtnr1c* has no significant effect on locomotor activity or sleep. (A) Average locomotor activity over two days (unshaded segments) and nights (shaded segments) of 5- to 6-dpf *mtnr1c+/+* (black), *mtnr1c+/-* (olive), and *mtnr1c-/-* (light green) siblings. **(B)** Same as in (A) but measuring average sleep (average number of consolidated ≥1-minute inactive bouts per 10 minutes). **(C-D)** Box plot of locomotor activity measurements during day 6 (C) and night 6 (D) of *mtnr1c+/+* (black), *mtnr1c+/-* (olive), and *mtnr1c-/-* (light green) siblings. **(E-F)** Box plot of sleep amounts (minutes per hour) during day 6 (E) and night 6 (F) for each genotype. **(G-I)** Box plots of average sleep bout lengths (in minutes) (G), average number of sleep bouts per hour (H), and latencies to first sleep bout (in minutes) (I) during night 6 for each genotype. Data are pooled from 2 video-tracker experiments, n = 39 for *mtnr1c+/+*, 76 for *1c+/-*, 38 for *1c-/-*. Box plots use Tukey method for determining whiskers and outliers; outliers are not shown. ns = not significant ($P > 0.05$); Kruskal-Wallis test.

2.2 Sleep is defective in zebrafish lacking all three MT1 receptor paralogs

Since we found no phenotype among any single mutants, we considered the possibility that the receptors act redundantly to promote sleep or can compensate for one another if any one receptor is non-functional. To test this, we in-crossed mutants to generate a triple mutant *mtnr1aa; mtnr1ab; mtnr1al* line, which covers the three zebrafish MT1 receptor family paralogs, and a double mutant *mtnr1ba; mtnr1bb* line, which covers the two zebrafish MT2 receptor family paralogs. While it is certainly possible that receptors across the two families (MT1 and MT2) could be acting redundantly or compensate for each other, we hypothesized that MT1 and MT2 served distinct functions, and designed triple and double mutants accordingly. If neither the triple mutant *1aa; 1ab; 1al* line nor the double mutant *1ba; 1bb* line gave a sleep phenotype, we could try other combinations. While building these lines, we also generated mutants for all three combinations of double mutants for the MT1 receptor family: *mtnr1aa; mtnr1ab*, *mtnr1aa; mtnr1al*, and *mtnr1ab; mtnr1al*.

It is important to note that the genes encoding *mtnr1ab* and *mtnr1al* are located on the same chromosome. Accordingly, generating a line carrying a mutant allele in both genes was the product of homologous recombination, and in future experiments using these double mutant lines or the triple mutant line, these two alleles only rarely segregate from each other. This is fortuitous in that it reduces the number of genotypes yielded by certain crosses: i.e., a triple heterozygous *1aa+/-; 1ab+/-; 1al+/-* animal crossed to a triple null *1aa-/-; 1ab-/-; 1al-/-* animal yields 4 genotypes (2^2) rather than 8 genotypes (2^3). However, it is also a detriment because some control genotypes cannot be retrieved from certain crosses: i.e., within a clutch of siblings from the same cross mentioned above, we cannot determine the role of *mtnr1al* alone (by comparing heterozygous and homozygous

null animals at that locus) in an *mtnr1aa-/-; mtnr1ab-/-* background since it nearly always segregates with *mtnr1ab*.

To ensure that we would be able to detect potentially small sleep or wake phenotypes while working with the triple mutants, we wished to limit the number of genotypes used in our assay. To this end, we saved triple wild-type *mtnr1aa+/+; 1ab+/+; 1al+/+* animals and triple homozygous null *mtnr1aa-/-; 1ab-/-; 1al-/-* animals from the mixed progeny of a triple heterozygous *mtnr1aa+/-; 1ab+/-; 1al+/-* in-cross, and then we raised these fish to adulthood. Next, we in-crossed the *+/+; +/+; +/+* adults to generate a clutch of all triple wild-type larvae and, on the same day, also in-crossed *-/-; -/-; -/-* lines to generate a clutch of all triple null mutant larvae. The triple wild-type and triple null animals are 'cousins' of each other—rather than siblings—but were only 'in-bred' for one generation to avoid any confounding effects that in-breeding might have on behavior.

We found that the loss of all three MT1-type melatonin receptors in *mtnr1aa-/-; 1ab-/-; 1al-/-* mutants resulted in strong sleep defects at night compared to their wild-type cousins (Fig. 2.7 B, F). This was accompanied by a significant increase in locomotor activity during the night (Fig. 2.7 D), a significant decrease in the average length of sleep bouts (but also, interestingly, a small but significant increase in the number of sleep bouts) (Fig. 2.7 G-H), and a significantly longer latency to first sleep bout at night (Fig 2.7 I) in the triple mutants. We also observed a strong reduction in locomotor activity during the day (Fig. 2.7 A, C) and a small but significant increase in sleep during the day (Fig. 2.7 E). This day phenotype was observed in half of our experiments (2 of 4); in the other half of our experiments, there was no day phenotype. The sleep defect, however, was present in all experiments, and closely resembles the *aanat2-/-* phenotype, suggesting that melatonin is acting through MT1-type receptors.

Consistent with the notion that melatonin promotes sleep through the MT1 family of receptors, we found no significant changes in activity or sleep in *mtnr1ba-/-; mtnr1bb-/-* double mutant larvae, which lack both MT2 receptor paralogs, compared to their double heterozygous siblings (*mtnr1ba+/-; mtnr1bb+/-*) (Fig. 2.8). Thus, loss of both MT2-type receptors has no significant effect on sleep, whereas the loss of all three MT1-type receptors yields strong sleep phenotypes. Melatonin therefore likely functions through the MT1 receptor family to promote sleep.

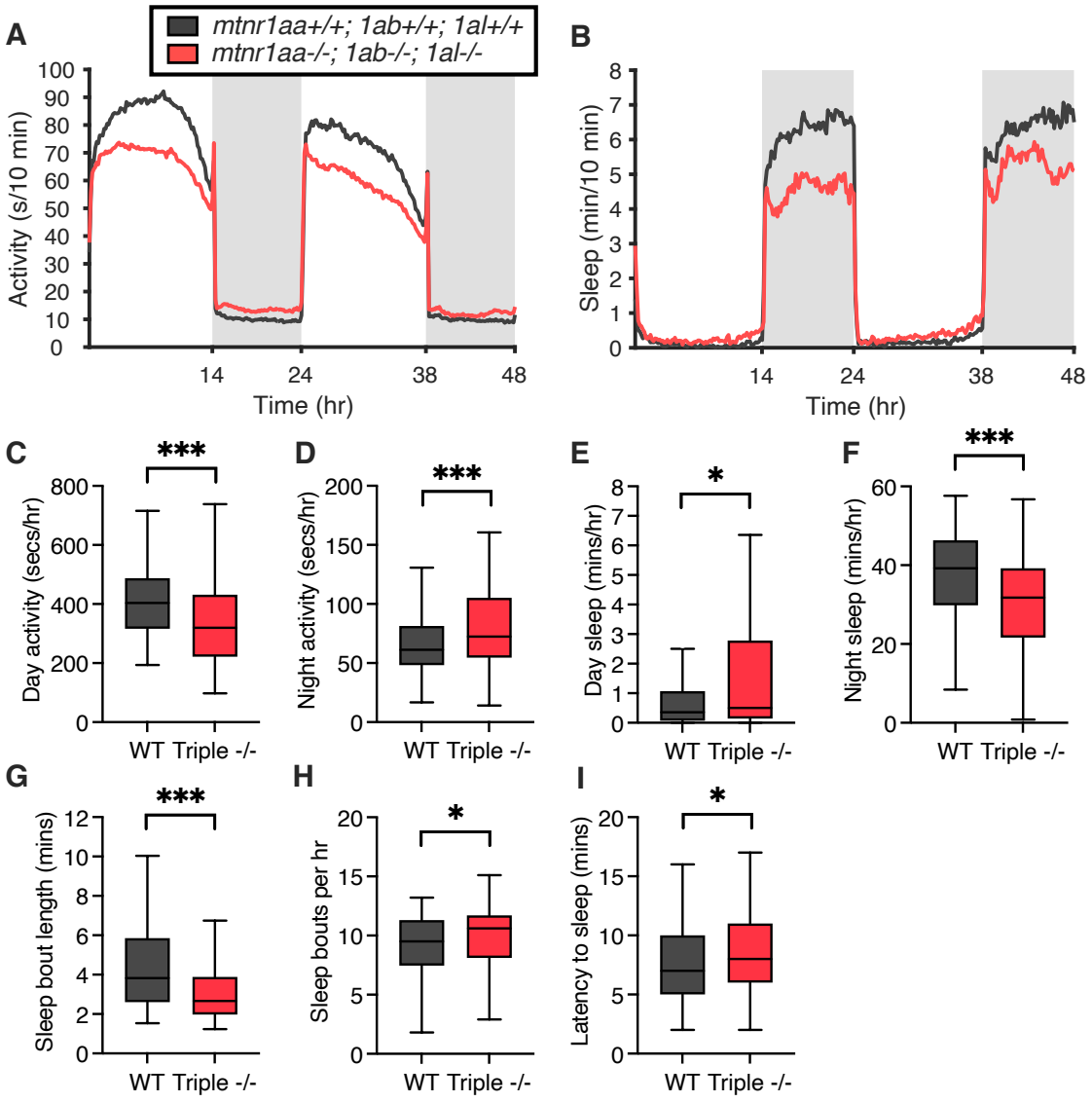

Figure 2.7. Zebrafish carrying mutations in all three MT1 paralogs are strongly impaired for sleep at night. (A-B) Average locomotor activity (A) and sleep (B) over two days (unshaded segments) and nights (shaded segments) of 5- to 6-dpf *mtnr1aa+/+; 1ab+/+; 1al+/+* (black; "triple *+/+*") and *mtnr1aa-/-; 1ab-/-; 1al-/-* (red; "triple *-/-*") cousins. **(C-D)** Box plot of locomotor activity measurements during day 6 (C) and night 6 (D) of triple *+/+* (black) and triple *-/-* (red) cousins. Triple *-/-* animals are significantly less active during the day and more active at night compared to triple *+/+* controls. **(E-F)** Box plot of sleep amounts during day 6 (E) and night 6 (F) for each genotype. Triple *-/-* animals sleep more during the day and less during the night than triple *+/+* controls. **(G-I)** Box plots of average sleep bout lengths (G), average number of sleep bouts per hour (H), and latencies to first sleep bout (I) during night 6 for each genotype. Triple *-/-* animals have shorter and more sleep bouts, and a longer sleep latency, than triple *+/+* controls. Data are pooled from 4 video-tracker experiments, n = 148 for *mtnr1aa+/+; 1ab+/+; 1al+/+*, 184 for *1aa-/-; 1ab-/-; 1al-/-*. Box plots use Tukey method for determining whiskers and outliers; outliers are not shown. ∗ = $P < 0.05$, ∗∗∗ = $P < 0.001$; Student's *t*-test.

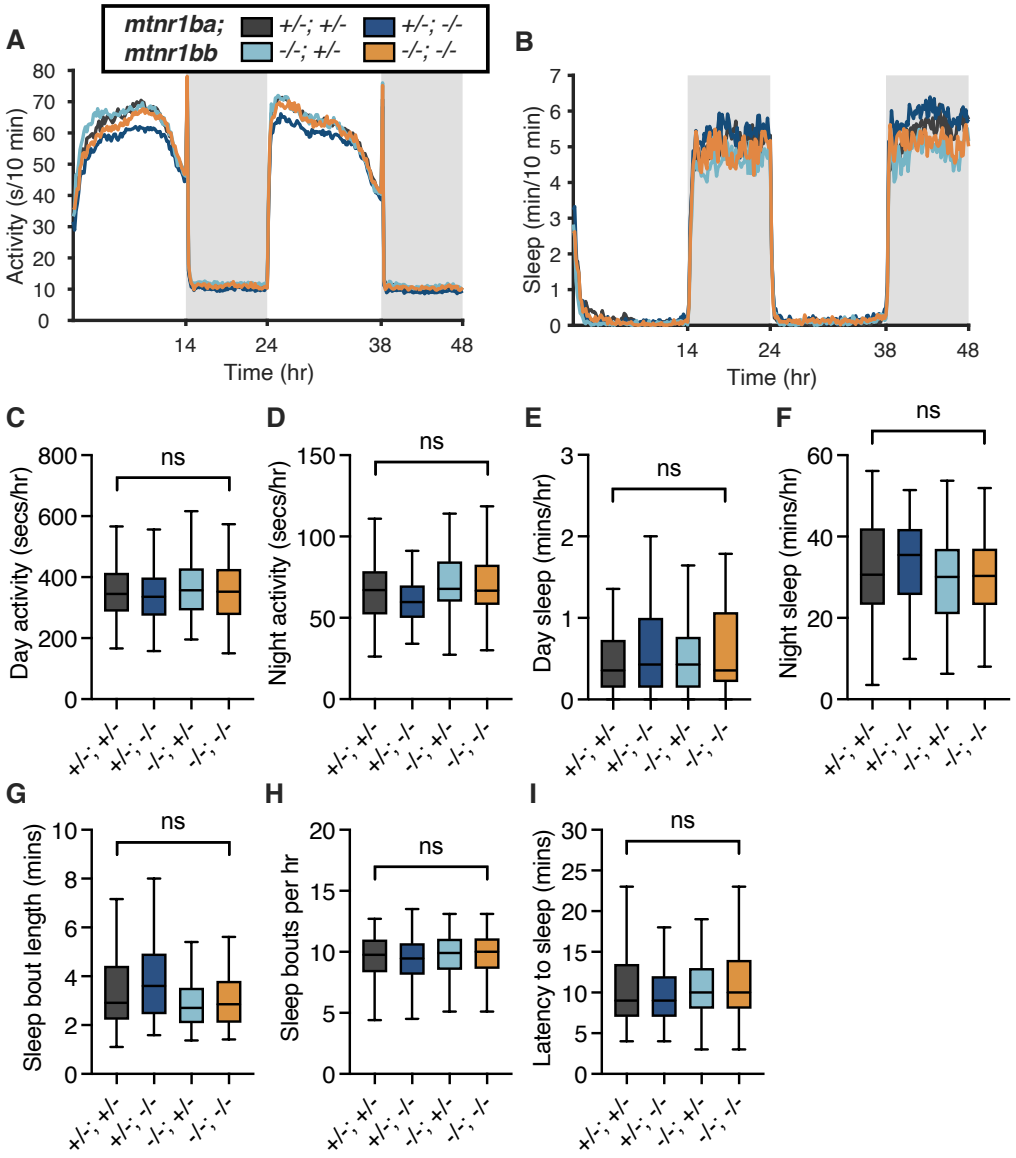

Figure 2.8. Mutation of both MT2 paralogs (*mtnr1ba-/-; mtnr1bb-/-*) has no significant effect on activity or sleep. (A-B) Average locomotor activity (A) and sleep (B) over two days (unshaded segments) and nights (shaded segments) of 5- to 6-dpf *mtnr1ba+/-; 1bb+/-* (black), *mtnr1ba+/-; 1bb-/-* (dark blue), *mtnr1ba-/-; 1bb+/-* (light blue), and *mtnr1ba-/-; 1bb-/-* (orange) siblings. **(C-D)** Box plot of locomotor activity measurements during day 6 (C) and night 6 (D) of *mtnr1ba+/-; 1bb+/-* (black), *mtnr1ba+/-; 1bb-/-* (dark blue), *mtnr1ba-/-; 1bb+/-* (light blue), and *mtnr1ba-/-; 1bb-/-* (orange) siblings. **(E-F)** Box plot of sleep amounts during day 6 (E) and night 6 (F) for each genotype. **(G-I)** Box plots of average sleep bout lengths (G), average number of sleep bouts per hour (H), and latencies to first sleep bout (I) during night 6 for each genotype. Data are pooled from 4 video-tracker experiments, n = 78 for *mtnr1ba+/-; 1bb+/-,* 86 for *mtnr1ba+/-; 1bb-/-*, 68 for *mtnr1ba-/-; 1bb+/-*, 79 for *mtnr1ba-/-; 1bb-/-*. Box plots use Tukey method for determining whiskers and outliers; outliers are not shown. ns = not significant ($P > 0.05$); Student's *t*-test.

2.3 Sleep induced by exogenous melatonin is suppressed in MT1 receptor triple mutants but not by MT2 receptor double mutants

As mentioned above, we anticipated that larvae carrying mutations in the melatonin receptors relevant for sleep would also be resistant to sleep induced by exogenous melatonin. Based on our finding that MT1 receptor triple mutants, but not MT2 receptor double mutants, were impaired for normal nightly sleep, we hypothesized that the MT1 receptor triple mutants would be less likely to sleep after administration of 1 μM melatonin, which quickly and reliably induces sleep in larval zebrafish. The effect of melatonin on sleep during the day is robust enough that we anticipated being able to interpret differences among 4 genotypes; accordingly, we used the progeny from a triple heterozygous *1aa+/-; 1ab+/-; 1al+/-* to triple homozygous null *1aa-/-; 1ab-/-; 1al-/-* cross. To record behavior for these experiments, we used the same video-tracking system, and, as before, loaded 4-dpf larvae into the 96-well plates and began recording on the night of 4 dpf. On 6 dpf, around noon, 150 μL of a 4.3X concentrated solution of melatonin was added to the media in each well, for a final concentration of 1 μM, and larvae were then left unperturbed.

Upon delivery of melatonin, we observed a strong reduction in locomotor activity (Fig. 2.9 A-C) and increase in sleep (Fig. 2.9 D-F) in the triple heterozygous control fish, as expected. The *1aa+/-; 1ab-/-; 1al-/-* mutants also showed strong reductions in locomotor activity, similar to the triple heterozygotes (Fig. 2.9 C), and were very mildly but not significantly impaired for induction of sleep by melatonin (Fig. 2.9 F). On the other hand, we found that triple homozygous MT1 receptor mutants (*1aa-/-; 1ab-/-; 1al-/-*) were highly resistant to melatonin's effects on locomotor activity and sleep, consistent with our unperturbed behavioral assays and with the notion that melatonin promotes sleep through

the MT1 family of melatonin receptors. Surprisingly, *1aa-/-; 1ab+/-; 1al+/-* mutants were also strongly resistant to the activity decrease and sleep induced by melatonin. They did not suppress the effects of melatonin on locomotor activity quite as strongly as did the triple null mutants (Fig. 2.9 A, C), but both genotypes were comparable in their suppression of melatonin-induced sleep (Fig. 2.9 D, F).

We observed that the effect of melatonin treatment on locomotor activity and sleep in *mtnr1aa-/-; 1ab+/-; 1al+/-* animals is comparable to that of *mtnr1aa-/-; mtnr1ab-/-; mtnr1al-/-.* The simplest interpretation from these data is that melatonin induces sleep exclusively through *mtnr1aa*, and that the other MT1 receptors are largely dispensable. However, we note that *mtnr1aa-/-* mutants are not defective for sleep (Fig. 2.1), and we only observe sleep defects in animals that have mutations in all three MT1 paralogs (Fig. 2.7). Thus, while *mtnr1aa* appears to play a primary role in mediating melatonin's effects, there must be an interaction between *mtnr1aa* and the other MT paralogs. In the next section, we measure the responses of MT1 double mutants to melatonin treatment to examine this relationship.

We also tested the effect of melatonin treatment on MT2 family receptor mutants. Among the four genotypes, we found no significant difference in their responses to melatonin (Fig. 2.10). We conclude that melatonin promotes sleep primarily, and perhaps exclusively, via MT1 receptors.

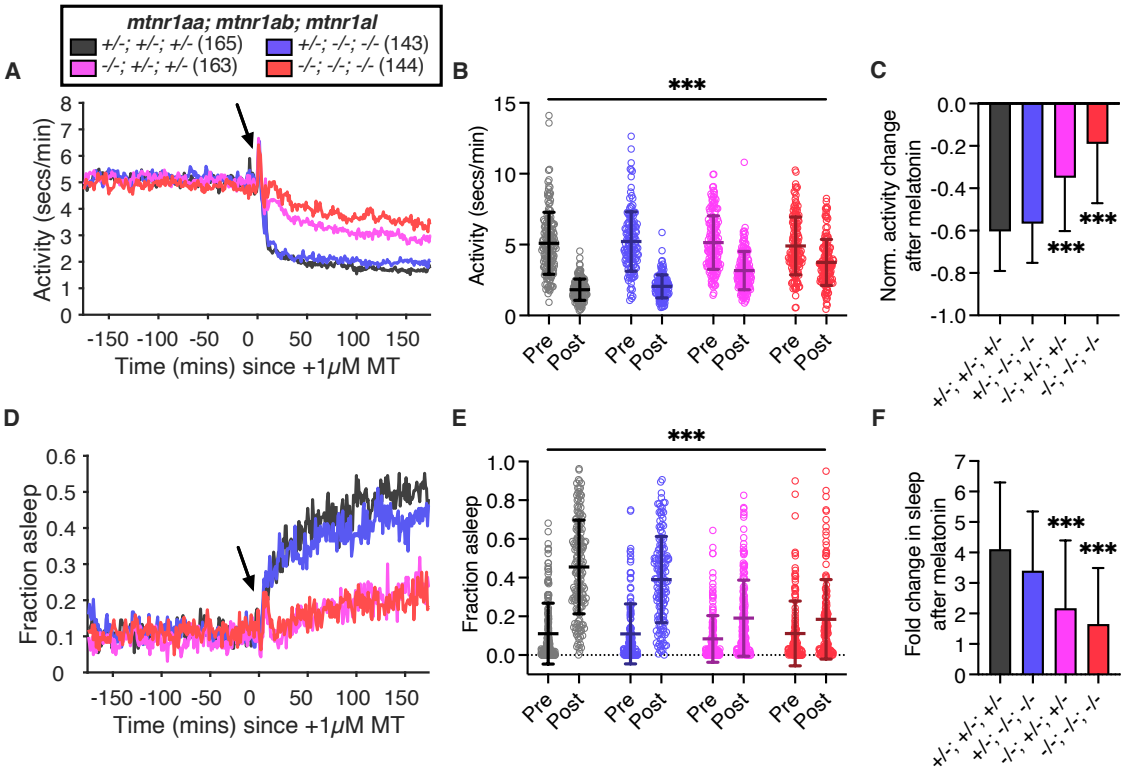

Figure 2.9. Induction of sleep by melatonin treatment requires MT1 paralogs. (A) Average locomotor activity of 6-dpf *mtnr1aa+/-; 1ab+/-; 1al+/-* (black), *mtnr1aa+/-; 1ab-/-; 1al-/-* (blue), *mtnr1aa-/-; 1ab+/-; 1al+/-* (pink), and *mtnr1aa-/-; 1ab-/-; 1al-/-* (red) larvae during 3 hours before and after administering 1 μM melatonin (MT) (at t = 0; indicated by the black arrow). **(B)** Locomotor activity for larvae of each genotype during 3 hrs before ('Pre') and after ('Post') MT administration, not including the 20 mins preceding or following MT administration. Lines represent mean +/- s.d. **(C)** Mean locomotor activity changes after MT administration normalized to pre-treatment baseline locomotor activity ((post-MT mean activity – pre-MT mean activity) / pre-MT mean activity). Error bars represent s.d. *mtnr1aa-/-; 1ab+/-; 1al+/-* and *mtnr1aa-/-; 1ab-/-; 1al-/-* animals are significantly impaired for MT-induced locomotor activity decrease compared to the triple heterozygous sibling controls. **(D)** Fraction of larvae that are asleep per minute for 3 hrs before and after MT administration (black arrow). **(E)** Same as in (B) but fraction of animals asleep. **(F)** Fold change in sleep amount after MT administration (post-MT sleep amount of each larva / pre-MT mean sleep amount of genotype). Compared to triple heterozygous controls, which show a ~4-fold increase in sleep after MT administration over the 3-hr period, *mtnr1aa-/-; 1ab+/-; 1al+/-* and *mtnr1aa-/-; 1ab-/-; 1al-/-* larvae show strongly suppressed MT-induced sleep. Data are pooled from 8 video-tracker experiments. Number of animals represented in each genotype are indicated in parentheses in legend. For panels (B) and (E), $***$ = $P < 0.001$; Two-way ANOVA. For panels (C) and (F), $***$ = $P < 0.001$ vs. *mtnr1aa+/-; 1ab+/-; 1al+/-*; Kruskal-Wallis test with Dunn's multiple comparisons test. Non-significant comparisons to the control genotype (*+/-; +/-; +/-*) are not annotated.

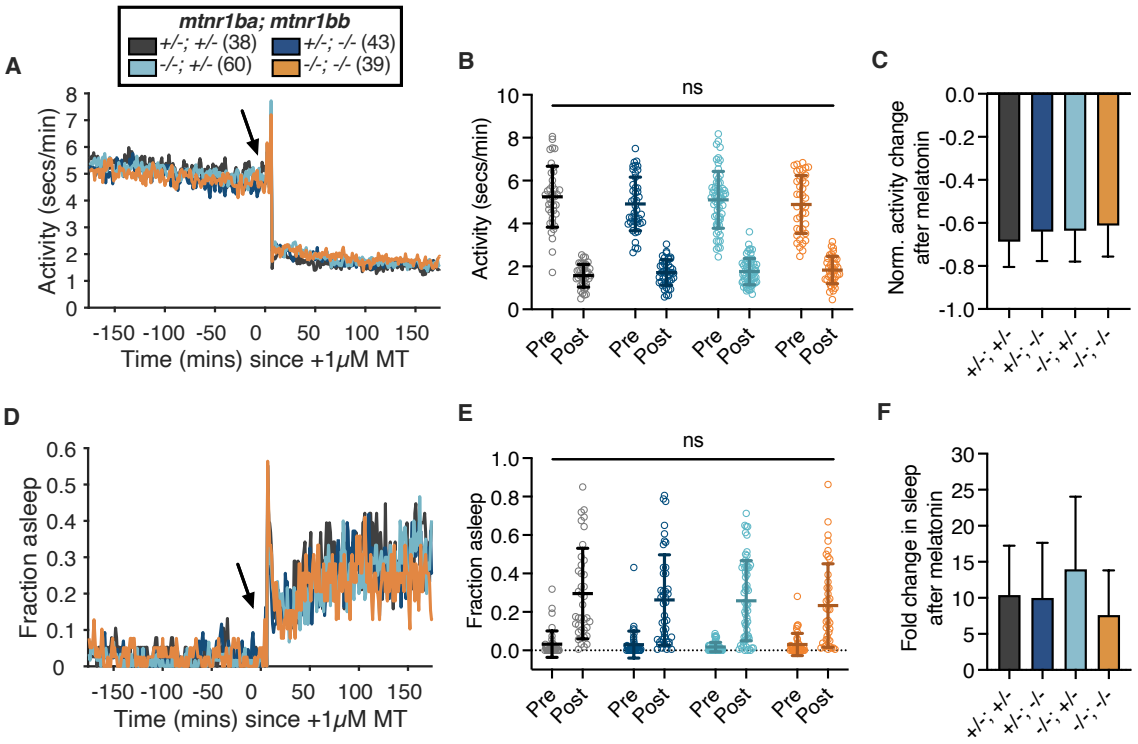

Figure 2.10. MT2 paralogs are dispensable for sleep induced by exogenous melatonin. (A) Average locomotor activity of 6-dpf *mtnr1ba+/-; 1bb+/-* (black), *mtnr1ba+/-; 1bb-/-* (dark blue), *mtnr1ba-/-; 1bb+/-* (light blue), and *mtnr1ba-/-; 1bb-/-* (orange) larvae during 3 hours before and after administering 1 μM melatonin (MT) (at t = 0; indicated by the black arrow). **(B)** Locomotor activity for larvae of each genotype over the 3 hrs before ('Pre') and after ('Post') MT administration, not including the 20 mins preceding or following MT administration. Lines represent mean +/- s.d. **(C)** Mean locomotor activity changes after MT administration normalized to pre-treatment baseline locomotor activity ((post-MT mean activity – pre-MT mean activity) / pre-MT mean activity). Error bars represent s.d. **(D)** Fraction of larvae that are asleep per minute for 3 hrs before and after MT administration (black arrow). **(E)** Same as in (B) but fraction of animals asleep. **(F)** Fold change in sleep amount after MT administration (post-MT sleep amount of each larva / pre-MT mean sleep amount of genotype). Data are pooled from 2 video-tracker experiments. Number of animals represented in each genotype are indicated in parentheses in legend. For panels (B) and (E), ns = not significant ($P > 0.05$); Two-way ANOVA. For panels (C) and (F), no genotype was significantly different from the control genotype (*+/-; +/-*); Kruskal-Wallis test with Dunn's multiple comparisons test.

2.4 Loss of two copies of *mtnr1aa* and one copy of either *mtnr1ab* or *mtnr1al* is sufficient to block the effect of melatonin treatment on locomotor activity.

The results of the exogenous melatonin experiments revealed something that the sleep assays in unperturbed mutants did not: loss of both copies of *mtnr1aa* in an *mtnr1ab+/-; mtnr1al+/-* background is sufficient to block the sleep-inducing effect of melatonin. From single mutant experiments, we know that loss of *mtnr1aa* alone does not yield a sleep phenotype (Fig. 2.1), nor does it block the effect of melatonin treatment (Fig. 2.11 A-B). To clarify which of the other two MT1 receptor paralogs, if not both, interacts with the loss of both copies of *mtnr1aa*, we examined the responses of MT1 double mutants to 1 μM melatonin administration. For each experiment, we either crossed *+/-; +/-* parents to *-/-; -/-* parents or we crossed *+/-; -/-* parents to *-/-; +/-* parents, which, in either case, yields four genotypes.

As expected from the triple mutant experiments, all genotypes among the *1ab; 1al* progeny exhibited similar responses to melatonin (Fig. 2.11 G-H). More interestingly, while *1aa+/-; 1ab+/-* and *1aa+/-; 1ab-/-* larvae had normal responses to melatonin (i.e., their locomotor activity levels decreased immediately after melatonin administration), both *1aa-/-; 1ab+/-* and *1aa-/-; 1ab-/-* animals showed a small but significant suppression of melatonin-induced activity decrease (Fig. 2.11 C-D). This suggests that loss of both copies of *mtnr1aa* and one copy of *mtnr1ab* is sufficient to decrease responses to melatonin treatment. We note, however, that neither the *1aa+/-; 1ab-/-* nor *1aa-/-; 1ab-/-* double mutants were as resistant to the effect of melatonin treatment as were the triple homozygous null mutants, and that *1aa-/-; 1ab-/-* animals did not show enhanced suppression compared to *1aa-/-; 1ab+/-* animals. We observed a similar result for the *1aa; 1al* progeny, but with important distinctions. Specifically, responses to melatonin were

noticeably impaired in *1aa-/-; 1al+/-* larvae (although not statistically significant, *P* = 0.0693), and responses were strongly and significantly impaired in *1aa-/-; 1al-/-* larvae (Fig. 2.11 E-F). The diminished responses to melatonin treatment among *1aa-/-; 1al-/-* larvae resembles those of the triple mutant, suggesting that most, if not all, of the effects of melatonin administration on locomotor activity is mediated by *mtnr1aa* and *mtnr1al*, and not by *mtnr1ab.*

To summarize the findings of these experiments, we interpret the 'hierarchy' of MT1 paralogs in mediating melatonin's effect on behavior accordingly: Melatonin acts primarily through the receptor encoded by *mtnr1aa*, and as long as one copy of *mtnr1aa* is functional, *mtnr1ab* and *mtnr1al* are dispensable. This is evidenced by the wild-type responses to melatonin treatment in *mtnr1aa+/-; 1ab-/-; 1al-/-* and *mtnr1ab-/-; mtnr1al-/-* animals (Fig. 2.9, 2.11 G-H). Loss of both copies of *mtnr1aa* in an otherwise wild-type background yields no phenotype, as evidenced by the wild-type levels of locomotor activity and sleep and the wild-type responses to melatonin treatment in *mtnr1aa-/- (1ab+/+; 1al+/+)* mutants (Fig. 2.1, 2.11 A). However, in an *mtnr1aa-/-* background, loss of just one copy of either *mtnr1ab* or *mtnr1al* is sufficient to reduce the effect of exogenous melatonin (Fig. 2.11 C-F). Therefore, we hypothesize that *1ab* and *1al* each compensates for the loss of *1aa*, and that this compensation is only "complete" if both copies of *1ab* and both copies of *1al* are present. Interestingly, suppression of melatonin's effects in *1aa-/-; 1ab+/-* are not dissimilar from *1aa-/-; 1al+/-* (Fig. 2.11 D, F), although *1aa-/-; 1al+/-* did not reach statistical significance compared to *1aa+/-; 1al+/-* controls. This would suggest that *mtnr1ab* and *mtnr1al* are possibly "equipotent" in compensating for loss of both copies of *mtnr1aa.* However, the doubly null *1aa-/-; 1al-/-* show very strong suppression of melatonin's effect, reminiscent of the suppression by *1aa-/-; 1ab-/-; 1al-/-* animals, whereas suppression by *1aa-/-; 1ab-/-* is not nearly as strong. Thus, *mtnr1al* seems to

play a greater role in mediating the effects of melatonin treatment than *mtnr1ab,* but this requires closer examination. The relative contributions of *1ab* and *1al* are difficult to clarify because of their position on the same chromosome, which makes it nearly impossible to accurately measure, among siblings, differences between *mtnr1aa-/-; 1ab+/-; 1al+/-* and, for example, *mtnr1aa-/-; 1ab-/-; 1al+/-.*

On the basis of our findings using exogenously delivered melatonin, as well as the observation that triple null *mtnr1aa-/-; 1ab-/-; 1al-/-* were strongly defective for sleep compared to their *1aa+/+; 1ab+/+; 1al+/+* cousins, we anticipated that we could detect differences among the genotypes from a cross between triple heterozygous MT1 receptor parents to triple null MT1 receptor parents. Indeed, we observed that *mtnr1aa-/-; 1ab-/-; 1al-/-* animals were significantly more active at night (Fig. 2.12 A, D) and significantly impaired for sleep at night (Fig. 2.12 B, F) compared to triple heterozygous siblings. Triple null mutants had decreased sleep bout lengths and were slower to fall asleep (Fig. 2.12 G, I); these effects on sleep architecture are consistent with those observed in the 2-genotype cousin experiment (Fig. 2.7 G-I). While not statistically significant, *mtnr1aa-/-; 1ab+/-; 1al+/-* animals also exhibited increased activity and decreased sleep at night, as well as shorter sleep bouts and longer latency to sleep, relative to triple heterozygotes. This is consistent with the hypothesis that *mtnr1aa* is the primary MT1 paralog for the sleep-promoting actions of melatonin, and that compensation by *mtnr1ab* and/or *mtnr1al* is less effective in a *1ab+/-; 1al+/-* background.

We note that, for most metrics, the *mtnr1aa+/-; mtnr1ab-/-; mtnr1al-/-* siblings showed an intermediate effect between triple heterozygous controls and the *mtnr1aa-/-; mtnr1ab+/-; mtnr1al+/-* siblings. This is inconsistent with our observations using exogenous melatonin, and points to some contribution of *mtnr1ab* and/or *mtnr1al* in regulating sleep that is independent of their roles in compensating for loss of *mtnr1aa.*

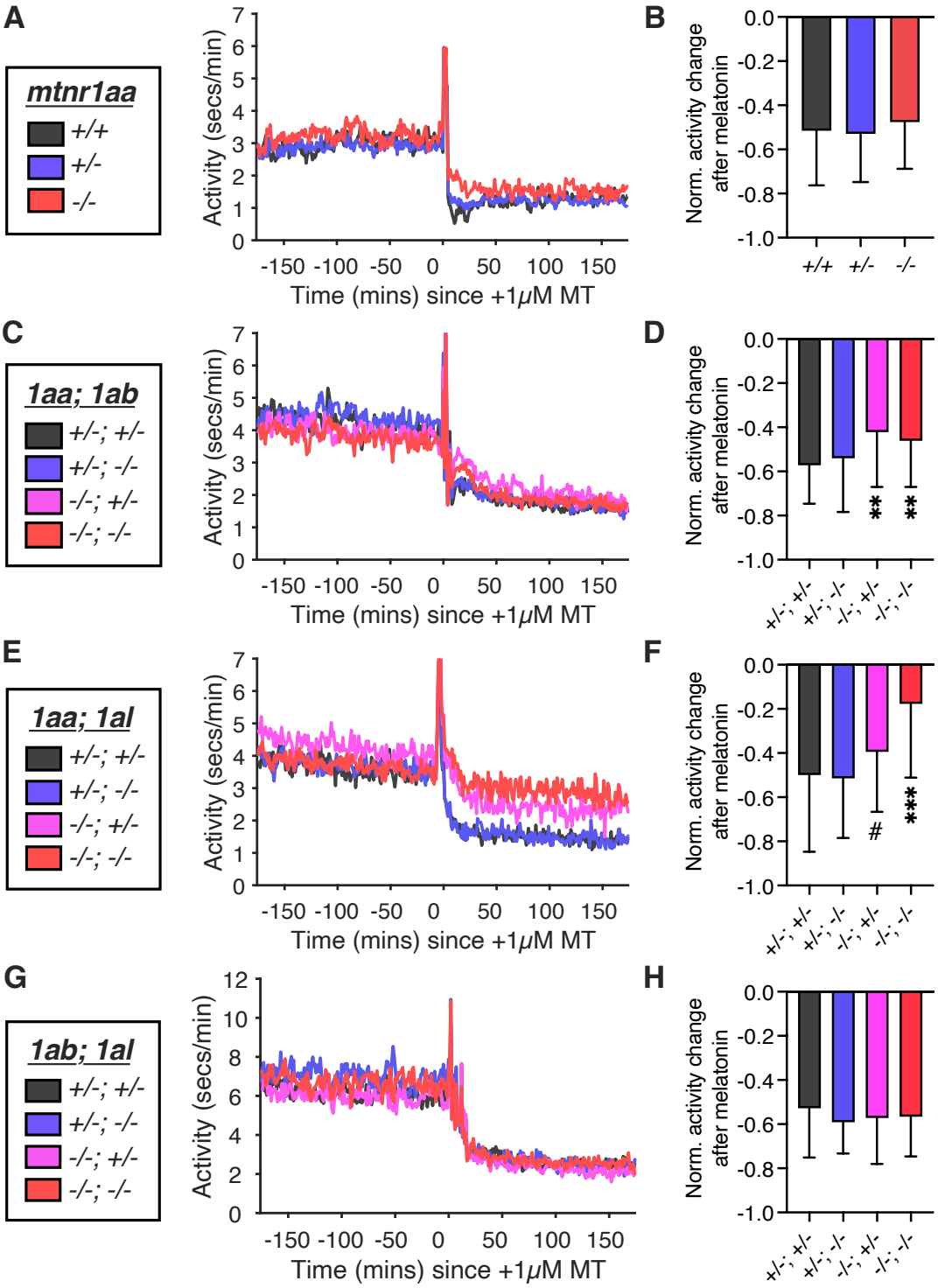

Figure 2.11. Locomotor activity changes induced by exogenous melatonin are suppressed upon mutation of both copies of the *mtnr1aa* gene *and* mutation of one or more copies of *mtnr1ab* or *mtnr1al*. (Continued on next page.)

Figure 2.11 (cont'd). (A) Average locomotor activity of 6-dpf *mtnr1aa+/-* (black), *mtnr1aa+/-* (blue), and *mtnr1aa-/-* (red) larvae during 3 hours before and after administering 1 μM melatonin (MT) at t = 0. **(B)** Mean locomotor activity changes after MT administration normalized to pre-treatment baseline locomotor activity ((post-MT mean activity – pre-MT mean activity) / pre-MT mean activity). Mean activities are calculated over the 3-hr windows pre- and post-MT plotted in (A) but exclude 20 mins preceding and following time of MT administration. Error bars represent s.d. **(C-D)** Same as in (A) and (B), respectively, but for *mtnr1aa+/-; 1ab+/-* (black), *mtrn1aa+/-; 1ab-/-* (blue), *mtnr1aa-/-; 1ab+/-* (pink), and *mtnr1aa-/-; 1ab-/-* (red). Mutation of *mtnr1aa* in a *1ab+/-* or *1ab-/-* background is sufficient to significantly inhibit the effect of MT treatment on locomotor activity. **(E-F)** Same as in (A) and (B), respectively, but for *mtnr1aa+/-; 1al+/-* (black), *mtrn1aa+/-; 1al-/-* (blue), *mtnr1aa-/-; 1al+/-* (pink), and *mtnr1aa-/-; 1al-/-* (red). Mutation of *mtnr1aa* in a *1al-/-* background is sufficient to significantly inhibit the effect of MT treatment on locomotor activity. **(G-H)** Same as in (A) and (B), respectively, but for *mtnr1ab+/-; 1al+/-* (black), *mtrn1ab+/-; 1al-/-* (blue), *mtnr1ab-/-; 1al+/-* (pink), and *mtnr1ab-/-; 1al-/-* (red). ∗∗ = $P < 0.01$, ∗∗∗ = $P < 0.001$ vs. control genotypes (black bar in each panel); Kruskal-Wallis test with Dunn's multiple comparisons test. # indicates $P = 0.0693$. All other comparisons to the control genotypes are not significant and not annotated.

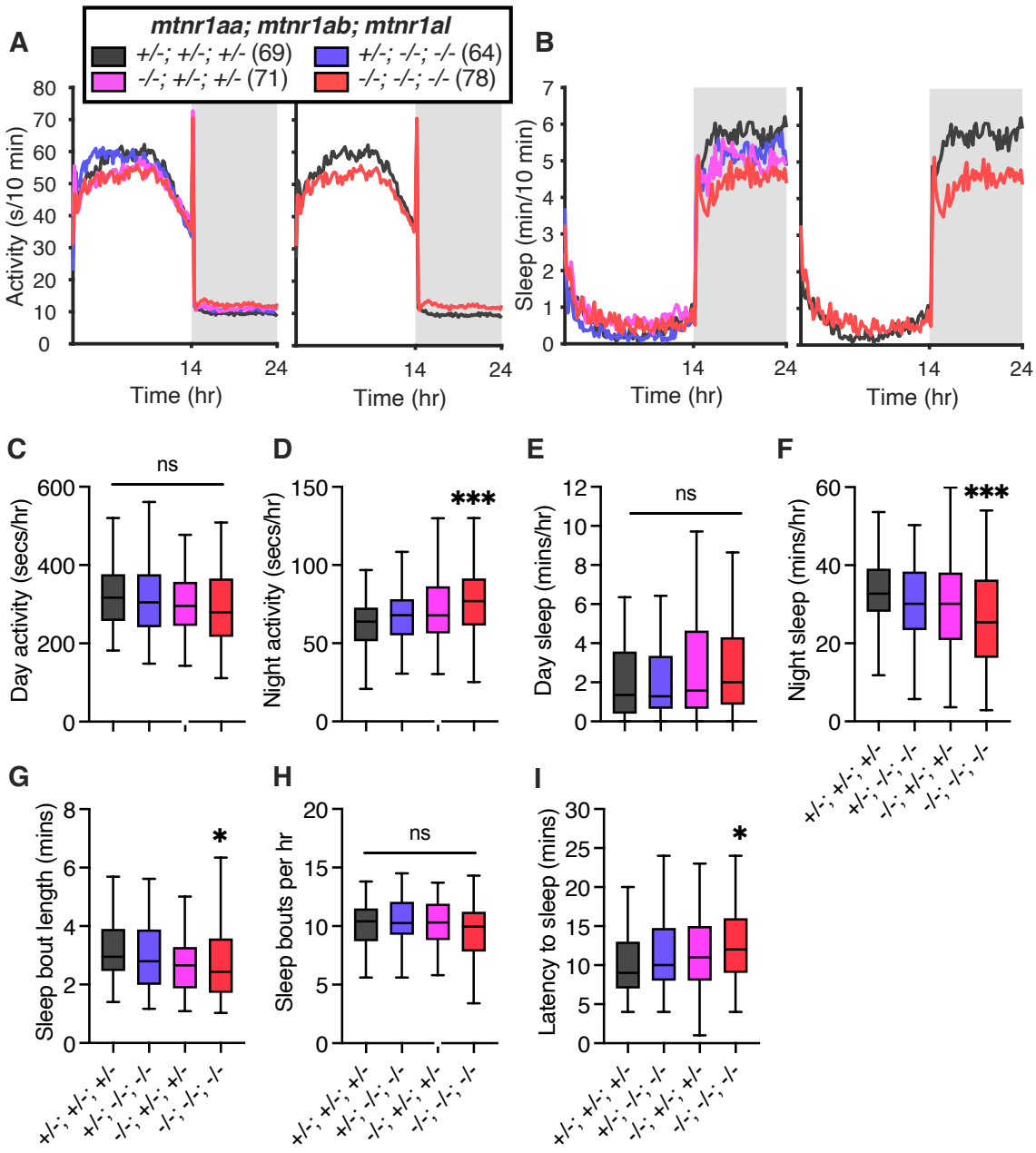

Figure 2.12. Mutation of all three MT1 paralogs is required for significantly reduced sleep at night compared to triple heterozygous siblings. (Continued on next page.)

Figure 2.12 (cont'd). (A-B) Average locomotor activity (A) and sleep (B) during day 5 (unshaded segment) and night 5 (shaded segment) of *mtnr1aa+/-; 1ab+/-; 1al+/-* (black), *mtnr1aa+/-; 1ab-/-; 1al-/-* (blue), *mtnr1aa-/-; 1ab+/-; 1al+/-* (pink), and *mtnr1aa-/-; 1ab-/-; 1al-/-* (red) siblings. Left plots show traces for all four genotypes, and right plots show only the triple heterozygous and triple homozygous mutant genotypes for clarity and for comparison to the traces in Figure 2.7. **(C-D)** Box plot of locomotor activity measurements during day 5 (C) and night 5 (D) for each genotype. *mtnr1aa-/-; 1ab-/-; 1al-/-* are significantly more active at night compared to triple heterozygotes. **(E-F)** Box plot of sleep amounts during day 5 (E) and night 5 (F) for each genotype. *mtnr1aa-/-; 1ab-/-; 1al-/-* animals sleep significantly less at night compared to triple heterozygotes. **(G-I)** Box plot of average sleep bout lengths (G), average number of sleep bouts per hour (H), and latencies to first sleep bout (I) during night 5 for each genotype. Triple null siblings have significantly shorter sleep bouts and significantly longer sleep latency compared to triple heterozygotes. Data are pooled from 4 video-tracker experiments. Number of animals represented in each genotype are indicated in parentheses in legend. Box plots use Tukey method for determining whiskers and outliers; outliers are not shown. ns = not significant ($P > 0.05$); ∗ = $P < 0.05$, ∗∗∗ = $P < 0.001$ vs. *mtnr1aa+/-; 1ab+/-; 1al+/-*; Kruskal-Wallis test with Dunn's multiple comparisons test. All other comparisons to the control genotype are not significant and not annotated.

2.5 A whole exon knockout allele of *mtnr1aa* phenocopies MT1 receptor triple mutants

Our observation that *mtnr1aa-/-* mutants had no sleep phenotype, nor did they show any suppression of melatonin-induced sleep as single mutants (i.e., in a *mtnr1ab+/+; mtnr1al+/+* background), but that *mtnr1aa-/-; 1ab+/-; 1al+/-* were resistant to the effects of melatonin treatment, suggested to us that *mtnr1aa* may be the primary receptor through which melatonin exerts its effects, and that its loss can be compensated by its paralogs *mtnr1ab* and *mtnr1al*. Genetic compensation is a process by which mRNA transcripts containing premature termination codons (PTCs) (due to a nonsense or frameshift mutation, for example) are marked for degradation, and mRNA fragments resulting from this degradation drive the up-regulation of similar genes on the basis of their homology to the degraded transcripts' fragments (Wilkinson, 2019; El-Brolosy et al., 2019). These up-regulated genes could then substitute in function for the mutated gene.

Genetic compensation depends on the transcription and subsequent detection of PTC-bearing transcripts. For genes where nonsense or frameshift mutations would result in genetic compensation by paralogous genes, it was shown that by deleting (knocking out) the entire coding region of that gene, where no erroneous transcript can be synthesized, genetic compensation was abolished (El-Brolosy et al., 2019). We wished to test the hypothesis that *mtnr1ab* and *mtnr1al* are compensating for the loss of *mtnr1aa*, and that the heterozygosity at either *mtnr1ab* or *mtnr1al* renders this compensation significantly less effective, which results in the emergence of behavioral phenotypes. To this end, we created a 'whole-exon knockout' (Ex1-KO) allele of *mtnr1aa* using CRISPR guides that are designed to target 50 bp upstream of the predicted transcription start site (based on Ensembl RNA-Seq models) and 380 bp downstream of the first (of two) *mtnr1aa*

exon (Table 2.1). Because the intron between the two exons that comprise *mtnr1aa* is 83.7 kbps in length, we chose to knock out only the first exon rather than the entire gene. From CRISPR injections, we recovered two lines of *mtnr1aa(Ex1-KO)* mutants in which the DNA sequence between the CRISPR guides' targets (i.e., the region encoding *mtnr1aa* exon 1) was apparently absent, as determined by PCR. We therefore anticipate that *mtnr1aa* mRNA is not transcribed in homozygous *mtnr1aa(Ex1-KO)-/-* animals and that genetic compensation of *mtnr1aa* by *mtnr1ab* and *mtnr1al* should be absent. If the lack of phenotype in the *mtnr1aa* 20-bp deletion (d20) mutant (Fig. 2.1) is due to genetic compensation, we expect *mtnr1aa(Ex1-KO)-/-* larvae to phenocopy the *mtnr1aa-/-; 1ab-/-; 1al-/-* triple mutant animals. If, on the other hand, *mtnr1aa(Ex1-KO)-/-* larvae resemble the *mtnr1aa(d20)-/-* mutant and exhibit no sleep phenotype, then the relationship between *mtnr1aa*, *mtnr1ab*, and *mtnr1al* is more complicated.

Homozygous mutant *mtnr1aa(Ex1-KO)* larvae were significantly more active (Fig. 2.13 A, D) and slept less (Fig. 2.13 B, F) at night compared to their wild-type siblings. The sleep defect was accompanied by a significant decrease in average sleep bout length (Fig. 2.13 G) and a longer latency to sleep (Fig. 2.13 I). These phenotypes resemble those of *mtnr1aa-/-; 1ab-/-; 1al-/-* animals (Figs 2.7 and 2.12).

Because the sleep phenotype of the *mtnr1aa(Ex1-KO)* mutant resembles that of the triple null mutant, we conclude that melatonin primarily promotes sleep via *mtnr1aa*, and that *mtnr1ab* and *mtnr1al* provide genetic compensation in the *mtnr1aa(d20)* mutant. However, because we have not directly compared *mtnr1aa(Ex1-KO)* mutants to triple null MT1 receptor mutants or to melatonin-deficient *aanat2* mutants, nor have we generated *mtnr1ab* or *mtnr1al* whole-exon deletion lines, we cannot completely rule out other configurations.

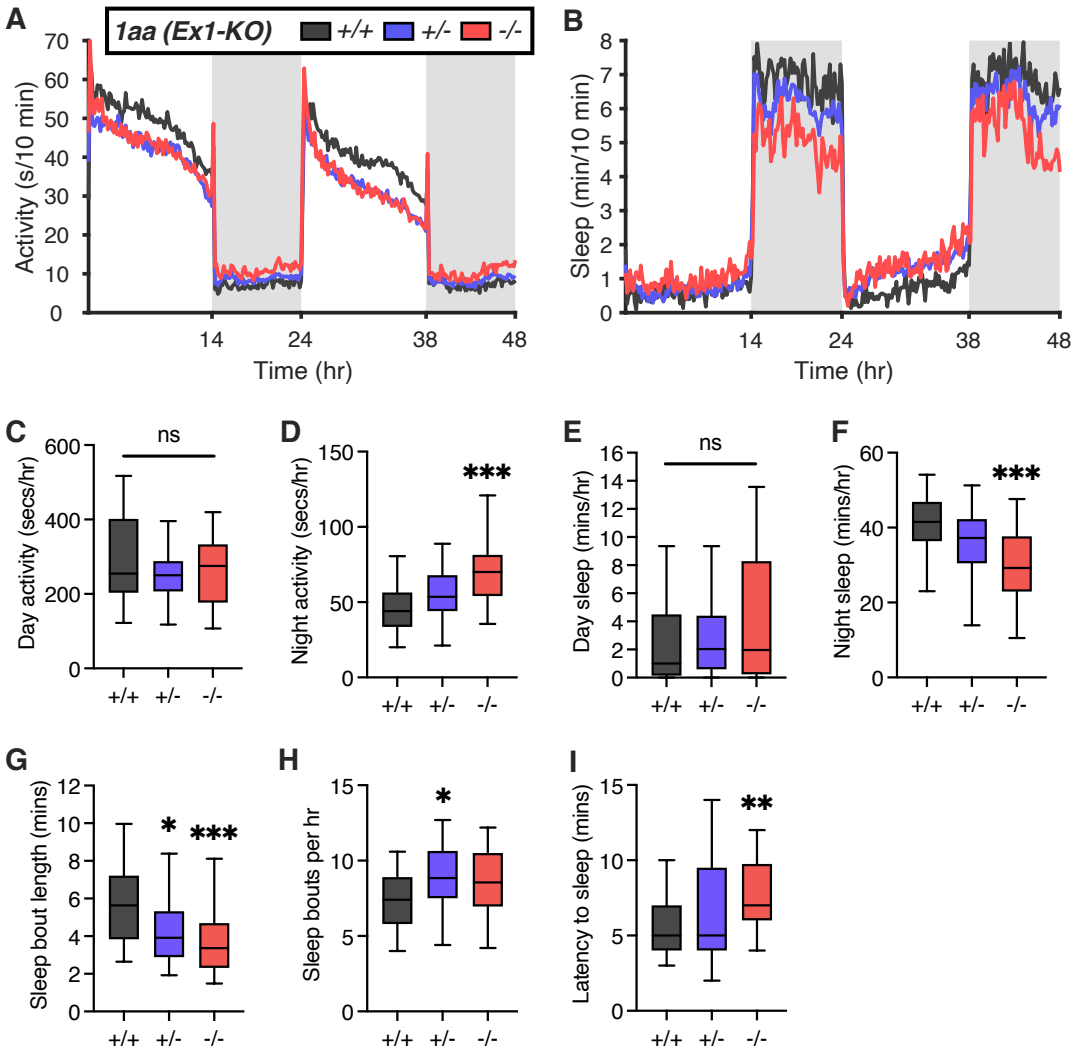

Figure 2.13. Deletion of the first exon of *mtnr1aa* causes significant defects in sleep. **(A-B)** Average locomotor activity (A) and sleep (B) over two days (unshaded segments) and nights (shaded segments) of 6- to 7-dpf *mtnr1aa+/+* (black), *mtnr1aa+/-* (blue), and *mtnr1aa-/-* (red) whole exon deletion (Ex1-KO) mutants. **(C-D)** Box plot of locomotor activity measurements during day 6 (C) and night 6 (D) of *mtnr1aa(Ex1-KO)+/+* (black), *+/-* (blue), and *-/-* (red) siblings. *mtnr1aa(Ex1-KO)-/-* mutants are significantly more active during the night compared to *+/+* controls. **(E-F)** Box plot of sleep amounts during day 6 (E) and night 6 (F) for each genotype. *mtnr1aa(Ex1-KO)-/-* mutants sleep less during the night than *+/+* controls. **(G-I)** Box plot of average sleep bout lengths (G), average number of sleep bouts per hour (H), and latencies to first sleep bout (I) during night 6 for each genotype. *mtnr1aa(Ex1-KO)-/-* mutants have significantly shorter sleep bouts and significantly longer sleep latency compared to *+/+* controls. Data are pooled from 2 video-tracker experiments, n = 23 for *1aa(Ex1-KO)+/+,* 56 for *1aa(Ex1-KO)+/-*, and 36 for *1aa(Ex1-KO)-/-*. Box plots use Tukey method for determining whiskers and outliers; outliers are not shown. ns = not significant ($P > 0.05$), ∗ = $P < 0.05$, ∗∗ = $P < 0.01$, ∗∗∗ = $P < 0.001$ vs. *+/+*; Kruskal-Wallis test with Dunn's multiple comparisons test. All other comparisons to the control genotype are not significant and not annotated.

2.6 MT1 receptors are expressed broadly throughout the larval zebrafish brain

We anticipated that the site(s) of melatonin receptor expression, specifically that of the MT1 family (i.e., *mtnr1aa, mtnr1ab,* and *mtnr1al*), would reveal which regions of the brain are immediately affected by melatonin and therefore provide insight as to *how* melatonin promotes sleep, a process that is very poorly understood. Melatonin receptors are G-protein coupled receptors (GPCRs) and are reported to interact with $G_{i/o}$ proteins; thus, activation of melatonin receptors results in the reduction of cyclic AMP in a cell that expresses it. We hypothesized that areas of brain that show high melatonin receptor expression would be involved in 'wake' or 'active behaviors,' and that the nocturnal release of melatonin from the pineal would promote sleep by inhibiting neuronal activity in these regions.

GPCRs tend to be expressed at low levels and may share high sequence similarity with other GPCRs, and each of these characteristics makes it difficult to confidently detect melatonin receptor transcripts by traditional *in situ* hybridization (ISH) techniques. First, using ISH to detect lowly expressed transcripts may require longer periods of incubation at various steps in the ISH process, which can also enhance background signal or signal from non-specific binding. Second, in transcripts that share high homology with other genes of the same class or family, it is sometimes impossible to synthesize a specific riboprobe that is (a) long enough to offer enough substrate for detection, while (b) specific enough to protect against off-target binding. Moreover, we found that in zebrafish (see below), as in other species (from more recent reports), melatonin receptors have a relatively widespread and diffuse expression pattern in the brain, which adds to the aforementioned difficulty in detecting their expression by obscuring the distinction between relevant signal and background. As a result of these complications, we iterated

through many different types of ISH techniques to identify, with high confidence, the parts of the zebrafish brain that express melatonin receptors. This section is sub-divided based on the four different techniques that were used: fluorescence ISH (FISH), chromogenic ISH, hybridization chain reaction (HCR), and reporter gene fusion using BAC recombineering. Each of these ISH methods were useful in deciphering the expression pattern of the receptors.

Fluorescence ISH. As a first attempt to determine the expression pattern of each melatonin receptor, we generated 1,000~1,500-bp riboprobes that included as much 5' and 3' untranslated region (UTR) of the target transcript as possible; however, for some targets we were limited by the length of transcripts and by the availability of curated UTR sequence. We used a standard FISH protocol on dissected larval brains (see 'Materials and methods'). Figure 2.14 shows the fluorescence detected using *mtnr1aa* riboprobes in three separate planes of the same representative brain. We found widespread signal across many regions of the brain, with some regions showing more concentrated or higher levels of expression. Most notably, we observed high expression in the cell body layer of the optic tectum (the zebrafish homolog of the mammalian superior colliculus), and in a bilateral cluster of cells in the dorsal hindbrain (Fig. 2.14 A, B). In more ventral planes, we find less concentrated signal, but from later ISH approaches and analyses (see below), we note that there is consistent fluorescence in bilateral anterior-to-posterior stripes that cover the dorsal-most region of the preoptic area and ventral-most part of the posterior tuberculum (Fig. 2.14 C). The pattern was consistent across all brains where signal was observable. We also labeled *mtnr1ab* transcripts in dissected brains using the same FISH protocol (data not shown). Like *mtnr1aa*, *mtnr1ab* is also expressed at relatively high levels in the optic tectum. We also found *mtnr1ab* signal in the hindbrain, in four clusters

that surround the *mtnr1aa*-expressing cells. We could not detect any signal by FISH using riboprobes designed to target *mtnr1al*.

Chromogenic ISH. The signal that we observed using FISH was widespread, diffuse, and did not seem to fill cell bodies like we anticipated based on *in situ* data for other genes, like neuropeptide-encoding genes (see positive control in Fig. 2.15 C, for example). This is likely due to these genes being expressed at low levels. As an alternative *in situ* approach to clarify and/or confirm the expression pattern, we used the same riboprobes but with alkaline phosphatase-conjugated antibodies, which, when exposed to the colorless substrate NBT/BCIP, converts the substrate to a blue dye. Typically, FISH is considered more sensitive, but chromogenic ISH lends itself well to longer signal development, using a modified protocol, which we had hoped would reveal a more accurate expression pattern, or confirm the pattern revealed by FISH.

After incubating dissected brains with riboprobe, we allowed the chromogenic signal to develop in a 1:10 diluted NBT/BCIP staining buffer for 5 days in darkness at 4C. Although we were unable to survey the expression pattern with fine resolution using chromogenic ISH, particularly in the dorsal-ventral axis, we found a similar pattern by chromogenic ISH for both *mtnr1aa* and *mtnr1ab* as that which we detected by FISH (Figs. 2.15-2.16). In particular, for *mtnr1aa*, we observed strong signal (relative to other brain regions) throughout the optic tectum and in a small bilateral cluster of cells in the hindbrain. The pattern for *mtnr1ab* also was comprised of optic tectum signal, which we noticed was less intense and possibly more spatially restricted than *mtnr1aa*, as well as the hindbrain populations we observed by FISH. Thus, although the riboprobes used here were the same as those used for FISH, and we cannot therefore rule out non-specificity or another probe-related problem, we detected a common pattern by fluorescent and chromogenic ISH for both *mtnr1aa* and *mtnr1ab*, suggesting that the signal is likely authentic.

Using riboprobes targeting *mtnr1al* and the same prolonged signal development protocol, we were able to detect faint staining in the optic tectum, suggesting that all three MT1-type receptors are expressed in this region, just at different levels (Fig. 2.16 A-C). An alternative explanation is that the faint signal we observe in *mtnr1al* riboprobe-treated brains is the result of weak off-target binding to a different melatonin receptor, such as *mtnr1aa*.

Hybridization chain reaction. As a third ISH-based approach to determine the expression pattern of these receptors, we used hybridization chain reaction (HCR) (Choi et al., 2018). HCR utilizes a set of ~20 pairs of short DNA probes (~20 base pairs long), where each pair of probes recognizes adjacent sequences in the target transcript. Only when a pair of probes binds its respective pair of sequences—adjacent to one another—will a set of fluorescently-tagged DNA hairpins bind the probe pair and then initiate an amplification process by which the hairpins polymerize, enhancing the fluorescent signal. Thus, HCR is not only highly specific, as it requires two specific probes to bind their targets immediately adjacent to one another to recruit the fluorescent hairpins, but it is also potentially useful for detecting transcripts expressed at low levels, as the fluorescent signal is amplified via polymerization of multiple fluorescent hairpins.

We obtained a set of probes designed to target *mtnr1aa* and *mtnr1ab*. The sequences that the probes are designed to bind were queried against the zebrafish genome and chosen on the basis of their specificity, further reducing the chance of signal from off-target binding. However, a probe set recognizing *mtnr1al* could not be designed because the available coding sequence did not contain enough sequence that was unique to *mtnr1al*; there was too much overlap with other *mtnr* genes. We noticed that the available *mtnr1al* sequence was particularly lacking in 3' UTR sequence. In an effort to uncover more 3' UTR sequence, which could be used to generate an *mtnr1al* HCR probe

set, we conducted a series of PCR reactions from cDNA template with a constant 5' primer positioned in the first exon of the gene, paired with different 3' primers spaced along a putative 3' UTR. We were able to amplify a product using reverse primers that targeted up to 1,025 bp after the stop codon, suggesting the presence of at least 1,025 bp of 3'UTR. Even when using a probe set designed around this longer sequence, however, we observed no signal by HCR for *mtnr1al*.

Encouragingly, we found an expression pattern for *mtnr1aa* and *mtnr1ab* by HCR that matched the patterns we observed by fluorescent and chromogenic ISH, and we were able to detect that pattern using whole larvae, for which the HCR protocol is optimized (as opposed to dissected brains in the ISH attempts above) (Fig. 2.17). This gave us the opportunity to look for expression in parts of the brain that can be damaged or missing due to dissection (namely, the most dorsal and posterior regions), as well as in anatomical regions outside of the brain (i.e., the retina). It also gave us the opportunity to register HCR-labeled larvae to a reference larva, which we could then input to a MATLAB-based ZBrainViewer that allows us to identify the specific brain regions where we see the highest expression (Fig. 2.17 A', B', C') (Randlett et al., 2015).

Once again, the signal was punctate (i.e., did not fill cell bodies) and widespread, which, in the areas of lowest and most diffuse expression, still made it difficult to distinguish the signal from background. Nevertheless, we noted consistent, concentrated labeling for *mtnr1aa* in the following regions: the cell body layer of the optic tectum (Fig. 2.17 A, B), the same bilateral cluster of hindbrain cells we observed by previous ISH techniques (which are just dorsal to the octavolateralis efferent neurons (OENs)) (Fig. 2.17 A, A'), bilateral stripes in the preoptic area / posterior tuberculum (Fig. 2.17 C, C'), the habenula (not shown), the inferior olive (Fig. 2.17 B, B'), and in two cell layers of the retina: the retinal ganglion cell (RGC) layer and the inner nuclear layer (INL) (Fig. 2.18 C, C'). We

note that this is very likely not an exhaustive list of regions where *mtnr1aa* transcripts are present but instead are the areas with the highest observable expression.

Although we have not registered *mtnr1ab* HCR-labeled larvae to the reference larva, we observed expression in the following regions: the cell body layer of the optic tectum (Fig. 2.18 A-B), four hindbrain clusters that straddle the *mtnr1aa* cluster laterally and rostrocaudally Fig. 2.18 A), the cerebellum (not shown), the habenula (not shown), and in the same two layers of the retina (RGC and INL) that express *mtnr1aa* (Fig. 2.18 C, C''). We saw less *mtnr1ab* signal in more ventral parts of the brain compared to *mtnr1aa.* Because of the punctate nature of the fluorescence, we could not judge whether or not regions where we saw signal for both *mtnr1aa* and *mtnr1ab* (i.e., the optic tectum, retina, and habenula) contained cells that co-expressed both receptors, even when labeling both in the same larva.

Moreover, we could not confidently ascertain the neurotransmitter identify of most *mtnr1aa*-expressing cells through colocalization experiments, again due to the nature of the *mtnr1aa* HCR signal. The *mtnr1aa* hindbrain cluster is the most 'cellular' appearing signal, and we therefore tried to gauge its overlap with fluorescence from HCR riboprobe sets that target (1) *chata* and *chatb*, markers for cholinergic neurons, (2) *gad1b* and *gad2*, markers for GABAergic neurons, (3) *vglut1, vglut2a,* and *vglut2b*, markers for glutamatergic neurons, and (4) *glyt2*, a marker for glycinergic neurons. We noticed that the *mtnr1aa* signal seemed to be apposite to, and not overlapping, the nearby *chata/chatb* and *gad1b*/*gad2* signal in the hindbrain, suggesting that the cells comprising the *mtnr1aa* hindbrain cluster are neither cholinergic nor GABAergic (Fig. 2.19). The *mtnr1aa* hindbrain cells are also not glycinergic, as we found no *glyt2*-positive cells nearby (data not shown). We suspect that the *mtnr1aa*-expressing hindbrain cells are therefore glutamatergic, but the *vglut1/2a/2b* probe set yielded no signal, and we could not look for co-localization as

a result. We also tried a different HCR probe set, with sequences targeting *vglut2a* used in a published study (Lovett-Barron et al., 2020), but again could not identify any signal.

Reporter gene fusion by BAC recombination. As a complementary approach that would also provide potentially useful genetic access to *mtnr1aa*-expressing cells, we wished to generate a transgenic reporter line by placing GAL4 under control of *mtnr1aa* regulatory sequence to support its expression in *mtnr1aa*-positive cells. To help ensure that all of the sequence elements required to fully recapitulate *mtnr1aa* expression are present in the transgenic construct, we opted to recombine the GAL4 coding sequence into a bacterial artificial chromosome (BAC) containing ~160 kb of genomic sequence surrounding and including the *mtnr1aa* locus (Fig. 2.20 A) (Bussmann & Schulte-Merker, 2011). GAL4 was placed in-frame at the translational start site of *mtnr1aa* in the BAC construct, and Tol2 donor sites were placed in the 'backbone' of the BAC to facilitate insertion using Tol2 transposase-mediated transgenesis (Fig. 2.20 A). The confirmed and purified *mtnr1aa:GAL4* BAC was injected into *UAS:GFP* embryos at the 1-cell stage along with Tol2 mRNA.

We recovered two stable lines carrying a heritable *mtnr1aa:GAL4* transgene, each of which surprisingly supported expression of the *UAS:GFP* effector in distinct and limited anatomical regions. In the first, GFP was exclusively expressed in the INL of the retina (Fig. 2.20 B). *In situ* data validates this expression since we found *mtnr1aa* signal in this layer of the retina by HCR (Fig. 2.18 C, C'). In the second line, we observed strong GFP expression in the torus longitudinalis (TL) and some GFP-positive cells in the habenula (Fig. 2.20 C). Habenula expression had been observed by HCR, but we had not identified expression in the TL from *in situ* experiments, likely because the structure is near the dorsal surface of the brain. In dissected brains, the TL may be easily damaged during dissection, and in whole mount, the structure curves along the dorsal surface of the larva,

making it difficult to assign any signal in that area specifically to the TL. We verified the presence of *mtnr1aa* transcripts in the TL by HCR in the TL-GFP-positive *mtnr1aa:GAL4; UAS:GFP* line, and we indeed saw co-localization of *mtnr1aa* and GFP fluorescence, although the expression domains did not perfectly overlap (Fig. 2.20 D-D'').

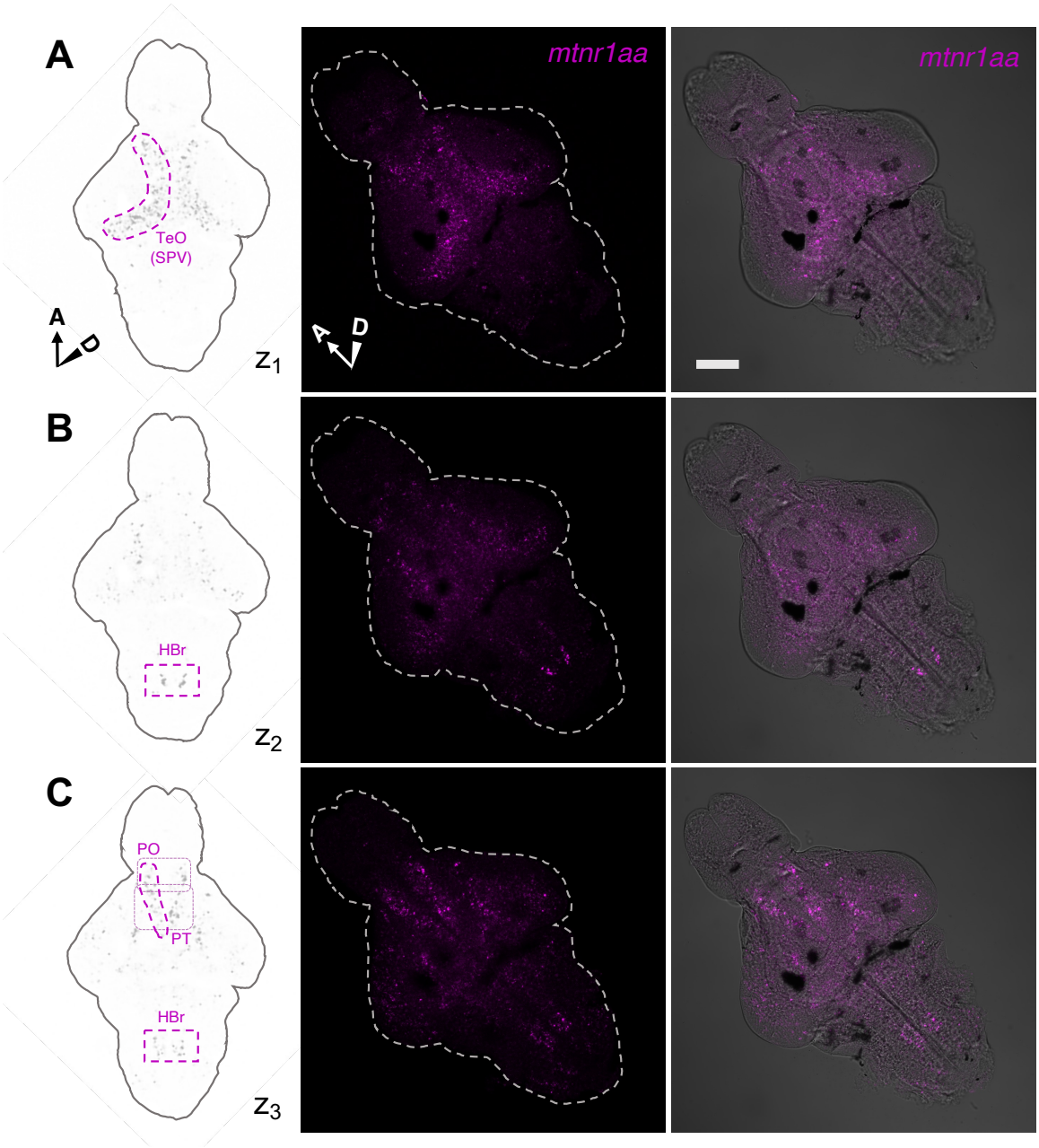

Figure 2.14. Expression pattern of *mtnr1aa* using fluorescence *in situ* hybridization (FISH). (A) (Left) Illustration of the signal depicted in the right panels. There is prominent expression in the cell body layers (stratum periventriculare, SPV) of the optic tectum (TeO). (Center) Fluorescence from riboprobes targeting *mtnr1aa* in a dissected wild-type brain. (Right) Same as (center) but with bright-field image overlaid to show anatomy. Dark spots are pigmented melanocytes from residual skin. **(B)** Same as in (A) but a more ventral plane, which shows *mtnr1aa* expression in the ventral layers of the TeO and in a bilateral cluster of cells (indicated by square) in the hindbrain. **(C)** Same is an (B) but a more ventral plane, which shows *mtnr1aa* expression in two stripes that pass through the preoptic area (PO) and the posterior tuberculum (PT). Scale bar = 100 μm. A = anterior, D = dorsal; z_1 - z_n refer to different z-axis planes of the same brain.

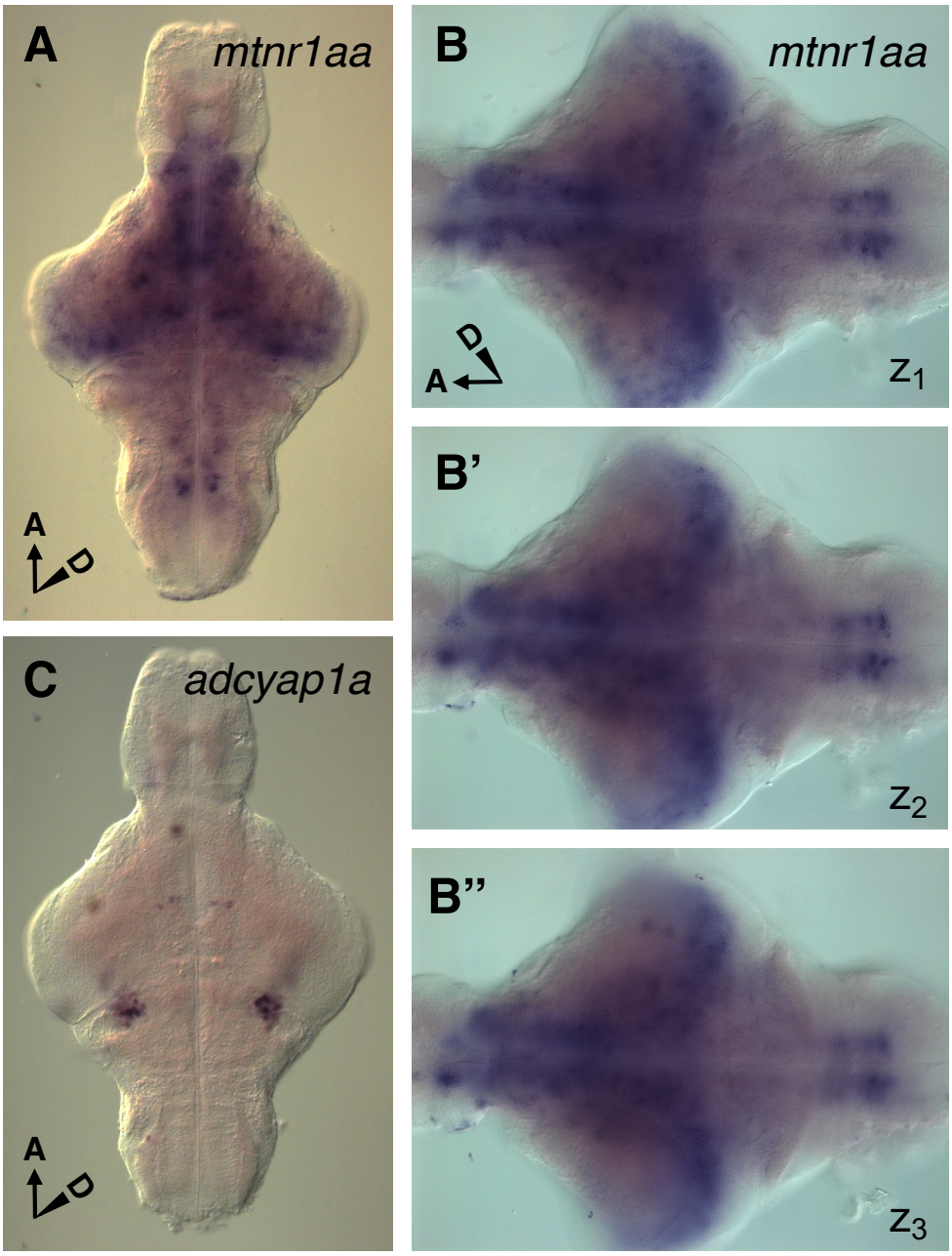

Figure 2.15. Expression pattern of *mtnr1aa* using chromogenic *in situ* hybridization. **(A)** Bright-field image of chromogenic signal in a dissected wild-type brain treated with *mtnr1aa*-targeting riboprobes. Signal is evident in the optic tectum and in a bilateral cluster of cells in the hindbrain. **(B-B")** DIC images showing *mtnr1aa* chromogenic signal across three focal planes in the same dissected wild-type brain. **(C)** Bright-field image of chromogenic signal using a riboprobe targeting the neuropeptide-encoding gene *adcyap1a*. This is provided as an example of a discrete, 'cellular'-appearing pattern by *in situ* hybridization. A = anterior, D = dorsal; z_1 - z_n refer to different z-axis planes of the same brain.

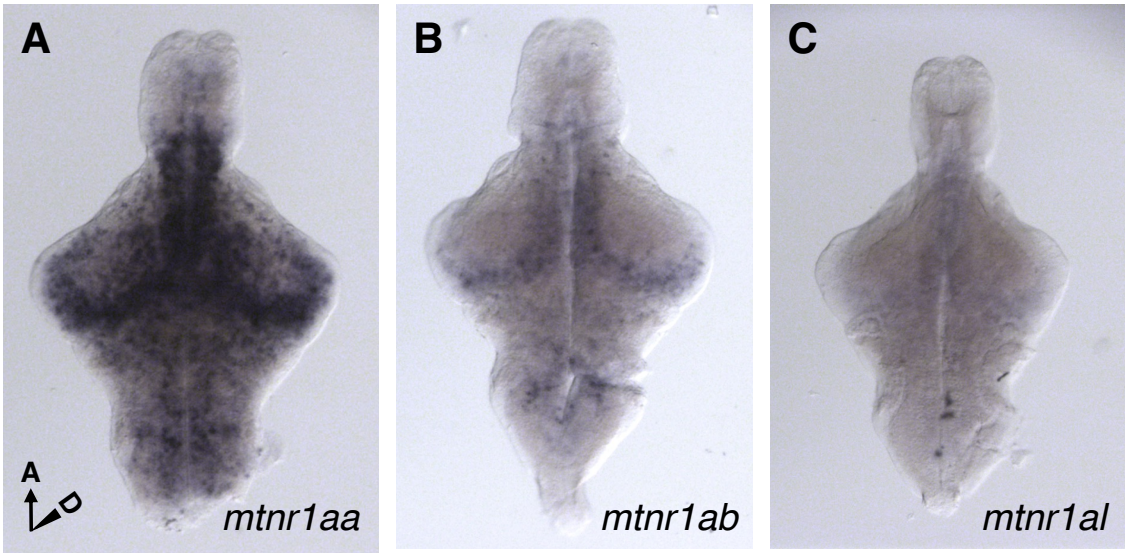

Figure 2.16. Expression pattern of *mtnr1aa, mtnr1ab,* and *mtnr1al* using chromogenic *in situ* hybridization. Bright-field images of chromogenic signal in dissected wild-type brains treated with riboprobes targeting either *mtnr1aa* (A), *mtnr1ab* (B), or *mtnr1al* (C). All three riboprobes yielded signal in the optic tectum, with differing intensities (*mtnr1aa* > *mtnr1ab* > *mtnr1al*). *mtnr1ab* signal was observed in the hindbrain, adjacent to the *mtnr1aa*-expressing cluster. A = anterior, D = dorsal.

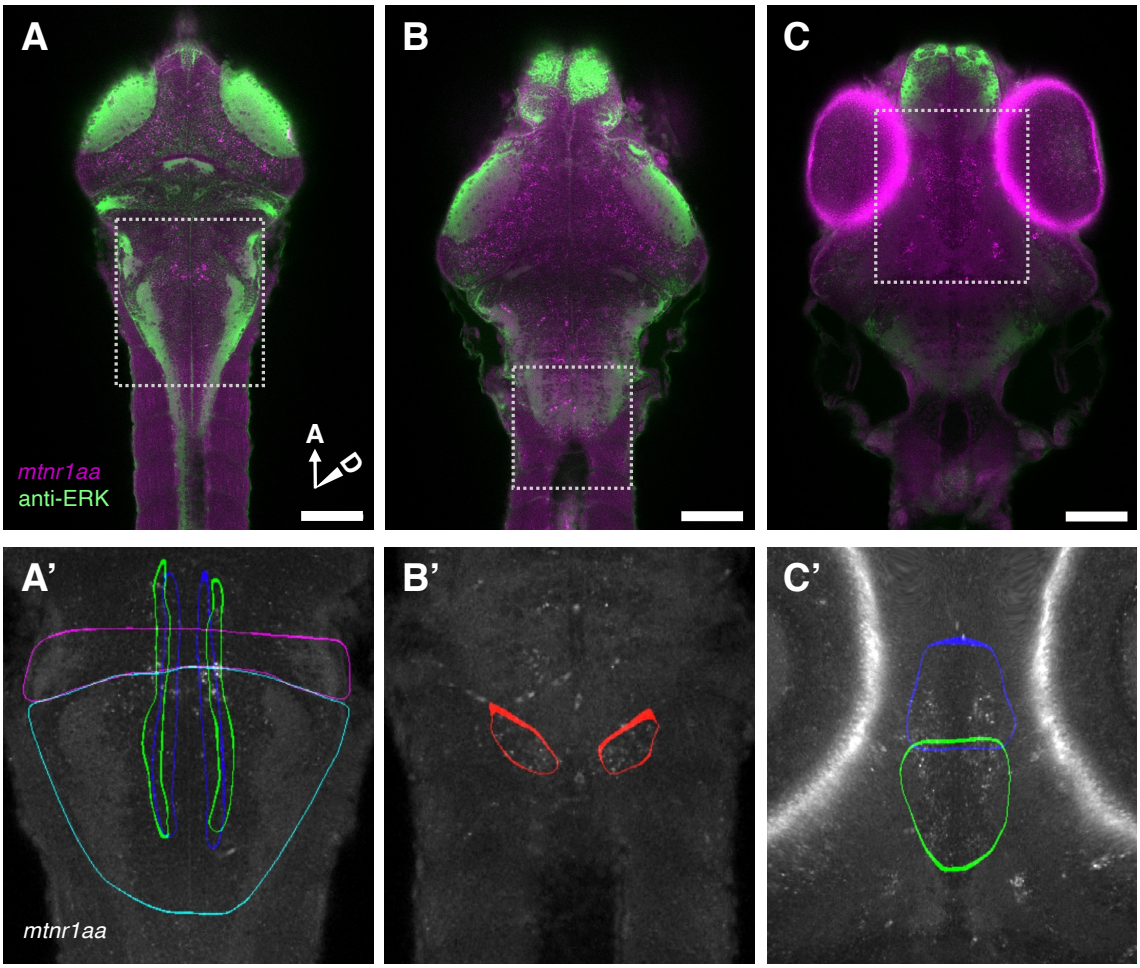

Figure 2.17. Expression pattern of *mtnr1aa* using hybridization chain reaction (HCR) ISH. (A) Confocal image showing fluorescence from *mtnr1aa* HCR (magenta) and anti-ERK antibody (green) in a wild-type larva. *mtnr1aa* expression was observed in the optic tectum and in a cluster of cells in the hindbrain, corroborating the pattern produced by other ISH methods. ERK co-staining was performed in order to register the larva to a reference atlas (ZBrain), which was used to more confidently assign the *mtnr1aa* signal to specific anatomical regions. A magnified section (dotted square) is shown in (A') after registration, which shows the *mtnr1aa* hindbrain cluster placed between rhombomeres 6 (magenta) and 7 (cyan) and straddling specific neurotransmitter-expressing stripes referred to as '*vglut2* stripe3' (green) and '*glyt2* stripe3' (blue) by the ZBrain atlas. **(B)** Same as in (A) but a more ventral focal plane. The magnified, registered region in (B') shows the *mtnr1aa* signal localized to the inferior olive (red). **(C)** Same as in (B) but a more ventral focal plane. The magnified, registered region in (C') shows the *mtnr1aa* signal spanning the preoptic area (blue) and posterior tuberculum (green). Scale bar = 100 μm. A = anterior, D = dorsal.

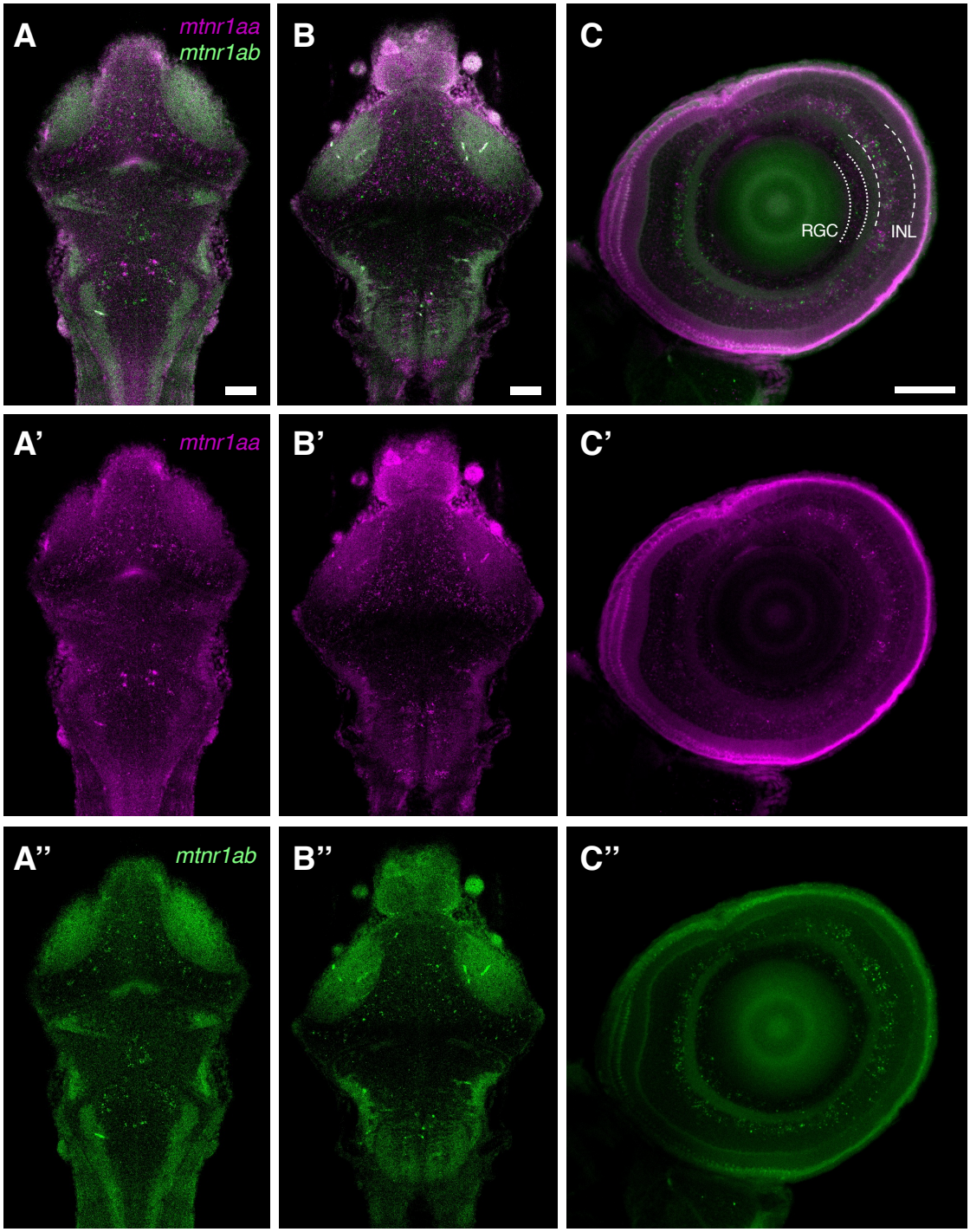

Figure 2.18. Expression pattern of *mtnr1aa* and *mtnr1ab* using hybridization chain reaction (HCR) ISH. Confocal images showing fluorescence from *mtnr1aa* HCR (magenta) and *mtnr1ab* HCR (green) in two focal planes (A, B) and in the retina (C). Both *mtnr1aa* and *mtnr1ab* signal is observed in the optic tectum, in non-overlapping clusters in the hindbrain, and in both the inner nuclear layer (INL; example segment labeled with dashed line) and retinal ganglion cell (RGC; example segment labeled with dotted line) layer of the retina. *mtnr1aa* signal is also present in the inferior olive. Scale bar = 50 μm.

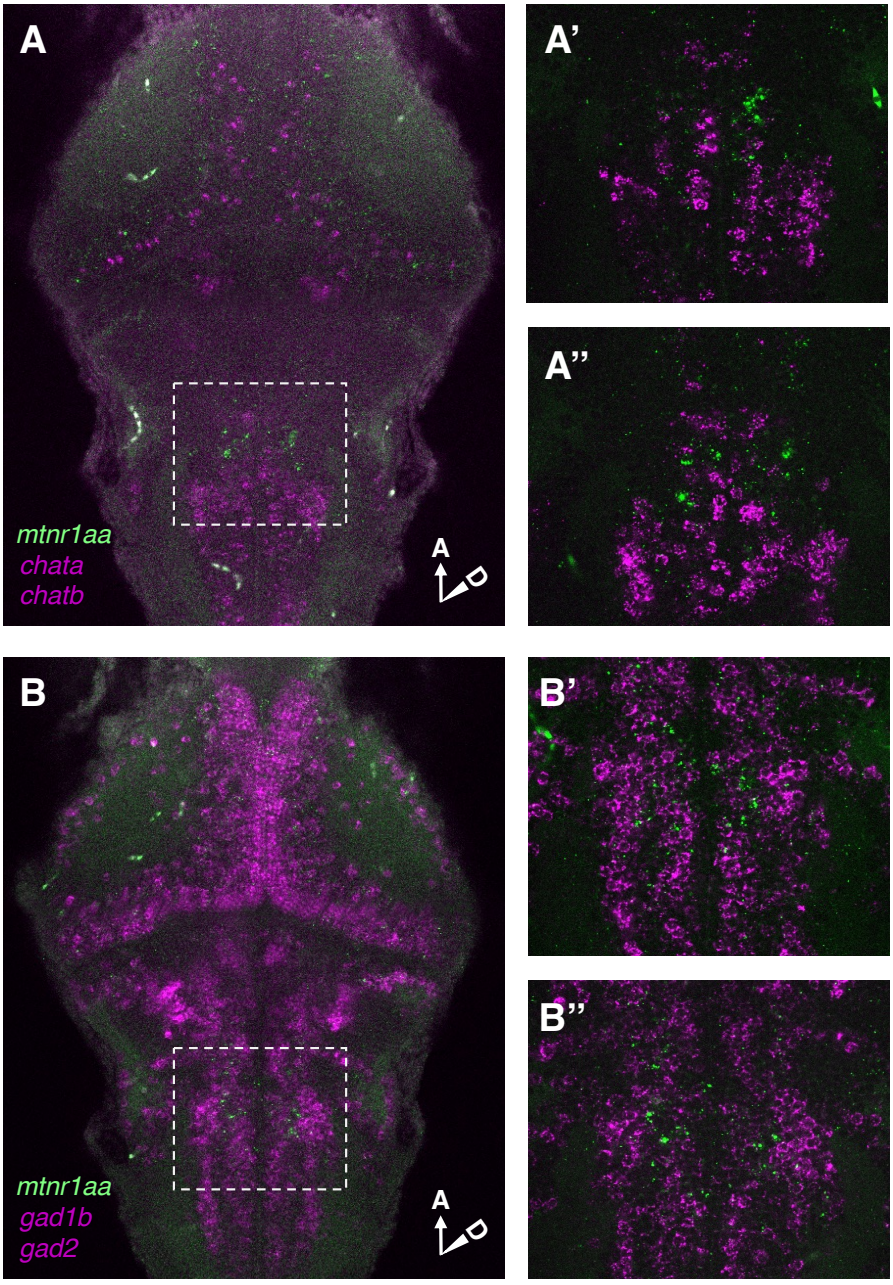

Figure 2.19. *mtnr1aa* HCR fluorescence in the hindbrain does not overlap with markers for acetylcholinergic neurons or GABAergic neurons. (A) Confocal images showing fluorescence from *mtnr1aa* HCR (green) and both *chata* and *chatb* (combined) HCR (magenta). The *mtnr1aa*-positive hindbrain cells do not overlap with *chata/chatb* cells. Images acquired with a 20X objective are shown in (A), while magnified images (40X objective) of area inside dashed square in two separate larvae are shown in (A'-A''). **(B)** Confocal images showing fluorescence from *mtnr1aa* HCR (green) and both *gad1b* and *gad2* (combined) HCR (magenta). The *mtnr1aa*-positive hindbrain cells do not overlap with *gad1b/gad2* cells. Images acquired with a 20X objective are shown in (B), while magnified images (40X objective) of area inside dashed square in two separate larvae are shown in (B'-B''). A = anterior, D = dorsal.

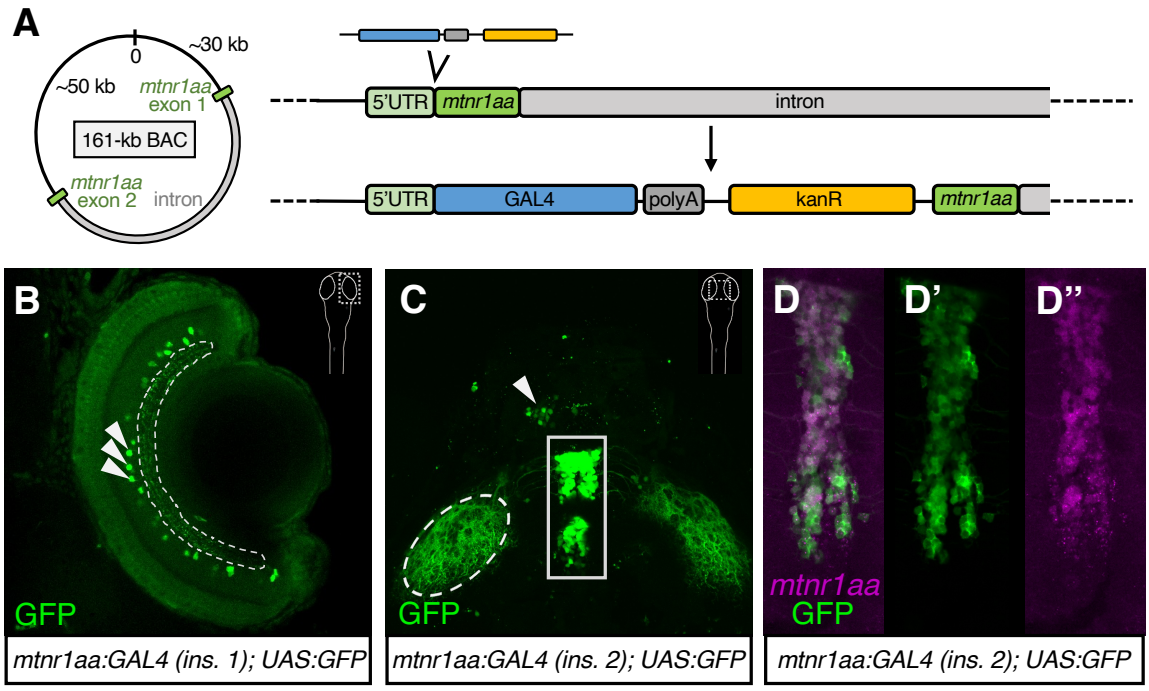

Figure 2.20. *Tg(mtnr1aa:GAL4)* transgenic lines show expression in the retina and the torus longitudinalis. (A) Schematic of the ~160-kb bacterial artificial chromosome (BAC) that contains the entire *mtnr1aa* locus and ~80-kb surrounding sequence (left), and the recombination strategy wherein the GAL4 transgene was inserted in-frame at the translational start site of *mtnr1aa* (right). **(B)** GAL4-driven expression of *UAS:GFP* in the retina in one *Tg(mtnr1aa:GAL4); Tg(UAS:GFP)* line ("*ins. 1*"). GFP fluorescence is exclusive to cells of the inner nuclear layer (arrowheads) and projections in the inner plexiform layer (dashed line). **(C)** GAL4-driven expression of *UAS:GFP* in the torus longitudinalis (TL, squared region) and in some cells of the habenula (arrowhead) in another *Tg(mtnr1aa:GAL4); Tg(UAS:GFP)* line ("*ins. 2*"). GFP fluorescence in optic tectum neuropil (dashed circle) is from TL projections. In panels (B) and (C), images are of live embedded larvae. **(D)** *mtnr1aa* (magenta) and GFP (green) HCR signal overlap the TL of a fixed *mtnr1aa:GAL (ins. 2); UAS:GFP* larva, confirming expression of *mtnr1aa* in the TL.

2.7 Behavioral responses to visual stimuli are suppressed in awake melatonin-treated larvae

We reasoned that melatonin may be acting to promote sleep, at least in part, by acting on the visual system. This hypothesis is based on the following observations: First, the melatonin receptors that we have shown are responsible for melatonin's somnogenic effect are relatively concentrated in the retina, the primary visual organ, and in neuronal populations that are known to play a role in visual processing, namely the optic tectum (Northmore, 2011), torus longitudinalis (Northmore, 2011), and inferior olive (Felix et al., 2021; Yang et al., 2022) (Figs. 2.17, 2.18, 2.20). Second, one of the hallmarks of the sleep state is a reduced sensitivity to sensory stimuli, which includes but is not limited to visual stimuli. The molecular and neuronal mechanisms that are responsible for dampening sensory responsiveness during sleep are not well-understood, and melatonin is an intriguing candidate as one such regulator. However, we note that the role of melatonin in promoting sleep cannot be fully attributed to any role it might play in the visual system because our lab has shown that loss of melatonin signaling results in a drastic impairment in sleep at night, when larvae are in completely dark video-trackers (Gandhi et al., 2015). If melatonin strictly promoted sleep through suppression of the visual system, we would expect no phenotype in the absence of visual cues.

We first asked if zebrafish larvae treated with exogenous melatonin are less likely to respond behaviorally to visual stimuli than are vehicle-treated control larvae. We predicted that if melatonin indeed acts on the visual system, then melatonin-treated larvae would be less likely than controls to respond to a mild visual stimulus to which ~50% of control larvae normally react. In developing an assay to test this prediction, several considerations needed to be addressed. Most importantly, since treatment with melatonin

potently induces sleep, and since sleep is *necessarily* associated with an increased arousal threshold, it was crucial to ensure that the assay was designed and interpreted in such a way that avoids confounding melatonin's sleep-promoting role with its potential role in regulating visual responses. Any non-responsive, melatonin-treated fish may not have responded to visual stimulation because (a) its ability to respond to visual stimuli was attenuated by melatonin or because (b) it was asleep and therefore less likely to respond to any mild stimulus, regardless of any specific effect melatonin might have had on its visual system. More simply, we needed to control for sleep.

We designed a stimulation regime comprised of three 1-second 'dark flashes,' each spaced twenty seconds apart (Fig. 2.21 A-B). During each dark flash, the white lights in the video-tracker are reduced from 100% (the value to which larvae are normally exposed during 'day' conditions) to either 0% or 10% for 1 second and are then restored to 100%. We delivered the following sequence of flashes:

1. 0% '**dark flash'** for 1 second, followed by 100% light for 19 seconds.
2. 10% '**dim flash'** for 1 second, followed by 100% light for 19 seconds.
3. 0% '**dark flash'** for 1 second.

The first 0% dark flash serves as a strong visual stimulus, which we used to identify larvae that (a) can respond to visual stimuli, (b) were, with certainty, awake at least 20 seconds preceding the subsequent 10% dim flash, and (c) were *likely* awake at the time of the subsequent dim flash, based on the operational definition of sleep bouts as one-minute periods of inactivity. The 10% dim flash serves as a milder stimulus, which in preliminary experiments we found to elicit a response in about 50% of untreated wild-type larvae. We expected that the 10% dim flash would be sensitive enough to reveal potential differences in response probabilities between vehicle- and melatonin-treated larvae. The final 0% dark flash is intended to demonstrate that animals remained responsive over the 40-sec series

of flashes, which helps to ensure that any differences in response to the 10% dim flash are not due to rapid habituation or some other immediate loss of responsiveness.

After loading 6-dpf larvae into 96-well behavior plates, we allowed them to acclimate in the video-tracker at 100% light for two and a half hours before the first series of dark flashes (trial 1). Then, we repeated the dark flash sequence (trial 2) after a 29-minute interim period at 100% light (Fig. 2.21 A-B). After trial 2, we delivered melatonin (to 1 μM final concentration) or a vehicle equivalent into alternating rows of wells, waited another two and a half hours, then subjected larvae to two more dark flash sequences (trials 3 and 4), again spaced 29 minutes apart.

We classified larvae as responders or non-responders to the flashes based on whether or not the locomotor activity exhibited by each larva during the 1-second dark or dim flash exceeded a certain threshold (Fig. 2.21 C-D) (see 'Materials and methods' for threshold determination). Briefly, we generated a set of 1-second activity changes to 100 random timepoints over a 10-minute window preceding each series of visual stimuli for each treatment group. From this set, the 95th percentile of activity changes was chosen as the response threshold.

To control for sleep, we took two approaches in analyzing responses to the dim flash. First, we quantified the likelihood of response to the 10% dim flash *only among larvae that responded to the first 0% dark flash.* Thus, if a larva did not respond to the 10% dim flash, it was not likely due to that larva being asleep since it responded to the 0% dark flash 20 seconds earlier. We found that, prior to treatment with vehicle or melatonin, the fraction of larvae that responded to the initial dark flash *and* the 10% dim flash was around 0.5 for each group (0.53 for the pre-vehicle group and 0.47 for the pre-melatonin group) (Figs. 2.21 E, 2.22 B). Upon delivery of melatonin, the fraction of responders to both flashes diminished to 0.27 (pre- v. post-MT: $P = 0.0016$, paired *t*-test), while the vehicle-

treated group response fraction diminished non-significantly to 0.43 (pre- v. post-vehicle: $P = 0.203$, paired *t*-test) (Figs. 2.21 F, 2.22 B). Melatonin-treated larvae were significantly less likely to respond to the dim flash than were vehicle-treated larvae (post-vehicle v. post-MT: $P = 0.0053$, paired *t*-test), suggesting that melatonin can suppress behavioral responses to a light stimulus, and that this effect is independent from melatonin's role in sleep since we only considered animals' responses to the dim flash if they responded to a stronger dark flash 20 seconds earlier. This effect of melatonin is also not likely attributable to any change in motor control because the magnitudes of the activity changes during the dim flash were the same for vehicle- and melatonin-treated dim flash responders (Fig. 2.22 C).

It is still possible that larvae that were responsive to the 0% dark flash may have entered sleep in the 20 seconds between the initial 0% dark flash and the 10% dim flash. It is also possible that, despite not being in a *bona fide* sleep state, melatonin-treated larvae might still be *sleepier* than vehicle controls in the seconds preceding the dim flash. Indeed, we found that melatonin-treated larvae that responded to the dark flash were still overall less active after the dark flash than vehicle-treated controls, pre- or post-dark flash, which suggests that the dark flash does not fully restore locomotor activity levels of melatonin-treated fish to that of controls (Fig. 2.22 A). Thus, we took a second approach to more confidently exclude any sleeping fish. Instead of restricting analysis to larvae that responded to the dark flash, we restricted analysis to larvae in both treatment groups that exhibited relatively high levels of locomotor activity during the 15 seconds preceding the 10% dim flash. We calculated the mean locomotor activity level for each larva during these 15 seconds, and only considered larvae from both groups that showed activity levels between the mean and 1 standard deviation above the mean of vehicle-treated larvae. In other words, we controlled for variance in locomotor activity among vehicle- and

melatonin-treated larvae by only comparing locomotor activity-matched groups (Fig. 2.22 D). This approach makes it very likely that we only analyzed larvae that were awake at the time of the dim flash.

In 8 independent experiments, we found that out of 44 “active” melatonin-treated larvae that meet the above criteria, only 11 (0.25) responded to the dim flash, whereas out of 113 “active” vehicle-treated control larvae, 52 (0.46) responded to the dim flash (post-vehicle v. post-MT: $P = 0.0186$, Fisher’s exact test) (Fig. 2.22 D-E). Thus, even among larvae that were similarly active just prior to a visual stimulus, melatonin-treated larvae were significantly less likely to respond to the stimulus, further suggesting that melatonin suppresses visual responses, and that it does so independently of sleep.

We considered the possibility that the reduced likelihood to respond to visual stimulation that we observe in melatonin-treated larvae may be an outcome of any sleep-promoting manipulation, not necessarily a specific effect of melatonin on the visual system. We tested this possibility by asking if larvae treated with quipazine, a serotonin receptor agonist that strongly promotes sleep, also show reduced responsiveness to visual stimuli using the same assay. If the effect that we observe with melatonin is not a result of melatonin’s effect on the visual system but is instead an effect of any sleep-promoting agent, then we expect quipazine-treated animals to be similarly inhibited in their responses to the dim flash. Conversely, if the effect of melatonin on visual responsiveness is specific to melatonin, we expect to find no difference in dim flash responses between quipazine- and vehicle-treated animals. Importantly, previous experiments in the Prober lab have demonstrated that the serotonergic system promotes sleep via a mechanism that is independent of melatonin (Oikonomou et al., 2019).

We found that, among larvae that responded to the initial dark flash, treatment with 1-μM quipazine caused an inconsistent and non-significant reduction in the fraction

of responsive larvae (pre-quipazine (0.52) v. post-quipazine (0.42): $P = 0.24$, paired *t*-test), not dissimilar to the effect of vehicle (pre-vehicle (0.46) v. post-vehicle (0.46): $P = 0.91$, paired *t*-test) (Fig. 2.23 A-B). Additionally, there was no significant difference between quipazine-treated larvae and vehicle-treated controls in responsiveness to the dim flash (post-vehicle (0.46) v. post-quipazine (0.42): $P = 0.66$, paired *t*-test) (Fig. 2.23 A-B). When we compared the responses of *activity-matched* quipazine- and vehicle-treated larvae across 6 experiments, we found that 43 (0.51) out of 85 "active" vehicle-treated fish responded to the dim flash, and 12 (0.52) out of 23 "active" quipazine-treated fish responded to the same flash (post-vehicle v. post-quipazine: $P > 0.99$, Fisher's exact test) (Fig. 2.23 C-D). Thus, treatment with quipazine, despite being a potent inducer of sleep, has no effect on the visual responses of zebrafish larvae to the dim flashes presented in our assay. These data indicate that the effect of melatonin on visual responsiveness is not an effect observed with any sleep-promoting manipulation, supporting the notion that melatonin specifically suppresses behavioral responses to visual stimuli.

If melatonin's effect on visual responses is separable (although, in our view, not entirely distinct) from its sleep-promoting role, then it is possible that the effect of melatonin on visual responses is mediated by receptors that are different from those that mediate its role in sleep. If our assay is properly controlling for sleep by ruling out inactive larvae at the time of the dim flash, then by subjecting melatonin receptor mutants to the same assay, we can ascertain which receptors mediate melatonin's effect on visual responses. When we examined dim flash responses among activity-matched triple MT1 receptor mutants (*mtnr1aa-/-; mtnr1ab-/-; mtnr1al-/-*), we found no significant difference between vehicle- and melatonin-treated larvae: 24 (0.44) out of 55 "active" vehicle-treated mutant larvae responded to the dim flash, and similarly, 19 (0.39) out of 49 melatonin-treated mutant larvae showed responses (post-vehicle v. post-melatonin: $P = 0.6916$, Fisher's exact test)

(Fig. 2.22 F-G). Thus, melatonin's effect on the visual system appears to be mediated by the MT1 receptor family, which is consistent with the expression pattern of these receptors and is also consistent with the notion that melatonin's role in inhibiting visual responses is *part of* its role in promoting sleep.

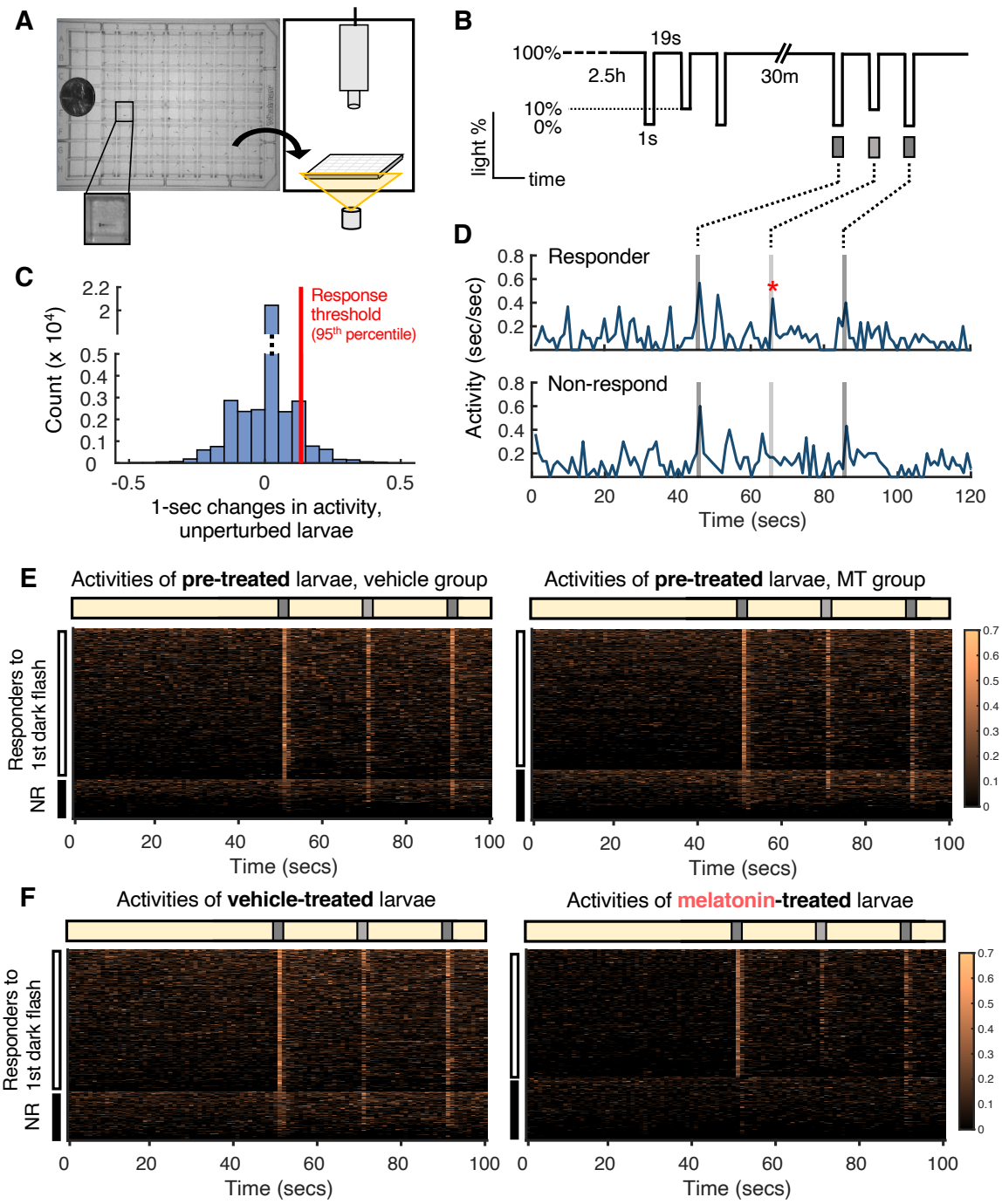

Figure 2.21. A locomotor assay to test the visual responsiveness of vehicle- and melatonin-treated larvae. (Continued on next page.)

Figure 2.21 (cont'd). (A) Illustration of a 96-well plate containing zebrafish larvae. The 96-well plate is placed into a video-tracker that continuously records fish locomotor activity using infrared light and an infrared camera and delivers visual stimuli by controlling the intensity of white lights. **(B)** A diagram of the visual stimuli sequence used to test fish responsiveness. The lighting schedule consists of a 1-sec 'dark flash,' where light is reduced from 100% to 0%, followed 20 seconds later by a less intense 1-sec 'dim flash,' where light is reduced from 100% to 10%, and then another 'dark flash' 20 seconds later. **(C)** Histogram of 1-second locomotor activity changes collected over a 10-min window preceding an example stimuli trial. For each trial and each treatment group/genotype, the 95^{th} percentile of activity changes was chosen as the response threshold. **(D)** Example activity traces of a larva that responded to the dim flash (above; response marked with red asterisk) and a larva that did not respond to the dim flash, despite responding to the dark flash earlier (below). **(E)** Heat map of locomotor activity per second for all larvae in the vehicle group (left) and melatonin group (right), prior to treatment, 50 seconds preceding and 50 seconds following the start of the stimuli trial (first dark flash occurs at t = 50). Larvae are sorted as responders or non-responders ("NR") to the first dark flash, then sorted by mean activity over the 100-sec window. **(F)** Same as in (E), but after delivery of vehicle (DMSO, left) or 1 μM melatonin (right). For (E) and (F), color bar represents locomotor activity per second.

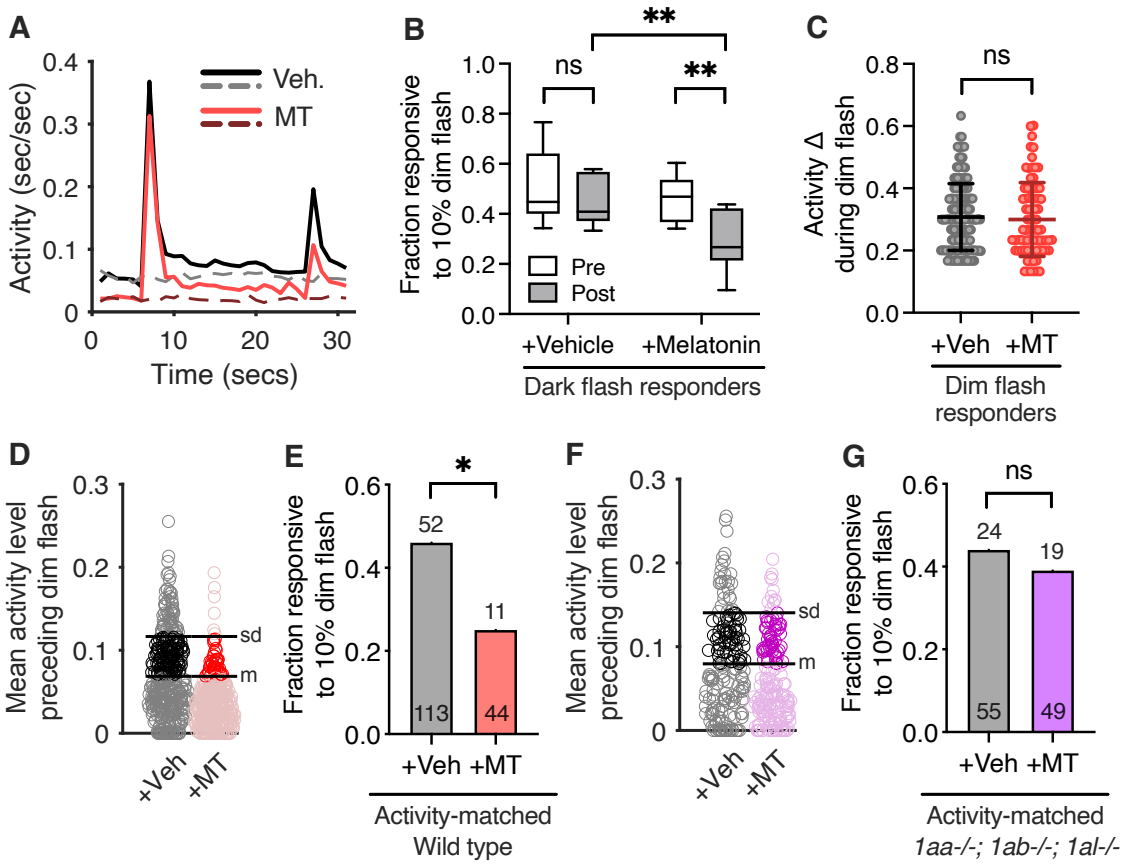

Figure 2.22. Melatonin treatment decreases behavioral responses to a visual stimulus. (A) Mean activity levels of vehicle-treated (black) and melatonin-treated (red) larvae that responded to the dark flash. Solid lines show locomotor activity over 30 seconds covering the dark and dim flashes, while dashed lines show the baseline locomotor activity 30 seconds preceding the dark flash. Note that the dark flash increases the locomotor activity of melatonin-treated larvae compared to its baseline but does not restore it to wild-type levels. **(B)** Fraction of vehicle- and melatonin-treated larvae that responded to the dim flash pre- and post-treatment across 8 experiments. Only larvae that responded to the dark flash are included. **(C)** Locomotor activity changes of vehicle-treated and melatonin-treated dim flash responders at the time of dim flash (1 s). Means ± s.d. are shown. **(D)** Mean locomotor activity for each larva during 15 seconds between the dark and dim flashes. Each circle represents a single fish. Values representing the mean ('m') and 1 standard deviation above the mean ('sd') for vehicle-treated fish are indicated. Larvae with locomotor activity levels that fall between 'm' and 'sd' are used for activity-matched analysis shown in (E). **(E)** Fraction of activity-matched vehicle- or melatonin-treated larvae that responded to the dim flash. Number of responsive larvae for each group is displayed above each bar, and total number of activity-matched larvae for each group is displayed inside each bar. **(F-G)** Same as in (D-E) but using *mtnr1aa-/-; mtnr1ab-/-; mtnr1al-/-* mutant larvae. For panel B, ns = not significant ($P > 0.05$), ∗∗ = $P < 0.01$; paired *t*-test. For panel C, ns = not significant ($P > 0.05$); Student's (unpaired) *t*-test. For panels E and G, ns = not significant ($P > 0.05$), ∗ = $P < 0.05$; Fisher's exact test.

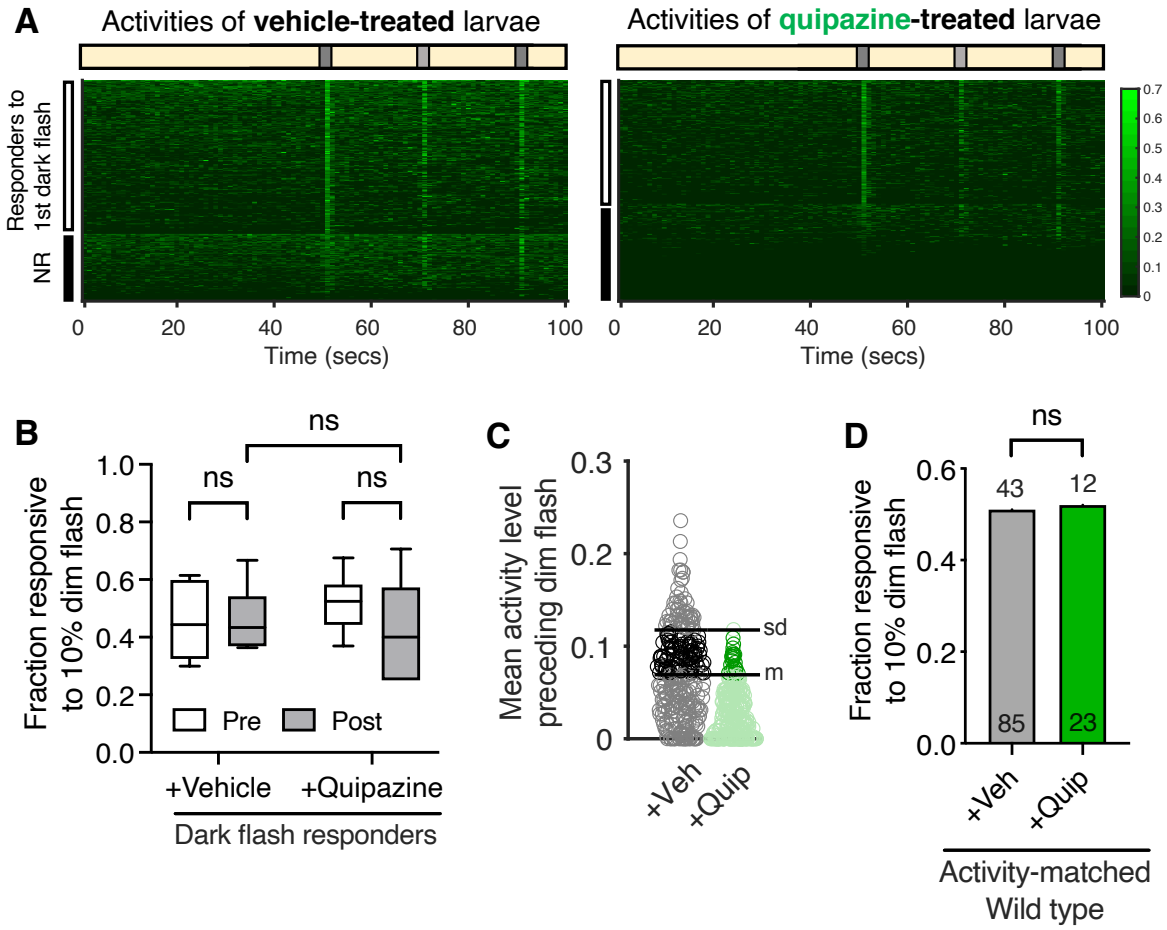

Figure 2.23. Quipazine treatment promotes sleep but does not affect behavioral responses to a visual stimulus. (A) Heat map of locomotor activity per second for all vehicle-treated (left) and quipazine-treated (right) larvae, 50 seconds preceding and 50 seconds following the start of the stimuli trial (first dark flash occurs at t = 50). Larvae are sorted as responders or non-responders ("NR") to the first dark flash, then sorted by mean activity over the 100-sec window. **(B)** Fraction of vehicle- and quipazine-treated larvae that responded to the dim flash pre- and post-treatment across 6 experiments. Only larvae that responded to the dark flash are considered. **(C)** Mean locomotor activity for each larva during 15 seconds between the dark and dim flashes. Each circle represents a single fish. Values representing the mean ('m') and 1 standard deviation above the mean ('sd') for vehicle-treated fish are indicated. Larvae with locomotor activity levels that fall between 'm' and 'sd' are used for activity-matched analysis shown in (D). **(D)** Fraction of activity-matched vehicle- or quipazine-treated larvae responding to the dim flash. Number of responsive larvae for each group is displayed above each bar, and total number of activity-matched larvae for each group is displayed inside each bar. For panel B, ns = not significant ($P > 0.05$); paired *t*-test. For panel D, ns = not significant ($P > 0.05$); Fisher's exact test.

2.8 Loss of melatonin signaling restores behavioral responses to certain visual stimuli at night

While the experiments above demonstrate that melatonin, when exogenously delivered to the larvae, *can* inhibit behavioral responses to a visual stimulus, we wished to test whether a *normal* function of melatonin is to suppress visual responses during sleep at night. Specifically, we wanted to measure responsiveness to visual stimuli at night in *aanat2-/-* mutants, which do not synthesize melatonin, compared to their melatonin-proficient siblings. Because melatonin is synthesized only at night, and because light exposure causes degradation of the AANAT enzyme, we could not deliver dark or dim flashes during otherwise constant light at night, and we therefore modified our assay accordingly.

We developed a 'lights-ON' / 'lights-OFF' assay modeled after similar experiments described by Emran et al. (2010), which showed that larval zebrafish become unresponsive to visual stimuli at night due to a loss of synaptic transmission in the retina (Emran et al., 2010). In this assay, animals are exposed to 5 minutes of 100% light during the night and subjective day (i.e., circadian day but with the lights still off), which allows us to measure behavioral responses to both the lights turning on (0% → 100%; 'lights-ON') and the lights turning off (100% → 0%; 'lights-OFF'). 6-dpf larvae that were raised in normal light-dark cycles were moved to video-trackers in the afternoon, and at 11:00 pm (the normal start of night), the lights were turned off and, outside of stimulation periods, remained off until the end of the experiment the following afternoon. We delivered five 5-min 100% light bouts during the 14-h experiment, each beginning at the following times: 1:30 am (night), 3:00 am (night), 4:30 am (night), 11:00 am (subjective day), and 1:30 pm (subjective day) (Fig. 2.24 A).

During a typical 5-min light bout, we found that wild-type animals, on average, exhibit immediate 1-second peaks in locomotor activity within 1 second of the lights-ON transition during both subjective day and night and then show gradual increases in locomotor activity over a 10-second window (Fig. 2.24 D). After a lights-ON transition during the day, the elevated activity remains stable over the entire duration of the 5-min 100% light period (Fig. 2.24 C). In contrast, during the night, the elevated activity returns to pre-lights-ON levels over approximately 2 minutes (Fig. 2.24 B). The decline in activity at night is likely explained by the larvae returning to sleep.

At the end of the 5-min light period, the lights-OFF transition elicits remarkably different behavioral responses during subjective day versus during the night (Fig. 2.24 E). To a daytime lights-OFF transition, wild-type animals again show a brief 1-second increase in locomotor activity within 1 second after the light turns off (observed in the previous 'dim flash' experiments, as well) and then show a gradual increase in locomotor activity, similar to that of the post-lights-ON response, except slightly more rapid and with greater amplitude. The higher level of locomotor activity post-lights-OFF does not return to pre-lights-ON levels for approximately 13 minutes. Strikingly, the equivalent lights-OFF transition during the night, despite also being preceded by 5 minutes of light, elicits virtually no response from wild-type larvae. This behavior was also shown by Emran et al., and, with other experimental results, supported their claim that zebrafish 'lose vision' at night. Some of our findings differ from that of Emran et al., however, in that we do observe reliable responses to a lights-ON transition at night, whereas Emran et al. reported no response.

While Emran et al. uncovered subcellular changes in the retina that may underlie the differences in visual responsiveness during day and night (Emran et al., 2010), we wondered if the lack of a lights-OFF response during the night was also due to the

presence of melatonin at night. This is not an unreasonable idea since blind fish are still able to detect changes in light and dark, presumably due to the functions of deep brain photoreceptors. Based on our finding that melatonin impairs behavioral responses to 'dim flashes' in awake larvae during the day, we hypothesized that endogenously secreted melatonin similarly 'impairs' light responses during the night and, accordingly, that a loss of melatonin signaling would result in subjective day-like responses to lights-OFF transitions at night. Thus, we predicted that *aanat2-/-* larvae would show wild-type responsiveness to lights-ON and lights-OFF transitions during the day (when melatonin is not present) but would be more likely than controls to respond to lights-OFF transitions at night. There was not a very strong difference in wild-type responses to lights-ON during subjective day versus night (Fig. 2.24 D); thus, it was not clear whether or not time of day similarly attenuates responses to that particular stimulus.

We found that, compared to *aanat2+/-* control siblings, which had a wild type-like lack of response to lights-OFF at night, *aanat2-/-* mutant larvae showed a robust response to the transition (Fig. 2.24 B, G). The immediate *aanat2-/-* response to the lights-OFF transition was similar to that of control larvae during the subjective day (Fig. 2.24 E, G), when melatonin is absent, consistent with the notion that melatonin mediates the suppression of the lights off response. We noted that after the immediate response, *aanat2-/-* mutant larvae returned to their baseline activity level. This is unlike the dynamics of *aanat2+/-* controls during subjective day, where locomotor activity remains higher for several minutes, suggesting that there are non-melatonin-related factors driving the prolonged elevated locomotor activity during circadian day (Fig. 2.24 E, G). Both *aanat2+/-* and *aanat2-/-* animals responded similarly to the lights-ON transition at night (Fig. 2.24 F).

The enhanced response of *aanat2-/-* animals to lights-OFF at night may simply be explained by the fact that melatonin-deficient animals sleep less at night, and as a result

of being awake, they are more easily aroused by the lights-OFF transition. Indeed, during the 5-min light period preceding the lights-OFF transition, *aanat2-/-* animals are noticeably more active than the *aanat2+/-* control siblings (Fig. 2.24 B, G). Thus, in order to control for sleep, we took a similar approach as in the 'dim flash' experiments described in Chapter 2.7. We only considered animals with mean locomotor activities during a 15-sec window just preceding the lights-OFF transition that fall within a certain range that we arbitrarily defined as 'more active,' or, at the very least, 'not sleeping' (Fig. 2.24 H). By only comparing larvae that could be deemed 'more active,' we reasoned that any differences in the responses between similarly active *aanat2+/-* and *aanat2-/-* larvae could not be attributed to one group being asleep or inactive.

Then, as before, we defined a response to lights-OFF as an above-threshold change in locomotor activity during the 1 second following the lights-OFF transition. The threshold for a response was calculated in a similar manner as in the 'dim flash' experiments, but with two differences. First, because lights-OFF transitions are preceded by 5 minutes of light, and we wished to collect 'baseline' locomotor activity changes within this limited window, 1-second activity changes were collected over a shorter window preceding the stimulus (100 'mock' timepoints over 200 seconds prior to lights-OFF). Second, we used the top 90th percentile (instead of 95th) from this collection of baseline activity changes to define the threshold; this decreased stringency was to enhance number of responders, which we found to be lower in this experiment compared to the dim flash experiment.

Among similarly active *aanat2+/-* and *aanat2-/-* siblings, *aanat2-/-* larvae still showed an obvious peak in locomotor activity at the time of the lights-OFF transition, whereas *aanat2+/-* larvae showed no response, despite being equivalently active, on average, as the *aanat2-/-* mutants (Fig. 2.24 J). For the first trial at night (at ZT 16.5), 4

(0.16) out of 25 “active” *aanat2+/-* larvae responded to the lights-OFF transition, while 17 (0.35) out of 48 “active” *aanat2-/-* larvae responded (Fig. 2.24 H-I, left). This difference was not statistically significant (*aaant2+/-* v. *aanat2-/-*: $P = 0.1057$, Fisher’s exact test), likely due to a low sample size. We also found a difference among the two genotypes in lights-OFF responses at the end of the second 5-min window (at ZT 18), which also was not statistically significant (*aanat2+/-* (0.19 responsive) v. *aanat2-/-* (0.33 responsive): $P = 0.2769$, Fisher’s exact test) (Fig. 2.24 H-I, right). For the third 5-min window (at ZT 19.5), larvae from both genotypes were mostly non-responsive for unclear reasons (*aanat2+/-* (0.13 responsive) v. *aanat2-/-* (0.22 responsive): $P = 0.53$, Fisher’s exact test). When data from all three time points are combined, the difference between the two genotypes is statistically significant (*aanat2+/-* (0.16 responsive) v. *aanat2-/-* (0.30 responsive: $P = 0.03$, Fisher’s exact test).

To summarize, we showed in section 2.7 that administration of melatonin can suppress responses to visual stimuli independently of its effects on sleep. As a corollary experiment, we examined the visual responsiveness of melatonin-deficient larvae and hypothesized that they would be more responsive to visual stimuli at night compared to larvae that do synthesize melatonin. We found that, at night, melatonin-deficient *aanat2-/-* mutant larvae are more likely to respond to a lights-OFF stimulus than their melatonin-proficient *aanat2+/-* siblings, even when controlling for the sleep defect cause by loss of melatonin in the *aanat2-/-* mutants. This difference is clear in the behavioral traces (Fig. 2.24 G, J), but did not reach statistical significance for individual timepoints (Fig. 2.24 I), likely due to the small sample size imposed by restricting the entire sample set to activity-matched groups. More experiments are necessary, perhaps with further optimization of responder determination, to confirm the initial findings presented here.

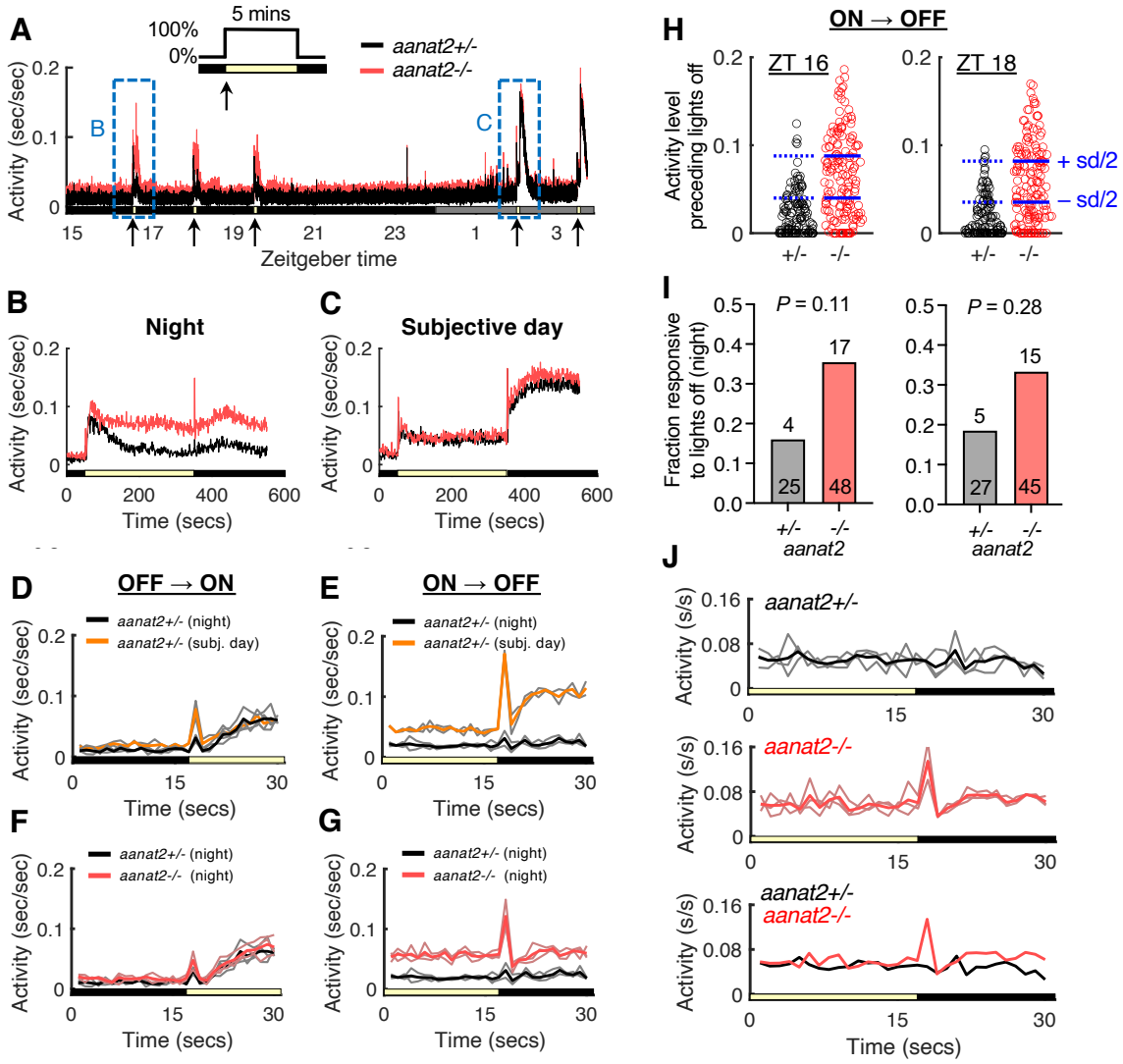

Figure 2.24. *aanat2* mutants but not controls respond to a lights off stimulus at night. (Continued on next page.)

Figure 2.24 (cont'd). (A) Average locomotor activity of 6-dpf *aanat2+/-* (black) and *aanat2-/-* (red) animals over the 14-hr experiment. Start of stimulation windows (5 mins lights on) are indicated by arrows. **(B)** Average locomotor activities of *aanat2+/-* and *aanat2-/-* animals during a representative stimulation window at night (ZT 16.5; blue box in panel A). **(C)** Same as in (B) but during subjective day (ZT 2). **(D-E)** Comparison of responses of *aanat2+/-* control larvae to OFF→ON (D) and ON→OFF (E) light transitions during night (black) or subjective day (orange). Thin and thick lines indicate individual trials and average of all trials, respectively. **(F-G)** Same as (D-E) but comparing *aanat2+/-* controls (black) to *aanat2-/-* mutants (red) during each transition at night. Note that the *aanat2-/-* responses at night are similar to the responses of controls during subjective day. **(H)** Mean locomotor activity for each larva during 15 seconds preceding the ON→OFF transition at ZT 16.5 (left) and ZT 18 (right). Larvae with mean activity levels within +/- 0.5*s.d. of *aanat2-/-* animals (blue lines) were used for analysis in (I) and (J). **(I)** Fraction of activity-matched *aanat2+/-* and *aanat2-/-* larvae that are responsive to the ON→OFF transition at night. Number of responsive larvae for each genotype is displayed above each bar, and total number of activity-matched larvae for each genotype is displayed inside each bar. **(J)** Locomotor activity traces of activity-matched *aanat2+/-* (black) and *aanat2-/-* (red) larvae at the time of the ON→OFF transition. Averages for the three stimulation periods at night across experiments are shown in lighter traces. Note the absence of a response in melatonin-proficient *aanat2+/-* animals, despite having an equivalent average locomotor activity as *aanat2-/-* siblings prior to the light transition.

2.9 Neuronal activity in the optic tectum is attenuated by melatonin

On the basis of our findings above, where we show that melatonin suppresses behavioral responses to visual stimuli, and again considering the expression of melatonin receptors in regions of the brain that govern visual processing, we asked whether melatonin could suppress visually-evoked neuronal responses in brain regions that are (a) responsive to visual stimuli and (b) express melatonin receptors, such as the optic tectum. We reasoned that any melatonin-induced diminished responses to visual stimuli in these brain areas may be the neurophysiological basis for the behavioral effects of melatonin treatment that we describe above. Further, the identification of neuronal populations that are responsive to light stimuli and attenuated by melatonin might provide a useful model for understanding how sleep dampens sensory inputs in the central nervous system.

To this end, we are using 2-photon selective plane microscopy (2P-SPIM) to image the brains of larvae that express the calcium sensor GCaMP7f pan-neuronally (i.e., under control of the *elavl3* promoter) (Fig. 2.25 A-B). We use 2P-SPIM to image larvae for 3 reasons: First, the use of a single plane, or sheet, of excitation light affords imaging speeds appropriate for capturing relatively fast neuronal responses across several z-planes, which allows us to survey large volumes in one experiment. Second, light sheet illumination allows us to excite only the focal plane being imaged, which protects the sample against photo-bleaching. Third, use of two-photon excitation light (920 nm), a wavelength not visible to zebrafish larvae, ensures that the larvae are not visually stimulated by the excitation light, and responses we record are therefore spontaneous or stimulus-evoked.

In a preliminary experiment, shown in Figure 2.25, a 6-dpf larva was embedded in 1% low-melting agarose and affixed to a custom-built ‘caddy’ that is designed to fit in the imaging chamber of the 2P-SPIM microscope. The agarose was cut away from the head

and the tail, leaving only a block of agarose near the base of the head and at the swim bladder to secure the larva in place. While we did not record tail movements in this assay, we freed the face and tail of the larva to minimize stress that may result from its confinement. The larva was then loaded into the larval media-filled microscope imaging chamber.

We recorded from 10 z-planes covering a 50-μm volume that encompassed most of the optic tectum. Each volume was captured at a rate of 1 volume every 2 seconds. To generate visual responses we delivered 625-nm LED light flashes, which would not interfere with GCaMP signal. The LED light source faced the left eye of the mounted larva (Fig. 2.25 B). As we were unsure of the appropriate range of LED powers that, when delivered to the larva, would elicit reliable responses but also be sensitive enough to detect changes caused by melatonin, we tested 5 LED intensities, from 0.2-4.0 μW/cm^2, in a shuffled order (i.e., not in a series of increasing or decreasing intensities) (Fig 2.25 B, see inset). Each LED flash was spaced 2 minutes apart, and each series (10 minutes) of 5 LED intensities was repeated 6 times, for a total recording time of 1 hour, during the day. After the first 1-hr recording, we removed half of the media in the chamber and replaced it with a 2X solution of melatonin, then resumed recording and delivered another 1-hr series of LED flashes.

We observed reliable changes in GCaMP7f fluorescence in response to LED stimuli that generally scaled with LED intensity (Fig. 2.25 F-G). The most striking change that we observed after addition of melatonin was a decrease in baseline fluorescence ('F_0'), which may represent an effect of melatonin on intracellular calcium levels (perhaps via adenylate cyclase inhibition), or a reduction in spontaneous firing rates of tectal neurons, or both (Fig. 2.25 C, E). We noted that the changes in fluorescence (F-F_0) in response to the LED stimulation were diminished after treatment with melatonin (Fig. 2.25

F), but that after normalizing to baseline ($F-F_0 / F_0$), these differences were largely absent (Fig. 2.25 G). Thus, the raw, but not normalized, increases in fluorescence in response to LED stimuli were decreased after melatonin treatment, and this difference is most apparent at lower LED intensities. Taken together, melatonin may reduce both baseline calcium levels and stimulus-evoked calcium changes in optic tectum neurons, or it may only influence baseline calcium levels in these neurons, which, by itself, might limit stimulus-evoked changes in calcium due to a decreased availability of calcium within the neuron. Moreover, since we observed the strongest effect of melatonin treatment on responses to the lowest LED intensity presented, we posit that the range of intensities used in this experiment were generally sufficient to elicit responses in spite of melatonin's effects, which may be subtle.

While promising, these data are preliminary and difficult to interpret without repetition and proper controls (i.e., vehicle-treated). Current efforts in the lab are aimed toward: generating stimulus-response curves to ensure that the LED intensities being used cover an appropriate range to detect melatonin-induced changes, recording tail movements and heart rate to infer the timing of melatonin's effect for each experiment, and using computational methods to segment and cluster neurons based on their activities. Preliminary evidence from these efforts suggests that the effect of melatonin on stimuli-evoked responses is not apparent when analyzing bulk fluorescence but is evident in subsets of neurons that are responsive to the light stimuli. Thus, the use of cell segmentation and clustering algorithms may help detect melatonin-induced changes in neuronal responses, which are possibly limited to only a subset of light-responsive tectal neurons.

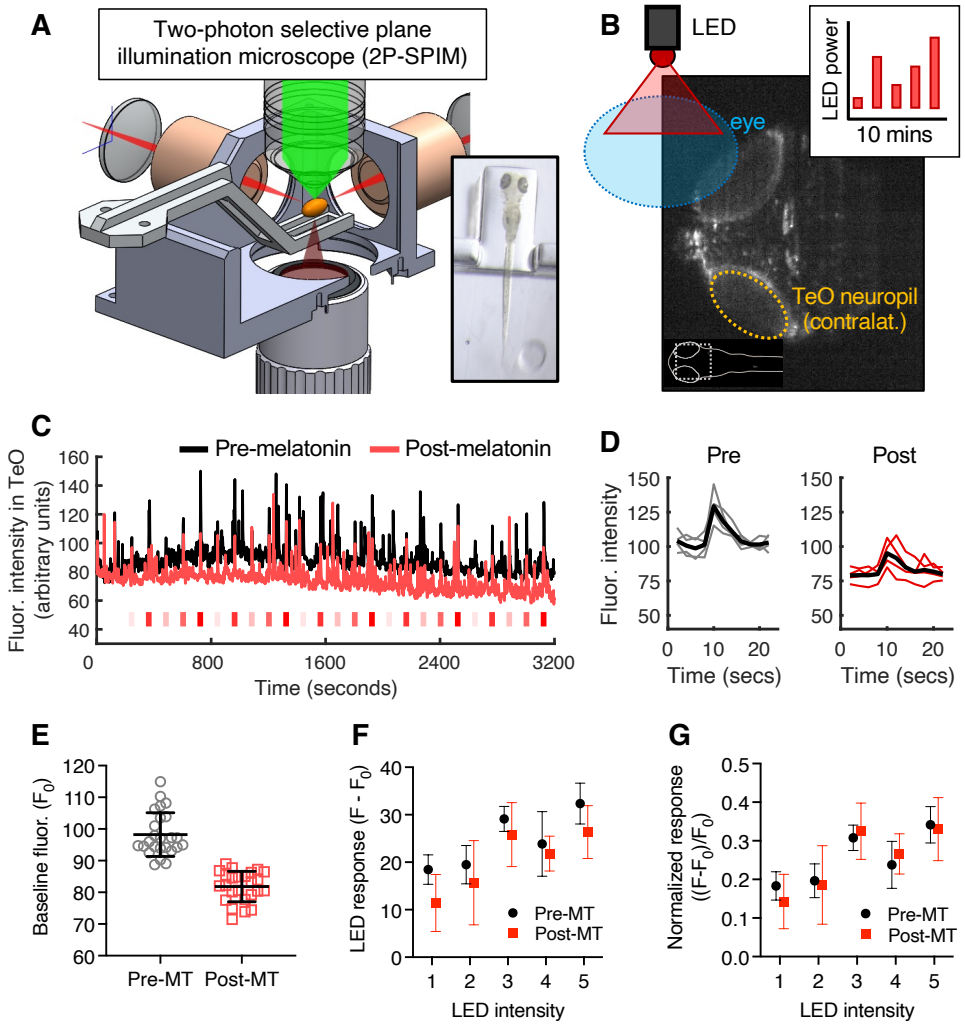

Figure 2.25. Neuronal activity in the optic tectum is attenuated by melatonin treatment. (A) Schematic of the 2-photon selective plane illumination microscope (2P-SPIM). A larva embedded in agarose is depicted in the inset. **(B)** A representative image depicting a 6-dpf *Tg(elavl3:GCaMP7f)* larva being recorded. Diagrams show the relative position of the LED light source, which faces the left eye of the larva (image is flipped), and the contralateral tectal neuropil from which fluorescence values were measured. Inset shows the sequence of 5 LED powers, which were delivered 2 min apart. **(C)** Background-subtracted fluorescence intensity values pre- (black) and post-melatonin (red) administration over 5 cycles of LED stimulation. Stimuli are represented by differently shaded red markers. **(D)** Single trial (thin lines) and average of all trials (thick lines) showing responses to the lowest LED intensity stimulation pre- and post-melatonin treatment in a representative z-plane. **(E)** All baseline fluorescence (F_0) values pre- and post-melatonin treatment. F_0 is calculated as the mean fluorescence over a 3-sec window starting 5 sec prior to an LED stimulus. **(F)** Average change in fluorescence ($F-F_0$) for each LED intensity pre- and post-melatonin. **(G)** Same as (F) but normalized to baseline fluorescence. For data in panels (C-G), a single, representative plane was used. In panels (E-G), bars represent s.d.

DISCUSSION

The molecular and neuronal mechanisms by which melatonin promotes sleep are poorly understood. While melatonin-binding GPCRs have been identified, and various effects of melatonin receptor activation have been characterized at the intracellular level, it remained to be determined which receptors—if any—are required for melatonin's role in sleep, and how their activation promotes sleep at the neuronal and behavioral levels.

Using genetic loss-of-function experiments, we have shown that melatonin acts through MT1 receptors for normal night-time sleep and for sleep induced by exogenous melatonin. We found that MT2 was dispensable for sleep in both of these assays. We found that, among the zebrafish MT1 receptor paralogs *mtnr1aa*, *mtnr1ab*, and *mtnr1al*, melatonin likely promotes sleep primarily through *mtnr1aa*, while *mtnr1ab* and *mtnr1al* can compensate for mutation of *mtnr1aa*. This is evidenced by loss-of-function phenotypes in *mtnr1aa-/-* mutants where the entire first exon was deleted and in triple mutant *mtnr1aa-/-; mtnr1ab-/-; mtnr1al-/-* animals, but a lack of phenotype in *mtnr1aa-/-* mutants that contain a smaller frame shifting deletion, which are predicted to generate premature termination codon-bearing transcripts that trigger genetic compensation.

We note that, although the data presented here suggests that *mtnr1aa* is likely the primary transducer of melatonin's role in sleep, we have not definitively ruled out contributions by the other receptors. Future experiments will test the overall contribution of *mtnr1aa* to melatonin's role in sleep. Specifically, we wish to assay behavior in *mtnr1aa(Ex1-KO); aanat2* double mutants. If *mtnr1aa* is the sole receptor through which melatonin promotes sleep, there should be no difference in sleep at night between *mtnr1aa(Ex1-KO)-/-; aanat2+/-* versus *mtnr1aa(Ex1-KO)-/-; aanat2-/-* siblings. On the other hand, if the *mtnr1aa(Ex1-KO)-/-* sleep defect is enhanced in the *aanat2-/-*

background, then other receptors must also contribute to melatonin's effect on sleep. If necessary, to determine the contributions of *mtnr1ab* and *mtnr1al*, we can also compare sleep defects in *mtnr1aa(Ex1-KO)-/-; mtnr1ab+/-; mtnr1al+/-* versus *mtnr1aa(Ex1-KO)-/-; mtnr1ab-/-; mtnr1al-/-* siblings.

Studies that attempt to implicate a melatonin receptor subtype in sleep have been carried out using receptor-specific agonists and receptor KO mutations in nocturnal rodents (Fisher & Sugden, 2009; Ochoa-Sanchez et al., 2011; Comai et al., 2013). The authors of these studies concluded that MT1 receptors regulate REM sleep, whereas MT2 receptors regulate NREM. While our behavioral assays are unable to distinguish between REM and NREM sleep, we found no role for MT2 receptors in sleep, which raises interesting questions. It is possible that NREM- and REM-like sleep stages do not exist in zebrafish, and it may therefore be apt that only one receptor type is essential for zebrafish sleep. Alternatively, if zebrafish sleep is indeed comprised of NREM and REM sleep, it is possible that one is permissive for the other, and that manipulations affecting only MT2 receptors do not yield phenotypes outside of MT1-mediated sleep. Until NREM- and REM-like states are convincingly identified in zebrafish, the notion that each melatonin receptor serves sleep stage-specific roles, as the rodent literature suggests, cannot be substantiated in zebrafish.

We detected MT1 receptors by *in situ* hybridization in tissues known to play a role in visual processing, namely the retina, optic tectum, torus longitudinalis, and inferior olive. We also detected transcripts in the posterior tuberculum, which is part of a loosely defined "wider" thalamus-equivalent structure in zebrafish (Mueller, 2012). This is worth noting, as the mammalian thalamus is considered an important relay point in sensory processing and is a site of melatonin receptor expression (Lacoste et al., 2015).

On the basis of the expression pattern of melatonin receptors, we reasoned that a function of melatonin may be to attenuate neuronal circuits involved in visual processing, which would facilitate sleep. To test this hypothesis, we subjected melatonin-treated wild-type larvae and melatonin-deficient mutant larvae to behavioral assays that test their responsiveness to visual stimuli. The results from those experiments suggest that melatonin does indeed play a role in suppressing responses to these stimuli. We tried, as much as possible, to minimize the confounding effect that sleep itself has on sensory systems. We knew that melatonin promotes sleep, and that a component of sleep is an increased arousal threshold to sensory stimulation, including visual stimulation. Thus, it was predictable that melatonin would suppress responses to visual stimuli. We rule out this interpretation in three ways. First, we measured the likelihood of a response to a visual stimulus only among larvae that had responded—i.e., were awake—20 seconds earlier to a stronger stimulus. Second, we compared response likelihoods among larvae that were similarly active and above the mean activity level of vehicle-treated controls. And finally, we showed that not any sleep-promoting manipulation (i.e., activation of serotonin signaling using the serotonin receptor agonist quipazine) will yield a defect in visual responses in awake larvae. These modifications point to a role for melatonin in regulating vision outside of—but potentially a part of—its role in sleep.

Whether or not melatonin's function in visual processing is permissive, instructive, or unrelated to its sleep-promoting function is difficult to test without genetic access to melatonin receptor-expressing cells, and without knowing which neuronal populations mediate its effects on sleep and which mediate its effects on responses to visual stimuli (if they are separable). If these populations could be independently targeted, site-specific knock-out of MT1 receptors in the visual-related tissues would allow us to test the ability

of visual perturbations to arouse larvae that are defective in MT1 signaling but with intact sleep.

Melatonin plays a well-documented role in retinal physiology, which might support the notion that melatonin's role in vision is independent from its role in sleep. Retinal melatonin is secreted by photoreceptor cells at night and opposes dopaminergic signaling to promote 'dark adaptation' in the eye, which includes increasing sensitivity to light (Wiechmann & Sherry, 2013). The diminished responses of larvae treated with exogenous melatonin may be a consequence of supraphysiological levels of melatonin in the eye. However, in our loss-of-function assay, we used *aanat2-/-* mutants, which are predicted to retain melatonin signaling in the eye, as its paralog *aanat1* is likely still functional in *aanat2-/-* mutants and is exclusively expressed in the zebrafish eye (Gandhi et al., 2015). Melatonin produced in the eye appears to be dispensable for melatonin's function in regulating sleep, as *aanat1* mutants exhibit normal sleep/wake states and do not enhance the *aanat2* mutant sleep phenotype (Gandhi et al., 2015). Nevertheless, more experiments are needed to determine if the changes in light responsiveness that we observe in melatonin-treated or -defective animals are purely reflective of established melatonin signaling pathways in the eye. In addition, whether melatonin affects behavioral responses to visual stimuli via neurons in the eye or the brain can be directly addressed by calcium imaging experiments (see below).

Melatonin receptors are reported to be highly expressed in the superior colliculus of mammals (Lacoste et al., 2015), and we detect *mtnr1aa* and *mtnr1ab* transcripts in the equivalent brain region of zebrafish, the optic tectum. Interestingly, among other functions, the superior colliculus has been shown to play a role in a phenomenon known as 'masking,' or the ability of light and dark to directly promote sleep or wake (Zhang et al., 2019). Specifically, ablation of GABAergic neurons in the superior colliculi of mice

abolished dark-induced waking during the light phase but had no effect on light-induced sleep during the dark phase. We also find melatonin receptors in the torus longitudinalis, which is a brain structure exclusive to teleosts. At least some neurons in this structure, which sends projections to the optic tectum, are also activated by dim light and darkness (Northmore, 2011). Thus, we point out a trend where melatonin receptors are expressed in neuronal populations responsive to dark stimuli. One function of melatonin, which could still be concordant with its function in sleep, might be to suppress dark-activated neuronal circuits during the night, when those populations would otherwise be unnecessarily active. The torus longitudinalis-specific *mtnr1aa:GAL4* line that we generated may be useful in testing hypotheses related to this potential function of melatonin.

We also point out that the optic tectum is involved in the processing of multiple sensory modalities, including auditory and somatosensory stimuli (Thompson et al., 2016). While we have focused on visual stimulation in this study, we do not dismiss a role for melatonin in inhibiting responses to other stimuli, as well. The most prominent expression of *mtnr1aa* is in a bilateral population of cells adjacent to the octavolateralis efferent neurons (OENs), which may indicate a role for melatonin in suppressing acoustic stimuli (Odstrcil et al., 2022). We are devising follow-up experiments, modeled after the light stimulation assays reported here, to test whether or not melatonin attenuates responses to acoustic and somatosensory stimulation. Exploring the role of melatonin in suppressing responses to these stimuli would reveal whether melatonin specifically affects visual responses or is more broadly responsible for the increased arousal threshold to all stimuli that is a hallmark of sleep.

Finally, in early experiments using 2-photon selective plane illumination microscopy (2P-SPIM), we report a reduction in baseline GCaMP7f fluorescence and a possible reduction in light-evoked responses in the optic tectum neuropil after treatment

with melatonin. While this result is preliminary and does not distinguish between melatonin's effects on vision versus sleep, it nevertheless supports the notion that melatonin suppresses visual responses. We are optimizing imaging and stimulation parameters to more judiciously validate these preliminary findings.

The rapidly reversible dampening of sensory input (or increased arousal threshold) is a hallmark of sleep, and the mechanisms by which the nervous system accomplishes this are poorly understood. Our observation that melatonin, a potent inducer of sleep, has effects on the visual responsiveness of zebrafish, offers a potential model through which we can test hypotheses about how this feature of sleep is achieved. By using 2P-SPIM to record GCaMP fluorescence, before and after administering melatonin, in neuronal populations that act at different steps in a sensory circuit, we hope to gain new insights as to how the nervous system regulates this aspect of the sleep state.

MATERIALS AND METHODS

Animal husbandry. Wild-type zebrafish (*Danio rerio*) used in this study are the cross-progeny of the Tupfel long fin (TL) line and the AB line, called TLABs. Zebrafish are maintained in accordance with the California Institute of Technology Institutional Animal Care and Use Committee (IACUC) and Office of Laboratory Animal Resources (OLAR) guidelines. Embryos and larvae were housed in petri dishes containing E3 larval media (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$). Dishes contained ≤ 50 larvae and were stored in a 28.5 ºC incubator on a 14h:10h light:dark schedule (same as in the fish facility).

Mutant zebrafish. All melatonin receptor (*mtnr)* mutant zebrafish described in this study were generated using the Alt-R CRISPR/Cas9 system by IDT (Integrated DNA Technologies). In this method, an RNA molecule containing sequence that recognizes the desired target ('crRNA') is hybridized with a universal 'tracrRNA' to form the complete guide RNA ('gRNA') molecule that recruits Cas9 to the DNA target. A protocol for using this system in zebrafish embryos was previously described (Essner, 2016) and was followed here, with the only modification of adding 0.5 μL phenol red to the final injection mix to aid in visualizing the injection. 1-2 μL of the injection mix was injected directly into the cell of a wild-type embryo at the 1-cell stage.

CRISPR guide target sequences were selected using the online tool CHOPCHOP (https://chopchop.cbu.uib.no/) (Labun et al., 2019). The crRNAs were synthesized by IDT using the target sequences described in Table 2.1.

Mutagenicity of each CRISPR guide RNA was confirmed in ~3-4-dpf larvae by T7 endonuclease I treatment. Briefly, injected larvae were pooled in groups of 5-10, and genomic DNA ('gDNA') was extracted using proteinase K treatment. A short (~200-bp) region surrounding the target site was amplified from each pool of DNA by PCR. 10 μL of PCR product was added to 2 μL 10X Buffer 2 (NEB) and 5 μL water, heated to 95 ºC for 5 minutes to de-anneal DNA strands, then moved to room temperature to allow re-annealing of DNA. 0.5 μL of T7 endonuclease I was added to each sample, incubated at 37 ºC for 20 minutes, then samples were loaded onto a 2-4% agarose gel, and fragments

were separated by electrophoresis. In pooled DNA samples containing mutagenized DNA, hetero-duplexes between mutant and wild-type DNA fragments are expected to form when the DNA is allowed to cool, and these mismatches are cut by T7 endonuclease I, resulting in multiple bands on the gel. Guide RNAs that were found to be mutagenic were re-injected into embryos, and the injected larvae were raised to adulthood.

Potential 'founders' (adults injected as embryos) were out-crossed to wild-type zebrafish, and the progeny (F1s) were again screened by T7 endonuclease I to identify founders carrying heritable mutant alleles. Mutant F1s were raised to adulthood and then fin-clipped, whereby a small piece of the caudal fin is excised and used as a source of genomic DNA. Heterozygous (i.e., mutant-bearing) F1s were identified by PCR, isolated, and the exact nature of the DNA lesion in each F1 was identified by Stratacloning and sequencing. Each mutant line was out-crossed twice more such that the first generation used for behavior analyses were the product of F3 in-crosses.

Mutants were routinely genotyped by PCR using the primer sets described in Table 2.2. For genotyping Ex1-KO mutants, two PCR reactions were performed: one using a primer (Rev1) that binds within exon 1 and is predicted to amplify only when the exon is present (i.e., in wild-type and heterozygous fish), and one using a primer (Rev2) that binds in the intronic region downstream of exon 1 and is predicted to amplify a 250 bp band only in fish carrying the *Ex1-KO* deletion allele.

aanat2 mutants have been previously described (Gandhi et al., 2015).

Transgenic zebrafish. The bacterial artificial chromosome (BAC) encompassing the zebrafish *mtnr1aa* gene (clone CH211-26A7) was obtained from BACPAC Genomics (Emeryville, CA) as a stab culture. Recombination of Tol2 long terminal repeats in the BAC vector backbone (to facilitate Tol2 transposase-mediated insertion into the genome) and recombination of the GAL4 coding sequence in the *mtnr1aa* coding region were performed as described in Bussmann & Schulte-Merker (2011). The recombined *mtnr1aa:GAL4* BAC was injected into UAS:GFP embryos at the 1-cell stage. 1-2 μL of an injection mix (50 ng/μL BAC, 1 μL (final 50 ng/μL) Tol2 mRNA, 0.5 μL phenol red, water to 10 μL) was

injected into the cell. Injected larvae were screened for any fluorescent signal, then raised, and their progeny were screened for fluorescence or for GAL4 by PCR.

The *Tg(HuC:GCaMP7f); casper-/-* fish were a gift from the lab of Misha Ahrens.

Video-tracker experiments. Depending on the experiment, 4-, 5-, or 6-dpf larvae were loaded individually into each well of a 96-well plate (7701-1651, Whatman, Pittsburgh, PA, U.S.A.), containing 650 μL of E3 larval media, and sealed using an optically transparent adhesive film (4311971, Applied Biosystems). The plate was loaded into a "ZebraBox" video-tracker (Viewpoint Life Sciences, Lyon, France), which monitors locomotor activity via constant infrared light illumination while under a controlled visible light regime (14h:10h light:dark cycles for standard behavior experiments, but modified for other experiments as explained in-text). The housing for the 96-well plate is located within a chamber filled with re-circulating water to maintain a constant temperature of 28.5 ºC.

For video-tracker experiments with melatonin or quipazine treatment, the 96-well plates were left unsealed, and, for experiments that spanned several days, D.I. water (~150 μL) was added to each well on each day to replenish evaporated media. On the day of drug treatment, water was not added, and instead, 150 μL of a 4.3-μM solution of melatonin or quipazine in E3 larval media was added to each well, then topped up to 650 μL (the well capacity) by eye, for a final concentration of 1 μM. 150 μL of an equivalent amount of DMSO diluted in E3 larval media was added to control wells.

Pharmacology. Melatonin and quipazine were prepared from powder stocks (Sigma-Aldrich) by dissolving in DMSO to 50 mM and 100 mM stock concentrations, respectively.

Analysis of behavior. The video-tracker reports locomotor activity as the cumulative duration of frames in which sufficient pixel changes occurred from the previous frame over a 1-second or 1-minute bin, depending on the experiment. A frame is captured every 33 milliseconds. Data from the video-tracker experiments were analyzed using MATLAB scripts previously described (Lee et al., 2017); these scripts convert the video-tracker output into a format usable by subsequent MATLAB scripts (see below) and provide quantifications of certain sleep metrics (e.g., see Figure 2.1 C-I). Sleep bouts are defined

as a continuous string of 1-min bins where no detectable activity occurred. This threshold is based on the observation that zebrafish larvae exhibit an increased arousal threshold after 1-minute of inactivity (Prober et al., 2006). Sleep latency is defined as the length of time from lights off to the start of the first sleep bout.

For video-tracker experiments designed to test the behavioral responses to melatonin, dark/dim flash responses, or 5-min lights on responses, data were first analyzed using the scripts described above, then further analyzed using custom MATLAB code.

Threshold determination for determining whether a fish responded to light stimuli were calculated as follows: A series of 100 evenly spaced 'mock' time points were chosen over a 10-min (for dark/dim flash assay) or 200-sec (for 5-min lights on assay) window preceding each stimulus. At each mock time point *t*, the locomotor activity change from *t* to *t*+1 was calculated. The cumulative activity change values for all fish were compiled (100**n* values), and the 95th percentile (dark/dim flash) or 90th percentile (5-min lights on) of compiled activity changes was chosen as the threshold value (see Figure 2.21 C for example histogram of activity changes). We reasoned that activity changes that exceed this value are rare enough that, when coincident with a stimulus presentation, are likely representative of a response to the stimulus and not an otherwise random movement. Thresholds were re-determined for each time point during the assay and determined separately for each group (i.e., vehicle- or drug-treated), reasoning that the baseline of activities are likely different for different groups and different over the course of the experiment. Thresholds were validated by re-analyzing data sets with a slight time offset in the stimulus times. These offsets resulted in drastic loss of responders, confirming that our threshold determinations were properly judging responders/non-responders.

In situ *hybridization.* The *in situ* hybridization protocols used in this study are adapted from Thisse & Thisse (2008). Riboprobes were designed to target as much 5'- and 3'-UTR of the target transcript as possible. Table 2.2 shows the primer sequences used to generate each of the riboprobes.

Riboprobes were synthesized from purified PCR product containing the relevant target sequence and a T7 transcription start site added via the 3' PCR primer. RNA (antisense

to the target) was transcribed *in vitro* using T7 polymerase and digoxigenin-labeled nucleic acid mix (Roche). After transcription, RNA was purified using DNAse I (Roche) and column purification (Quick Spin RNA column, Roche), then ethanol precipitated and resuspended in 50% hybridization (Hyb) buffer to a concentration of ~50 ng/μL. Riboprobes were stored at -20 ºC.

For both fluorescence and chromogenic *in situ* hybridizations, 6-dpf larvae were fixed in a solution of 2% paraformaldehyde (PFA) and 4% sucrose in PBS-Tween (PBS-T; PBS containing 0.1% Tween-20) overnight at room temperature. Brains were then dissected and equilibrated in 5 changes of 100% methanol, then stored in methanol overnight (at minimum) in a -20 ºC incubator. Brains were re-hydrated, washed, treated with 10-μg/mL proteinase K in PBS-T for 5 mins, then re-fixed in 4% PFA for ≥ 20 mins at room temperature. Brains were then moved to 50% Hyb buffer in a 68 ºC microsample incubator for ≥ 1 hr, then incubated in pre-warmed probe solution containing 2.5 ng/μL probe overnight at 68 ºC.

For chromogenic *in situs*: samples were incubated for ≥ 2 hrs at room temperature in a blocking solution containing 2 mg/mL BSA and 5% normal goat serum in PBS-T. Blocking solution was exchanged for an antibody solution containing alkaline phosphatase (AP)-conjugated sheep anti-digoxigenin Fab fragments (Roche) diluted 1:2000 in blocking solution, then incubated overnight, nutating, at 4 ºC. Samples were then washed in 2 mg/mL BSA in PBS-T at room temperature, then equilibrated in NTMT buffer. Brains were then incubated in NBT/BCIP staining solution and covered to avoid light exposure. Development was allowed to proceed for ~1-2 hrs at room temperature, then the solution was exchanged for a fresh 1:10 dilution of staining solution and moved to 4 ºC, still covered, where the development proceeded for 4-5 days. Developed brains were re-fixed and stored in 80% glycerol at 4 ºC until imaged.

For fluorescence *in situs*: samples were incubated for ≥2 hrs at room temperature in a blocking solution containing 1X Blocking Reagent (Roche) in maleic acid buffer. Blocking solution was exchanged for an antibody solution containing horse radish peroxidase (POD)-conjugated sheep anti-digoxigenin Fab fragments (Roche) diluted 1:400 in blocking solution, then incubated overnight, nutating, at 4 ºC. Samples were washed in

PBS-T, then incubated in a tyramide solution containing 1:300 Cy3 tyramide in 1X amplification buffer (Perkin Elmer) for 20 mins in the dark. Brains were stored in Vectashield (Vector Labs) at 4 ºC until imaged.

For HCR *in situ* hybridization, the v3.0 protocol for whole mount zebrafish larvae by Molecular Technologies was followed with the following modifications: (1) In lieu of 1-phenyl 2-thiourea (PTU) treatment, larvae were 'bleached' in a solution of 0.5 mL 30% hydrogen peroxide and 1.0 mL 10% potassium hydroxide in water for ~30 mins until all pigment was gone. This step followed fixation but preceded methanol dehydration. (2) Larvae were not treated with proteinase K. (3) 2.5 μL (5 pmol) of each probe set ('odd' and 'even') were used. (4) Hybridization was allowed to proceed for 36 hours.

For HCR and ERK antibody co-labeling, after the HCR protocol, larvae were treated with 0.05% trypsin-EDTA on ice for 45 mins, then incubated in blocking solution (1% BSA, 2% normal goat serum, 2% DMSO, and 0.25% Triton-X in PBS) for 1 hr at room temperature. Larvae were then incubated in 1:500 mouse anti-ERK antibody in blocking solution at 4 ºC for 2-3 days, nutating, then in 1:500 AlexaFluor488-conjugated goat anti-mouse secondary antibody in blocking solution at 4 ºC for 2-3 days, nutating. Larvae were washed, then stored in Vectashield until imaged.

All HCR probe sets were designed and synthesized by Molecular Technologies, and the sequences of the probes are made available by Molecular Technologies upon request: mtnr1aa-B2, mtnr1ab-B3, vglut1/vglut2a/vglut2b-B6, gad1b/gad2-B7, chata/chatb-B8, glyt2-B9, GFP-B3. The fluorescent hairpins used in this study were: B2-AlexaFluor546, B2-AlexaFluor488, B3-AlexaFluor488, B6-AlexaFluor594, B7-AlexaFluor594, B8-AlexaFluor594, B9-AlexaFluor594.

Chromogenic *in situ* hybridization samples were imaged using a Zeiss Axoimager compound microscope with an EC Plan-Neofluar 10x/0.30 NA air objective or a Plan-Apochromat 20x/0.8 NA air objective. Fluorescent *in situ* samples, including HCR, and *Tg(mtnr1aa:GAL4); Tg(UAS:GFP)* larvae were imaged using a Zeiss LSM 780 or LSM 880 confocal microscope with a Plan-Apochromat 10x/0.45 NA air objective, a W Plan-

Apochromat 20x/1.0 NA water objective, or an LD C-Apochromat 40x/1.1 NA water objective.

2P-SPIM imaging. Live imaging was performed using a custom-built two-photon selective plane illumination microscope (2P-SPIM) (Truong et al., 2011) with 920-nm excitation pulsed laser light (Alcor), focused by spherical optics to yield a focused beam waist of approximately 4 μm and total power of 100 mW. Fluorescence signal was collected by a water-immersion objective (Olympus XLUMPLFLN-W 20x/1.0 NA), sCMOS camera (Hamamatsu ORCA-Flash 4.2), and appropriate spectral filters. For delivery of visual stimuli, 625-nm light was presented using an LED light source (Thorlabs), which was programmed to deliver a sequence of 100-ms LED flashes of the following intensities (in $\mu W/cm^2$): 0.2, 2.0, 0.4, 1.0, 4.0. Image acquisition and stimuli delivery were controlled using MATLAB and MicroManager software.

6-dpf *Tg(elavl3:GCaMP7f); nacre-/-* larvae were briefly anesthetized in tricaine (100 mg/L), embedded in 1% low-melting agarose, and mounted on a custom-built 'caddy,' which was submerged in the microscope imaging chamber containing 40 mL E3 larval media. Larvae were allowed to recover ~30 minutes prior to the start of imaging. During imaging, 10 focal planes spaced 5 μm apart, encompassing 50 μm of larval brain just beneath the dorsal surface of the head, which includes much of the optic tectum, were captured every 2 seconds. LED stimuli were presented in the above sequence of intensities, spaced 2 min apart. Each 5-flash sequence was repeated six times, for a total imaging session of 1 hour. After 1 hour of imaging and stimulus presentation, 20 mL of E3 media in the chamber was pipetted out of the chamber, and 20 mL of a 20 μM (2X) melatonin solution was pipetted into the chamber, for a final melatonin concentration of 10 μM. After melatonin delivery, larvae were left unperturbed for ~30 minutes, then imaging and stimulus presentation was resumed for 1 hour. In both the pre- and post-melatonin treatment imaging sessions, the first series of LED flashes (10 mins) was discarded from analysis.

GCaMP data was analyzed using Fiji/ImageJ and MATLAB. ROIs covering the optic tectum neuropil contralateral to the eye facing the LED light source were hand-drawn at 5 focal planes. Background subtraction was performed by subtracting the fluorescence intensity values of a small ROI drawn in the corner of the imaging frame.

Statistical analyses and figure preparation. All statistical analyses were carried out in GraphPad Prism. Figures were generated using Fiji/ImageJ, MATLAB, GraphPad Prism, and Microsoft PowerPoint.

Primers used for genotyping mutants:			
Gene	**Allele**	**Fwd/ Rev**	**Sequence (5' → 3')**
mtnr1aa	d20	Fwd	GTCAATGCCAAAACCCATTATT
		Rev	ACTGATCTGGCAGTGCATGTAG
mtnr1ab	d11a	Fwd	CATTGTGGATAATGGAGACCAG
		Rev	TTTACAGTGCCTGCAATTGTTT
mtnr1al	i5	Fwd	GATGGCGATGCCTGTAATGTTG
		Rev	CAGGAAATGCCTTTGTGGTGAG
mtnr1ba	d5	Fwd	TGTCTCCCTCATCAGGAACC
		Rev	GTGCCCTCCGTAAACATTCT
mtnr1bb	d11b	Fwd	GTGTAAAATCAGCGGCTTCC
		Rev	CTCGAGGGTCGTAACTCAGC
mtnr1c	d99	Fwd	TTTGTGGTGAGTCTTTCTGTGG
		Rev	GGTGAAGGTGCAGGAGAAAA
mtnr1aa	Ex1-KO	Fwd	TAATGAATGAGGGGACGACAG
		Rev1	CGACCCGGACAACTTTCC
		Rev2	TTATATGACTGAACTGGGAACTG
Primers used for riboprobe synthesis templates:			
Target		**Fwd/ Rev**	**Sequence (5' → 3')** (T7 transcription start site underlined)
mtnr1aa		Fwd	CCGTGGATGTCATGTACCAA
		Rev	GAATTGTAATACGACTCACTATAGGGTTGTTTAAATCCCGGTGGAG
mtnr1ab		Fwd	GCCTGGCAGTCGCTATTAAC
		Rev	GAATTGTAATACGACTCACTATAGGGATAAAACGCTCCCAAACAGC
mtnr1al		Fwd	GCCGCTGTCCTGTTATGATG
		Rev	GAATTGTAATACGACTCACTATAGGGCGAAAGTTGTGGTTTAGCACTC

Table 2.2. Primer sets used in this study. (Top) PCR primers for genotyping mutants used in behavioral assays. (Bottom) primers used to amplify templates from which riboprobes were synthesized. For each riboprobe, the T7 transcription start site, underlined, is positioned on the 3' primer such that an antisense probe is transcribed.

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