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Physiologically Relevant Changes in Serotonin Resolved by Fast Microdialysis

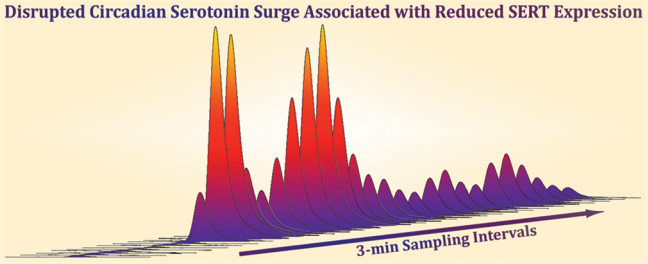
Hongyan Yang,[†] Andrew B. Thompson,[†] Bryan J. McIntosh,[∥] Stefanie C. Altieri,[†] and Anne M. Andrews[†,‡,§,*]

[†]Semel Institute for Neuroscience & Human Behavior and Hatos Center for Neuropharmacology, David Geffen School of Medicine, [‡]Department of Chemistry and Biochemistry, and [§]California NanoSystems Institute, University of California, Los Angeles, California, United States

[∥]Eicom Corporation, San Diego, California, United States

Supporting Information

ABSTRACT: Online microdialysis is a sampling and detection method that enables continuous interrogation of extracellular molecules in freely moving subjects under behaviorally relevant conditions. A majority of recent publications using brain microdialysis in rodents report sample collection times of 20−30 min. These long sampling times are due, in part, to limitations in the detection sensitivity of high performance liquid chromatography (HPLC). By optimizing separation and detection conditions, we decreased the retention time of serotonin to 2.5 min and the detection threshold to 0.8 fmol. Sampling times were consequently reduced from 20 to 3 min per sample for online detection of serotonin (and dopamine) in brain dialysates using a commercial HPLC system. We developed a strategy to collect and to analyze dialysate samples continuously from two animals in tandem using the same instrument. Improvements in temporal resolution enabled elucidation of rapid changes in extracellular serotonin levels associated with mild stress and circadian rhythms. These dynamics would be difficult or impossible to differentiate using conventional microdialysis sampling rates.



KEYWORDS: *mice, behavior, no net flux, serotonin transporter, knockout, circadian*

The neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) mediates a wide range of physiological functions in the central and peripheral nervous systems, as well as in peripheral organs.[1−5] Serotonin is thought to act principally as a modulatory neurotransmitter via interactions with its six families of G-protein-coupled receptors (5-$HT_{1-2,4-7}$).[6,7] Ultrastructural studies show that serotonin transporters (SERT) are located extrasynaptically, suggesting a volume transmission mode of action.[8] Furthermore, despite the fact that serotonin-selective reuptake inhibitors (SSRIs) block serotonin uptake almost immediately, antidepressant effects are often not apparent until several weeks after initiating treatment.[9,10] Delayed therapeutic outcomes contribute to the perception that serotonin acts as a tonic regulator of neurotransmission.

Evidence also suggests a broader role for serotonin. Serotonin axons directly form axo-dendritic and axo-axonal connections.[11−14] In addition to characteristic tonic firing patterns, some serotonergic neurons have been reported to exhibit burst firing in vivo.[15−17] Moreover, 5-HT_3 receptors, which are ligand-gated ion channels, allow serotonin to induce rapid changes in membrane potentials in excitatory and inhibitory neurons in hippocampus and cortex, respectively.[18,19] SSRIs and other antidepressants cause changes in firing rates and electrophysiological characteristics of serotonergic neurons in the dorsal raphe,[20,21] as well as nonserotonergic neurons in other brain areas involved in emotional responses, including prefrontal cortex,[22] mesoaccumbens,[23] ventral tegmental area,[24] hippocampus,[25] and locus coeruleus.[26] Thus, in addition to acting as a neuromodulator, serotonin appears to function as a fast-acting "classical" neurotransmitter, making it necessary to investigate serotonin neurotransmission with high temporal resolution. The ability to measure transient changes in extracellular serotonin will also be important for discovering how behaviorally relevant information is encoded in serotonergic signaling.

Conventional microdialysis, which is well suited for measuring basal neurotransmitter levels, allows for sampling over periods ranging from minutes to months, and permits continuous measurement of neurotransmitters in the extracellular fluid.[27,28] No-net-flux (NNF) microdialysis, also referred to as zero-net-flux, is used to determine basal extracellular concentrations corrected for in vivo extraction fraction.[29−36] In the past decade, programmable infusion pumps and autoinjectors, and advanced analytical instrumenta-

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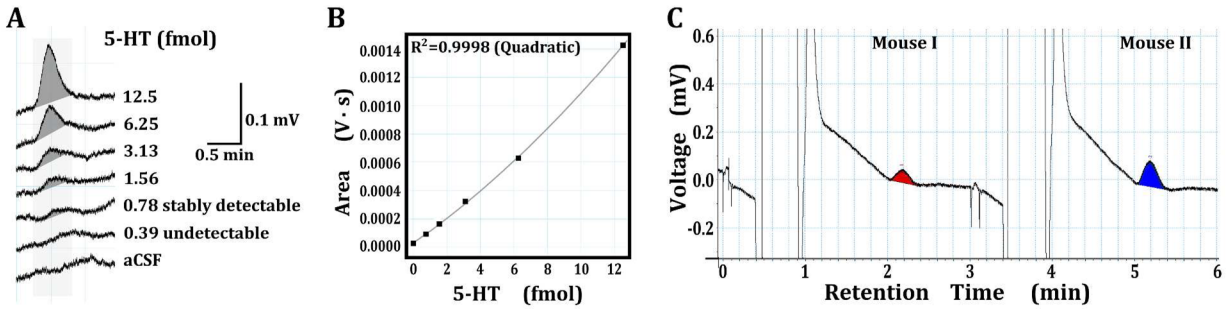

Figure 1. Sensitivity and tandem "fast" microdialysis for serotonin. (A) Representative chromatograms of serotonin standards. Gray areas depict integrated peak areas. The lowest level at which serotonin could be reliably detected was ~0.8 fmol. (B) Quadratic curve-fit of standards. (C) Basal dialysate samples from two tandem-linked SERT+/+ mice were injected 3 min apart. Fast separation enabled 6 min dialysate sampling with 3 min online analysis of two samples in the same chromatogram. The retention time of serotonin was 2.2 min. Basal serotonin levels were 3 fmol (red peak, 0.16 nM) and 9 fmol (blue peak, 0.47 nM) in 18 μL dialysate samples from two mice without correction for probe recovery.

tion have enabled the rapid adoption of online microdialysis. Here, dialysate samples from awake animals are directly transferred to instrumentation, as opposed to collection, storage, and later analysis.[37] These advances have made it possible to couple neurochemical measurements with behavioral analyses.[38,39] Additionally, progress has been made for highly time-resolved measurements of many neurotransmitters, some with resolution in the range of seconds, using online microdialysis.[40]

Our colleagues and we recently reported significant improvements in the separation and detection of serotonin in dialysates collected offline via high pressure and temperature separations using custom instrumentation.[41,42] Thus, the stage is set for combining advances in chromatographic analysis of serotonin with online microdialysis for rapid detection of transient changes in extracellular serotonin concentrations. Here, we report on progress in this regard. Using fast online microdialysis, we observe brief, physiologically coupled changes in extracellular serotonin levels. The methods described take advantage of improvements in commercial instrumentation and therefore, are readily accessible to other neuroscientists.

RESULTS AND DISCUSSION

Sampling Times for in Vivo Brain Microdialysis over the Past 30 Years. Using the keywords "brain" and "microdialysis", we performed a literature survey on PubMed to investigate the sampling times most commonly used in preclinical in vivo microdialysis research over the past 30 years (Table S1, Supporting Information). We established three epochs for our search based on publication patterns: the "pioneer" or early period (1984−1987), the "widespread use" period (1988−2000), and the "recent" period (2001−2012). For detailed information regarding the selection and sampling of these epochs, see Table S1 in Supporting Information. Within each epoch, representative search periods each yielded 15−41 results from which to examine sampling times (Table S1).

The survey revealed a trend in microdialysis research toward longer sampling rates over time. In the pioneer period, 20% of published articles used 5 min-sampling intervals. This dropped to 6% of articles in 2000, and by 2012, no papers were found that sampled at this high rate among representative papers. At the same time, the percentage of studies that used sampling times of 20 min or more increased, from 35% in the pioneer period to 82% in 2012.

At least three major factors appear to contribute to this shift. First, studies from the pioneer period primarily focused on amino acids such as glutamate, aspartate, and γ-aminobutyric acid (GABA). Concentrations of these amino acids are relatively high in brain dialysates (>1 μM). As a result, small dialysate volumes and hence, short sampling times could be employed because the concentrations of these amino acid neurotransmitters were within the detection limits of contemporaneous HPLC instrumentation. The development of more sensitive instruments enabled researchers to investigate molecules with lower basal concentrations, including the monoamine neurotransmitters norepinephrine (NE), dopamine (DA), and serotonin. These transmitters are found at nanomolar to picomolar concentrations in dialysate samples. However, to detect these low concentrations, longer sampling times were needed.

In addition to the challenges posed by the low physiological concentrations of monoamine neurotransmitters, sampling rates have been impacted by the increased availability of microdialysis techniques. In the 1980s, investigators who developed the technique carried out microdialysis. These investigators had strong knowledge of the related methodologies, and used them optimally. Once in vivo microdialysis became a more common method, many practitioners presumably had less expertise regarding separations and detection, and were hampered by limitations of commercially available instrumentation.

Finally, the shift toward longer sampling times has been encouraged by the development of reverse microdialysis, which allows introduction of exogenous substances into the extracellular space through the microdialysis probe.[43,44] One advantage of this method is that a drug can be delivered into a precise brain region. It is also straightforward to manipulate the dose and time course of drug effects compared to systemic administration. Changes in the concentrations of endogenous substances in response to administration of a drug can be monitored simultaneously. As a result, microdialysis has been commonly used in pharmacological and toxicological studies, in which high temporal resolution is generally not necessary.[45]

Faster Online Microdialysis for Serotonin (and Dopamine). By optimizing analysis conditions, we have reduced our detection threshold for serotonin from ~5[32,34] to 0.8 fmol (Figure 1A,B). Simultaneously, we decreased the retention time for serotonin from 17−20 min[32,34] to ~2 min (Figure 1C). Using similar analysis conditions, both dopamine and serotonin were resolved with retention times of 1.2 and 2.6

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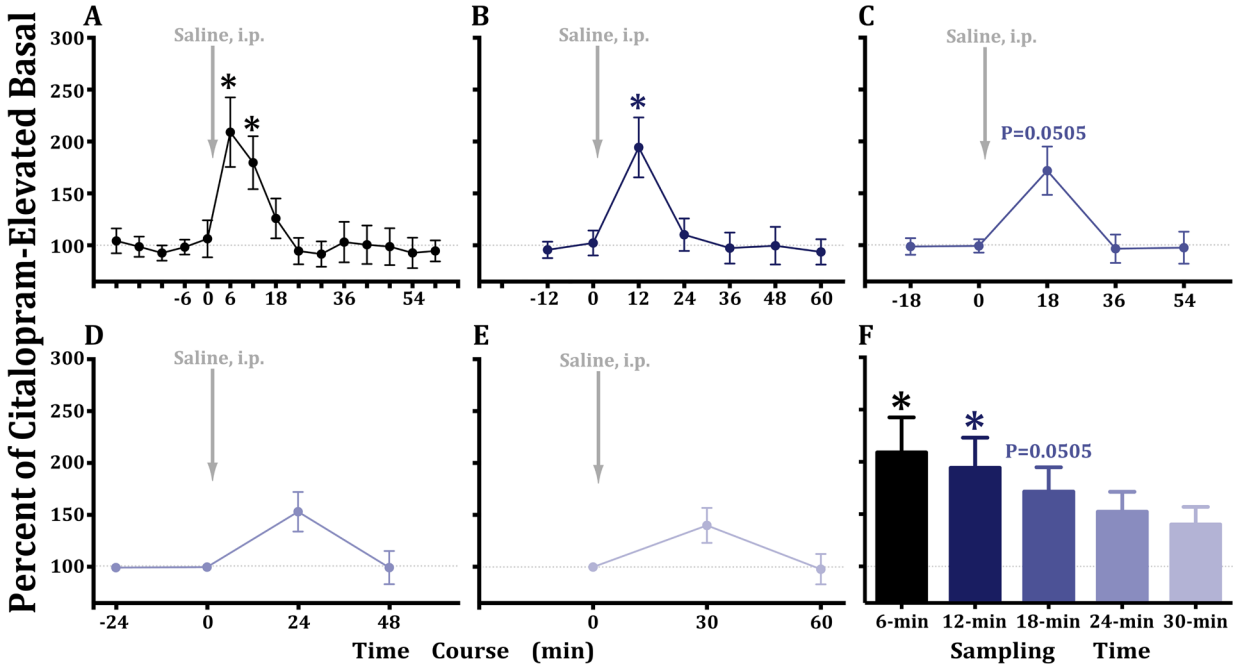

Figure 2. Serotonin release in response to saline injection. (A) Basal serotonin dialysate samples were collected for 30 min in the presence of 1.2 μM citalopram. A single intraperitoneal injection of saline (1 mL/kg body weight) was then administered with additional sampling for 60 min. For each subject, the mean serotonin concentration in the first 5 samples (30 min) was delineated as 100% CIT-baseline. Samples were analyzed relative to this baseline to assess the effects of injection on extracellular serotonin. The dialysate sampling rate was 6 min. In (B)-(E), samples were combined to simulate the effects of saline injection at longer sampling times, i.e., 12, 18, 24, and 30 min, respectively. (F) Comparison of apparent peak values at each sampling interval. The ability to detect rapid physiological changes in extracellular serotonin decreases with increasing sampling times. Basal serotonin was 0.83 ± 0.1 nM, which was increased by CIT perfusion to 7.0 ± 2 nM. Data are from SERT+/+ mice (N = 7). *P < 0.05.

min, respectively (Figure S1A). This enabled concurrent analysis of both neurotransmitters by online microdialysis in mice with a sampling time of 3 min.

Taking advantage of decreased retention times and increased sensitivity, we developed a tandem online injection setup that allowed a single HPLC instrument to analyze samples from two subjects simultaneously and in an automated manner. Here, dialysate samples from each mouse were collected at 6 min intervals via two online autoinjectors (Figure S2). Injections were staggered by 3 min such that two samples (one from each mouse) were analyzed in a single 6 min chromatogram (Figure 1C). The retention time for serotonin was 2.2 min and the serotonin peaks from each mouse were offset by 3 min. In experiments in which higher temporal resolution is not needed, including those designed to measure tonic shifts, slow responses, or changes in basal neurotransmitter concentrations, this tandem injection method increases the number of subjects that can be investigated using a single instrument, significantly reducing the time and costs associated with an experiment.

Dialysate Serotonin Levels Increase in Response to Saline Injection. Improvements in microdialysis detection limits and reduced online sampling times have enabled measurements that more closely approximate real-time neurochemical activity. Using this approach, we observed physiologically relevant changes in extracellular serotonin levels that were not detected at 20 min sampling intervals. Debate remains as to whether a single injection of vehicle is a mild stressor.[46,47] Despite this, saline injection is often used as a control condition to differentiate the effects of a drug from those of the injection itself. In previous studies, changes in extracellular serotonin levels in response to intraperitoneal (ip) saline injection have not been observed when dialysate samples were collected every 20 min in striatum (unpublished observations). Here, high temporal resolution revealed brief but measurable increases in dialysate serotonin levels in the hippocampus following ip saline (Figure 2).

Collection of dialysate samples began 30 min prior to saline injection and continued for 60 min thereafter. Sampling occurred at a rate of 6 min per sample. Artificial cerebrospinal fluid (aCSF) containing 1.2 μM S-citalopram (CIT) was continuously infused into the ventral hippocampus beginning 120 min prior to injection and throughout the duration of the experiment. S-Citalopram is an SSRI antidepressant widely used in humans.[48] We previously found that a 30−40 min infusion of 1.2 μM CIT is sufficient to elevate the local extracellular serotonin level to a new equilibrium that is approximately 6× higher than the original basal level. Basal serotonin levels in the absence of CIT are near the limit of detection, precluding quantitative analysis of reductions in these levels. Thus, local CIT infusion was used to raise extracellular serotonin levels so that potential increases *or* decreases in response to saline injection could be investigated.

In the first two 6 min samples after saline injection, there was a significant increase in dialysate serotonin levels to 210 ± 30% (P < 0.001) and 180 ± 30% (P < 0.01) of baseline (Figure 2A). Increases were no longer statistically significant by the third sample (13−18 min postinjection, 130 ± 20%, P > 0.05) (Figure 2A). When samples were combined during data analysis to mimic the results of longer sampling times, injection-induced increases in extracellular serotonin were diminished below significance. (Figure 2F) These data show that saline injection induces brief increases in extracellular serotonin despite SERT inhibition, suggesting that the effects of injection involve increases in serotonin release. The effects may

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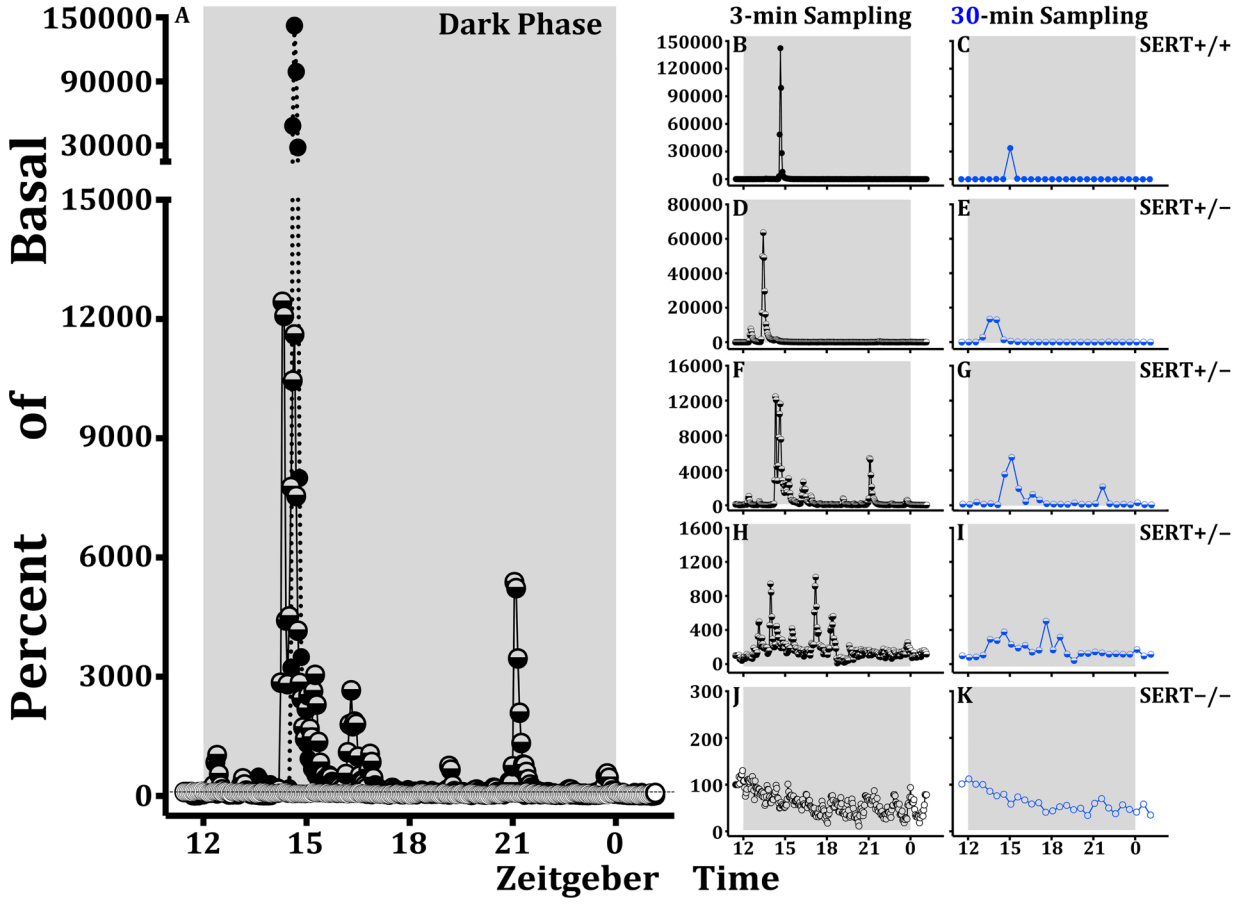

Figure 3. Reduced SERT expression produces different patterns of spontaneous oscillation in extracellular serotonin levels during the dark phase. (A) Overlay of typical examples from mice of each genotype. Spontaneous serotonin surges during the dark phase were large in SERT+/+ mice (solid circles, approximately 1500 times the basal level), while SERT−/− mice (open circles) did not show surges but rather serotonin fluctuated around basal levels. Spontaneous serotonin surges during the dark phase in SERT+/− mice (half-filled circles) fell between those of the SERT+/+ and SERT−/− mice. There was usually only one large serotonin surge in SERT+/+ mice during ZT13−18, while multiple intermediate-sized serotonin surges occurred in SERT+/− mice throughout the dark phase, with more changes tending to cluster during ZT13−18. Typical examples of circadian serotonin surges (black symbols) measured using 3 min sampling from (B) a SERT+/+ mouse, (D,F,H) three different SERT+/− mice, and (J) a SERT−/− mouse. (C,E,G,I,K) Samples are combined to simulate 30 min sampling (blue symbols). Basal serotonin levels were 0.58 ± 0.02 nM (N = 14), 0.65 ± 0.01 nM (N = 19), and 1.8 ± 0.1 nM (N = 15) for SERT+/+, SERT±, and SERT−/− mice, respectively, without correction for probe recovery.

be specific to hippocampus and/or the presence of an SSRI because even with 6 min sampling, no change in extracellular serotonin was observed in the nucleus accumbens after ip saline.[49]

Spontaneous Circadian-like Serotonin Surges Occur in the Dark Phase. In addition to revealing transient serotonin responses to saline injection, we detected rapid surges in serotonin levels linked to circadian rhythms by fast microdialysis. The primary circadian "pacemaker" in mammals is the suprachiasmatic nucleus (SCN) of the hypothalamus. Neurons in this nucleus receive input from light-sensitive retinal ganglion cells[50] and maintain an intrinsic cyclic firing pattern over a period of approximately 24 h. This pattern can persist in the absence of external cues, but it is entrained by natural variation in light throughout the day to maintain synchrony between the SCN and the external environment. Serotonin neurons originating in the dorsal and median raphe are thought to modulate SCN activity.[51,52]

Due to circadian rhythms, time of day is an important consideration when performing research on living subjects.[53] Jürgen Aschoff, one of the founders of chronobiology, introduced the concept of "zeitgeber [time-giver] time" in the 1960s to simplify the reporting of results that were impacted by circadian-linked changes. Using this system, the time at which daylight emerges or the lights turn on in the laboratory is referred to as zeitgeber time zero (ZT0).

We observed spontaneous spiking in serotonin levels associated with the light-dark cycle by continuous sampling of dialysate at 3 min intervals for 20 h and throughout the dark phase. Basal extracellular serotonin concentrations were more stable during the light phase (ZT0−12) than the dark phase (ZT12−24) (Figure 3), in agreement with the majority of previous studies on neurotransmitter levels in nocturnal rodents.[54−57] Differences in patterns of spontaneous serotonin oscillation during the dark phase were observed between wildtype and SERT deficient mice. Continuous recording from SERT+/+ mice revealed large, spontaneous surges in serotonin levels during the first half of the dark phase (ZT13−18), when spontaneous activity and body temperature are highest (Figure 4, dark gray bar B). These surges reached concentrations that were 700−1500 times higher than basal levels (Figure 3B). Extracellular serotonin levels in mice with one copy of the *Sert*

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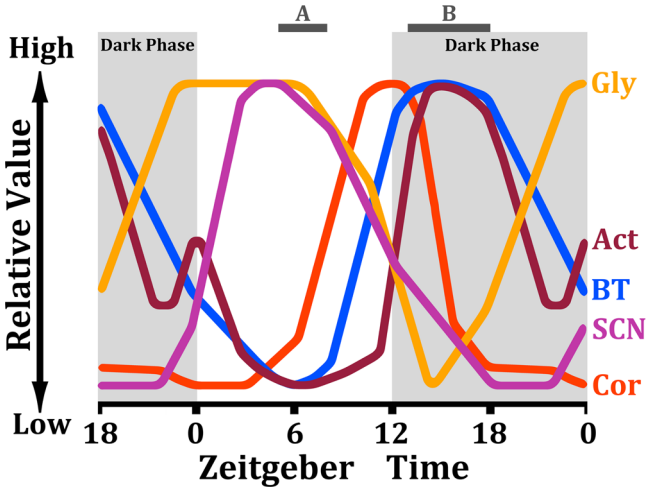

Figure 4. Schematic of circadian rhythms. Data are graphed using relative values. The circadian rhythms of brain glycogen (Gly), spontaneous activity (Act), body temperature (BT), SCN neuron activity (SCN), and plasma corticosterone (Cor) are represented by orange, burgundy, blue, purple, and red lines, respectively. Brain glycogen serves as secondary energy storage and changes in glycogen concentrations are markers of global brain activity.[59] The light gray areas represent the dark phase during a 12/12 h light/dark cycle. The dark gray bar "A" represents the time frame for potential circadian shifts in serotonin levels during the light phase (refer also to Figure 5). The dark gray bar "B" represents the time frame over which spontaneous serotonin surges were observed in SERT+/+ and SERT +/− mice (refer also to Figure 3).

gene inactivated (SERT+/− mice) showed smaller, irregular surges (Figure 3D, F, H). Here, intermediate serotonin surges (1-800-fold) occurred throughout the dark phase, with larger surges tending to occur during the first half of the dark phase (ZT13−18). Mice lacking both copies of the *Sert* gene (SERT−/− mice) showed no evidence of similar serotonin surges, though small fluctuations around basal levels were observed (Figure 3J).

Microdialysis experiments investigating circadian patterns are often carried out using sampling times of 30 min or longer due to the long time frame, that is, 24 h, over which circadian changes are measured. To assess the impact of a prolonged sampling time on the ability to detect rapid circadian-linked changes in extracellular serotonin levels, samples were combined into contiguous groups of 30 min samples during data analysis (Figure 3C, E, G, I, K). Serotonin surges that could be resolved at 3 min sampling rates were diminished when data were grouped into 30 min bins. In SERT+/− mice, multiple surges merged into a single, wider peak when the sampling time was increased from 3 to 30 min (Figure 3E, G, I). In SERT+/+ mice, the magnitudes of serotonin surges were greatly decreased when samples were combined (Figure 3C). These findings indicate that slow sampling during circadian research is likely to miss important aspects of the circadian profile of serotonin, and possibly of other neurotransmitters.

To assess the behavioral relevance of these serotonin surges, we compared their time course to those of other circadian patterns. A schematic of mammalian circadian changes in SCN activity,[58] brain glycogen concentrations,[59] plasma corticosterone levels,[60] body temperature,[61] and spontaneous locomotor activity[62] is shown in Figure 4. Phasic serotonin surges in SERT+/+ and SERT+/− mice appear to be correlated with increases in brain activity, spontaneous locomotor activity, and plasma corticosterone concentrations. This suggests that serotonin may play an essential role in the generation and regulation of the circadian rhythmicity of these physiological processes. Compared to wild type mice, SERT−/− mice exhibit altered sleep patterns,[63] reduced brain glucose utilization,[64] and hypolocomotion in the open field test,[65] but not in the home cage.[66] The lack of spontaneous serotonin surges in SERT−/− mice may contribute to these differences. Rats and mice exposed to serotonin reuptake inhibitors during early postnatal development also show disrupted sleep patterns thought to model changes observed in patients with mood disorders.[67−72] Given the links between alterations in the serotonin system and sleep regulation,[63] circadian changes in the regulation of serotonin levels might be involved in disrupted sleep patterns in patients with mood and/or sleep disorders.

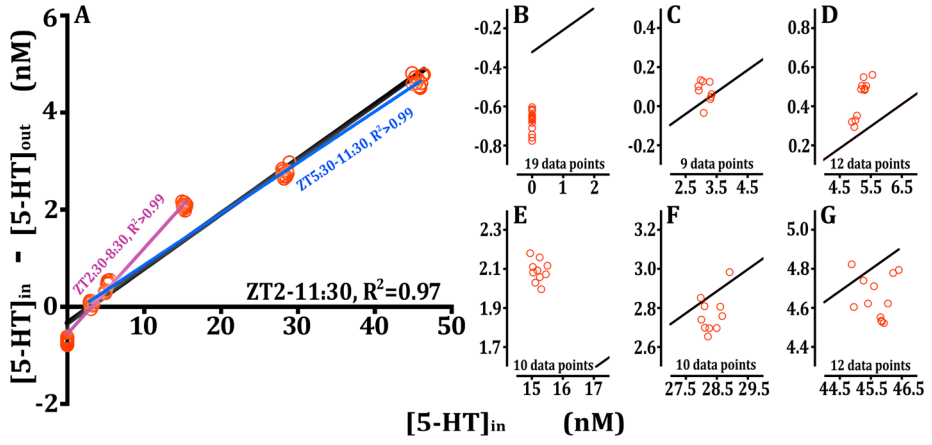

Figure 5. No-net-flux analysis of extracellular serotonin levels. In this example, the order of infusion of serotonin concentrations ($[5\text{-HT}]_{in}$) was 0, 15, 2.5, 5, 50, and 30 nM. (A) The overall R^2 of the linear regression (black line) was 0.97 for all 6 infused concentrations across ZT2-11:30. The R^2 value was improved by analyzing data from either the first 6 h (purple line, $R^2 > 0.99$, ZT2:30−8:30 for 4 concentrations) or the last 6 h (blue line, $R^2 > 0.99$, ZT5:30−11:30 for 4 concentrations). (B)−(G) Each serotonin infusion concentration generated 9−12 data points. Individual points are shown for increasing $[5\text{-HT}]_{in}$. Distributions of the data at each infusion concentration are tightly clustered showing the precision of the measurements. The overall regression line is shown in black.

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NNF Analysis of Extracellular Serotonin. To investigate basal serotonin concentrations further and to avoid spontaneous serotonin surges during the dark phase, we conducted NNF analyses during the light phase. Using a tandem online injection method, which enables a single HPLC instrument to simultaneously analyze dialysate samples from two subjects, we carried out NNF using 6 min sampling on more than 100 mice during 6 months. Faster sampling also allowed more data points to be collected for each 90 min infusion of different concentrations of serotonin (usually 10−14 points per concentration (C_{in}), Figure 5B−G). This enabled greater accuracy in estimating the recovered concentration (C_{out}). The average R^2 value of all data collected at 6 min per sample during ZT2-11:30 was 0.97 ± 0.002 (Figure 5A). At a collection rate of 20 min per sample, only 5−6 data points can be obtained in a 90 min serotonin infusion, and the overall R^2 value was ∼0.90.[32,34]

The R^2 value was improved (>0.99) when the first 6 h (ZT2:30−8:30 for 4 concentrations) or the last 6 h (ZT5:30−11:30 for 4 concentrations) were analyzed separately. This could be due to subtle circadian shifts in basal serotonin levels during the light phase (Figure 4, dark gray bar A). These findings suggest that when NNF experiments are confined to a shorter time span and a greater number of samples per infusion concentration are analyzed, estimations of basal extracellular serotonin concentration are improved. Greater accuracy enables small but biologically important differences in extracellular serotonin to be detected. Based on the current findings, the time for each infusion of serotonin can be reduced to 45 min using 3 min sampling, or to 60 min using 6 min sampling. This would yield 5−6 data points per concentration and allow completion of a NNF experiment within 6 h, avoiding major circadian changes.

■ CONCLUSIONS AND FUTURE PROSPECTS

Fast microdialysis can reveal new information about neurotransmitter signaling. The role of glutamate as a primary excitatory transmitter is supported by microdialysis findings using short sampling times.[73,74] It is likely that rapid measurement of serotonin by microdialysis will similarly elucidate physiologically important dynamics associated with serotonergic neurotransmission. We anticipate that temporally resolved microdialysis[41,42] will enable investigation of serotonin release and reuptake kinetics in mice with different levels of SERT expression,[75] which will inform us about the effects of variations in serotonin signaling in humans with native *SERT* gene polymorphisms.[76] Additionally, online microdialysis can be performed in experimental animals during behavioral tasks.[38,39] High temporal resolution and multiplexed measurements will be vital to understanding how behaviorally relevant information is encoded in chemical neurotransmission.[77,78]

■ METHODS

Animals. Female and male mice from a SERT-deficient linage on a mixed CD1 × 129S6/SVev background at 10−14 months of age were used for all experiments.[79] Three different genotypes (SERT+/+, SERT+/−, and SERT−/−) were generated from SERT+/− mating pairs, and were maintained at the University of California, Los Angeles (UCLA). All mice were housed in groups of 2−4 same-sex siblings per cage until guide cannula implantation. Food and water were available ad libitum, and the light−dark cycle (12/12 h) was set with lights on at 0400 h (ZT0). The same light schedule was strictly maintained in the room where microdialysis was performed. UCLA is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All animal care and use met the requirements of "The Guide for the Care and Use of Laboratory Animals", revised 2011. The UCLA Chancellor's Research Program preapproved all procedures.

High Performance Liquid Chromatography. An Eicom integrated HPLC system (HTEC-500, Eicom Corporation, San Diego, CA) was used with an Eicompak PP-ODS II stationary phase (4.6 mm i.d. × 30 mm, 2 μm particles), a WE-3G graphite-working electrode, and two EAS-20s online injectors. This instrument has been optimized to reduce overall dead volume, and noise from the electrochemical cell. To prevent oxidation of analytes in dialysate samples, the manufacturer has replaced the majority of metal parts in the flow stream with inert materials. The composition of the mobile phase was 96 mM NaH_2PO_4 (Fluka Cat#17844), 3.8 mM Na_2HPO_4 (Fluka Cat#71633), pH 5.4, 2.8% MeOH (EMD Cat#MX0475-1), 50 mg/L EDTA·Na_2 (Fluka Cat#03682), and 500−1000 mg/L sodium decanesulfonate (TCI Cat#I0348) in water purified via a Milli-Q Synthesis A10 system (EMD Millipore Corporation, Billerica, MA). Separation occurred at a flow rate of 450−750 μL/min, and the column temperature was maintained at 25−30 °C. Electrochemical detection occurred at an applied potential of +450 mV at the working electrode vs Ag/AgCl.

Guide Cannulae Implantation. Mice were rendered unconscious by 5% isoflurane for ∼5 min in an induction chamber. A surgical anesthetic plane was then maintained with 2% isoflurane throughout the surgery. Animals were mounted on a stereotaxic frame (Kopf Instruments, Tujunga, CA). Eyes were protected from corneal dehydration using sterile ophthalmic ointment. The fur over the surgery area was shaved, and 0.05−0.1 mL bupivacaine was injected subcutaneously (sc) at the surgical site to provide local analgesia during surgery. An injection of 5 mg/kg ketoprofen was administered sc at the nape of the neck to provide postsurgical analgesia.

The surgery site was sterilized by applying 3 alternating scrubs with Betadine and 70% isopropanol prior to exposing the skull with a 5 mm × 5 mm circular incision. The skull was cleaned and dehydrated with 2% medical grade H_2O_2. One medical grade flat-tip stainless steel anchor screw (Eicom Corp., San Diego, CA) was affixed to the skull, approximately 2 mm ventral to the guide cannula implantation site to anchor the dental resin and guide cannula to the skull. A 0.7 mm diameter burr hole was drilled in the skull (coordinates relative to Bregma for left ventral hippocampus: AP −2.8 mm, ML −3.5 mm, DV −2.2 mm; left ventral striatum: AP +1.2 mm, ML −1.2 mm, DV −3.5 mm).

A sterilized 32-gauge needle was inserted into the hole to pierce the dura mater without damaging the surface of the brain. A guide cannula for a CMA/7 microdialysis probe (CMA/Microdialysis, Harvard Apparatus, Holliston, MA) was slowly lowered into the hole. Dental resin (Trim II from Bosworth Company, Skokie, IL) was used to secure the guide cannula to the surrounding exposed skull and to seal the exposed area. A stylet was inserted into the guide cannula to keep it free from debris until the microdialysis probe was inserted. Mice were allowed to recover from surgery for at least 3 days prior to microdialysis.

Dialysis Configuration. Two EAS-20s autoinjectors (Eicom Corp., San Diego, CA) were linked in tandem to enable HPLC analysis of dialysate samples from two mice in a single chromatogram (Figure 1C). The linking arrangement for the autoinjectors is shown in Figure S2 in the Supporting Information. The flow diagram for the dialysis setup is shown in Figure S3. One CMA/400 syringe pump (4-channel) was used to deliver aCSF at a constant flow rate to two mice (147 mM NaCl (Fluka Cat#73575), 3.5 mM KCl (Fluka Cat#05257), 1.0 mM $CaCl_2$ (Aldrich Cat#499609), 1.2 mM $MgCl_2$ (Aldrich Cat#449172), 1.0 mM NaH_2PO_4 (Fluka Cat#17844), 2.5 mM $NaHCO_3$ (Fluka Cat#88208); pH 7.3 ± 0.03 using NaOH (Aldrich Cat#415413), modified from Trillat et al.[80] and Mathews et al.).[32] Additionally, two CMA/102 programmable syringe pumps were used to deliver aCSF containing different concentrations of serotonin (2.5, 5, 15, 30, and 50 nM) for NNF experiments in a preprogrammed order. The 2.5, 5, and 15 nM concentrations were infused using pseudorandomization across subjects, followed by 30 and 50 nM

infusion on an alternating schedule. A series of CMA/110 zero-dead-volume liquid switches was used to permit manual switching between solutions to deliver the desired solutions to the microdialysis probes during the course of experiments without introducing air into the infusion route (Figure S3).

Microdialysis. During ZT10−12 on the day before microdialysis, each mouse was lightly anesthetized with isoflurane, and a 2 mm CMA/7 microdialysis probe was inserted slowly into the brain. The animal was allowed to recover for 30 min while aCSF was flushed through the probe at 3 μL/min. The aCSF flow rate was then lowered to 1.1 μL/min for overnight perfusion (except for circadian studies, see below). Microdialysis experiments were carried out during ZT2−8 on the second day. The aCSF flow rate was increased to 3 μL/min 30−60 min before the first dialysate sample was collected for analysis. After collecting basal serotonin dialysate samples for 120 min, aCSF containing 1.2 μM CIT was infused into the brain through the microdialysis probe for another 120 min. Then, a single ip injection of sterile saline (1 mL/kg) was administered. Samples were collected at 6 min intervals. Serotonin measurements were normalized relative to the mean serotonin concentration of the 5 samples (30 min) prior to saline injection (100% CIT-elevated baseline).

For circadian studies, during ZT6−7 on the first day of the experiment, mice were lightly anesthetized with isoflurane. A 2 mm CMA/7 microdialysis probe was inserted through the guide cannula, and aCSF was infused through the probe at 3 μL/min throughout the experiment. Mice were allowed to recover from acute tissue damage due to probe insertion for at least 3 h before data collection. Circadian microdialysis experiments were carried out from ZT11 on the first day through ZT5−6 on the second day. Samples were collected every 3 min. The data were normalized relative to mean serotonin concentrations in samples collected during ZT11−12 (before lights off).

For NNF, different concentrations of serotonin in aCSF were delivered in a predetermined order using a programmable CMA/102 infusion pump. Prior to collecting dialysate samples, each serotonin solution was infused through all dialysate tubing (except the probe), collected by the autoinjector, and analyzed by HPLC. This method of analyzing serotonin concentrations under conditions approximating the dialysis setup enables more accurate estimates of C_{in}, as previously described.[32]

No-net-flux was carried out during ZT2−11:30 on the second day at a flow rate of 3 μL/min for 6 different concentrations of serotonin. Basal serotonin levels were measured for 120 min using aCSF devoid of serotonin. Then, aCSF solutions containing 2.5, 5.0, or 15 nM serotonin were delivered for 90 min each in a predetermined order by CMA/102 programmable syringe pumps. The concentrations of serotonin exiting the probe were measured. After this, higher concentrations of serotonin (30 or 50 nM) were delivered for 90 min per concentration in a predetermined order. The collection time for each NNF sample was 6 min.

Histological Confirmation of Probe Placement. After microdialysis, mice were lightly anesthetized with isoflurane for probe removal, and sacrificed by cervical dislocation. Brains were removed and preserved in 7% paraformaldehyde in phosphate buffer (PFA-PB) for 48−72 h at room temperature on an orbital shaker. Brains were then transferred to 30% sucrose-PB. Preserved brains were sectioned at 50 μm using a refrigerated cryostat. Sections were stained by cresyl violet. Probe position was examined using a light microscope. Only data from brains in which the probe was correctly located in the ventral hippocampus or ventral striatum were included in the analysis.

Statistics. All data were analyzed by GraphPad Prism v6.0b. The effects of saline injection were analyzed by Student's unpaired two-tailed *t* tests. Data for NNF were analyzed by linear regression to generate *x*-intercepts (extracellular serotonin concentrations) for individual subjects. All values are presented as means ± SEMs. $P < 0.05$ was considered statistically significant. Significance is indicated in figures as $***P < 0.001$, $**P < 0.01$, and $*P < 0.05$.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table containing the results of the literature search for historical sampling times, representative chromatograms of dopamine and serotonin separation in ventral striatum, and schematics of the tandem autoinjector setup and no-net-flux liquid switch configurations. This material is available free of charge via the Internet at http://pubs.acs.org/.

■ AUTHOR INFORMATION

Corresponding Author

*Mailing address: Neuroscience Research Building, 635 Charles E. Young Dr. South, Box 957332, Los Angeles, CA 90095-7332. E-mail: aandrews@mednet.ucla.edu. Phone: 310-794-9421.

Author Contributions

HY and AMA designed the experiments, which were carried out by HY and ABT. SCA provided the test subjects. HY and BJM designed the tandem HPLC system. HY, ABT, SCA, and AMA wrote the manuscript.

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Notes

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Mental Health or the National Institutes of Health. The authors declare the following competing financial interest(s): HY, ABT, SCA, and AMA declare no conflicts of interest. Eicom Corporation employs BJM.

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