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Prefrontal Cortex Metabolome Is Modified by Opioids, Anesthesia, and Sleep

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Obtundation of wakefulness caused by opioids and loss of wakefulness caused by anesthetics and sleep significantly alter concentrations of molecules comprising the prefrontal cortex (PFC) metabolome. Quantifying state-selective changes in the PFC metabolome is essential for advancing functional metabolomics. Diverse functions of the PFC suggest the PFC metabolome as a potential therapeutic entry point for countermeasures to state-selective autonomic dysfunction.

behavioral state control; computational modeling; state-selective physiology; systems biology; visceral cortex

Introduction

Anatomically distributed neurons that remain active during the absence of attention-demanding tasks comprise default mode networks (1). Additional networks have been discovered that continue to be active even during the loss of wakefulness caused by sleep (2), conscious sedation (3), or anesthesia (4). Persisting network activity is difficult to reconcile with impairments in physiology that occur during the obtundation of wakefulness caused by opioids and the loss of wakefulness caused by volatile anesthetics and sleep. The prefrontal cortex is a component of multiple default mode networks with multiple integrative functions (5). There is consensus that the prefrontal cortex modulates autonomic physiology (6–8) and behavioral states of sleep (9–12), anesthesia (13–16), pain (17), and nociception (18). Additional evidence indicates that the prefrontal cortex also contributes to the process of opioid abuse (19–21). A second context for this focus on the prefrontal cortex derives from recent discoveries that concentrations of molecules comprising the prefrontal cortical metabolome are significantly altered during the loss or obtundation of wakefulness (11, 22–24).

Physiological studies seek a multiscale understanding that aims to vertically integrate data from lower level to higher level phenotypes, some of which manifest as emergent phenomena. Such a multiscale approach was described in 2009 as a Systems Biology (25). This "new biology" shares many goals of the American Physiological Society (APS), founded in 1887 (26, 27), such as advancing scientific discovery, understanding life, and improving health (28). The breadth of systems biology defies a single, unifying definition (29–33). Systems biology eschews conceptual and statistical assumptions of independence. Large and complex datasets are welcomed, and computational, predictive modeling is used to analyze and interpret multi-omics data (34–36). Systems biology embraces complexity that manifests as a structural or functional change emerging as a summation of its constituent parts (37, 38). The foregoing features stand in contrast to limitations noted previously (32, 34) of hyperreductionistic studies that may produce detailed but sometimes disjointed information. Efforts to apply control theory to systems physiology also confront complexities due to changes that emerge across time. Intracellular membrane potentials of trigeminal motoneurons were discovered in the late 1970s to change from excitation to inhibition during the transition from the state of non-REM sleep to the state of REM sleep (39). This finding indicated that conclusions regarding cellular excitability based on measures made during one physiological state may not apply during another physiological state. Subsequent studies have generalized nonlinear, state-selective biology to clinically relevant systems physiology (40).

This Introduction would be incomplete without acknowledging ongoing controversies regarding conceptual and methodological approaches to the study of nonlinear, state-selective biology. The statistician George Box has been paraphrased as noting that all models are wrong, but some are useful (41). It is easier to appreciate this quip as a cautionary phrase than to confront evidence that the assumptions of the general linear model (GLM) (42) are commonly violated by complex, nonlinear, biological systems. At present, scientists long-committed to the GLM interact with colleagues who have benefited from formal training in data analytics and computational biology. Even the introduction to a successful MatLab book notes that some biologist "of yore resent what they perceive to be a hostile takeover of the field" (43). Such concerns are

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not paranoid fantasies given that storied Departments of Physiology have been renamed Departments of Systems Biology. One aim of this review is to advocate for combining the analytic and conceptual approaches of systems and reductionistic biology. The combined approach discussed here is focused on neurochemistry of the prefrontal cortex (11, 22, 23), a brain region interacting with multiple networks exhibiting state-selective activation and deactivation (2–4).

First highlighted are changes in brain chemistry and breathing relative to opioid-induced respiratory depression. The second section reviews recent findings regarding neurotransmitter reorganization during the loss of wakefulness caused by isoflurane anesthesia. The third section compares changes in the prefrontal cortex metabolome that occur during the loss of wakefulness associated with sleep and with anesthesia. Opioids, anesthesia, and sleep produce altered states of consciousness that are characterized by clinically significant autonomic dysfunction. Each of these three sections outlines analytic chemical and computational approaches that enabled measuring and modeling a chemical connectome underlying state-selective physiology. Time-dependent analyses are a unifying feature of the studies reviewed below (11, 22–24). Evaluation of time as a putative causal variable is essential for efforts to understand complex systems (38). The concluding Perspectives places this work in a historical context and points to exciting future opportunities.

Prefrontal Cortex Opioids Alter Neurochemistry and Breathing

The most dangerous adverse effect of opioids is respiratory depression (44). Opioid overdose is a leading cause of premature death in the United States (45). The economic cost of this opioid crisis was projected to be about $500 billion in 2020 (46). The significant problem of opioid-induced respiratory depression is not limited to the abuse of diverted or illegally produced opioids (47). Even opioids administered acutely in a perioperative setting can be “a significant cause of preventable morbidity and mortality” (48). Furthermore, among patients who receive opioids over prolonged intervals of time, opioid-induced respiratory depression is “unpredictable” (49). This is directly relevant to the finding that among 6% of U.S. surgical patients, a common postsurgical complication is persistent opioid use 90 days after surgery (50). The exponential increase in opioid-induced mortality is reflected by the fact that the U.S. consumes ~80% of the world opioid supply while comprising <5% of the world population (51). The public health burden of opioid-induced respiratory depression is emphasized by evidence that the physiological and psychological manifestations of opioid use disorder are worsened by the ongoing coronavirus pandemic (52–55).

Respiratory rhythm generation arises from neuronal networks in the ponto-medullary brain stem (FIGURE 1A) (56–59). Although the prefrontal cortex contains no neurons that generate breathing, it has been known since the 19th century that breathing is altered by electrically stimulating the prefrontal cortex [see Lépine (1875) in Ref. 60]. Pathway mapping studies since the 1980s have consistently documented neuronal projections connecting prefrontal cortex with brain regions that regulate breathing. As recently reviewed (61), this top-down modulation of breathing involves sensory and motor pathways connecting the prefrontal cortex with respiratory neurons in the pontine and medullary brain stem. Extensive pathways also connect the prefrontal cortex with hypoglossal, laryngeal, pharyngeal, trigeminal, and facial motoneurons that enable breathing-based vocalization (62). Via additional pathways, the prefrontal cortex is connected with periaqueductal gray and with hypothalamic and limbic nuclei that enable eupneic breathing to rapidly change in response to environmental stimuli involving nociception and/or perceived threat (63). Furthermore, the prefrontal cortex is involved in bottom-up modulation during which respiratory afferents alter brain and behavior (64). Data from humans show that the prefrontal cortex tracks respiratory sensation and modulates arousal and cortical excitability (65). Breathing can significantly modulate pain (66), the cortical electroencephalogram (67), emotional states (68), and levels of behavioral arousal (69). Readers interested in mind-body integration are referred to an excellent review (70) on the basic and clinical relevance of pulmonary afferent input.

Neuroanatomical substrates linking the prefrontal cortex to control of breathing and states of arousal provide a context for evaluating the hypothesis that opioids alter breathing and prefrontal cortex neurochemistry. As schematized, (FIGURE 1B, LEFT) these studies used dialysis delivery of morphine directly into the prefrontal cortex of C57BL/6J (B6) mice (24, 71). Measures were made of eight neurotransmitters (FIGURE 2, A–H) shown previously to modulate breathing and sleep (13, 14, 18, 72–77). Morphine administration caused a significant increase in the concentration of acetylcholine (FIGURE 2A) and a significant decrease in the concentration of adenosine (FIGURE 2D). Comparable studies in rat showed that morphine decreased prefrontal cortex ACh release when delivered to basal forebrain (78) and increased local ACh release when administered into the trigeminal motor nucleus (79) and the hypoglossal nucleus (80). Respiratory measures revealed that dialysis delivery of morphine to prefrontal cortex of B6 mouse also significantly decreased breathing frequency, tidal volume, and minute ventilation (FIGURE 2, I–K). These novel neurotransmitter measures are consistent with evidence across species that prefrontal cortical areas interact with brainstem regions regulating autonomic function (24, 59, 81). FIGURE 2 results show that

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prefrontal cortex administration of morphine significantly depressed breathing and changed concentrations of prefrontal cortex neurotransmitters. These findings encourage similar studies of different opioids across a range of concentrations.

In addition to causing respiratory depression, opioids administered to humans cause a blunting of wakefulness characterized by eyelid closure and diminished responsiveness to environmental stimuli. For many years these traits were mistakenly interpreted as signs of sleep. Clinical and preclinical studies using polysomnography have shown that opioids inhibit sleep and disrupt the normal periodicity of the sleep-wake cycle (18, 76, 82, 83) Misinterpreting opioid-induced blunting of wakefulness as sleep has been facilitated by the fact that opioids cause a dose-dependent dissociated state that comprises a mixture of traits that do not normally occur together (cf. FIGURE 3, A AND B).

FIGURE 2, L AND M, illustrates the results of Poincaré analyses in which the distribution of points

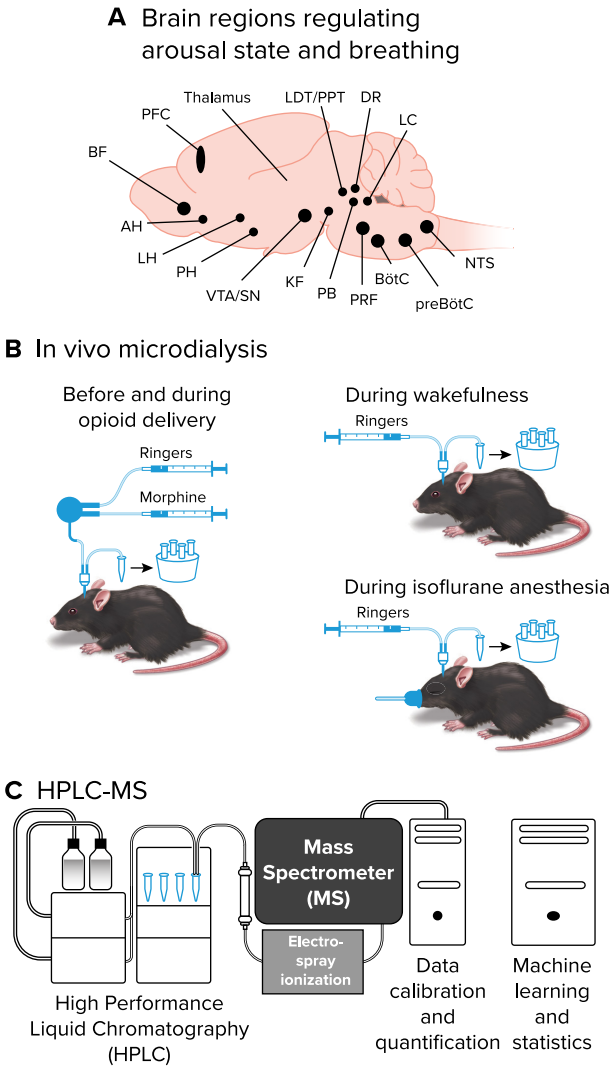

FIGURE 1. **Brain regions and experimental design.**
A: sagittal diagram of mouse brain schematizes the location of key brain regions that regulate states of arousal and/or breathing. Regions known to regulate sleep and wakefulness include the basal forebrain (BF), anterior hypothalamus (AH), lateral hypothalamus (LH), posterior hypothalamus (PH), thalamus, laterodorsal tegmental nucleus (LDT), pedunculopontine tegmental nucleus (PPT), dorsal raphe nucleus (DR), locus coeruleus (LC), and the pontine reticular formation (PRF). Areas generating breathing include the parabrachial nuclei (PB), Kölliker-Fuse nucleus (KF), Bötzinger complex (BötC), pre-Bötzinger complex (preBötC), and nucleus tractus solitarius (NTS). The prefrontal cortex (PFC) provides descending modulation of sleep and breathing. *B*, *LEFT*: in vivo microdialysis was used to collect endogenous neurotransmitters from the prefrontal cortex of awake mice before (*TOP* syringe) and during (*BOTTOM* syringe) delivery of morphine to the prefrontal cortex. Use of a liquid switch (sphere) permitted a within-subjects design. *B*, *RIGHT*: in a separate study, microdialysis also was used to collect endogenous neurotransmitters and cellular metabolites from awake mice (*TOP*) and mice anesthetized with isoflurane (*BOTTOM*) using a between-subjects design. *C*: the accuracy and high resolution of high-performance liquid chromatography-mass spectrometry (HPLC-MS) made it possible to quantify (nM) multiple neurotransmitters that were collected simultaneously from the same brain region in the same mouse. These measures were then analyzed by machine learning approaches to identify transmitters that predicted the state of isoflurane anesthesia. For additional studies, HPLC-MS was used to identify differences between relative amounts of cellular metabolites during wakefulness and anesthesia. *B* and *C* were adapted from Ref. 22 with permission from *Journal of Neurophysiology*.

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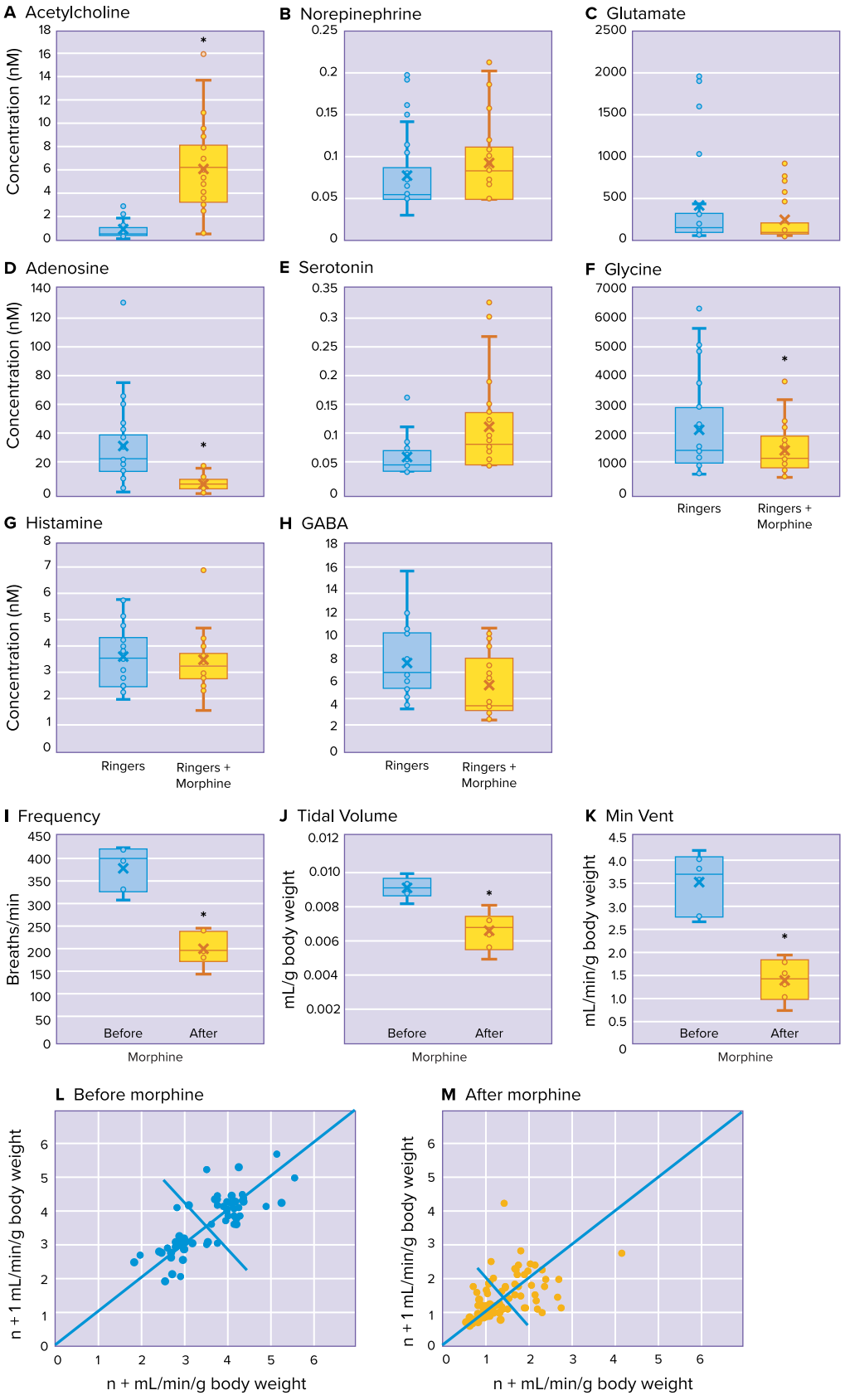
perpendicular to the line $x = y$ represents breath to breath variability, while the point distribution along the line $x = y$ represents breathing variability across the duration of the experiment. The data show that dialysis delivery of morphine to the prefrontal cortex of B6 mice caused a decrease in breathing variability (71). This finding is consistent with evidence that microinjection of morphine into prefrontal cortex of intact



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behaving mice decreases minute ventilation variability (84). Additional data (FIGURE 3C) show that systemic administration of fentanyl to B6 mice also causes a decrease in the variability of breathing. Efforts to identify brain sites of action of systemically administered drugs can be facilitated by comparing the effects of the same drug delivered systemically and delivered to a specific brain region (78). Buprenorphine delivered systemically to B6 mice (85) decreased minute ventilation variability, as did fentanyl (FIGURE 3C). Opioids delivered into the prefrontal cortex by microdialysis (FIGURE 2M) or microinjection (84) depress breathing variability. The results of these two independent studies using two modes of CNS drug delivery support the interpretation that the prefrontal cortex is one brain region contributing to opioid-induced respiratory depression (24, 59, 86).

FIGURE 3, A AND B, plots spectrograms of electroencephalogram (EEG) power for a range of EEG frequencies as a function of time (87, 88). The spectrograms show that compared with saline (FIGURE 3A), fentanyl (FIGURE 3B) increased EEG power (deep red color) in the delta range (0.5 to 4 Hz). EEG delta activity normally does not occur during wakefulness. Thus these results show that fentanyl causes a dissociated state of wakefulness characterized by an EEG trait that normally occurs during the nonrapid eye movement (NREM) phase of sleep. The EEG spectrograms (FIGURE 3) are consistent with studies in a different group of B6 mice (89, 90) showing that fentanyl causes a dissociated state of wakefulness that includes increased EEG slow-wave activity in the 0.5- to 4-Hz delta range. This dissociated state of wakefulness is of interest relative to evidence that the prefrontal cortex influences drug seeking behavior and addiction (20, 21, 91, 92). Interestingly, fentanyl causes significantly greater EEG slow wave activity after self-administration compared with passive administration in heroin-dependent individuals (93).

Prefrontal Cortex Neurochemistry Reconfigures during the Anesthesia-Induced Loss of Wakefulness

Human studies from the 1960s demonstrated that loss of wakefulness diminishes the drive to breath. In contrast to overventilated and anesthetized humans, overventilation during wakefulness was not followed by apneic breathing when overventilation was discontinued (94). This discovery led to the conclusion that respiration is stimulated by factors other than CO_2 and "that the cerebral activity associated with wakefulness is a component of the normal respiratory drive" (94). The wakefulness stimulus for breathing is a construct (95) that is clinically relevant for pulmonology (96), anesthesiology (97), sleep disorders medicine (98), addictionology (99), and clinical neuroscience (70). A corollary to the wakefulness stimulus for breathing is that breathing will be altered by disrupting neuronal networks that promote wakefulness.

Breathing in B6 mice, as in humans, is depressed during the loss of wakefulness caused by isoflurane anesthesia (100, 101). These relationships led us to compare the effects of opioids and isoflurane anesthesia on prefrontal cortex neurotransmitter concentrations. Extracellular fluid was collected by microdialysis from prefrontal cortex of intact, B6 mice during wakefulness and during the elimination of wakefulness by isoflurane (FIGURE 1B, RIGHT) (22). Liquid chromatography-dual mass spectrometry (FIGURE 1C) provided measures of eight neurotransmitters collected simultaneously from mouse prefrontal cortex. FIGURE 4 shows the concentrations of those eight neurotransmitters collected during wakefulness (blue) and during isoflurane anesthesia (red).

Inferential statistics demonstrated that isoflurane significantly decreased acetylcholine (FIGURE 4A) and significantly increased adenosine (FIGURE 4D) (22). These studies unmasked differences between the effects of isoflurane and morphine on prefrontal cortex neurotransmitter concentrations. Morphine significantly increased acetylcholine (FIGURE 2A) and decreased adenosine (FIGURE 2D). The present studies were not designed to elucidate the mechanism underlying the different effects of isoflurane and morphine on prefrontal cortex neurotransmitters. Different effects of isoflurane and morphine are not surprising given that even full mu agonists have differential effects. For example, fentanyl, but not morphine, has affinity for monoamine receptors and transporters (102). As described in the following paragraph, differing effects of morphine and isoflurane on prefrontal cortex neurotransmitters likely reflect a differential reorganization of neurotransmitter networks.

FIGURE 5 summarizes analyses showing a reorganization of neurotransmitter interactions by isoflurane.

FIGURE 2. **Dialysis delivery of morphine to prefrontal cortex alters neurotransmission and breathing.** Dialysis with Ringer's (vehicle control, blue) and with Ringer's containing morphine (yellow) made it possible to measure 8 simultaneously collected neurotransmitters (*A*–*H*) in every dialysis sample. The *BOTTOM* and *TOP* of each box indicate the 1st and 3rd quartiles, respectively. Inside each box, the horizonal line shows the median, and the **x** plots the mean. Whiskers indicate the lowest data point within 1.5 times the interquartile range (IQR) of the lower quartile, and the highest data point within 1.5 times IQR of the upper quartile. Outliers are indicated by small dots above and/or below the whiskers. Morphine depressed: breathing frequency (*I*), tidal volume (*J*), and minute ventilation (*K*). The Poincaré plots show that compared with Ringer's before morphine (*L*), minute ventilation variability was depressed by morphine (*M*). *Significantly different from control. Adapted from Ref. 24 with permission from *Journal of Neurophysiology*.

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FIGURE 5 also illustrates that the interactions among measured neurotransmitters mathematically predicted the presence or absence of wakefulness. This study (22) combined hypothesis testing via inferential statistics with predictive modeling by artificial intelligence (AI). This targeted metabolomics approach (103, 104) was followed by data analyses using supervised machine learning. Measuring neurotransmitter concentrations during wakefulness and during the isoflurane-induced loss of wakefulness enabled predictive model development. Heatmap plots of nondirectional Pearson correlations values (FIGURE 5, A AND B) illustrate neurotransmitter relationships during wakefulness and isoflurane anesthesia. Overall, the heatmaps reveal fewer high positive correlations between pairs of neurotransmitters during anesthesia than during wakefulness. FIGURE 5, C AND D, shows networks for neurotransmitter pairs that had Pearson correlation coefficients of 0.5 or greater. Those networks included four of

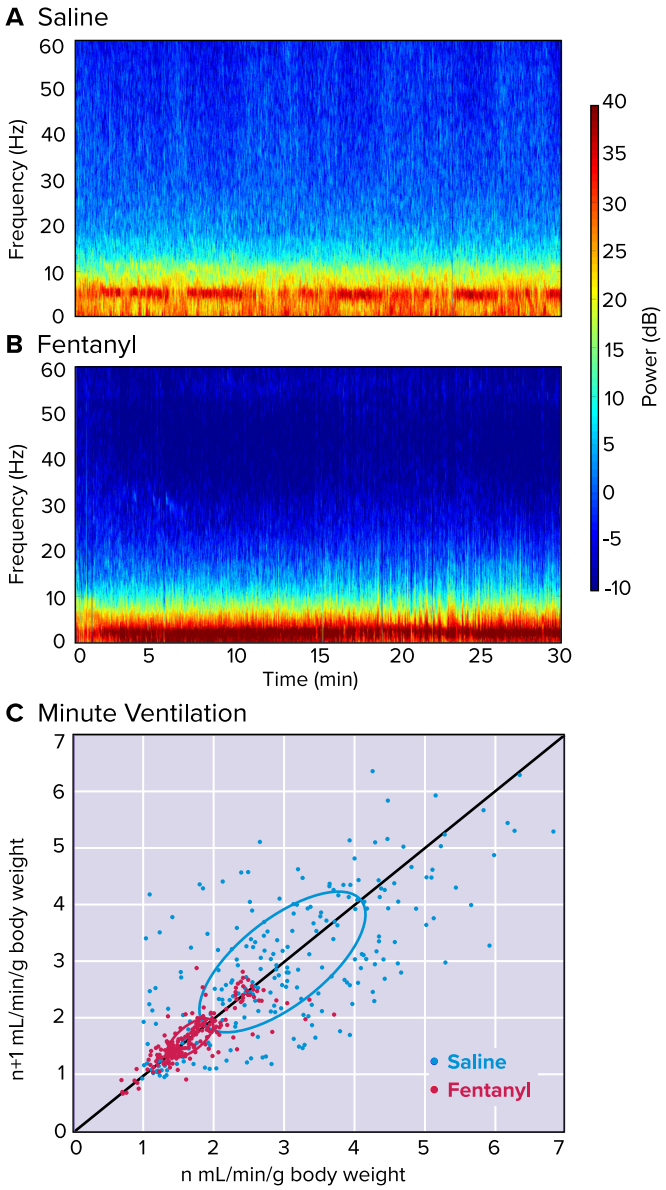

FIGURE 3. **Systemic fentanyl dissociates behavioral states and electroencephalographic (EEG) traits while decreasing minute ventilation variability.**
Tapered spectrograms of EEG power after systemic injection of saline (*A*; vehicle control) or fentanyl (*B*). Color bar at *RIGHT* shows EEG power in decibels (dB). EEG frequency (*LEFT* ordinate, Hz) and power are plotted for the initial 30 min after injection (abscissa). Recordings from the same mouse were made on different days during wakefulness. Spectrograms show that fentanyl (3 mg/kg) increased EEG power in the 0.5 to 4 Hz delta range relative to control. Poincaré plot of minute ventilation (*C*) recorded from 4 mice shows that compared with saline administration (blue dots), systemic fentanyl (red dots) decreased minute ventilation variability. The SD of the dots perpendicular to the line $x = y$ is referred to as SD1 and reflects breath-to-breath variability of minute ventilation. SD1 is indicated by the short radius of the blue ellipse (saline) and red ellipse (fentanyl). The SD of the points along the line $x = y$ is referred to as SD2 and reflects the variability in minute ventilation across the 60 min of recording. SD2 is indicated by the long radius of each ellipse. This Poincaré plot shows a smaller width and length of the red ellipse (fentanyl) compared with the blue ellipse (saline).

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the eight neurotransmitters during wakefulness (nodes illustrated as blue ovals) that were all correlated (edges illustrated as connecting lines). During isoflurane anesthesia the four-transmitter network fractionated into two different networks, one of which comprised only GABA and glutamate. During wakefulness and during isoflurane anesthesia, the most highly correlated neurotransmitters were those shown by many laboratories to contribute to generating and maintaining wakefulness (74, 105, 106).

Networks unmasked by Pearson correlations are limited to identifying linear, pairwise relationships. Those network relationships encouraged additional analyses using an iterative random forest (iRF) algorithm to identify linear and nonlinear relationships (22). The models were trained with 75% of the data and the other 25% of the data were used to evaluate the accuracy of the model. During wakefulness, seven of the eight neurotransmitters formed two distinct networks. Norepinephrine, adenosine, dopamine, and GABA comprised a network in which norepinephrine concentration predicted the concentrations of the other three transmitters (FIGURE 5E). Acetylcholine, histamine, and serotonin comprised a separate network. During the loss of wakefulness caused by isoflurane (FIGURE 5F), the two neurotransmitter networks were reorganized. Acetylcholine and serotonin were added to one network and that addition changed the network relationships. During isoflurane anesthesia, GABA and glutamate comprised a separate network. The double headed arrow schematizes that the concentrations of GABA and glutamate were reciprocally predictive.

The iRF (FIGURE 5G) illustrates results from a supervised machine learning algorithm that identified neurotransmitters predicting the state of isoflurane anesthesia. FIGURE 5G shows that the neurotransmitters adenosine, norepinephrine, and acetylcholine (*TOP ROW* of nodes) predicted neurotransmitter relationships (*MIDDLE ROW* of nodes). Additionally, the

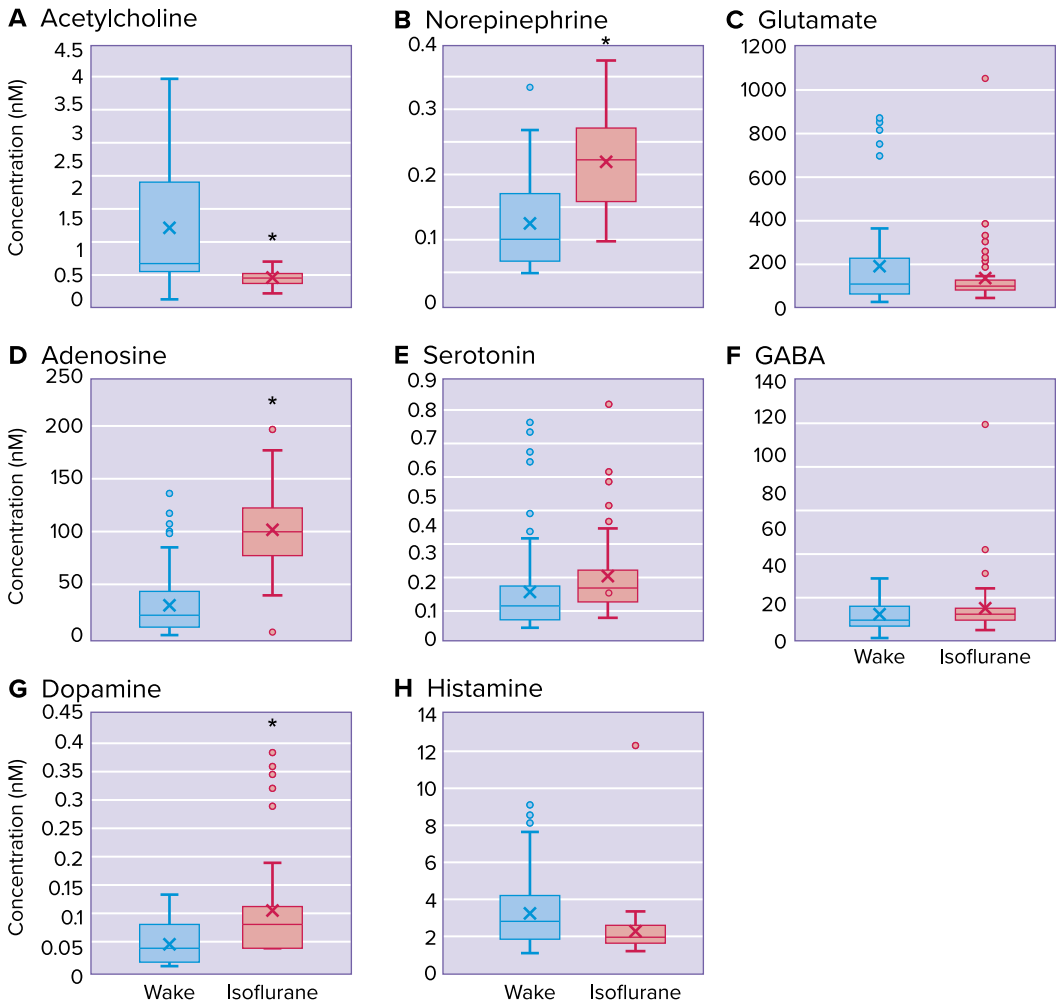

FIGURE 4. **Concentrations of prefrontal cortex neurotransmitters measured during wakefulness and during isoflurane anesthesia.**
Box plots illustrate the concentration (nM) of 8 neurotransmitters (*A–H*) simultaneously collected by microdialysis during wakefulness (blue) and during isoflurane anesthesia (red). The horizonal line within each box indicates the median (2nd quartile), and the **x** plots the mean. Whiskers illustrate data within 1.5 times the interquartile range. Small dots above or below the whiskers show outliers. *Significantly different from control. From Ref. 22 with permission from *Journal of Neurophysiology*.

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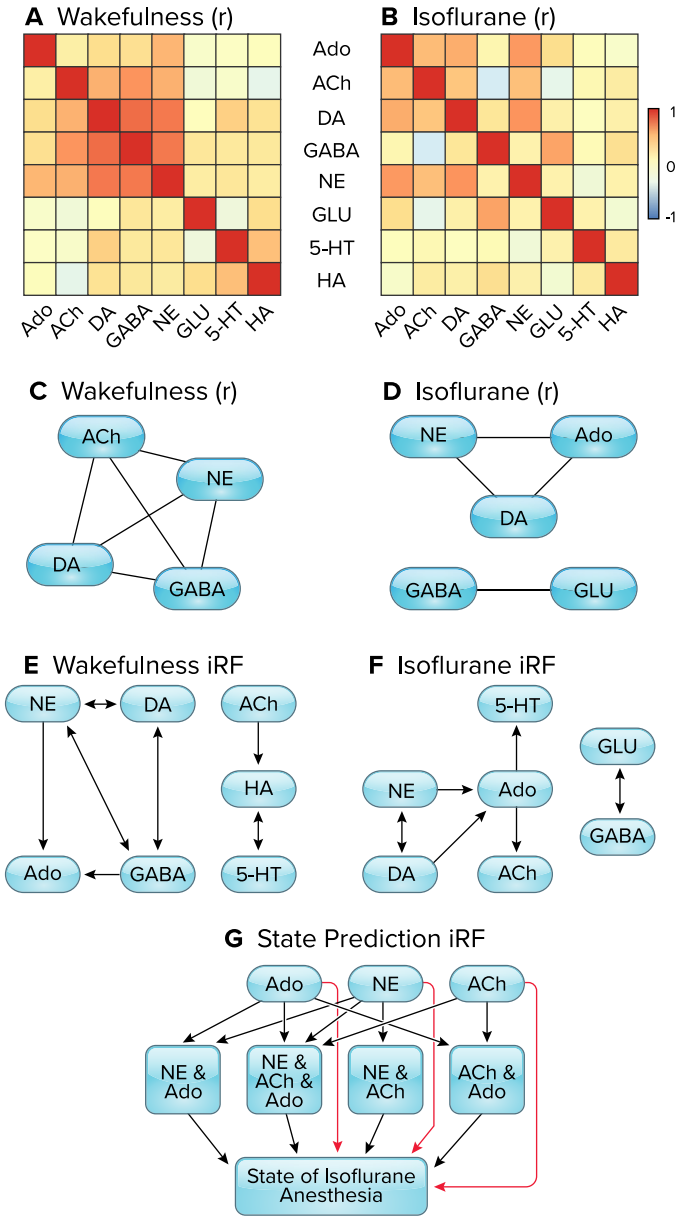

FIGURE 5. **Artificial intelligence algorithms show that isoflurane anesthesia reorganizes neurotransmitter interactions and that neurotransmitter concentrations predict state of consciousness.** Heatmaps illustrate the correlation (*r*) between neurotransmitter pairs during wakefulness (*A*) and isoflurane (*B*) anesthesia. The color key at *RIGHT* shows Pearson correlations ranging from −1 (blue) to +1 (red). Heatmap colors represent the strength of the pairwise correlations between neurotransmitters. Comparison of the waking and isoflurane heatmaps shows that the pairwise relationships between these 8 neurotransmitters were altered during the isoflurane-induced loss of wakefulness. Neurotransmitters with a Pearson *r* value of 0.5 or greater exhibit different network configuration during wakefulness (*C*) and isoflurane anesthesia (*D*). Iterative random forest (iRF) analyses made it possible to identify neurotransmitters that predicted the concentrations of other neurotransmitters during states of wakefulness (*E*) and isoflurane anesthesia (*F*). The results revealed both unidirectional (single arrowhead) and bidirectional (double arrowhead) predictive relationships. The iRF state prediction algorithm identified 3 neurotransmitters (*G*, *TOP ROW*), the concentrations of which accurately predicted (red arrows) the state of isoflurane anesthesia. *G*, *MIDDLE ROW*: illustration of pairs of neurotransmitters that also enabled state prediction. ACh, acetylcholine; Ado, adenosine; DA, dopamine; GABA, gamma-aminobutyric acid; GLU, glutamate; HA, histamine; NE, norepinephrine; 5-HT, serotonin. Adapted from Ref. 22 with permission from *Journal of Neurophysiology*.

neurotransmitter interactions in FIGURE 5G, *middle row of nodes*, differentiated the state of wakefulness from the state of isoflurane anesthesia. The red arrows illustrate that the concentrations of adenosine, norepinephrine, and acetylcholine independently predicted the loss of wakefulness caused by isoflurane anesthesia. The finding that prefrontal cortex concentrations of adenosine and acetylcholine predicted states of consciousness (FIGURE 5G) is consistent with previous evidence that adenosine A_1 and A_{2A} receptors in mouse prefrontal cortex modulate acetylcholine release and levels of EEG and behavioral arousal (107). To our knowledge, these findings (22) are the first to show that an isoflurane-induced loss of

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Table 1. ***Prefrontal cortex metabolites differed during sleep and isoflurane anesthesia relative to wakefulness***

Metabolite	Sleep	Anesthesia	*P*	Metabolic Pathway
D-gluconate	↓	↑ *q* = 7.60E-05	<0.0001	Pentose phosphate
Glutamate	↓	↑ *q* = 1.42E-04	<0.0001	Amino acid (AA)
Homovanillic acid	↓	↑ *q* = 5.24E-06	<0.0001	Organic acid
Lactate	↓	↑ *q* = 1.75E-07	<0.0001	Glycolysis
N-acetyl-β-alanine	↓	↑ *q* = 1.46E-06	<0.0001	*N*-acetylated AA
N-acetyl-glutamine	↓	↑ *q* = 7.32E-01	<0.0001	*N*-acetylated AA
Orotate	↓	X *q* = 9.18E-01	0.9177	Pyrimidine metabolism
Pyruvate	↓	X *q* = 9.66E-02	0.0761	Glycolysis
Succinate/methylmalonate	↓	↑ *q* = 1.95E-06	<0.0001	TCA cycle
Tryptophan	↓	↓ *q* = 3.37E-08	<0.0001	Amino acid
Uridine	↓	↑ *q* = 5.11E-05	<0.0001	Pyrimidine metabolism

Levels of 11 prefrontal cortex metabolites measured during sleep (11) and during isoflurane anesthesia (23). Symbols indicate significant decreases (↓), increases (↑), or no change (X) in metabolites relative to wakefulness. *P* values are from unadjusted mixed model ANOVA, and *q* values give the Benjamini-Hochberg false discovery rate-adjusted probabilities. Adapted from Ref. 23 with permission from *Journal of Neurophysiology*.

wakefulness caused a reorganization of neurochemical networks in mouse prefrontal cortex.

Opportunities for future research include experiments designed to simultaneously sample multiple brain regions before, during, and after different drugs. Such studies also are feasible using lipidomic and proteomic analyses. The following section highlights results from parallel studies of prefrontal cortex metabolites that were collected during wakefulness, sleep, and anesthesia.

The Metabolome of Mouse Prefrontal Cortex Is Differentially Altered during Sleep and Anesthesia

Sleep and anesthesia are distinctly different states, yet both states are characterized by disruptions of autonomic physiology. The prefrontal cortex is normally deactivated during NREM sleep relative to wakefulness (9, 18, 108, 109), and some sleep disorders are characterized by prefrontal cortex deactivation or overactivation (110). Sleep apnea can contribute to cognitive impairment involving prefrontal cortex dysfunction (111), as indicated by two lines of evidence. First, sleep apnea is associated with alterations in human brain chemistry (112). Second, diffuse brain damage in the prefrontal cortex has been observed in some sleep apnea patients (113, 114). Recent efforts to understand prefrontal cortex neurochemistry during sleep focused on the metabolome (11). Microdialysis samples were collected from B6 mice during EEG-defined states of NREM sleep and wakefulness. An untargeted metabolomics approach (115) was used to measure those dialysis samples with ultra-performance liquid chromatography-high-resolution mass spectrometry (UPLC-HRMS). A brief summary of the results (Table 1) showed that 11 of 36 identified molecules comprising the prefrontal cortex metabolome were significantly decreased during NREM sleep relative to wakefulness (11).

The finding of sleep-selective decreases in prefrontal cortex metabolites encouraged study of the prefrontal cortex metabolome during the isoflurane-induced loss of wakefulness (23). Microdialysis samples were obtained from mouse prefrontal cortex during wakefulness and during isoflurane anesthesia (FIGURE 1B, RIGHT) (23). Analyses using UPLC-HRMS detected 2153 molecules, 91 of which could be identified. Analytes were grouped as detected during both wakefulness and anesthesia (n = 61) and as unique to wakefulness (n = 23) or anesthesia (n = 7). During anesthesia relative to wakefulness there was a significant, 4-fold change in 21 of the metabolites. During anesthesia 11 of these 21 molecules decreased and 10 molecules increased (23).

Multivariate analyses revealed significant separation of molecules detected during wakefulness and anesthesia (FIGURE 6). A plot of partial least squares discriminate analysis (PLS-DA) score (FIGURE 6A) shows lack of overlap between metabolites collected during wakefulness (blue triangles) and during isoflurane anesthesia (red circles). The 95% confidence interval for the state-space distribution of metabolites is illustrated by the blue and red ellipses. The PLS-DA distributions were confirmed by fuzzy k-means cluster analysis on principal component analyses scores (FIGURE 6B). The volcano plot (FIGURE 6C) shows, relative to wakefulness, molecules that significantly decreased (≥4-fold) during anesthesia (green, n = 11) and molecules that significantly increased (≥4-fold) during anesthesia (orange, n = 10). Between the green and orange points are gray points representing 40 molecules that did not change by 4-fold during anesthesia compared with wakefulness.

FIGURE 7 summarizes changes in 21 metabolites during anesthesia (red) relative to wakefulness (blue). During isoflurane administration there was a decrease

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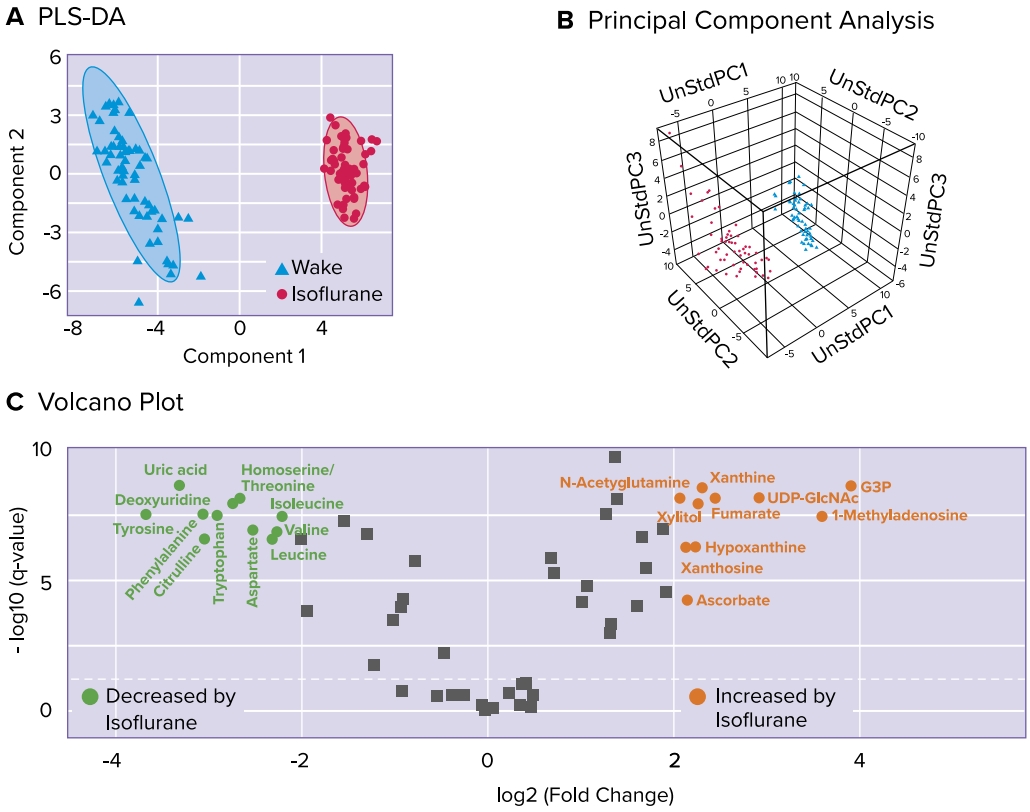

FIGURE 6. **The prefrontal cortex metabolome differs during states of wakefulness and isoflurane anesthesia.** *A* and *B*: partial least squares discriminate analysis (PLS-DA; *A*) and principal component analyses (*B*) confirmed state-selective differences in metabolites. *C*: the volcano plot illustrates molecules with a 4-fold significant decrease (green) and 4-fold significant increase (orange) during isoflurane anesthesia compared with wakefulness. In the volcano plot, gray squares represent 40 molecules that did not exhibit a significant change during isoflurane. All 3 analyses confirmed state-selective separation of metabolites. UnStdPC, unstandardized principal components; G3P, *sn*-glycerol-3-phosphate; UDP-GlcNAc, uridine diphosphate-*N*-acetylglucosamine. Adapted from Ref. 23 with permission from *Journal of Neurophysiology*.

in levels of amino acids and an increase in purines. Studies from many laboratories report that the loss of wakefulness is associated with brain site-specific decreases in monoaminergic transmission (74). Tryptophan is a precursor required for the biosynthesis of the monoamines, serotonin and melatonin. Numerous studies have shown that wakefulness is promoted by serotonin and sleep is increased by melatonin. As summarized in Table 1, of all the metabolites measured in both studies, only prefrontal cortex tryptophan was significantly decreased during the loss of wakefulness leading to sleep (11) and during the loss of wakefulness caused by isoflurane anesthesia (23). The results of these two studies show that states of sleep and anesthesia are more different than similar with regard to the prefrontal cortex metabolome of B6 mice (Table 1).

The aspirational goal of standardizing brain metabolomics (116) must solve complexities of scale in spatial, temporal, and magnitude domains, all of which are unresolved limitations of the data reviewed here. The prefrontal cortex is spatially complex and comprised of multiple subregions (117, 118). In the temporal domain is the persisting problem of time required to obtain biological samples from intact, behaving animals. Even near real-time-sampling of the extracellular space provided by capillary electrophoresis-mass spectrometry (119) is slow, relative to synaptic processing time involved in network communication. The metabolome is estimated to comprise more than 200,000 molecules (120) many of which remain unidentified. Even the scores of identified molecules measured during states of NREM sleep (11) and anesthesia (23) represent a small fraction of the brain metabolome.

Perspectives

Everything Old Is New Again

The Introduction placed the present findings within a context that advocates unified approaches associated with systems biology and classical neurophysiology. Three decades ago a “White Paper” produced by the APS Long Range Planning Committee recommended that the discipline of physiology be defined as “Integrative Biology” (121). Many concepts shared by both contemporary physiology and systems biology date to the 19th century. Claude Bernard’s text (122) on experimental medicine is regarded as a classic for

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conveying the relationships between physiological states and symptoms of disease progression. The physiologist Henry Bowditch worked with Bernard at Collège de France and, upon returning to the United States, Bowditch recruited Walter Cannon to physiology. Links between Bernard's and Cannon's concepts of homeostatic regulation are reviewed elsewhere (123). Bernard and Cannon recognized that afferent and efferent limbs of feedback control are nonlinear and exhibit state-selective regulation. Physiological "states" and "systems" are central to Cannon's concept of homeostasis. Five of Cannon's six postulates regarding homeostatic control refer to states of the organism (124). Cannon's first postulate describes the body as "an open system." From a thermodynamic perspective an open biological system is actively engaged in metabolism that involves energy transfer with the environment (125).

Recognition that the principles of physics are manifest in biology dates to the 19th century work of Helmholtz and the discoveries of du Bois-Reymond on "animal electricity" (126). For an accessible consideration of the relationship between physics and computational biology see Stevens (127). Physiological changes caused by opioids (FIGURES 2 AND 3), anesthesia (FIGURES 4–7), and NREM sleep (Table 1) are accompanied by changes in energy metabolism (128–130). There is compelling evidence that the capacity for energy transfer is a significant determinate of organismal health (131). Research on complexity makes clear that seemingly disparate physiological control systems commonly are not independent (37). Bernard understood that the brain influences the heart and contemporary studies have identified the prefrontal cortex as a brain region that modulates cardiac function (132). For example, Takotsubo cardiomyopathy is commonly preceded by emotional or physical stress (133). Emotional states (63) and multiple visceral control systems (64) are modulated by the prefrontal cortex. Among the most prevalent examples of state-selective physiology are the ~80 visceral, somatic, and cognitive disorders associated with sleep (40).

Eight years of Cannon's research career were devoted to studies aiming to understand the mechanisms that regulate "stable states of the organism" (134). Stability, of course, is relative to time scale. Process biology emphasizes that each physiological measure is a point sample representing continuous flux, ongoing at all organismal levels (135). Characterizing state-selective changes in the prefrontal cortex metabolome (11, 22, 23) is a technologically updated emulation of Cannon's studies with Arturo Rosenblueth on the chemical mediation of homeostasis (136). Rosenblueth extended the state concept from physiology to machines (137), thereby contributing to the development of cybernetics (138). The cyberneticist Ross Ashbey (139) recognized that "the state of a system is at any given instant the set of numerical values which its variables have at that instant." The data reviewed here show that a machine learning algorithm reliably predicted states of consciousness based on prefrontal cortex concentrations of neurotransmitters (22).

The construct of a wakefulness stimulus for breathing is a story model that emerged from comparisons of breathing during wakefulness and breathing during the anesthesia-induced loss of wakefulness (94). There is evidence that cholinergic neurotransmission in the prefrontal cortex contributes to the wakefulness stimulus for breathing. Muscarinic cholinergic receptors of the M2 subtype modulate activation of prefrontal cortical EEG and acetylcholine release (140, 141). The muscarinic cholinergic agonist carbachol administered to prefrontal cortex of anesthetized rat promotes behavioral arousal (142), and breathing is enhanced by delivery of the acetylcholinesterase inhibitor neostigmine into mouse prefrontal cortex (143).

The approaches described in this review demonstrate the feasibility of curating and analyzing neurochemical data from multiple networks, brain regions, and across multiple organisms. We speculate that these approaches ultimately will enable a quantitative model of brain regions and molecules that reliably predict state-selective changes in breathing. The state concept also has explanatory power across organismal clades, representatives of which vary widely in neuronal scale. States of wakefulness, non-REM sleep, and REM sleep are actively generated by the human brain, which is estimated to contain 100 billion neurons (144). Even oscillating states of consciousness generated by the overwhelmingly complex human brain can be conceptualized as dynamic, state space models (145). Organisms such as *Drosophila melanogaster* have ~100,000 neurons and the nematode *Caenorhabditis elegans* possess ~300 neurons. Flies (146) and worms (147) also are open biological systems that display states and features homologous to human states of neurobehavioral arousal.

Conclusions, Tensions, and Future Directions

Electrochemical transmission is the canonical mode of information processing by the nervous system (148, 149). The evidence in this review demonstrates the feasibility of combining targeted and untargeted metabolomics to identify candidate molecules that modulate visceral functions of the prefrontal cortex. The discoveries reviewed here are consistent with evidence that the prefrontal cortex contributes to regulation of behavioral states (9, 12, 20, 150, 151) and breathing (7, 24, 59, 65, 76, 143, 152, 153).

Microdialysis delivery of morphine to the prefrontal cortex changed neurotransmitter concentrations within the prefrontal cortex (FIGURE 2, A–H) and significantly depressed breathing (FIGURE 2, I–M). Systemic fentanyl administration caused a dissociated state of wakefulness characterized by increased delta

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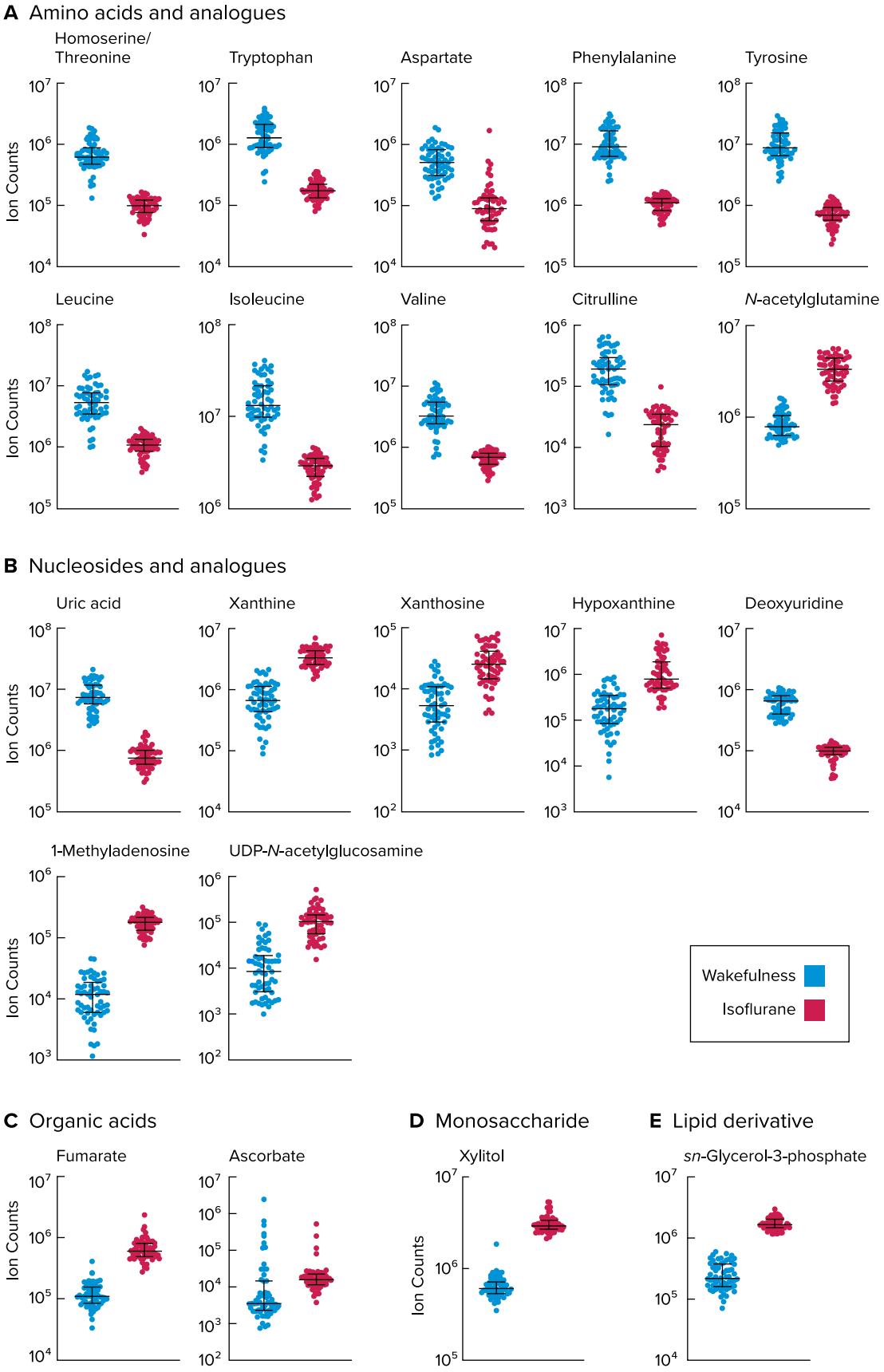

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wave activity (FIGURE 3B). The morphine-induced decrease in respiratory variability (FIGURE 2M) was replicated by systemic administration of fentanyl (FIGURE 3C). The finding that opioids administered systemically and directly into the prefrontal cortex each caused decreased respiratory variability is consistent with the interpretation that the prefrontal cortex may be one brain region contributing to opioid-induced depression of respiratory variability.

Targeted metabolomic studies (FIGURE 4) of prefrontal cortex neurotransmitters during wakefulness and the isoflurane-induced loss of wakefulness show that state-selective changes in prefrontal cortex neurotransmitters are not limited to opioid-induced obtundation of wakefulness. Machine learning analyses (FIGURE 5) revealed that prefrontal cortex concentrations of adenosine, norepinephrine, and acetylcholine reliably predicted the state of isoflurane anesthesia. Quantifying multiple neurotransmitters in each microdialysis sample led to the novel finding of state-selective reconfiguration of neurotransmitter interactions. This discovery has significant implications for approaches that measure one analyte at a time across states of consciousness. The discovery of neurotransmitter reconfiguration (FIGURE 5) represents the plasticity of networks within the prefrontal cortex. Network reconfiguration suggests a foundational change for interpreting neurochemical data. Alterations in a neurochemical variable may result from second-order effects (FIGURE 5) reflecting network reconfiguration.

The neurochemical data reviewed here highlight tensions along three fronts. First, there are tensions between the approaches of systems biology versus reductionistic physiology. Disciplinary status was achieved by physiology (26) more than a century before systems biology was referred to as a new biology (25). In fact, understanding physiological complexity is one of the goals of the Institute for Systems Biology (154). The importance of nominalism in science is well documented (30), but technology rather than disciplinary ideology is the unifier that bridges organizational silos. The present discoveries regarding the prefrontal cortex metabolome were enabled by measurements using orbitrap mass spectrometry (155) and by analyses that employed machine learning and predictive AI algorithms. A distinction between physiology and systems biology is repudiated by the fact that computational biology shares a lineage between physiology and cybernetics. The potential benefits of approaches that combine physiology, systems biology, and medicine are illustrated by the ability of predictive modeling to generate testable hypotheses regarding relationships between the SARS-CoV-2 virus, gene expression networks, angiotensin-bradykinin, and severe symptoms of COVID-19 (156).

A second tension concerns value judgments regarding hypothesis directed versus hypothesis neutral studies. The data reviewed here show that combining untargeted and targeted metabolomics for studies of prefrontal cortex (11, 22, 23) enabled insights not possible via a single-minded commitment to one approach. Advances from multidisciplinary studies are so rapid that even their descriptions often require neologisms (30). For example, "chemoconnectomics" proposes using *Drosophila* to map the "entirety of all neurotransmitters, neuromodulators, neuropeptides, and their receptors to trace neural circuitry anatomically and functionally" (157). The *Drosophila* proposal is an extension of the brain activity map outlined almost a decade ago as a way to apply functional connectomics to mammalian brain (158), including the entire brain studied at a mesoscopic scale (159, 160). Neurochemical connectivity databases show good progress toward achieving such ambitious goals (cf. Table 1 in Ref. 161). The ChemNetDB database (http://www.chemnetdb.org) has curated and organized 50 yr of neuroanatomical track-tracing and neurochemical measurements from 36,464 rats (162). The rat genome database (https://rgd.mcw.edu), extant since 1999, now includes a multispecies knowledgebase (163). Discovery science has been promoted for more than a decade (164) by aggregating human brain imaging data (http://fcon_1000.projects.nitrc.org/). Interpretation of the prefrontal cortex neurochemical data reviewed here (11, 22, 23) relied heavily on the Human Metabolome Database and the Kyoto Encyclopedia of Genes and Genomes.

A future-looking symposium in February 2020 was cosponsored by the National Academies to mark the landmark 1945 report by Vannevar Bush entitled "Science, the Endless Frontier" (165). Diverse symposium speakers encouraged training in mathematics and computational approaches. Mathematics has been described as biology's next microscope because of the ability to reveal previously obscured relationships (166). Present investigators are the beneficiaries of more than three decades of increased computing power at decreasing costs (167).

All life sciences confront unresolved tensions due to information overload. The preface of a valued book on the prefrontal cortex notes that between the 2008

FIGURE 7. **Metabolites in prefrontal cortex that significantly increased or decreased during isoflurane anesthesia (red) relative to wakefulness (blue).**
Median and interquartile range for the 21 molecules in FIGURE 6C that significantly ($q < 0.0001$) increased or significantly decreased during wakefulness (blue) and isoflurane anesthesia (red). The metabolite ion counts (*A*–*E*) are organized by biofunction using the Human Metabolome Database and the Kyoto Encyclopedia of Genes and Genomes for mouse. During isoflurane anesthesia, 9 of the 10 amino acids were significantly decreased, and 5 of 7 nucleosides and analogs were significantly increased. From Ref. 23 with permission from *Journal of Neurophysiology*.

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and 2015 editions, the PubMed database regarding the prefrontal cortex increased by more than 14,000 articles (5). A search on Semantic Scholar (168) for "prefrontal cortex" on December 1, 2020 returned ~486,000 citations. This list comprises only 0.02% of the 2 billion citations currently available on Semantic Scholar. This example illustrates why programs such as the Defense Applied Research Projects Agency (169) and the Allen Institute (170) aim to make all digitized content machine readable. Current estimates from the Cisco Global Cloud Index indicate that in 2021 ~94% of all workloads will be cloud based and comprise a volume of ~7.2 zettabytes (171). One Zettabyte equals 10^{21} bytes of data, equivalent to one trillion gigabytes. Directly relevant to physiology are healthcare data estimated worldwide for 2020 to comprise ~25,000 petabytes (172). The information genie is out of the bottle. The ability to access, analyze, and interpret this volume of information, however, is limited by the human capacity for signal processing. Even 75 yr ago Bush noted that our information has become so complex we need mechanized records if we are to push experiments to a logical conclusion and not become bogged down by overtaxing our limited memory (173). We confront the paradox that information overload hopefully will be mitigated by inclusion of "mathematical, statistical, and computational methods into mainstream biological training" (174). ■

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R.L. and H.A.B. conceived and designed research; R.L. and H.A.B. analyzed data; R.L. and H.A.B. interpreted results of experiments; R.L. and H.A.B. prepared figures; R.L. and H.A.B. drafted manuscript; R.L. and H.A.B. edited and revised manuscript.

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