学位論文

SCOP/PHLPP1β in the basolateral amygdala regulates circadian expression of mouse anxiety-like behavior

(扁桃体基底外側核に存在するSCOP/PHLPP1βによる
マウス不安様行動の概日制御)

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SCOP/PHLPP1β in the basolateral amygdala regulates circadian expression of mouse anxiety-like behavior

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"*Education is what remains after one has forgotten what one has learned in school.*"

I am already looking forward to whatever that would remain after I forget everything presented in this dissertation. Hopefully, quite soon. Thank you guys, I love you.

ABSTRACT

The daily solar cycle has enabled organisms to equip with an internal circadian clock, allowing the synchronization of bodily functions according to the temporal niches they live in. Sleep/wake cycles, feeding patterns, and body temperature are among the most widely known physiological functions under the regulation of the circadian clock. Circadian regulation of physiology extends to higher brain functions including cognition and memory, such that time-of-day-dependent variations in cognitive performance and memory formation efficiency have been described in various species ranging from insects to humans. Adding to the growing list, the past decade has seen a huge leap in the understanding of the functional relationships between the circadian clock and animal emotionality/affect. While it had been long known that disturbances in human activity rhythms such as those arising from rotating shift work or jet lag increase the risk for mood disorders and that disrupted circadian rhythms are associated with major affective disorders, their causal relationship remained unknown until relatively recently.

Recent studies using rodent models have at least in part provided an explanation by describing the affective phenotypes arising from clock dysfunction, where perturbation of the circadian clock triggers a spectrum of affective abnormalities ranging from hyperactive mania to mixed anxiety states to anhedonia. While reports linking clock gene mutations and the resulting affective disorders are abundant today, however, the physiological roles of the circadian clock in affect regulation remain unexplained. In other words, the defects in affective behaviors associated with clock gene mutations are inconsistent with the circadian defects associated therewith, and, combined with the also inconsistent brain region-specific effects of clock dysfunction, addressing physiological affect regulation by the clock solely based on affective abnormalities observed in mice carrying clock gene mutations has proven insufficient.

Here, in order to more fully understand how the clock maintains affective behaviors at physiological levels, I characterized the time-of-day-dependent regulation of anxiety-like behavior in *Mus musculus*. I show that mouse anxiety-like behavior exhibits circadian rhythmicity and demonstrate that the circadian clock in the dorsal telencephalon (dTel) is required for the rhythmic expression of anxiety-like behaviors. I identify SCOP (suprachiasmatic nucleus circadian oscillatory protein; also known as PHLPP1β, pleckstrin homology domain leucine rich repeat protein phosphatase 1β) as an essential intracellular signaling molecule mediating the regulation of anxiety-like

behaviors downstream of the clock. Using viral-mediated, basolateral amygdala (BLA)-specific knockout of *Scop*, I demonstrate that SCOP in the BLA serves an anxiogenic function on the elevated plus maze. Collectively, I conclude that the circadian expression of SCOP in the BLA plays a pivotal role in generating the circadian rhythmicity in the anxiety-like behavior. My results demonstrate SCOP as a novel regulator of affect and reveal its key roles in the anxiogenic functions of the BLA and, together with the identification of dTel clock machineries in affect regulation, provide important insights into the mechanisms governing mammalian affective behaviors as well as into the pathology of affective disorders.

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5-HT	5-Hydroxylamine (Serotonin)
AAV	Adeno-associated virus
AKT	Protein kinase B/V-Akt Murine Thymoma Viral Oncogene Homolog
a.u.	Arbitrary unit
BLA	Basolateral amygdala
BMAL1	Brain and muscle arnt-like 1
CeA	Centromedial amygdala
cKO	Conditional knockout
CLOCK	Circadian locomotor output cycles kaput
CNS	Central nervous system
CT	Circadian time
DA	Dopamine
DAPI	4',6-diamidino-2-phenylindole
DBP	D site of albumin promoter binding protein
DRN	Dorsal Raphe nucleus
dLL	Constant dim light
dTel	Dorsal telencephalon
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
EGTA	Ethylene glycol tetraacetic acid
EPM	Elevated plus maze
ERK	Extracellular signal-regulated kinase
GSK3β	Glycogen synthase kinase 3β
iCre	Codon-improved Cre recombinase
kDa	Kilodalton
KO	Knockout
LC	Locus coeruleus
LD	Light/dark cycle
L-ITR	Left inverted terminal repeat
LTP	Long-term potentiation
NP-40	Nonident P-40

pA	Poly-adenosine
PFC	Prefrontal cortex
PH	Pleckstrin homology
PHLPP	Pleckstrin homology leucine-rich protein phosphatase
PMSF	Phenylmethanesulfonyl fluoride
OF	Open field
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
R-ITR	Right inverted terminal repeat
REV-ERBα	Nuclear receptor subfamily 1 group D member 1 (NR1D1)
RRE	HIV-1 Rev response element
SCN	Suprachiasmatic nucleus
SCOP	Suprachiasmatic nucleus circadian oscillatory protein
Tris	Tris(hydroxymethyl)aminomethane
VTA	Ventral tegmental area
WPRE	Woodchuck hepatitis posttranscriptional regulatory element
WT	Wild-type
ZT	Zeitgeber time

INTRODUCTION

Approach and avoidance behavior constitutes a core component of animal decision-making (Hull, 1952; Tooby and Cosmides, 1990). Approach behavior in the presence of rewarding stimuli such as food and sexual partners and avoidance behavior in the presence of aversive stimuli such as predators can be seen across phylogeny, and in humans, this can be seen in the form of emotional or affective behaviors. Emotion in humans fundamentally shapes their behavioral tendencies to approach or avoid by modulating appetitive-aversive motivation (Adams Jr et al., 2006; Corr, 2013). Fear or anxiety, for example, elicits a set of defensive behavioral responses toward an imminent or anticipatory aversive stimulus, respectively, and can be triggered by internal and/or external cues (Tovote et al., 2015). Recent studies utilizing optogenetic tools have identified various important brain regions and circuitries underlying fear/anxiety responses, including neuronal subpopulations in the basolateral amygdala (BLA) (Tovote et al., 2015). The amygdala is thought to be central to the regulation of fear/anxiety, and activation of BLA projections to the centromedial amygdala (CeA) elicits anxiolytic responses, whereas somatic activation of BLA neurons is anxiogenic (Tye et al., 2011). Nevertheless, how the regulatory machineries converge to execute affective modulation of behavior remains largely unknown. Molecular mechanisms underlying affect regulation are much less well understood, and currently available anxiolytic medications produce their effects through pharmacological mechanisms of action yet to be fully understood (Farach et al., 2012). In order to more fully understand mammalian affect, addressing affect regulation from an alternative perspective is imperative.

In light of this, recent findings on the functional relationship between the circadian clock and affective behaviors could provide an important clue. The circadian clock is an organism's internal pacemaker system with an intrinsic period of *circa* 24 hours, where the "master" clock in the hypothalamic suprachiasmatic nucleus (SCN) receives input from retinal photoreceptors and accordingly synchronizes "slave" clocks distributed throughout the body, driving diverse physiological phenomena (See Fig. 1 for molecular components). Dysfunctions of the circadian clock such as those arising from shift work or jet lag have been linked to a variety of affective disorders (Scott, 2000). Conversely, abnormalities in the circadian rhythmicity of various physiological measures have been observed in patients diagnosed with major affective disorders (McClung, 2007). In rodents, perturbations of the circadian clock by means of surgical, genetic, pharmacological, light-induced, or behavioral manipulations lead to a spectrum of affective abnormalities (Table 1) (Gessa et al., 1995;

Tataroğlu et al., 2004; McClung, 2007; O'Donnell and Gould, 2007; Roybal et al., 2007; Hampp et al., 2008; LeGates et al., 2012; Spencer et al., 2013; Chung et al., 2014).

Recent evidence points to a mechanism by which dysfunctions in the circadian clock lead to affective abnormalities through aberrant dopaminergic activity in the ventral tegmental area (VTA), a major dopaminergic nucleus (Mukherjee et al., 2010; Chung et al., 2014). Despite the established roles of dopamine and other monoamine systems in affect regulation, their causality in affective disorders and sufficiency in the regulation of affect *per se* have been questioned (Krishnan and Nestler, 2008; Maes et al., 2011; Sanacora et al., 2012). Furthermore, while these studies provide important insights into affective abnormalities arising from clock dysfunction, the diversity of the affective phenotypes observed in multiple mouse lines carrying mutations in clock genes remains unexplained. For example, a loss-of-function mutation in the *Clock* gene results in mania-like behaviors including hyperactivity, reduced sleep, reduced anxiety-like and depression-like behaviors, and enhanced preference for cocaine and sucrose, whereas loss-of-function or null mutations in the *Per1* and/or *Per2* genes result in elevated anxiety-like behavior and reduced depression-like behavior (Roybal et al., 2007; Spencer et al., 2013). Lesioning of the SCN leads to decreased depression-like behavior, while *Cry1* and *Cry2* double knockout mice exhibit increased anxiety-like behavior and anhedonia (Tataroğlu et al., 2004; De Bundel et al., 2013). These results are further complicated by the inconsistent brain region-specific phenotypes such as the one triggered by knockdown of *Clock* in the VTA, which results in reduced anxiety-like behavior but enhanced depression-like behavior (Mukherjee et al., 2010).

Altogether, it is clear that the regulation of affect by the circadian clock comprises a complex system involving multiple brain regions, hindering our understanding thereof by the great complexity of associated phenotypes. Thus, in order to provide useful insights, the plethora of affective phenotypes arising from clock dysfunction need be factorized. To this end, I focused on the straightforward but as-yet-established possibility of time-of-day-dependent regulation of mouse anxiety-like behavior, an affective behavior partly dependent on the amygdala function (Tovote et al., 2015). The efficiency of fear memory formation in mice, another amygdala-dependent function, has been shown to exhibit time-of-day-dependent variations (Chaudhury and Colwell, 2002), whereas fear-eliciting stimuli synchronize the circadian expression of clock genes in the amygdala but not in the SCN (Pantazopoulos et al., 2011). In humans, both positive and negative affects are reported to display diurnal variation (Golder and Macy, 2011), whereas excessive diurnal variation in mood states are a hallmark of major depressive and bipolar disorders (Hall et al., 1964),

implicating the physiological importance of time-of-day-dependent regulation of affect. In the present study, I sought to unravel the neuronal and molecular mechanisms governing affect regulation by characterizing temporal regulation of mouse anxiety-like behaviors by the circadian clock and by elucidating molecular and neuro-anatomical components underlying the temporal anxiety regulation by the clock.

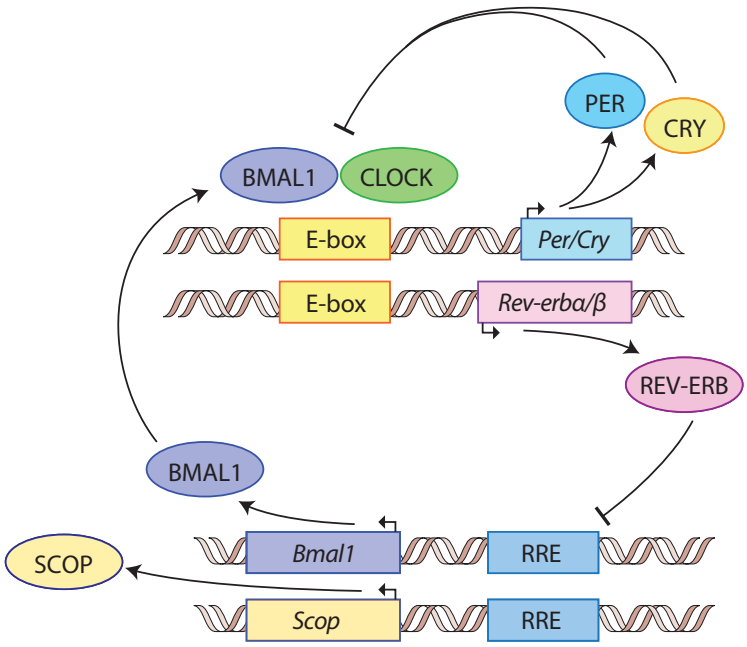

Figure 1. Molecular components of the mammalian circadian clock. The "molecular clock" consists of multiple autoregulatory transcriptional-translational negative feedback loops, of which two are shown here. The first loop comprises two transcription factors: BMAL1 and CLOCK, and two transcriptional repressors: PERIOD (PER) and CRYPTOCHROME (CRY). Two isoforms exist for each repressor. BMAL1 and CLOCK heterodimerize and activate transcription of various clock-controlled genes through binding to the E-box *cis* elements of the target genes. Transcription of *Per1/2* and *Cry1/2* is also activated by the BMAL1-CLOCK heterodimer. PER and CRY proteins accumulate during the day and translocate to the nucleus, where they repress the BMAL1-CLOCK-mediated transcription. This results in repressed transcription of E-box genes during the night, leading to a gradual reduction in the PER and CRY levels. Freed from repression by PER/CRY, BMAL1 and CLOCK then re-bind to the E-box elements and start a new cycle. One such cycle takes circa. 24 hours to complete, forming the basis for the cell-autonomous nature of the circadian clock. Zeitgebers, i.e. time cues such as light, can adjust the molecular clock through various means, including the induction of *Per* expression. The "subloop" consists of BMAL1-CLOCK-mediated E-box transcription of *Rev-erbα/β* and REV-ERB-mediated repression of RRE (HIV-1 Rev response element) *cis* element. RRE-regulated genes include *Bmal1*, whose expression peaks at dawn. Shown here is a simplified schematic, and each clock-controlled gene can have multiple regulatory *cis* elements, working in concert to determine the phase of the gene's rhythmic expression.

Table 1. Summary of clock-related gene alterations and associated phenotypes.
"Anxiety" denotes mouse anxiety-like behavior evaluated by an elevated plus maze test; "Depression" denotes mouse depression-like behaviors evaluated by tail-suspension and/or Porsolt forced swim tests.

Manipulation/Genotype	Affective Phenotype					Period
SCN lesion [1]	↑ Activity	↓ Depression	———			Arrhythmic
Clock^{A19} [2]	↑ Activity	↓ Depression	↓ Anxiety	↑ Cocaine pref.	↑ DA	Long
Clock KD in VTA [3]	↑ Activity	↑ Depression	↓ Anxiety			(Long?)
Per2$^{Brdm1/Brdm1}$ [4]	———	↓ Depression	———	↓ MAOA	↑ DA	Short
Per1$^{-/-}$/*Per2*$^{-/-}$ DKO [5]	Normal	———	↑ Anxiety			Arrhythmic
Per1/*Per2* KD in NAc [5]	Normal	———	↑ Anxiety			(Short?)
Cry1$^{-/-}$ or *Cry2*$^{-/-}$ [6]	Normal	Normal	↑ Anxiety			Long
Cry1$^{-/-}$ *Cry2*$^{-/-}$ DKO [6]	↓ Activity	Normal	↑ Anxiety	↓ Cocaine preference		Arrhythmic
Nr1d1 (*Rev-erbα*) KO [7]	———	↓ Depression	↓ Anxiety		↑ DA	Short
GSK3β^{S9A} overexpression [8]	↑ Activity	↓ Depression	———			Short
Fbxl3$^{Afh/Afh}$ [9]	Normal	↓ Depression	↓ Anxiety			Long

(VTA: Ventral Tegmental Area; NAc: Nucleus accumbens; MAOA: monoamine oxidase-A; DA: Dopamine)

References: 1. Tataroğlu et al., 2004; 2. Roybal et al., 2007; 3.Mukherjee et al., 2010; 4. Hampp et al., 2008; 5. Spencer et al., 2013; 6. De Bundel et al., 2013; 7. Chung et al., 2014; 8. Prickaerts et al., 2006; 9. Keers et al., 2012.

MATERIALS AND METHODS

Plasmids. pCAG-iCre was a generous gift from Dr. Takahiko Matsuda (Kyoto University Institute for Virus Research, Kyoto, Japan). The pCCALL2 vector was a generous gift from Dr. Andras Nagy (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada). To produce pCCALL2-mCherry, a fragment containing the *mCherry* gene from *ins:mCherry* (Pisharath et al., 2007) was inserted into pCCALL2 linearized with *Xho*I and *Bgl*II.

Animals and housing. All experiments were conducted in accordance with guidelines set by The University of Tokyo and approved by the Committee on Animal Care and Use of the Graduate School of Science at The University of Tokyo. One hundred wild-type male C57BL/6J mice were purchased from Tokyo Laboratory Animals Science Co., Ltd (Tokyo, Japan). *Bmal1*$^{fl/fl}$ mice (Shimba et al., 2011), kindly provided by Dr. Shigeki Shimba (Nihon University Department of Pharmacy, Japan), were crossed with *Emx1*$^{Cre/+}$ mice (Iwasato et al., 2000; Kassai et al., 2008), kindly provided by Dr. Atsu Aiba (Center for Disease Biology and Integrative Medicine, The University of Tokyo, Japan), to obtain dorsal telencephalon (dTel)-specific *Bmal1*$^{fl/fl}$ *Emx1*$^{Cre/+}$ conditional knockout (cKO) mice. *"fl"* denotes a floxed allele. *Scop*$^{fl/fl}$ mice (K. Shimizu, E. Nakatsuji, Y. Kobayashi, Y. Fukada, submitted) were crossed with *Emx1*$^{Cre/+}$ mice to obtain *Scop*$^{fl/fl}$ *Emx1*$^{Cre/+}$ conditional knockout (cKO) mice. Briefly, *Scop*$^{fl/fl}$ mice were generated by Dr. Kimiko Shimizu by flanking the exon 4 of *Scop* (*Phlpp1*) gene with loxP sequences; Cre-mediated recombination results in a frameshift mutation and a premature stop codon, producing a non-functional SCOP protein lacking the functional domains except for the PH domain (Shimizu et al., submitted). *Bmal1*$^{fl/fl}$ mouse line was on a C57BL/6J background; *Scop*$^{fl/fl}$ mouse line was on a C57BL/6N background. All animals were housed under a 12 hr light/ 12 hr dark cycle (lights on at 8 am; ca. 200 lux) in temperature- and humidity-controlled compartments with food and water available *ad libitum*.

Adeno-associated virus (AAV) vector production. To produce iCre::EGFP fusion protein construct, the *EGFP* gene was PCR amplified from pEGFP-C1 (Addgene 2487) using the primer set: 5'-CTGCT CGAGG ATGGG GACGG ACCGG TCGCC ACCAT GGTGA GCAAG-3' and 5'-CGAGG CGGCC GCTAT TACTT GTACA GCTCG TCCAT GCCGA GAGTG-3'. The fragment was digested with *Xho*I and *Not*I and inserted into pCAG-iCre digested with *Xho*I and *Not*I to produce pCAG-iCre::EGFP. The *iCre::EGFP* fragment including the nuclear-localization signal (NLS) was then PCR amplified using the primer set: 5'-CTGGA ATTCG CGGCC GCTAT TACTT GTACA GCTCG TC-3' and 5'-

TTCGG ATCCG CCACC ATGGT GCCCA AGAAG AA-3'. The fragment was then digested with *Bam*HI and *Eco*RI and inserted into the 4.5-kb fragment of pAAV-hSyn-hChR2 (H134R)-EYFP (Addgene 26973) digested with *Bam*HI and *Eco*RI to obtain pAAV-hSyn-iCre::EGFP-WPRE ("pAAV-Cre"). The resulting vector encodes a fusion protein of iCre and EGFP with a 5 amino acid linker in between (GPVAT); EGFP fusion at the C-terminus has been shown to not interfere with Cre recombinase activity (Berton et al., 2006). To produce pAAV-hSyn-EGFP-WPRE ("pAAV-GFP"), the *EGFP* gene was PCR amplified from pEGFP-C1 using the primer set: 5'-CGCTG GATCC ACCGG TCGCC ACCAT GGTGA GCAAG-3' and 5'-AGATG AATTC TATTA CTTGT ACAGC TCGTC CATGC CGAGA GTG-3'. The fragment was then digested with *Bam*HI and *Eco*RI and inserted into the 4.5-kb fragment of pAAV-hSyn-hChR2 (H134R)-EYFP digested with *Bam*HI and *Eco*RI.

To validate the functionality of iCre::EGFP fusion protein, pCAG-iCre::EGFP was transfected to HEK293T/17 cells with pCCALL2-mCherry, which contains floxed *β-geo* (*lacZ*/neomycin-resistance fusion gene) cassette in front of mCherry gene. Upon Cre-mediated recombination, the *β-geo* gene is excised out, and mCherry is expressed. pCAG-iCre served as a control for recombinase activity. Transfection was carried out using a standard polyethylenimine (PEI) method. Briefly, DNA (500 μg per 100-mm culture dish) was diluted in dH_2O, mixed with PEI (100 μg/mL final), incubated for 15 min at RT, and added to the culture medium. GFP and mCherry intensities were examined 48 hours after transfection under an inverted fluorescent microscope (Keyence, Osaka, Japan). To verify that the human synapsin (hSyn) promoter induces Cre expression in neuronal cells, Neuro-2a (N2a) cells were transfected with pAAV-Cre or pAAV-GFP using Lipofectamine 3000 reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). GFP and mCherry intensities were examined as described above.

Validated pAAV vectors were then transfected to HEK293T/17 cells, together with pACG2-Y730F (Zhong et al., 2008) and pHelper (Stratagene) plasmids, using a standard PEI method described above with minor modifications. The ratio of plasmid constructs transfected was as follows: pAAV-Cre or pAAV-GFP, 5 μg; pACG2, 5.5 μg; pHelper, 10 μg. Cells were harvested 72 hrs after transfection, collected in PBS, pelleted, and resuspended in 200 μl of Dulbecco's PBS (1 mM $MgCl_2$) per 100-mm culture dish (culture area: 60 cm^2). After 3 freeze-thaw cycles in liquid nitrogen and 37°C water bath followed by a centrifugation, the lysate was treated with 1.25 U/μL Benzonase (Merck, Darmstadt, Germany) for 30 min at 37°C and then subjected to serial centrifugation until the supernatant was clear. The final purified viruses were aliquoted and stored at -80°C until

use. The titers of my purified viral vectors were as follows: AAV-Cre, 8 x 10^{12}; AAV-GFP, 1 x 10^{13} genome copies/mL.

To verify the infectious ability, purified AAV viral vectors were transduced to primary hippocampal neurons. Primary hippocampal cultures were prepared as previously described (Shimizu et al., 2007). Purified AAV-Cre or AAV-GFP was added to primary cultures at 20 days *in vitro* (DIV), and GFP signal was examined at 40 DIV as described above.

Surgery. Male *Scop*$^{fl/fl}$ mice aged 8-10 weeks were deeply anesthetized with a mixture of ketamine (140 mg/kg) and xylazine (8.8 mg/kg) in bacteriostatic saline given intraperitoneally (20 ml/kg) and placed on a stereotactic apparatus (Narishige, Tokyo, Japan). The skull was exposed, and holes were drilled bilaterally above the basolateral amygdala. The coordinates relative to bregma were: anteroposterior, -1.65 mm; lateral, +3.30 mm; dorsoventral, -4.45 mm. Mice were bilaterally injected with 0.5 µl of either AAV-iCre::EGFP or AAV-EGFP over 5 min, and the needles were kept in place for an additional 5 min to ensure infusion.

Behavioral assays. Male mice aged 12-16 weeks (wild-type and AAV-injected *Scop*$^{fl/fl}$) or 20-25 weeks (*Bmal1* and *Scop* cKO) were subjected to behavioral tests. Littermate *Bmal1*$^{fl/fl}$ *Emx1*$^{+/+}$ or *Scop*$^{fl/fl}$ *Emx1*$^{+/+}$ mice were used for control. Prior to testing, all mice were singly housed, entrained to the LD cycle for >2 weeks, and handled daily for acclimation at random times of day. Mice that underwent stereotactic surgeries were allowed to recover for 4 weeks before the behavioral tests. Each mouse received one trial on each behavioral paradigm, with the elevated plus maze test always preceding the open field test. All behavioral assays were conducted under dim light at 4.0 ± 0.1 lux at the center of the apparatuses. 4 lux was the minimum illuminance required for video tracking in our set up. Apparatuses were cleaned and dried after every experimental trial. For BLA-specific KO experiments, behavioral data of AAV-injected mice were sorted according to their infection sites determined by fluorescent microscopy (see below). Behavioral data from mice with clear bilateral GFP signal in the basolateral amygdalar complex (BLA; comprising lateral, basolateral, basomedial nuclei) were adopted as AAV-Cre or AAV-GFP data. Data from mice injected with AAV-Cre but with no GFP signal in the BLA were treated as "OFF-BLA" data.

Elevated Plus Maze. Mice were placed in the center of an elevated plus maze (O'Hara & Co., Ltd., Tokyo, Japan) facing one of the closed arms. The maze has four arms (5 x 25 cm), the opposing two of which are protected with clear walls (16 cm high), and is elevated 50 cm from the ground. Mice were allowed to explore the maze for 5 min; their behavior

was monitored with an automated video tracking system, and the time spent on open arms and entries into open/closed arms were determined using TIME_EP software (O'Hara & Co., Ltd.) or manually by an analyst blinded to time-of-day information (see Data analysis).

Open Field. Mice were placed in a 45 x 45 cm open field (O'Hara & Co., Ltd.), and their behavior was monitored for 5 min with an automated video tracking system. The time spent in the center of the open field (30% area, circular) and the distance traveled were determined using ImageJ software (NIH, MD, USA) with OpenField plug-in (O'Hara & Co., Ltd.).

Tissue preparation. For mRNA and protein analyses, mice were sacrificed by rapid cervical dislocation. Brain sections of 1-mm thickness containing the amygdala were prepared using a brain matrix (ASI Instruments, Warren, MI, USA), and basolateral and centromedial amygdalar complexes (BLA and CeA, respectively) were dissected using surgical knives, rapidly frozen, and stored at -80°C.

For the validation of *Scop* BLA KO, *Scop*$^{fl/fl}$ mice transduced with AAV-Cre or AAV-GFP were sacrificed, and brain sections were prepared as described above. The slices were then mounted on a glass slide and analyzed for GFP signal under a fluorescent stereoscopic microscope (Leica, Wetzlar, Germany). GFP-positive regions of the BLA were cut out using surgical knives while mounted on the microscope, rapidly frozen, and stored at -80°C until subsequent RNA extraction.

For fluorescent microscopy, brain slices were prepared by one of the following two methods: 1. AAV-injected mice were sacrificed by rapid cervical dislocation, and the brain was removed, mounted on a brain matrix (ASI Instruments), and sliced into 1-mm thick sections. 2. AAV-injected mice that had undergone behavioral assays were deeply anesthetized as described above and transcardially perfused with 25 mL of 0.9% saline containing 2.0 mM EDTA followed by 50 mL of 3% paraformaldehyde in PBS. The brains were removed, postfixed for 4 hrs at 4°C, cryoprotected in 30% sucrose in PBS overnight at 4°C, rapidly frozen at -50°C, and sectioned into a series of 20 μm-thick free-floating coronal slices using a cryostat (Leica). The 1-mm-thick sections were analyzed for GFP signal under a fluorescent stereoscopic microscope (Leica). The 20 μm-thick sections were mounted on glass slides (MAS-coated; Matsunami Glass, Osaka, Japan) with VectaShield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and analyzed for GFP signal under an inverted fluorescent microscope (BZ-9000; Keyence, Osaka, Japan).

Quantitative reverse transcription PCR. To quantitate mRNA levels, brain tissues were homogenized in TRIzol (Ambion, Oakland, CA, USA) using 27 gauge needles, and total RNA was extracted and purified using RNeasy minElute columns (Qiagen #74204,

Valencia, CA, USA) according to the manufacturers' instructions. Extracted RNA was adjusted to 200 ng/μL, treated with 1U per μg RNA of DNaseI (Promega, Madison, WI, USA) for 30 min at 37°C, and reverse transcribed with GoScript Reverse Transcriptase (Promega) with an equimolar mixture of random hexamer and oligo-dT primers. Using the cDNA as a template, transcripts for *Rps29, Bmal1, Dbp, Scop,* and *Nr1d1* (*Rev-erbα*) were amplified with gene-specific primers using the GoTaq Master Mix (Promega #A6001) and a real-time qPCR thermal cycler (StepOne Plus, Life Technologies, MA, USA). Primers used for *Rps29, Bmal1* and *Dbp* have been previously described (Hirano et al., 2013). The primers for *Scop* were: 5'-CTCCC ACCAA ACCTT CTCAT-3' and 5'-GCAGG GTTTC CAGTT TGTTT-3' for Fig.. 8 and 5'-TTGAA CATCT GCCTG CCAAC-3' and 5'-GGGGG TTTGC CTTAG GAAGT T-3' for Fig. 9; the primers for *Nr1d1* were: 5'-CGTTC GCATC AATCG CAACC-3' and 5'-GATGT GGAGT AGGTG AGGTC-3'.

SDS-PAGE and Western blotting. BLA and CeA tissues were homogenized by syringing through 27 gauge needles in TNE buffer with NP-40 [20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40] with 5 mM EGTA, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and protease inhibitor cocktail (Complete EDTA-free, Roche, Basel, Switzerland). The homogenates were kept on ice for 15 min and spun at >10,000 x *g* for 15 min. The supernatants were subjected to SDS-PAGE and immunoblotting as previously described (Shimizu et al., 2007). Signal was visualized using a conventional enhanced chemiluminescence detection system (GE Healthcare, Waukesha, WI, USA) and quantified using ImageJ software (NIH) by densitometric analyses based on relative standards loaded on every polyacrylamide gel. Immunoblotting conditions were as follows: αCB rabbit polyclonal antibody against SCOP (Shimizu et al., 1999), 1/1,500 in 3% BSA/TBST; mouse monoclonal antibody against β-actin (Sigma #A2228), 0.4 μg/mL in 3% skim milk (Difco, Detroit, MI, USA)/TBST; blocking: 1 hr at RT, primary antibody: overnight at 4°C, secondary antibody: 2 hrs at RT.

Data analysis. Behavioral analyses were automated except for the elevated plus maze (EPM) test for wild-type C57BL/6J mice, for which all recorded behavioral data were shuffled into random orders, and entries into open and closed arms and the time spent on each arm were recorded by an operator blinded to time-of-day information for each subject. One-way ANOVA tests were used to examine the statistical significance of data consisting of three or more groups. Unpaired two-tail Student's *t*-tests were used for analysis on data with two groups. All data are presented as means with SEM. *P* values are presented as "*P*" for ANOVA tests and "*p*" for *t*-tests.

RESULTS

Circadian expression of anxiety-like behaviors in wild-type mice

In order to establish the basis for examining the temporal regulation of affective behaviors by the circadian clock, I profiled the time-of-day-dependent variations in anxiety-like behaviors of wild-type mice. To evaluate mouse anxiety-like behaviors, I utilized elevated plus maze (EPM) and open field (OF) tests. These paradigms assess anxiety-like behaviors based on the subject's exploratory activity in a novel, aversive environment and have been validated extensively (Pellow et al., 1985; Belzung and Griebel, 2001; Prut and Belzung, 2003). One group of mice ("LD group", $n = 24$) were tested under a light-dark (LD) cycle at one of four or eight zeitgeber times (ZTs; lights on at ZT0 and off at ZT12). To eliminate the effects of external light conditions and extract the regulation by the intrinsic time-keeping system, the other group ("dLL group", $n = 76$) were housed under a constant dim light condition ("dLL") for > 24 hrs and tested on the second day under dLL at one of four or eight projected circadian times (Fig. 2*A*) (CTs; subjective day starts at CT0 and ends at CT12). Testing CTs were projected using a circadian period (τ) of 25 hrs, as the C57BL/6J mouse strain used in this study consistently exhibited activity rhythms with a τ of 25.05 ± 0.07 hr s.e.m. ($n = 6$).

Anxiety-like behaviors, as evaluated both by the open arm entries on the EPM and by the time spent in the center of the OF, exhibited both diurnal and circadian rhythmicity under LD and dLL, respectively (Fig. 2*B,C,E*) (EPM % open entries: LD, $F_{3,20} = 7.32$, $P = 0.0017$, dLL, $F_{3,44} = 7.44$, $P = 0.0004$; EPM time on open arms, LD, $F_{3,20} = 7.15$, $P = 0.0019$, dLL, $F_{3,44} = 5.10$, $P = 0.0041$; OF center time, LD, $F_{3,20} = 4.08$, $P = 0.021$, dLL, $F_{7,68} = 2.41$, $P = 0.029$). Anxiety-like behaviors on the EPM and OF (hereafter referred to as "EPM anxiety-like" and "OF anxiety-like" behaviors, respectively) had rhythms almost anti-phasic to each other, supporting the notion that these anxiety-like behaviors reflect distinct physiological functions (Carola et al., 2002; Ramos, 2008; Ramos et al., 2008) (See Discussion). No significant changes in the numbers of total arm entries or in general locomotor activity levels were found across the day except for OF total distance under LD (Fig. 2*D,F*) (EPM total entries: LD, $F_{3,20} = 0.37$, $P = 0.78$, dLL, $F_{3,44} = 1.25$, $P = 0.30$; OF total distance, LD, $F_{3,20} = 4.61$, $P = 0.013$, dLL, $F_{7,68} = 1.63$, $P = 0.14$). The diurnal profiles were markedly similar between the LD and dLL groups, suggesting that mouse anxiety-like behaviors are under active regulation by the intrinsic circadian clock.

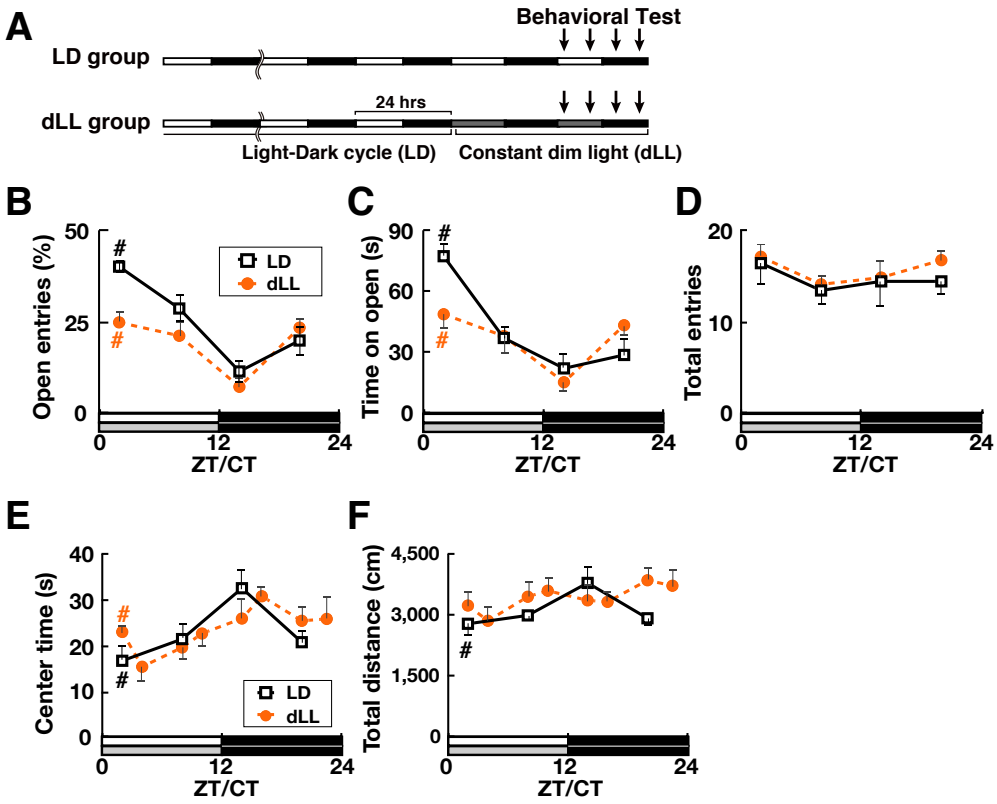

Figure 2. Diurnal and circadian expression of anxiety-like behaviors in wild-type mice on elevated plus maze (EPM, ***B-D***) and open field (OF, ***E,F***) tests under LD and dLL conditions. ***A,*** Timeline for behavioral assays. For LD tests, mice were kept under an LD cycle and tested at one of 4 or 8 ZTs (Zeitgeber Time; lights on at ZT0 and off at ZT12). For dLL tests, mice were placed under constant dim light 1 day prior to the testing and were assayed at one of 4 or 8 CTs (Circadian Time; subjective day starts at CT0 and ends at CT12). Each mouse received one trial for EPM and one trial for OF. ***B-D,*** Entries into open arms (***B***, "% Open entries", ratio of open:total entries) and the time spent on open arms (***C,*** "Time on open") represent anxiety-like behavior, while the total number of entries (***D***) represents general locomotor activity. ***E-F,*** The time spent on the center 30% area (***E,*** "Center time") and the total distance traveled (***F,*** "Total distance") are shown for the OF. White/black bars on the bottom represent the light/dark cycle (for LD) or subjective day/night (for dLL). # $P < 0.05$ between ZTs/CTs by one-way ANOVA. See Results for individual F and P values. $n = 6$ per data point for EPM LD and OF LD, $n = 12$ per data point for EPM dLL, $n = 7$-12 for OF dLL.

The circadian clock in the dorsal telencephalon is responsible for the rhythmic expression of anxiety-like behaviors

To directly examine the regulation of anxiety-like behaviors by the circadian clock, I generated *Bmal1*$^{fl/fl}$ *Emx1*$^{Cre/+}$ conditional knockout (cKO) mice, which lack functional BMAL1 protein in the dorsal telencephalon (dTel). BMAL1 (Brain and Muscle Arnt-Like 1; also known as Aryl Hydrocarbon Receptor Nuclear Translocator-Like, ARNTL), a basic helix-loop-helix transcription factor activating E-box-mediated transcription, constitutes the core transcriptional and translational feedback loop of the molecular circadian clock (Fig. 1), and cells lacking BMAL1 lose circadian rhythmicity (Bunger et al., 2000). This cKO allows to eliminate the effects from clock dysfunctions in the SCN, which leads to a

systemic loss of circadian rhythmicity, and in the monoamine-producing nuclei in the midbrain.

While *Bmal1* cKO mice exhibited normal activity rhythms and appeared physically normal (Fig. 3*E,F*), the circadian variations in anxiety-like behaviors were lost in *Bmal1* cKO mice (Fig. 3*A,C*) (EPM: WT, $p = 0.039$, cKO, $p = 0.45$; OF: WT, $p = 0.036$, cKO, $p = 0.86$). Anxiety-like behaviors remained high in *Bmal1* cKO mice (EPM, $p = 0.0032$ at CT2; OF, $p = 0.0020$ at CT14) across the day at levels comparable to the peak levels in wild-type mice. Hyperactivity or other mania-like behaviors were not evident, as *Bmal1* cKO had no significant effect on general locomotor activities (Fig. 3*B,D*) (EPM total entries CT2: $p = 0.59$; OF total distance CT14: $p = 0.73$) (EPM total distance traveled (cm): WT CT2, 1232 ± 67, CT14, 1129 ± 93; cKO CT2, 1226 ± 110, CT14, 1210 ± 124; $F_{3,31} = 0.26$, $P = 0.86$). The effects of *Bmal1* cKO did not exceed the range of the circadian variation in affective behaviors expressed by littermate control mice (*Bmal1*$^{fl/fl}$ *Emx1*$^{+/+}$) (Fig. 3*A,C*). These findings contrast with previous studies reporting for mice carrying whole-body mutations in clock genes a diverse range of affective abnormalities to varying degrees and directions (Roybal et al., 2007; Hampp et al., 2008; Mukherjee et al., 2010; De Bundel et al., 2013; Spencer et al., 2013; Chung et al., 2014). My results rather indicate that the circadian clock in the dTel functions to actively generate circadian rhythms in anxiety-like behaviors.

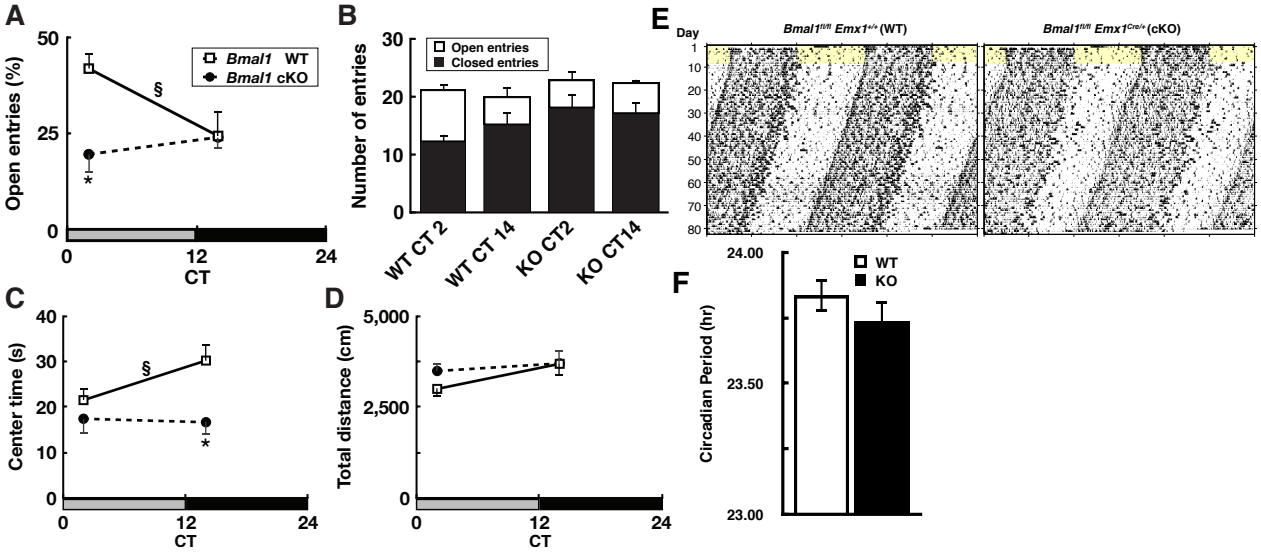

Figure 3. Circadian machineries in the dorsal telencephalon (dTel) regulate EPM (***A-B***) and OF (***C,D***) anxiety-like behaviors. ***A,*** % Open entries for *Bmal1* cKO mice (*Bmal1*$^{fl/fl}$ *Emx1*$^{Cre/+}$; open square) and littermate wild-type mice (WT, *Bmal1*$^{fl/fl}$ *Emx1*$^{+/+}$; filled circle) mice are shown. ***B,*** Numbers of entries into closed arms (filled) and open arms (open) are shown as stacked bars. ***C,D,*** The Center time for *Bmal1* cKO and littermate WT mice (***C***) and total distance traveled (***D***). *$p < 0.05$ vs WT at the corresponding CT, §$p < 0.05$ between CTs by Student's *t*-test. *n* for *Bmal1* WT: CT2, 9; CT14, 10. *n* for *Bmal1* cKO: CT2, 8; CT14, 8. ***E,F,*** Locomotor activity rhythms in *Bmal1* cKO and their littermate WT mice. ***E,*** Double-plot actogram showing 48 hrs of activity per line. Yellow squares denote the light phase during the LD cycle (7 days), below which is activity under constant darkness. ***F,*** Free-running circadian periods of *Bmal1* cKO ($n = 4$) and their littermate WT mice ($n = 5$) quantified from ***E***. $p > 0.3$ by unpaired Student's *t*-test.

SCOP in the dorsal telencephalon is required for circadian expression of anxiety-like behaviors

In order to gain further insights into the mechanisms governing the circadian regulation of anxiety-like behavior, I sought to identify the molecular components downstream of the circadian clock and hypothesized the involvement of SCN circadian oscillatory protein (SCOP/PHLPP1β). SCOP is a 183-kDa protein comprising Pleckstrin homology, leucine-rich repeat, protein-phosphatase 2C-like, glutamine-rich, and PDZ-binding domains (Fig. 4*A*; hence the alternate name PHLPP), and has been shown to regulate a range of intracellular signaling pathways (Fig. 4*B*) (Shimizu et al., 2003; Jackson et al., 2010; Shimizu et al., 2010). Originally identified as a gene product whose expression oscillates in a circadian manner in the rat SCN (Shimizu et al., 1999), SCOP plays an essential role in the consolidation of mouse long-term object recognition memory by serving as a reservoir for nucleotide-free K-Ras (Shimizu et al., 2003; 2007). SCOP and its shorter isoform PHLPP1α have been shown to regulate multiple signaling pathways including PI3K/AKT/GSK3β signaling through dephosphorylation of AKT, whereby SCOP and PHLPP1α exert tumor suppressive functions (Gao et al., 2005; Brognard and Newton, 2008).

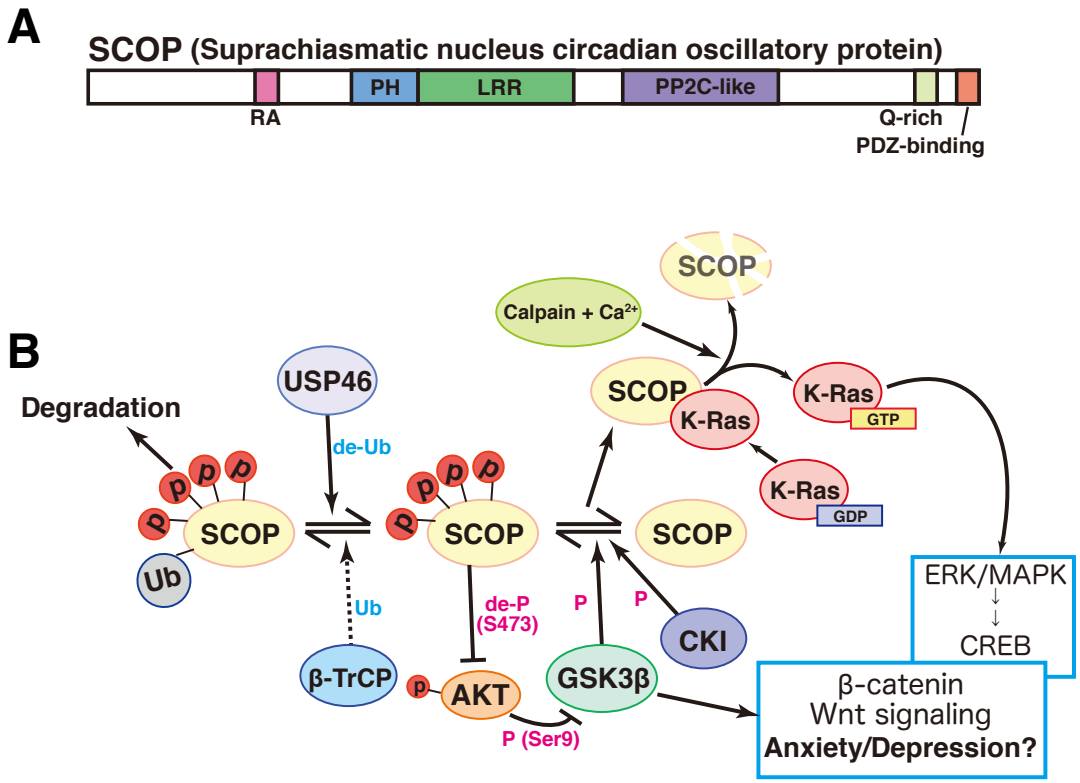

Figure 4. Model for SCOP function in anxiety regulation. ***A,*** Domain structure of SCOP. RA, putative Ras-association; PH, Plekstrin Homology; LRR, Leucine-rich repeat; PP2C, protein phosphatase 2C. ***B,*** Regulation of AKT/GSK3β and K-Ras/MAPK/CREB pathways by SCOP. SCOP dephosphorylates AKTs at the hydrophobic motif (S473 in mouse AKT1), suppressing its kinase activity. SCOP interacts with the nucleotide-free form of K-Ras and functions as a reservoir: Elevated intracellular Ca^{2+} levels trigger Calpain-mediated degradation of SCOP, releasing GTP-bound K-Ras to activate downstream MAPK cascades.

Given the highly enriched expression of SCOP in neurons (Shimizu et al., 1999) and its roles in the regulation of major intracellular signaling pathways, I examined the possibility of SCOP functioning as a circadian output pathway in the regulation of affective behaviors. I first generated dTel-specific *Scop* cKO mice (*Scop*$^{fl/fl}$ *Emx1*$^{Cre/+}$) and examined their anxiety-like behaviors. In both EPM and OF paradigms, *Scop* cKO mice failed to express circadian changes in anxiety-like behaviors (Fig. 5*A,C*) (EPM: WT, $p = 0.0014$, cKO, $p = 0.26$; OF: WT, $p = 0.049$, cKO, $p = 0.98$). In *Scop* cKO mice, EPM and OF anxiety-like behaviors remained constantly low throughout the day (EPM, $p = 0.0046$ at CT14), a phenotype distinct from that of *Bmal1* cKO mice (Fig. 3). Since whole-body *Scop* KO mice show no observable defects in circadian activity rhythms or sleep/wake cycles (Masubuchi et al., 2010) (Shimizu et al., submitted), SCOP is likely to function downstream of the circadian clock to express anxiety-like behaviors. OF anxiety-like behavior was less unequivocal in that while the circadian rhythmicity was lost, the effect of cKO was not statistically significant at either CT (Fig. 5*C*) ($p = 0.064$ at CT2). This further points to a differential regulation of EPM and OF anxiety-like behaviors. *Scop* cKO had no significant effects on general activity levels (Fig. 5*B,D*) (EPM total entries: $F_{3,41} = 1.56$, $P = 0.21$; EPM total distance: $F_{3,41} = 0.14$, $P = 0.93$; OF total distance: $F_{3,41} = 0.16$, $P = 0.92$).

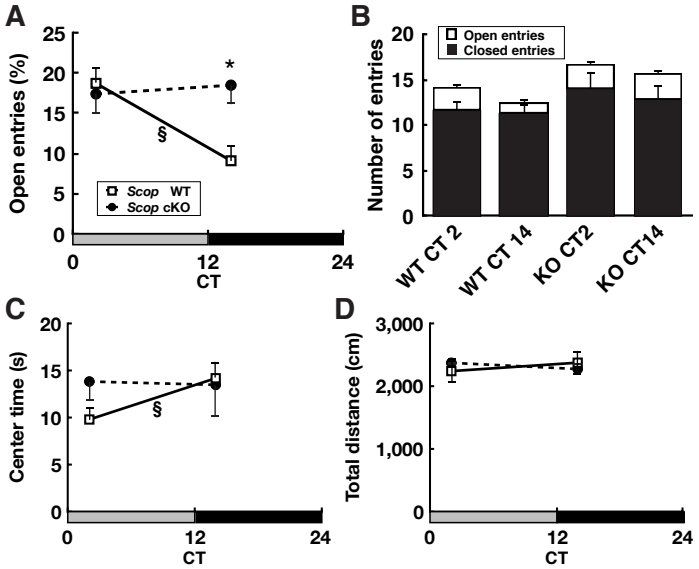

Figure 5. SCOP in the dTel is required for the circadian expression of EPM (***A-B***) and OF (***C,D***) anxiety-like behaviors. ***A,*** % Open entries for *Scop* cKO mice (*Scop*$^{fl/fl}$ *Emx1*$^{Cre/+}$; open square) and littermate WT mice (*Scop*$^{fl/fl}$ *Emx1*$^{+/+}$; filled circle) mice are shown. ***B,*** Numbers of entries into closed arms (filled) and open arms (open) are shown as stacked bars. ***C,D,*** The Center time for *Scop* cKO and littermate WT mice (***C***) and total distance traveled (***D***) are shown. *$p < 0.05$ vs WT at corresponding CTs and §$p < 0.05$ between CTs by Student's *t*-test. *n* for *Scop* WT: CT2, 14; CT14, 13. *n* for *Scop* cKO: CT2, 9; CT14, 9.

SCOP is expressed in a circadian manner in the basolateral amygdala

Given the requirement of SCOP in the dTel for the circadian expression of the anxiety-like behaviors, I sought to identify the brain region(s) within the dTel responsible for the SCOP-mediated regulation of anxiety-like behavior. *Emx1*^{Cre} expression is restricted to glutamatergic neurons and astrocytes in the dTel (Iwasato et al., 2004). Among the *Emx1*-expressing regions, the olfactory bulb, prefrontal cortex (PFC), hippocampus, and basolateral amygdala complex (BLA) have reported roles in anxiety-like behaviors. I first examined the expression of SCOP protein in these regions. PFC, hippocampus and BLA appeared to express SCOP in a circadian manner (Fig. 6*A,B*). In most of the regions tested, SCOP levels were higher at night. I then narrowed my focus on the amygdala and more fully profiled SCOP expression. Both protein and mRNA levels of SCOP exhibited circadian variations in the BLA, with the protein peaking at CT14 (Fig. 6*C-E*; mRNA: $F_{3,8} = 4.92$, $P = 0.032$; protein: $F_{3,8} = 21.5$, $P = 0.0004$). SCOP was also rhythmically expressed in the CeA, exhibiting a profile anti-phasic to that in the BLA (Fig. 6C-*E*; mRNA: $F_{3,8} = 5.71$, $P = 0.022$; protein: $F_{3,8} = 6.62$, $P = 0.015$), consistent with an earlier study reporting anti-phasic diurnal expression of the core clock component PERIOD2 in the BLA and CeA (Lamont et al., 2005).

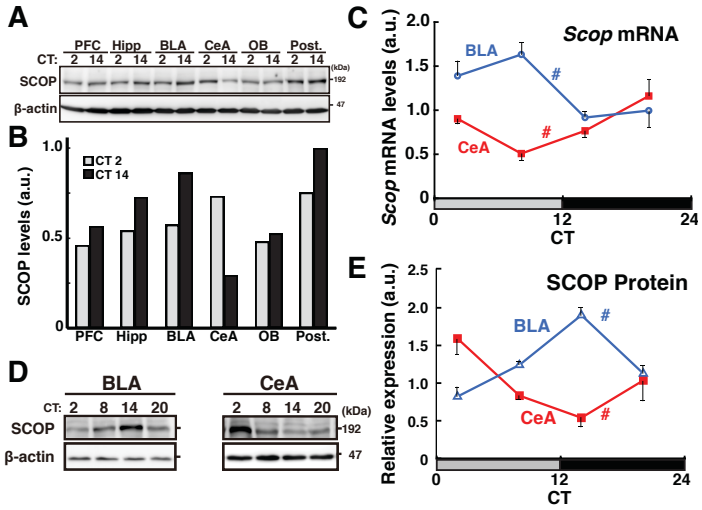

Figure 6. SCOP is expressed in a circadian manner in the basolateral amygdala (BLA). ***A,B,*** SCOP protein is widely expressed in the CNS (OB, olfactory bulb; PFC, prefrontal cortex; Hipp, hippocampus; CeA, centromedial amygdala; Post, posterior brain comprising midbrain, hindbrain, and cerebellum). ***A,*** Brain tissues were sampled at CT2 and CT14 and subjected to immunoblotting analyses against SCOP and β-actin. ***B,*** Quantification of (***A***) by densitometric analysis is shown. ***C-E,*** Levels of SCOP mRNA (***C***, quantitative PCR) and SCOP protein (***D,E,*** immunoblotting) in the BLA (Black) and CeA (Gray) were examined at 4 CTs. Representative immunoblots against SCOP and β-actin (***D***) and quantification of all samples (***E***) are shown. #$P < 0.05$ between CTs by one-way ANOVA. ***A,B,*** $n = 1$ per data point. ***C-E,*** $n = 3$ per data point. Data are means with SEM for ***C,E***.

I then examined the effects of *Bmal1* and *Scop* cKO on the temporal expression profiles of clock genes and *Scop* in the BLA. In the BLA of *Bmal1* cKO mice, *Bmal1* mRNA

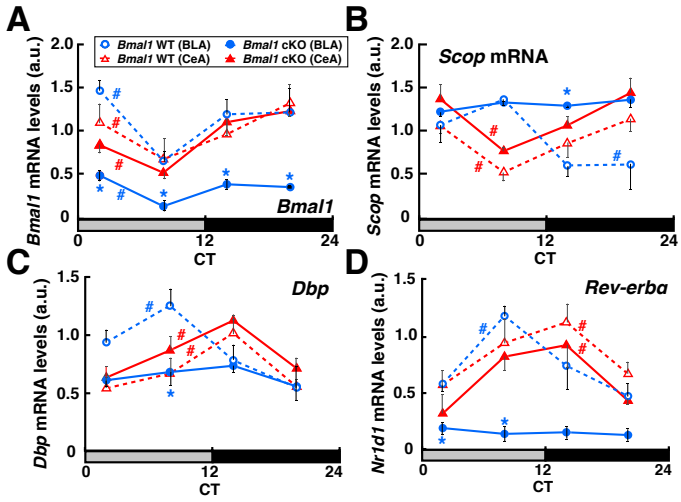

Figure 7. SCOP expression is upregulated in the BLA of *Bmal1* cKO mice. ***A-D,*** The BLA and CeA of *Bmal1* cKO mice (filled symbol, solid line) and littermate WT mice (open symbol, dashed line) sampled at 4 CTs were subjected to RT-qPCR analyses. Relative mRNA levels in the BLA (black circle) and the CeA (gray triangle) for *Bmal1* (***A***), *Scop* (***B***), *Dbp* (***C***), and *Nr1d1 (Rev-erbα)* (***D***) are shown normalized to *Rps29*. #$P < 0.05$ between CTs by one-way ANOVA. *$p < 0.05$ vs corresponding WT. $n = 3$ per data point. Data are means with SEM.

levels were downregulated by >3 fold (Fig. 7*A*), whereas *Scop* mRNA levels were constantly high throughout the day with no significant circadian rhythmicity (Fig. 7*B*; WT: $F_{3,8} = 4.06$, $P = 0.050$; cKO: $F_{3,8} = 0.86$, $P = 0.50$; WT vs cKO, $p = 6.8E\text{-}4$ at CTs 14 & 20). The E-box-regulated clock genes *Dbp* and *Rev-erbα/Nr1d1* were both expressed at low levels across the day in the BLA of *Bmal1* cKO mice (Fig. 7*C,D;* WT: *Dbp,* $F_{3,8} = 5.68$, $P = 0.022$; *Rev-erbα,* $F_{3,8} = 5.05$, $P = 0.030$; cKO: *Dbp*, $F_{3,8} = 0.73$, $P = 0.56$; *Rev-erbα*, $F_{3,8} = 0.27$, $P = 0.85$; WT vs cKO: *Dbp,* $p = 0.37$ at CT8; *Rev-erbα,* $p = 7.8E\text{-}4$ at CT8). These results suggest a *Bmal1*-mediated transcriptional repression of *Scop* presumably through a REV-ERB-mediated repression (Fig. 1) at the REV-ERB binding sites found in the intron 1 of *Scop* gene (Cho et al., 2012). In the BLA of *Scop* cKO mice, *Scop* mRNA levels were low across the day with a >2 fold reduction at CT8 (Fig. 8*B*; $p = 0.015$), whereas expression of *Bmal1* and *Dbp* was unaffected (Fig. 8*A,C*), supporting the function of SCOP downstream of the clock. In the CeA, *Bmal1* cKO had no observable effects on the expression of *Bmal1, Scop, Dbp,* or *Rev-erbα* (Fig. 7*A-D*), consistent with the lack of *Emx1* expression in ventral telencephalic tissues. *Scop* cKO also had no observable effects in the CeA on the expression of *Scop, Bmal1,* or *Dbp* (Fig. 8*A-D*).

SCOP in the BLA has an anxiogenic function and is essential for the circadian expression of EPM anxiety-like behavior

To investigate the function of SCOP in the BLA, I examined the effects of *Scop* knockout (KO) in the BLA. To this end, I constructed adeno-associated virus (AAV) expressing

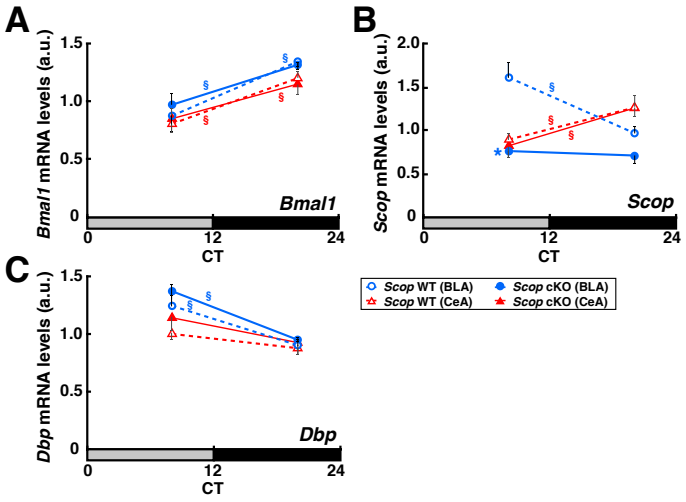

Figure 8. *Scop* cKO has no observable effects on the expression of clock genes in the BLA. ***A-C,*** The BLA and CeA of *Scop* cKO mice (filled symbol, solid line) and littermate WT mice (open symbol, dashed line) sampled at CTs 8 and 20 were subjected to RT-qPCR analyses. Relative mRNA levels in the BLA (black circle) and the CeA (gray triangle) for *Bmal1* (***A***), *Scop* (***B***), and *Dbp* (***C***) are shown normalized to *Rps29*. §$p < 0.05$ between CTs; *$p < 0.05$ vs corresponding WT by unpaired Student's *t*-test. $n = 3$ per data point. Data are means with SEM.

codon-improved Cre recombinase ("iCre") (Shimshek et al., 2002) fused to EGFP (iCre::EGFP) driven under human synapsin (hSyn) promoter ("AAV-Cre", Fig. 9*A*). *In* vitro analyses using cultured cells transiently transfected with plasmid constructs confirmed that iCre::GFP fusion protein had a recombinase activity comparable to native iCre protein in HEK293 cells (Fig. 9*B*) and that hSyn promoter induced expression of functional Cre protein in Neuro-2a cells (Fig. 9*C*). AAV-GFP control construct, which expresses EGFP under hSyn promoter (Fig. 9*A*) had no detectable Cre recombinase activity in transfected cells (Fig. 9*C*). AAV-Cre and AAV-GFP transduced to cultured primary hippocampal neurons induced iCre::EGFP and EGFP expression, respectively (Fig. 9*D*).

I then injected AAV-Cre or AAV-GFP bilaterally into the BLA of $Scop^{fl/fl}$ mice (*Scop* BLA KO; Fig. 9*K,L*) and examined the effects of *Scop* BLA KO on anxiety-like behaviors (Fig. 9*E*). Transduction with AAV-Cre resulted in a >3 fold reduction in *Scop* mRNA levels in the BLA of $Scop^{fl/fl}$ mice compared to the BLA of $Scop^{fl/fl}$ mice transduced with AAV-GFP ($p = 0.0017$) (Fig. 9*F*). Behaviorally, *Scop* BLA KO mice phenocopied *Scop* cKO mice, exhibiting lower anxiety-like behavior in the EPM test across the day (Fig. 9*G,H*). *Scop* BLA KO abrogated the circadian variation in open arm entries in the EPM test (WT: $p = 0.040$, BLA KO: $p = 0.38$) (Fig. 9*G*). The effects of *Scop* BLA KO on open arm entries at CT14 were significant compared to mice bilaterally transduced with AAV-GFP ($p = 0.014$) or to mice with AAV-Cre transduction outside of the BLA ("OFF-BLA"; $p = 0.031$). The total numbers of entries were unaffected ($F_{3,22} = 0.17$, $P = 0.92$, excluding OFF-BLA due to the insufficient

sample size) (Fig. 9*H*). AAV-GFP-transduced or OFF-BLA mice exhibited open arm entries at levels comparable to that in wild-type (Fig. 2*B*) or *Scop*$^{fl/fl}$ mice (Fig. 5*A*). *Scop* BLA KO did not have statistically significant effects on anxiety-like behavior in the OF test (Fig. 9*H,I*; $p = 0.28$ at CT2, $p = 0.80$ at CT14). These results demonstrate that SCOP in the BLA functions to elevate anxiety-like behavior in the EPM test.

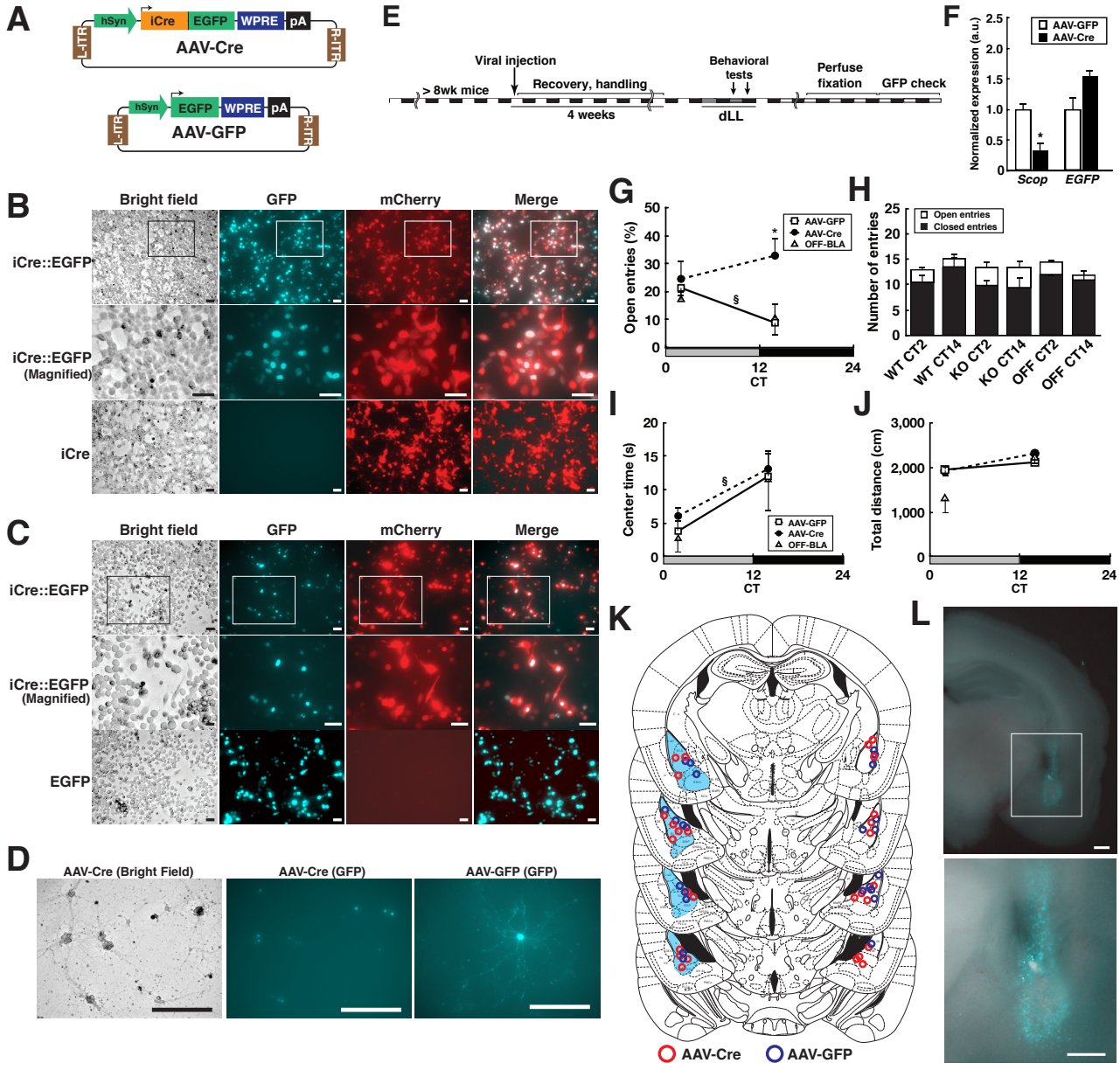

Figure 9. SCOP in the BLA is required for the circadian expression of EPM anxiety-like behavior. ***A,*** AAV vectors expressing iCre::EGFP fusion protein ("AAV-Cre") or EGFP ("AAV-GFP") were constructed (See Materials and Methods). ***B,*** Functionality of iCre::EGFP fusion construct was tested in HEK293T/17 cells. Transient expression of pCAG-iCre::EGFP together with Cre reporter construct pCCALL2-mCherry (top and middle rows), which expresses mCherry in the presence of Cre recombinase, resulted in enhanced mCherry signals at levels comparable to cells transfected with pCAG-iCre (bottom row). Insets represent the area magnified in the middle row. Almost all cells expressing iCre::EGFP also expressed mCherry (not quantified). *Continued on next page*

Figure 9 legend continued: ***C,*** Efficiency of human synapsin-1 (hSyn) promoter was tested in Neuro-2a cells. Transient expression of pAAV-hSyn-iCre::EGFP-WPRE together with pCCALL2 (top and middle rows) resulted in GFP and mCherry expression in transfected cells. Insets represent the area magnified in the middle row. Cre recombinase activity was not observed in cells transfected with pAAV-hSyn-EGFP-WPRE and pCCALL2-mCherry (bottom row). ***D,*** Primary hippocampal neurons were transduced with AAV-Cre (middle) or AAV-GFP (right), and GFP signal was examined 4 weeks after transduction. A bright field image for cells transduced with AAV-Cre is also shown in the left. ***E,*** Timeline for behavioral assays was as follows: *Scop*$^{fl/fl}$ mice aged >8 weeks were bilaterally injected with either AAV-Cre or AAV-GFP, and their anxiety-like behaviors were examined 4 wks after the surgery. Following behavioral assays, AAV transduction sites were determined by fluorescent microscopy, and behavioral data for mice with bilateral infection in the BLA were adopted. ***F,*** *Scop* and *EGFP* mRNA levels in the BLA of mice transduced with AAV-Cre (open bars) or AAV-GFP (filled bars) are shown. *$p < 0.05$ vs AAV-GFP. ***G-J,*** BLA-specific *Scop* KO attenuates EPM anxiety-like behavior. EPM % Open entries (***G***), total numbers of entries into closed and open arms (***H***), OF Center time (***I***), and OF Total distance (***J***) are shown for mice bilaterally infected with AAV-Cre (filled circle), mice bilaterally infected with AAV-GFP (open square), and mice with infection outside of the BLA ("OFF-BLA", filled triangle). ***K,*** Each circle represents the site of strongest GFP signal within the BLA (Shown in cyan) for each hemisphere of an individual mouse. Only mice with bilateral infection sites are shown. Red and blue circles denote infection sites for AAV-Cre and AAV-GFP, respectively. ***L,*** A representative infection in the BLA is shown. §$p < 0.05$ between CTs; *$p < 0.05$ vs AAV-GFP at corresponding CTs by unpaired Student's *t*-test. *n* for mice with bilateral transduction in the BLA: AAV-Cre, 8 (CT2) and 6 (CT14); AAV-GFP, 6 (CT2) and 6 (CT14). *n* for mice with AAV-Cre injection outside of the BLA, 5 (CT2) and 2 (CT14). Data are means with SEM. Scale bars, 50 μm for ***B-D,*** 500 μm for ***L.***

DISCUSSION

Taken together, my results demonstrate the function of SCOP in the mouse BLA in generating circadian rhythms in EPM anxiety-like behavior. I show that the clock machinery and SCOP in the dTel are required for the circadian expression of both EPM and OF anxiety-like behaviors (Figs. 3&5). *Bmal1* cKO leads to upregulation of SCOP expression in the BLA (Fig. 7*B*) and has anxiogenic effects, whereas *Scop* cKO has anxiolytic effects. Deletion of SCOP in the BLA abolishes circadian expression of EPM anxiety-like behavior on the EPM (Fig. 9*F*), signifying the role of SCOP in the BLA in driving EPM anxiety-like behavior. Collectively, I conclude that SCOP is a novel regulator of mouse affect functioning in anxiogenesis, driving anxiety-like behaviors in a circadian manner, and that the circadian expression of SCOP in the BLA confers rhythmicity on the anxiety-like behavior on the EPM.

An important finding in the present study is the identification of the dTel in mediating circadian regulation of affect (Figs. 3&5). Well-established affect-related brain regions such as the nucleus accumbens and other ventral striatal GABAergic nuclei, lateral habenula, and midbrain monoaminergic nuclei are not directly affected by *Bmal1* or *Scop* cKO. This is of particular importance because previous studies reporting affective abnormalities in mice harboring dysfunctional clock genes have focused on defective neurotransmitter systems, especially the dopaminergic neurons in the VTA (Hampp et al., 2008; Mukherjee et al., 2010; Chung et al., 2014). My study obviously does not exclude the possibility that dysfunctions of the circadian system in the dTel indirectly abrogate circadian functions of other brain regions. Nonetheless, systemic defects in the circadian rhythmicity in the monoamine systems are unlikely, as dopamine and 5-HT rhythms are required for the maintenance of locomotor activity levels (Hood et al., 2010) and photic entrainment of the circadian clock (Ciarleglio et al., 2011), respectively, which occurred normally in the *Scop* cKO mice used in this study. Furthermore, my finding that EPM and OF anxiety-like behaviors are differentially regulated by the circadian clock (Fig. 2*B,C,E*) marks it unlikely that rhythmic production and/or release of monoamines is a major contributor, as various manipulations of monoamine systems have been shown to act on these affective behaviors non-selectively (Pellow et al., 1985; Gross et al., 2002; Holmes et al., 2003; Prut and Belzung, 2003; Sidor et al., 2015). It is possible, however, that rhythmic release of monoamines in local brain regions other than the SCN and striatum is affected by the deletion of *Scop*.

On this basis, my results provide an important clue toward the mechanism by which dTel nuclei regulate affective behaviors, where SCOP under the regulation of the circadian clock plays an essential role. One tempting yet unlikely explanation would be that SCOP-mediated regulation of anxiety-like behaviors constitutes an affective regulatory unit acting independently of monoamine systems. Rather, I view the likely roles of SCOP as modulating cellular responses toward monoamine signaling. This is plausible because SCOP and its shorter variant PHLPP1α have been shown to regulate PI3K/AKT/GSK3β signaling through dephosphorylation of AKT, where signaling cascades downstream of dopamine D_2, 5-HT_{1A} and 5-HT_2 receptors converge, all of which have been implicated in anxiety disorders (Lanzenberger et al., 2007; Beaulieu, 2012; Cervenka et al., 2012; Quesseveur et al., 2012). Moreover, polymorphisms in *Akt1* are associated with elevated anxiety and depression; *Akt2* KO mice display elevated anxiety- and depression-like behaviors; and mice overexpressing a constitutively active form of GSK3β display mania-like behaviors (Prickaerts et al., 2006; Yang et al., 2012; Leibrock et al., 2013).

These lines of evidence lead me to a testable model in which activities of signaling pathways downstream of monoamine receptors are modulated by SCOP under circadian regulation, where acute affective responses are largely mediated by rapid activation/suppression of synaptic monoamine signaling, while milder, prolonged shifts in affective states are mediated by modulations in intracellular signaling pathways. Examining the effects of *Scop* deletion on neuronal firing and synaptic plasticity in response to monoamines would mark a first step. In fact, multiple studies have supported the roles of SCOP in the modulation of synaptic plasticity. Activity-dependent, calpain-mediated rapid degradation of SCOP both *in vivo* and in cultured neurons leads to a transient activation of ERK signaling necessary for LTP formation, followed by mTOR-dependent fast resynthesis of SCOP protein (Shimizu et al., 2003; 2007; Wang et al., 2014); inhibition of μ-calpain activity abolishes theta-burst-induced degradation of SCOP and LTP formation (Wang et al., 2014); and overexpression or deletion of *Scop* or inhibition of calpain abrogates long-term object recognition memory (Shimizu et al., 2007) (Shimizu et al., submitted). Modulation of synaptic plasticity in amygdala neurons has been repeatedly shown to modulate affective behaviors including anxiety, while emotionally arousing stimuli induce amygdala LTP (Shekhar et al., 2005; Pape and Pare, 2010). Moreover, my preliminary proteomics analyses suggest that SCOP localizes to the postsynaptic density (data not shown), in addition to the previously described localization to lipid rafts (Shimizu et al., 2003). Collectively, these data point to a possible role of SCOP in the modulation of

synaptic plasticity in the amygdala, although further study is required to attest such a model.

It is important to keep in mind, however, that my results do not exclude the roles of SCOP in affect regulation taking place in dTel regions other than the BLA. SCOP expression is ubiquitous in the CNS (Shimizu et al., 1999), in which many nuclei exhibit circadian expression of SCOP (Fig. 6*A,B*) (Shimizu et al., submitted), and I see it more likely that other dTel regions utilize SCOP for the circadian regulation of their functions. For example, while SCOP in the dTel is necessary for the circadian expression of OF anxiety-like behavior (Fig. 5*C*), *Scop* BLA KO did not have a significant effect (Fig. 9*I*), implicating the involvement of SCOP expressed in other dTel nuclei in the regulation of this behavior. In addition, SCOP in the hippocampal CA1 region drives mouse long-term recognition memory performance in a circadian manner (Shimizu et al., submitted). Furthermore, a recent study by Moriya and colleagues (Moriya et al., 2015) demonstrated that an aberrant 3.5 hr LD cycle, known to cause depression-like behaviors in mice (LeGates et al., 2012), attenuates diurnal expression rhythms of clock genes in the mouse hippocampus and amygdala. Conversely, dysfunction of USP46, a deubiquitinating peptidase that stabilizes SCOP protein, suppresses mouse depression-like behaviors (Tomida et al., 2009; Imai et al., 2012). Collectively, it is likely that SCOP function in the hippocampus, and presumably in other dTel brain regions under circadian regulation, contributes to the complex expression of affective behaviors. Examining the roles of SCOP in other dTel regions in the regulation of affective behaviors could provide valuable insights.

Importantly, the *Bmal1* or *Scop* cKO mice used in this study did not exhibit hyperactivity or extreme abnormalities in affective behaviors reported for several mouse lines carrying mutations in the clock genes. My results rather demonstrate that the defects caused by *Bmal1* or *Scop* cKO do not exceed the range of the circadian variation in anxiety-like behaviors expressed by wild-type mice (Figs. 3&5). Put another way, the loss of circadian rhythmicity in the dTel appears to "halt" the circadian expression of anxiety-like behaviors at a specific time of day. I am thus led to conclude that the circadian system in the dTel functions to confer circadian rhythmicity on mouse anxiety-like behaviors. This, together with my finding that the clock differentially regulates EPM and OF anxiety-like behaviors (Fig. 2*B,E*), suggests that the rhythmic expression of anxiety-like behaviors may play an important survival function. In this regard, examining whether and how this circadian expression is conserved in other species will be of particular interest.

It has to be noted that the distinct temporal profiles in the expression of anxiety-like behaviors in the EPM and OF tests came as no surprise, as these behaviors have been

repeatedly suggested to reflect distinct physiological phenomena. For example, factor analysis studies using rodents revealed that anxiety-related behaviors evaluated in the EPM and OF tests do not load on a common factor (Trullas and Skolnick, 1993; Ramos et al., 1998). Consistently, multiple pharmacological manipulations have been shown to produce test-selective effects on these anxiety-like behaviors (Vendruscolo et al., 2003). Hence, a current model considers the anxiety-like behaviors evaluated in the EPM and OF tests, as well as in other anxiety-related tests, as representing partially overlapping but distinct aspects of the multidimensional "anxiety", likely underlain by partially overlapping mechanisms (Ramos, 2008). Not only are my results (Fig. 2*B,E*) compatible with such a model, my finding that clock dysfunction in the dTel abolishes the distinct phase relationship between the EPM- and OF-anxiety-like behaviors (Fig. 3) suggests that the circadian clock is actively involved in the differentiation of these anxiety-like behaviors. Although further study is necessary to elucidate the underlying mechanisms, these results, combined with the selective effects of *Scop* BLA KO on the EPM but not the OF test (Fig. 9), provide an important foundation for unraveling the complexity of anxiety-like behaviors. Together, the differential regulation of EPM and OF anxiety-like behaviors by the clock and SCOP both poses a challenge that distinct neural correlates underlying these behaviors must be differentially addressed and respectively related to human anxiety and, by elucidating such complexity, raises the possibility to further factorize the pathology of affective disorders and lead to a more thorough understanding of mammalian affect regulation.

CONCLUSIONS

In summary, I have described the circadian expression of mouse anxiety-like behaviors, which requires clock machineries and SCOP function in the dTel, and demonstrated that SCOP in the BLA is expressed in a circadian manner and has anxiogenic effects on the EPM. While the molecular mechanisms remain to be established, I conclude that SCOP in the dTel plays an essential role in the circadian expression of anxiety-like behaviors, which likely represents an important function in animal survival. By characterizing the involvement of a novel, temporal axis in the regulation of mammalian affect, as well as by identifying key molecular and neuroanatomical players therein, my present study provides a new foundation for future research on animal emotionality and, given the intrinsic plasticity in affect regulation, potentially leads to the development of novel classes of treatment for affective disorders.

REFERENCES

Adams Jr RB, Ambady N, Macrae CN, Kleck RE (2006) Emotional expressions forecast approach-avoidance behavior. Motiv Emot 30:179-188.

Beaulieu J-M (2012) A role for Akt and glycogen synthase kinase-3 as integrators of dopamine and serotonin neurotransmission in mental health. J Psychiatry Neurosci 37:7-16.

Belzung C, Griebel G (2001) Measuring normal and pathological anxiety-like behaviour in mice: a review. Behav Brain Res 125:141-149.

Berton O, McClung CA, DiLeone RJ, Krishnan V, Renthal W, Russo SJ, Graham D, Tsankova NM, Bolaños CA, Rios M, Monteggia LM, Self DW, Nestler EJ (2006) Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. Science 311:864–868.

Brognard J, Newton AC (2008) PHLiPPing the switch on Akt and protein kinase C signaling. Trends Endocrinol Metab 19:223-230.

Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, Bradfield CA (2000) Mop3 is an essential component of the master circadian pacemaker in mammals. Cell 103:1009-1017.

Carola V, D'Olimpio F, Brunamonti E, Mangia F, Renzi P (2002) Evaluation of the elevated plus-maze and open-field tests for the assessment of anxiety-related behaviour in inbred mice. Behav Brain Res 134:49-57.

Cervenka S, Hedman E, Ikoma Y, Djurfeldt DR, Ruck C, Halldin C, Lindefors N (2012) Changes in dopamine D2-receptor binding are associated to symptom reduction after psychotherapy in social anxiety disorder. Transl Psychiatry 2:e120.

Chaudhury D, Colwell CS (2002) Circadian modulation of learning and memory in fear-conditioned mice. Behav Brain Res 133:95-108.

Cho H, Zhao X, Hatori M, Yu RT, Barish GD, Lam MT, Chong L-W, DiTacchio L, Atkins AR, Glass CK, Liddle C, Auwerx J, Downes M, Panda S, Evans RM (2012) Regulation of circadian behaviour and metabolism by REV-ERB-α and REV-ERB-β. Nature 485:123-127.

Chung S, Lee EJ, Yun S, Choe HK, Park S-B, Son HJ, Kim K-S, Dluzen DE, Lee I, Hwang O, Son GH, Kim K (2014) Impact of circadian nuclear receptor REV-ERBα on midbrain dopamine production and mood regulation. Cell 157:858-868.

Ciarleglio CM, Resuehr HES, McMahon DG (2011) Interactions of the serotonin and circadian systems: nature and nurture in rhythms and blues. Neuroscience 197:8-16.

Corr PJ (2013) Approach and avoidance behaviour: Multiple systems and their interactions. Emotion Review.

De Bundel D, Gangarossa G, Biever A, Bonnefont X, Valjent E (2013) Cognitive dysfunction, elevated anxiety, and reduced cocaine response in circadian clock-deficient cryptochrome knockout mice. Front Behav Neurosci 7:152.

Farach FJ, Pruitt LD, Jun JJ, Jerud AB, Zoellner LA, Roy-Byrne PP (2012) Pharmacological treatment of anxiety disorders: current treatments and future directions. J Anxiety Disord 26:833-843.

Gao T, Furnari F, Newton AC (2005) PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. Mol Cell 18:13-24.

Gessa GL, Pani L, Fadda P, Fratta W (1995) Sleep deprivation in the rat: an animal model of mania. Eur Neuropsychopharmacol 5 Suppl:89-93.

Golder SA, Macy MW (2011) Diurnal and seasonal mood vary with work, sleep, and daylength across diverse cultures. Science 333:1878-1881.

Gross C, Zhuang X, Stark K, Ramboz S, Oosting R, Kirby L, Santarelli L, Beck S, Hen R (2002) Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. Nature 416:396-400.

Hall P, Spear FG, Stirland D (1964) Diurnal Variation of Subjective Mood in Depressive States. Psychiatr Q 38:529-536.

Hampp G, Ripperger JA, Houben T, Schmutz I, Blex C, Perreau-Lenz S, Brunk I, Spanagel R, Ahnert-Hilger G, Meijer JH, Albrecht U (2008) Regulation of monoamine oxidase A by circadian-clock components implies clock influence on mood. Curr Biol 18:678-683.

Hirano A, Yumimoto K, Tsunematsu R, Matsumoto M, Oyama M, Kozuka-Hata H, Nakagawa T, Lanjakornsiripan D, Nakayama KI, Fukada Y (2013) FBXL21 Regulates Oscillation of the Circadian Clock through Ubiquitination and Stabilization of Cryptochromes. Cell 152:1106-1118.

Holmes A, Yang RJ, Lesch KP, Crawley JN, Murphy DL (2003) Mice lacking the serotonin transporter exhibit 5-HT(1A) receptor-mediated abnormalities in tests for anxiety-like behavior. Neuropsychopharmacology 28:2077-2088.

Hood S, Cassidy P, Cossette M-P, Weigl Y, Verwey M, Robinson B, Stewart J, Amir S (2010) Endogenous dopamine regulates the rhythm of expression of the clock protein PER2 in the rat dorsal striatum via daily activation of D2 dopamine receptors. J Neurosci 30:14046-14058.

Hull CL (1952) A behavior system; an introduction to behavior theory concerning the individual organism. New Haven, CT, US: Yale University Press.

Imai S, Mamiya T, Tsukada A, Sakai Y, Mouri A, Nabeshima T, Ebihara S (2012) Ubiquitin-Specific Peptidase 46 (Usp46) Regulates Mouse Immobile Behavior in the Tail Suspension Test through the GABAergic System. PLoS ONE 7:e39084.

Iwasato T, Nomura R, Ando R, Ikeda T, Tanaka M, Itohara S (2004) Dorsal telencephalon-specific expression of Cre recombinase in PAC transgenic mice. Genesis 38:130-138.

Iwasato T, Datwani A, Wolf AM, Nishiyama H, Taguchi Y, Tonegawa S, Knöpfel T, Erzurumlu RS, Itohara S (2000) Cortex-restricted disruption of NMDAR1 impairs neuronal patterns in the barrel cortex. Nature 406:726-731.

Jackson TC, Verrier JD, Semple-Rowland S, Kumar A, Foster TC (2010) PHLPP1 splice variants differentially regulate AKT and PKCα signaling in hippocampal neurons: characterization of PHLPP proteins in the adult hippocampus. J Neurochem 115:941-955.

Kassai H, Terashima T, Fukaya M, Nakao K, Sakahara M, Watanabe M, Aiba A (2008) Rac1 in cortical projection neurons is selectively required for midline crossing of commissural axonal formation. European Journal of Neuroscience 28:257-267.

Keers R, Pedroso I, Breen G, Aitchison KJ, Nolan PM, Cichon S, Nöthen MM, Rietschel M, Schalkwyk LC, Fernandes C (2012) Reduced Anxiety and Depression-Like Behaviours in the Circadian Period Mutant Mouse Afterhours. PLoS ONE 7:e38263.

Krishnan V, Nestler EJ (2008) The molecular neurobiology of depression. Nature 455:894-902.

Lamont EW, Robinson B, Stewart J, Amir S (2005) The central and basolateral nuclei of the amygdala exhibit opposite diurnal rhythms of expression of the clock protein Period2. Proc Natl Acad Sci USA 102:4180-4184.

Lanzenberger RR, Mitterhauser M, Spindelegger C, Wadsak W, Klein N, Mien LK, Holik A, Attarbaschi T, Mossaheb N, Sacher J, Geiss-Granadia T, Kletter K, Kasper S, Tauscher J (2007) Reduced serotonin-1A receptor binding in social anxiety disorder. Biol Psychiatry 61:1081-1089.

LeGates TA, Altimus CM, Wang H, Lee H-K, Yang S, Zhao H, Kirkwood A, Weber ET, Hattar S (2012) Aberrant light directly impairs mood and learning through melanopsin-expressing neurons. Nature 491:594-598.

Leibrock C, Ackermann TF, Hierlmeier M, Lang F, Borgwardt S, Lang UE (2013) Akt2 deficiency is associated with anxiety and depressive behavior in mice. Cell Physiol Biochem 32:766-777.

Maes M, Leonard B, Fernandez A, Kubera M, Nowak G, Veerhuis R, Gardner A, Ruckoanich P, Geffard M, Altamura C, Galecki P, Berk M (2011) (Neuro)inflammation and neuroprogression as new pathways and drug targets in depression: From antioxidants to kinase inhibitors. Prog Neuropsychopharmacol Biol Psychiatry 35:659-663.

Masubuchi S, Gao T, O'Neill A, Eckel-Mahan K, Newton AC, Sassone-Corsi P (2010) Protein phosphatase PHLPP1 controls the light-induced resetting of the circadian clock. Proc Natl Acad Sci USA 107:1642-1647.

McClung CA (2007) Circadian genes, rhythms and the biology of mood disorders. Pharmacol Ther 114:222-232.

Moriya S, Tahara Y, Sasaki H, Ishigooka J, Shibata S (2015) Housing under abnormal light-dark cycles attenuates day/night expression rhythms of the clock genes Per1, Per2, and Bmal1 in the amygdala and hippocampus of mice. Neurosci Res 99:16-21.

Mukherjee S, Coque L, Cao J-L, Kumar J, Chakravarty S, Asaithamby A, Graham A, Gordon E, Enwright JF, DiLeone RJ, Birnbaum SG, Cooper DC, McClung CA (2010) Knockdown of Clock in the ventral tegmental area through RNA interference results in a mixed state of mania and depression-like behavior. Biol Psychiatry 68:503-511.

O'Donnell KC, Gould TD (2007) The behavioral actions of lithium in rodent models: leads to develop novel therapeutics. Neurosci Biobehav Rev 31:932-962.

Pantazopoulos H, Dolatshad H, Davis FC (2011) A Fear-Inducing Odor Alters PER2 and c-Fos Expression in Brain Regions Involved in Fear Memory. PLoS ONE 6:e20658.

Pape HC, Pare D (2010) Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear. Physiol Rev 90:419-463.

Pellow S, Chopin P, File SE, Briley M (1985) Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 14:149-167.

Pisharath H, Rhee JM, Swanson MA, Leach SD, Parsons MJ (2007) Targeted ablation of beta cells in the embryonic zebrafish pancreas using E. coli nitroreductase. Mech Dev 124:218-229.

Prickaerts J, Moechars D, Cryns K, Lenaerts I, van Craenendonck H, Goris I, Daneels G, Bouwknecht JA, Steckler T (2006) Transgenic mice overexpressing glycogen synthase kinase 3beta: a putative model of hyperactivity and mania. J Neurosci 26:9022-9029.

Prut L, Belzung C (2003) The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur J Pharmacol 463:3-33.

Quesseveur G, Nguyen HT, Gardier AM, Guiard BP (2012) 5-HT2 ligands in the treatment of anxiety and depression. Expert Opin Investig Drugs 21:1701-1725.

Ramos A, Mellerin Y, Mormède P, Chaouloff F (1998) A genetic and multifactorial analysis of anxiety-related behaviours in Lewis and SHR intercrosses. Behav Brain Res 96:195–205.

Ramos A (2008) Animal models of anxiety: do I need multiple tests? Trends Pharmacol Sci 29:493–498.

Ramos A, Pereira E, Martins GC, Wehrmeister TD, Izídio GS (2008) Integrating the open field, elevated plus maze and light/dark box to assess different types of emotional behaviors in one single trial. Behav Brain Res 193:277–288.

Roybal K, Theobold D, Graham A, DiNieri JA, Russo SJ, Krishnan V, Chakravarty S, Peevey J, Oehrlein N, Birnbaum S, Vitaterna MH, Orsulak P, Takahashi JS, Nestler EJ, Carlezon WA, McClung CA (2007) Mania-like behavior induced by disruption of CLOCK. Proc Natl Acad Sci USA 104:6406-6411.

Sanacora G, Treccani G, Popoli M (2012) Towards a glutamate hypothesis of depression: an emerging frontier of neuropsychopharmacology for mood disorders. Neuropharmacology 62:63-77.

Scott AJ (2000) Shift work and health. Prim Care 27:1057-1079.

Shekhar A, Truitt W, Rainnie D, Sajdyk T (2005) Role of stress, corticotrophin releasing factor (CRF) and amygdala plasticity in chronic anxiety. Stress 8:209-219.

Shimba S, Ogawa T, Hitosugi S, Ichihashi Y, Nakadaira Y, Kobayashi M, Tezuka M, Kosuge Y, Ishige K, Ito Y, Komiyama K, Okamatsu-Ogura Y, Kimura K, Saito M (2011) Deficient of a Clock Gene, Brain and Muscle Arnt-Like Protein-1 (BMAL1), Induces Dyslipidemia and Ectopic Fat Formation. PLoS ONE 6:e25231.

Shimizu K, Mackenzie SM, Storm DR (2010) SCOP/PHLPP and its functional role in the brain. Mol Biosyst 6:38-43.

Shimizu K, Okada M, Takano A, Nagai K (1999) SCOP, a novel gene product expressed in a circadian manner in rat suprachiasmatic nucleus. FEBS Lett 458:363-369.

Shimizu K, Okada M, Nagai K, Fukada Y (2003) Suprachiasmatic nucleus circadian oscillatory protein, a novel binding partner of K-Ras in the membrane rafts, negatively regulates MAPK pathway. J Biol Chem 278:14920-14925.

Shimizu K, Phan T, Mansuy IM, Storm DR (2007) Proteolytic degradation of SCOP in the hippocampus contributes to activation of MAP kinase and memory. Cell 128:1219-1229.

Shimshek DR, Kim J, Hübner MR, Spergel DJ, Buchholz F, Casanova E, Stewart AF, Seeburg PH, Sprengel R (2002) Codon-improved Cre recombinase (iCre) expression in the mouse. Genesis 32:19-26.

Sidor MM, Spencer SM, Dzirasa K, Parekh PK, Tye KM, Warden MR, Arey RN, Enwright JF, Jacobsen JPR, Kumar S, Remillard EM, Caron MG, Deisseroth K, McClung CA (2015) Daytime spikes in dopaminergic activity drive rapid mood-cycling in mice. Mol Psychiatry 5:1406-1419.

Spencer S, Falcon E, Kumar J, Krishnan V, Mukherjee S, Birnbaum SG, McClung CA (2013) Circadian genes Period 1 and Period 2 in the nucleus accumbens regulate anxiety-related behavior. Eur J Neurosci 37:242-250.

Tataroğlu O, Aksoy A, Yilmaz A, Canbeyli R (2004) Effect of lesioning the suprachiasmatic nuclei on behavioral despair in rats. Brain Res 1001:118-124.

Tomida S, Mamiya T, Sakamaki H, Miura M, Aosaki T, Masuda M, Niwa M, Kameyama T, Kobayashi J, Iwaki Y, Imai S, Ishikawa A, Abe K, Yoshimura T, Nabeshima T, Ebihara S (2009) Usp46 is a quantitative trait gene regulating mouse immobile behavior in the tail suspension and forced swimming tests. Nat Genet 41:688-695.

Tooby J, Cosmides L (1990) The past explains the present: Emotional adaptations and the structure of ancestral environments. Ethol Sociobiol 11:375-424.

Tovote P, Fadok JP, Lüthi A (2015) Neuronal circuits for fear and anxiety. Nat Rev Neurosci 16:317-331.

Trullas R, Skolnick P (1993) Differences in fear motivated behaviors among inbred mouse strains. Psychopharmacology (Berl) 111:323–331.

Tye KM, Prakash R, Kim S-Y, Fenno LE, Grosenick L, Zarabi H, Thompson KR, Gradinaru V, Ramakrishnan C, Deisseroth K (2011) Amygdala circuitry mediating reversible and bidirectional control of anxiety. Nature 471:358–362.

Vendruscolo LF, Takahashi RN, Brüske GR, Ramos A (2003) Evaluation of the anxiolytic-like effect of NKP608, a NK1-receptor antagonist, in two rat strains that differ in anxiety-related behaviors. Psychopharmacology (Berl) 170:287–293.

Wang Y, Zhu G, Briz V, Hsu Y-T, Bi X, Baudry M (2014) A molecular brake controls the magnitude of long-term potentiation. Nat Comms 5:3051.

Yang C, Sun N, Ren Y, Sun Y, Xu Y, Li A, Wu K, Zhang K (2012) Association between AKT1 gene polymorphisms and depressive symptoms in the Chinese Han population with major depressive disorder. Neural Regen Res 7:235–239.

Zhong L, Li B, Mah CS, Govindasamy L, Agbandje-McKenna M, Cooper M, Herzog RW, Zolotukhin I, Warrington KH, Weigel-Van Aken KA, Hobbs JA, Zolotukhin S, Muzyczka N, Srivastava A (2008) Next generation of adeno-associated virus 2 vectors: point mutations in tyrosines lead to high-efficiency transduction at lower doses. Proc Natl Acad Sci USA 105:7827–7832.

Zirlinger M, Zirlinger M, Kreiman G, Kreiman G, Anderson DJ, Anderson DJ (2001) Amygdala-enriched genes identified by microarray technology are restricted to specific amygdaloid subnuclei. Proc Natl Acad Sci USA 98:5270–5275.

VITA

Jun Nakano was born in Tokyo, Japan on the 8th day of February, 1983 as the second son of Akihiko and Kyoko Nakano. He recalls to have enjoyed an active, relatively normal childhood. After graduating the Junior High School at Otsuka, University of Tsukuba, he dropped out of a private high school after two days of enrollment and started taking courses from the Global Home Education Program provided by Clonlara School, Ann Arbor, Michigan, where he realized his passion for the mystery and beauty of life sciences. In June 2002, he entered Diablo Valley College in Pleasant Hill, California to pursue a career as a biologist. He transferred to University of California, Los Angeles in 2005 with a major in Molecular, Cellular, and Developmental Biology and completed his bachelor's study in December 2007.

In the January of the following year, he started working as a laboratory technician at Dr. Gregory S. Payne's laboratory in Department of Biological Chemistry, David Geffen School of Medicine at University of California, Los Angeles, who later becomes the principal investigator for his Master's thesis study. In August 2008, while continuing his biochemistry work at the Payne Lab, focusing on the dynamics of intracellular protein trafficking in the budding yeast *Saccharomyces cerevisiae*, Jun moved his affiliation to Department of Biological Sciences, California State University, Los Angeles. In September 2010, he completed his Master's thesis study at the Payne Lab, UCLA, entitled: "Novel characterization of the dynamics of *Saccharomyces cerevisiae* AP-3-dependent trafficking to the vacuole," for which he received the degree Master of Science.

In October 1st, 2010, Jun returned to Japan for reasons he wishes to keep private, and continued following his passion for life sciences. He entered The University of Tokyo in April the following year, and started research on the field he had been irresistibly pulled towards for the past few years: animal emotionality. He launched a project focusing on the regulation of mouse affect by the circadian clock at the Fukada Laboratory, Department of Biophysics and Biochemistry (currently the Department of Biological Sciences), School of Science, The University of Tokyo. Upon acceptance of this dissertation, Jun will be completing his Ph.D degree.