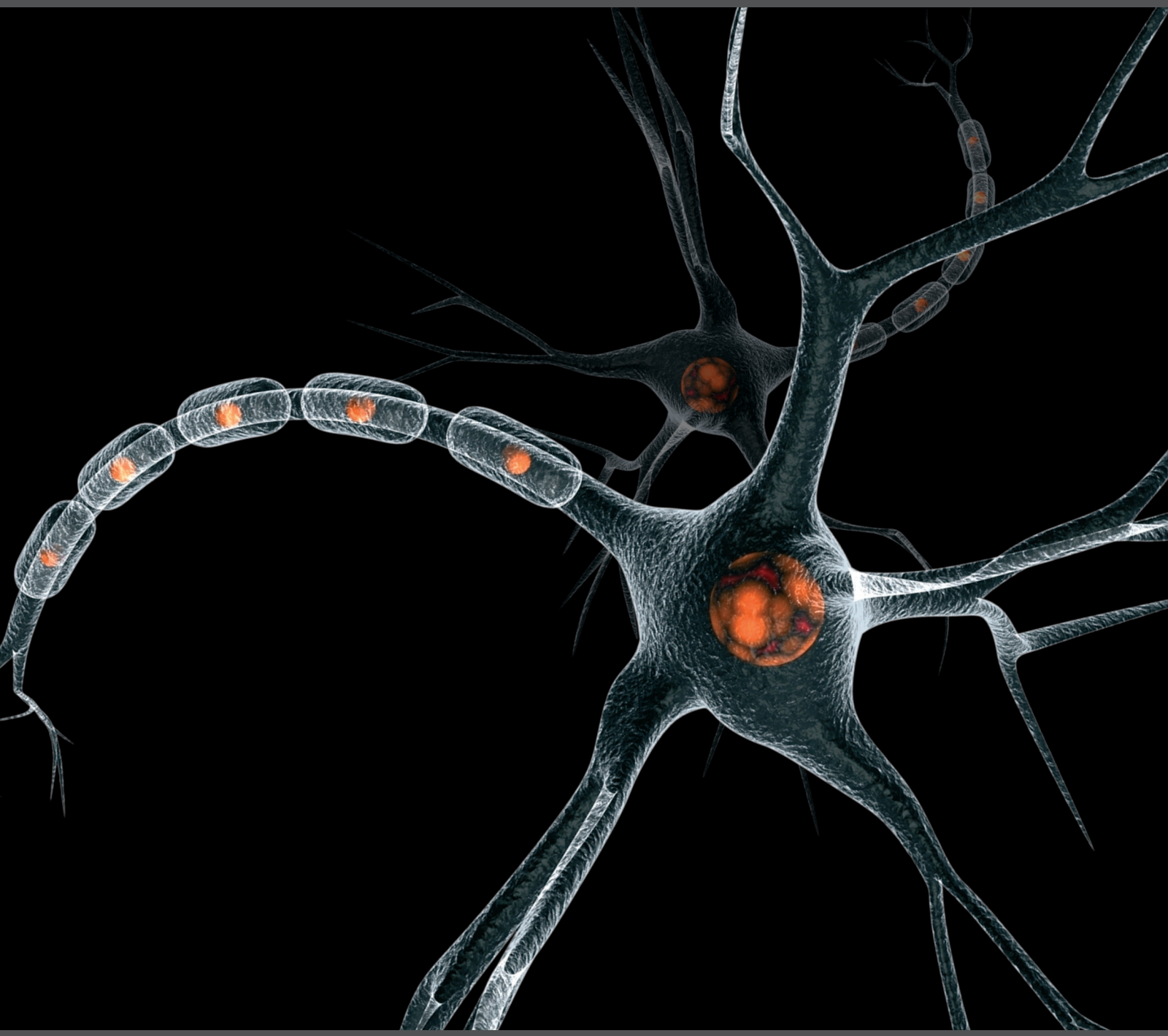
Neural Plasticity

Homeostatic Plasticity in the Nervous System

Guest Editors: Arianna Maffei, Dirk Bucher, and Alfredo Fontanini



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Editorial Board

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Editorial

Homeostatic Plasticity in the Nervous System

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Every organism relies on the maintenance of stable internal states, a phenomenon usually referred to as homeostasis. While homeostatic control of physiological functions like ion regulation has long been well described, homeostatic control of neuronal and network function is a relatively recent concept. It is intuitive to think that regulatory mechanisms must exist to preserve neural function in the face of constantly changing internal states and/or interactions with the environment. Growth and development, experience-dependent plasticity, and constant molecular turnover pose a challenge to the nervous system to keep all parameters within a functional range without overconstraining plastic processes vital for adaptive changes. Investigating the complexity of homeostatic regulation of single neurons and neural circuits is thus fundamental for understanding brain function.

In recent years, there has been a dramatic increase in the number of contributions to the study of mechanisms underlying homeostatic plasticity in the brain. One of the remaining challenges is to integrate findings from different levels of analysis, possibly providing a comprehensive theory that encompasses the complexity and multitude of findings, which at times may appear contradictory. Multiple levels of homeostatic regulation have now been identified in a variety of model systems. Neurons can preserve their excitability in a functional dynamic range by adjusting their intrinsic properties and the strength of their synapses in a cell-autonomous manner. A number of cellular and molecular mechanisms have been identified as regulators of cell-autonomous homeostatic plasticity. In most systems, the activation of these mechanisms occurs after a sensor detects deviations from expected levels of electrical activity and begins a cascade of events that compensates for these changes. Systems in which the expected set-point activity of a single neuron has been directly correlated with network function have allowed the identification of a number of parameters that can be adjusted to maintain stable levels of activity in the nervous system. Perhaps not surprising for such a fundamental property of biological systems, there is no unique pathway to homeostatic regulation of network activity even in relatively simple circuits. When looking at different neuronal types that make up neural circuits, it is often found that cell-autonomous mechanisms for homeostasis differ substantially. For example, chronic manipulations of activity result in changes in the excitability and synaptic properties of glutamatergic cortical neurons, consistent with a cell-autonomous regulation towards a set-point. In contrast, the same manipulations result in changes of GABAergic neuron properties that do not seem to promote maintenance of their own excitability. However, these changes may effectively favor maintenance of network activity by appropriately adjusting the global balance of excitation and inhibition in the circuit.

In this issue, G. Wang et al. review the current literature regarding homeostatic synaptic plasticity and the mechanisms regulating AMPA receptors trafficking and discuss these findings in the context of homeostatic regulation of the excitability of excitatory neurons. T. E. Krahe et al. report a role for CREB in homeostatic synaptic plasticity and propose that this set of plasticity mechanisms in the dorsal lateral geniculate nucleus plays a major role in regulating the sensitivity of cortical circuits to changes in sensory drive. F. C. Roth and A. Draguhn highlight the importance of the

regulation of GABA metabolism and transport for circuit homeostasis, and propose that inhibitory synapses may play a fundamental role that goes well beyond providing a brake on neural excitability.

The existence of multiple mechanisms for homeostatic plasticity is consistent with the possibility that each cell type may have an entire toolkit at its disposal to maintain a balanced level of activity, and it suggests a significant degree of flexibility in how a network can respond to different challenges. This diversity of mechanisms makes it difficult to identify general rules for homeostatic plasticity. Therefore, the investigation of interactions between different neuron types and their role in network function is crucial. Although the possibility that there are fundamental constraints to the degree of variability and of coordination of different homeostatic changes has been explored, this area of research is still in its infancy. Here, theoretical work is of fundamental importance for the integration of diverse experimental findings, with the goal to provide a general conceptual framework for homeostatic regulation of circuit excitability and function. In this context, B. N. Queenan et al. employ the principles of control theory to formally describe basic features of circuit homeostasis in the attempt to provide a comprehensive theory.

At the systems level, the interaction between cell-autonomous and circuit mechanisms may preserve stable sensory, motor, and cognitive functions. Investigating how homeostatic mechanisms observed at the single neuron and circuit level are integrated to regulate brain activity is extremely challenging. The complexity of interactions between different brain areas in sensory and cognitive processing and the difficulty of relating synaptic and intrinsic forms of plasticity to complex network functions have limited our ability to bridge cellular and system levels. Brainwide synchronization, as observed during sleep, has provided a good model for the study of interactions between synaptic plasticity and network state. A number of research groups have proposed different theories about how sleep affects brain activity and how it may contribute to the regulation of synaptic transmission. In this issue, the reviews by C. Cirelli and G. Tononi and by M. G. Frank present different viewpoints in this debate. The discussion generated by these reviews highlights the challenges that arise when attempting to connect findings from cellular, circuit, and system level approaches.

This special issue provides an up-to-date snapshot of a fundamental problem in neuroscience. All articles, while stating different opinions, converge in emphasizing the importance of homeostatic plasticity in maintaining stable performance of the nervous system. The richness of experimental and theoretical approaches now available will allow researchers to investigate neuron and network homeostasis in greater depth and detail and break new ground in the integration of findings from different levels of analysis. While the past years have been marked by several important discoveries in this field, there is still a long road ahead for connecting homeostatic mechanisms found at the cellular and network levels with actual maintenance of function at the systems level.

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Review Article

Wherefore Art Thou, Homeo(stasis)? Functional Diversity in Homeostatic Synaptic Plasticity

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Homeostatic plasticity has emerged as a fundamental regulatory principle that strives to maintain neuronal activity within optimal ranges by altering diverse aspects of neuronal function. Adaptation to network activity is often viewed as an essential negative feedback restraint that prevents runaway excitation or inhibition. However, the precise importance of these homeostatic functions is often theoretical rather than empirically derived. Moreover, a remarkable multiplicity of homeostatic adaptations has been observed. To clarify these issues, it may prove useful to ask: why do homeostatic mechanisms exist, what advantages do these adaptive responses confer on a given cell population, and why are there so many seemingly divergent effects? Here, we approach these questions by applying the principles of control theory to homeostatic synaptic plasticity of mammalian neurons and suggest that the varied responses observed may represent distinct functional classes of control mechanisms directed toward disparate physiological goals.

1. To Take Arms against a Sea of Troubles, and by Opposing End Them: Homeostatic Self-Regulation in Neurons

The concept of homeostasis has become a central tenet of physiology in the 80 years since its formal articulation [1]. Homeostatic regulation dynamically maintains the relatively fixed *milieu intérieur* which the French physiologist Claude Bernard defined as "the requirement for a free and independent life" [2]. However, the notion of neuronal homeostasis is a relatively new variation on this theme. In the past two decades, neurons and neuronal networks have been observed to self-regulate their output in a variety of in vitro and in vivo contexts. Despite (or because of) the explosion of research in recent years, homeostatic adaptation of neuronal synapses (known collectively as homeostatic synaptic plasticity or HSP) resists easy packaging into an overarching model, but instead seems splintered into a complex array of different factors and multiple mechanisms [3–5]. Here, we critically survey the literature and attempt to synthesize these varied observations into a more coherent picture by asking what purpose homeostatic adaptations serve. To limit the overwhelming number of questions raised by these issues, we restrict our focus to the best-characterized form of adaptation, the homeostatic responses occurring at excitatory synapses of the mammalian central nervous system (CNS). Other recent reviews have extensively covered other aspects such as intrinsic excitability [4, 6, 7], excitation-inhibition balance [4, 5], and the catalog of various molecules implicated in homeostatic adaptation [3, 8], and we have not attempted to provide a comprehensive review of these topics. To begin, we will apply the conceptual lens of control theory, which may provide a helpful framework in attempting to develop unifying organizational principles. We then attempt to explicate the variability of homeostatic responses as distinct methods of accomplishing multiple biological functions or goals in different cell types and circuits.

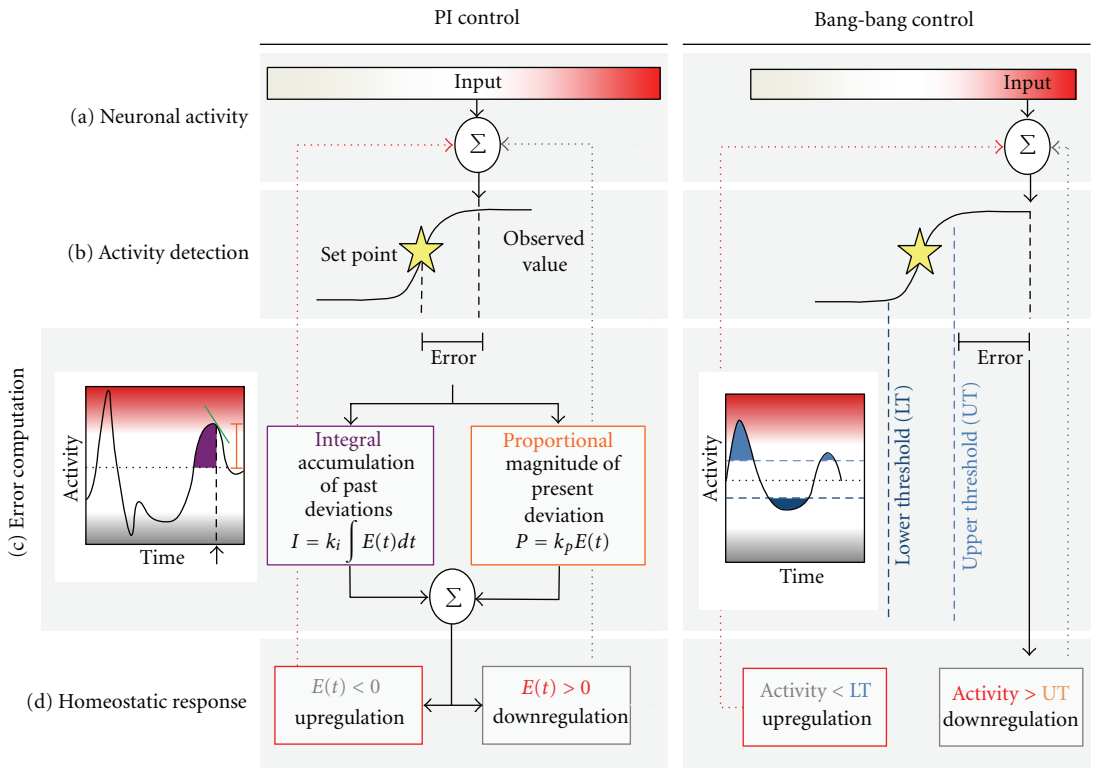

Figure 1: Closed-loop control in homeostatic regulation. In closed-loop control systems, observed activity values (a) are compared to a desired set point (yellow star) (b) and deviations are registered as errors (c). The homeostatic response program is calculated and initiated in response to the error signal (d). Many control strategies are possible, including proportional-integral (PI) control (left) and bang-bang control (right). *PI control*: PI controllers compute a compensatory response as a function of the properties of the error, namely, the proportional (orange, magnitude at $t = 0$ indicated with arrow) and integral (purple, cumulative error over time) components of the deviation. A variation of this regulation, the proportional-integral-derivative (PID) controller, also incorporates a derivative component that detects the rate of change of the deviation (green bar in activity trace, $D = k_D dE(t)/dt$). The initiated response is therefore tailored to the immediate degree of deviation from the set point (proportional), the cumulative magnitude of the deviation (integral), and the rate of change of the deviation (derivative). *Bang-bang control*: Bang-bang control consists of set compensatory responses which are initiated once a threshold is crossed (blue lines) and halted once the activity value returns to the acceptable range of values.

2. HSP: Lessons from Engineering

A critical question that often goes unanswered, or at least is left implicit, is that of the physiological importance of HSP. Theoretical network models suggest that HSP is a key ingredient for optimal information processing and stability. A popular view is that HSP is a requisite negative feedback "yin" to the "yang" of positive feedback-based associative, or Hebbian, plasticity mechanisms such as long-term potentiation (LTP) and long-term depression (LTD) [3, 5, 7, 9]. However, we can envision several scenarios in which different classes of homeostatic regulation may plausibly play biologically important roles, as discussed in the sections below.

In elucidating these functions, we propose to use the context of control theory [10–12]. Although such engineering models have been conceptually applied to homeostatic behavior of physiological systems [13–15] including neural ones [16, 17], in this paper we will systematically examine the literature via the lens of closed-loop control to explicate the abundant body of existing data. In a closed-loop control system, a *sensor* monitors system output and feeds the information back to a *controller* which adjusts one or more control parameters to maintain output at a desired level. This mode of regulation allows the controller to compensate dynamically for changes to the system by "looping back" to modulate control and contrasts with *open-loop* systems that lack such feedback mechanisms. Closed-loop regulation of neuronal activity can be broken down into the following parts: (1) detection of a specific output measure of activity (Figure 1(a)), (2) comparison to a set point representing "optimal" activity (Figure 1(b)), (3) calculation of "error," or the difference between detected and optimal levels, and computation of an appropriate homeostatic response program tailored to the error (Figure 1(c)), and (4) implementation of the compensatory homeostatic response (Figure 1(d)). The mechanisms of homeostatic control will likely depend critically and differentially on the physiological conditions that trigger different types of HSP in specific situations and contexts.

2.1. What Do We Mean by Activity? Publications in the field of homeostatic synaptic plasticity invariably begin with

the statement that neurons, faced with chronic changes in activity, alter their properties to return their output to normal levels. However, it is unclear what exactly is meant by terms such as "activity," "output," and "normal." Homeostatic regulation is predicated on the notion that biological systems have ideal set points for various parameters and that these set points are dynamically maintained to allow for continued function despite constantly fluctuating external environments. This notion seems intuitive for systems that require robust and relatively stable output, such as the neuromuscular junction [16, 18]. However, it is less obvious how homeostatic plasticity may be implemented in complex, highly plastic, and information-encoding environments such as the mammalian CNS.

It is immediately apparent that neuronal "activity" could be defined in a multitude of ways within a single (excitatory) cell, including currents or membrane potential fluctuations in postsynaptic, dendritic, or somatic membranes; calcium flux in dendritic spines, dendrites, or soma; action potential generation (i.e., firing rate) at the axon hillock; vesicular accumulation and release at presynaptic terminals; neurotransmitter concentration within or near the synaptic cleft, just to name a few. Each of these locations may have distinct set points and sensors for determining activity status, governing independent or concerted forms of plasticity that coexist within the neuron and that may be called upon during different functional requirements.

2.2. How Are Errors Detected? Neurons undergo fluctuations in synaptic input and action potential firing on a scale of seconds to minutes that are required for normal neuronal function. However, at what point has an activity regime switched over from "acute" to "chronic" or from "normal" to "excessive"? Alternatively, is there continual HSP adjustment occurring in proportion to activity levels? This measure will clearly depend on the cell type in question and its preferred firing rate/pattern, but are there general mechanisms that detect aberrant neuronal activity?

In man-made process control, several homeostatic strategies are widely applied depending on the particular requirements. Bang-bang control (Figure 1, right) is a relatively simple control strategy used by thermostats to homeostatically regulate temperature: compensatory responses turn on when a threshold is exceeded (or after a certain delay) and turn off when the set point is achieved. Another common approach is proportional-integral control (Figure 1, left), which initiates feedback tailored to the properties of the detected error. The proportional component reflects the current degree of deviation from the ideal, while the integral component senses accumulation of errors over time and exerts greater feedback as the sum of these errors increases. Thus, the compensatory response is a function of both the magnitude and persistence of the deviation from the set point. Computational studies on homeostatic neuronal activity regulation have demonstrated that directly linking an activity measure (somatic Ca^{2+} levels) to the conductances of ion channels confers integral control over the channel without explicit integration [19, 20]. It should be stressed that there is an infinite number of possible control methods and that these examples given are not necessarily the ones used in biology, but may serve to provide valuable conceptual guidance when approaching distinct types of homeostatic regulation.

2.3. What Are the Compensatory Responses? As we have outlined, the set point for neuronal "activity" could potentially consist of multiple parameters including synaptic currents, calcium levels, action potential firing rate, presynaptic vesicle number, or neurotransmitter concentration. Theoretically, these same parameters could be altered to homeostatically adjust neuronal "output," but this does not necessarily have to be the case. In order to regain appropriate activity state, neurons and networks can alter basically all of their components: passive and active membrane properties [6, 7, 21, 22], densities and conductances of ion channel subtypes [23, 24], efficacy and locations of inhibitory and excitatory connections [25–27], modulatory neurotransmitter (dopaminergic/serotonergic/acetylcholinergic) tone, and so forth. Indeed, network simulations suggest that a large number of combinatorial parametric modifications can yield equivalent neuronal firing pattern corrections [28]. These diverse participants in homeostatic adaptation no doubt contribute to the large number of mechanisms being uncovered, which may act coordinately or as multilayered back-up systems in case of failure or overload in primary ones. As we have stated earlier, here we will focus on a small sector of this parametric space, the changes occurring at excitatory synapses.

3. Whys and Wherefores of Excitatory HSP: Functional Classification

Activity at excitatory synapses consists primarily of excitatory postsynaptic currents (EPSCs) mediated by the AMPA subtype of fast glutamate receptors (AMPARs), and in the CNS occurs predominantly at small, motile protrusions called dendritic spines. Characteristics of AMPAR-mediated miniature EPSCs, the postsynaptic responses to release of a single presynaptic vesicle of glutamate, are widely used to infer information about synaptic properties. Increases in mEPSC amplitude are consistent with higher density/conductance of postsynaptic receptors at individual synapses [23]. Elevated mEPSC frequency is usually interpreted as increases in either the presynaptic release probability at existing sites (increases in the vesicular pool or vesicular turnover rate) [29] or in the number of functional synaptic sites (more dendritic spines or new synapses onto already established spines) [30]. Accordingly, decreases in mEPSC amplitude and frequency, observed in various overactivity paradigms, are interpreted as decreases in postsynaptic and presynaptic properties, respectively. However, caution is needed in attributing mEPSC alterations to exclusively pre/postsynaptic changes. For instance, mEPSC frequency and amplitude are not independent; practically speaking, once synapses become very small, current amplitudes from them fall below the threshold of detection and this leads to a decrease in the measured frequency. Additionally, unsilencing of so-called "silent" synapses that previously lacked AMPARs [30] is a postsynaptic effect which manifests as a change in mEPSC frequency.

Furthermore, there is evidence that pre- and postsynaptic development is coordinated [31, 32]. With these caveats in mind, it is still controversial whether changes in mEPSC amplitude or frequency are the predominant HSP response and which AMPAR subunits are the main players subject to regulation (e.g., [23, 24, 33–36]; see Table 1). As we shall see, careful consideration of experimental variables and biological functions may shed light on these and other controversies. In the following sections, we will explore several possible neuronal contexts for HSP, using control theory to dissociate the components involved and delineate the different functional classes (summarized in Table 2). In particular, we will examine various inactivity paradigms with respect to three variables: scope, synaptic locus, and degree. The scope of the inactivity may be either network-wide (Section 4), cell autonomous (Section 5), or synapse specific (Section 6). Within each section, we will examine divergent findings by grouping inactivity paradigms by synaptic locus (pre- or postsynaptic) and degree (reduced or abolished activity). The focus on inactivity regimes reflects the preponderance of studies using these experimental paradigms, although in some cases we will delve into the consequences of overactivity. When appropriate, we will distinguish between developing and established networks, as the developmental state of the network has consequences for functional regulation.

4. Network-Wide Inactivity

The most commonly used inactivity paradigms induce network-wide changes in activity state via bath application of drugs. In the following sections, we group the network-wide inactivity paradigms into functional classes (see Table 2) according to the locus (pre- or postsynaptic) and severity (reduced or abolished) of the inactivity

4.1. Reduced Presynaptic Input: Scaling and Synaptic Calibration. A seminal observation in the field was that simply seeding cultured hippocampal neurons at different densities caused reciprocal regulation of synaptic strength, with higher densities yielding weaker synapses and lower densities resulting in stronger synapses [59]. A similar result is found when cells are plated onto larger surface areas, even when the cell density remains constant: large networks have more heavily interconnected neurons with globally weaker excitatory connections and stronger inhibitory connections; conversely, smaller networks have less synaptic innervation but proportionally stronger constituent excitatory connections and weaker inhibitory ones [26]. The network size and the degree of innervation therefore control the range of synaptic strengths that are considered to be acceptable in the first place, presumably functioning as a guard to maintain stability during development of networks.

A global form of synaptic adaptation can also be induced pharmacologically, via application of the voltage-gated sodium channel blocker, tetrodotoxin (TTX), which prevents the firing of action potentials (APs) and thus greatly reduces the frequency of presynaptic vesicular release. Much of the early work on describing the effects of HSP on excitatory synapses was performed in young neurons, during the period of robust synaptogenesis, which occurs in vivo at 2 weeks postnatal [60] and at day in vitro (DIV) 10–14 [61]. In dissociated primary cultures of young cortical [38, 40, 41] or hippocampal [41, 44] neurons, TTX has been observed to cause a global increase in AMPAR-mEPSC amplitudes but no change in frequency, suggesting increased postsynaptic strength but not synapse number or release kinetics. Such a phenomenon has led to the prevailing notion of "synaptic scaling" [38], a neuron-wide, multiplicative change in synaptic strength at all synapses. The finding that networks calibrate the strength of their synaptic connections raises the possibility that TTX blockade during development, by reducing the frequency of synaptic inputs, "tricks" neurons into believing they are part of a less dense neuronal network. The resulting scaling in synaptic strength could therefore be considered part of a developmental synaptic calibration machinery.

How does the neuron sense its total endowment of synaptic innervation? In the case of global homeostasis, one possible activity sensor is postsynaptic firing rate, as compensatory neuron-wide HSP can be initiated by local application of TTX to neuronal cell bodies but not to portions of the dendritic tree [40]. However, sustained postsynaptic depolarization is sufficient to induce downregulation of synaptic strength independent of action potential firing [62]. This conclusion has been supported by a recent study demonstrating that chronic optogenetic overactivation of individual CA1 neurons in hippocampal organotypic slices induces cell autonomous homeostatic downregulation of postsynaptic strength [63]. This leads to the question of what is actually being measured and how this translates into an index of over- or underactivity. Somatic calcium levels appear to be an important activity sensor in this process [8, 40, 63], and L-type voltage-gated Ca^{2+} channels have been implicated as the mode of calcium entry [24, 39, 46, 63]. Downstream calcium-dependent second messengers such as calmodulin [64] or various enzymes (e.g., adenylyl cyclase [39]) could represent biochemical readouts of these calcium transients. Interesting examples of the latter category are α- and β-CaMKII, prominent Ca^{2+}/calmodulin-dependent postsynaptic kinases that are reciprocally downregulated and upregulated, respectively, during prolonged inactivity [51], and are associated with L-type voltage-gated Ca^{2+} channels [65].

The related family member CaMKIV also appears to be an important potential sensor, as its function is required for homeostatic downregulation in response to optogenetic hyperstimulation [63], while decreased nuclear CaMKIV activation mimics and occludes adaptation to neuronal inactivity [40]. Because gene transcription [40, 44] or protein translation [35, 49, 50, 66] is required for some forms of HSP, a potential integrative mechanism for registering and integrating errors in activity state could be based on the accumulation of activity-dependent mRNAs or proteins. Such a system may involve activity-inducible inhibitory factors such as the immediate early gene Arc [52, 67], inactivity-induced stimulatory factors, or both for optimum bidirectionality.

Table 1: Homeostatic synaptic adaptations to chronic inactivity. An overview of select references which have investigated the neuronal response to chronic inactivity via functional analyses of AMPA receptor-mediated excitatory synaptic transmission. References are arranged by cell type (column 1) and inactivity paradigm (column 2). Within each paradigm, studies are listed in ascending age order (column 3). ↑,↓ = significant change in mEPSC amplitude or frequency. — = no change in parameter. N/A = parameter was not reported. *mEPSC frequency was not directly measured.

Cell type	Inactivity paradigm	Days in vitro (DIV) or postnatal day (P)	Amp.	Freq.	Reference
		In vitro (dissociated culture)			
Spinal cord	CNQX + APV	DIV 10	↑	—	[23]
Cortex (Ctx)	CNQX + APV	DIV 21	↑	↑	[37]
Ctx	APV	DIV 7–9	—	—	[38]
Ctx	CNQX	DIV 7–9	↑	—	[38]
		DIV 14–17	↑	↑	[39]
Ctx	TTX	DIV 7–9	↑	—	[38]
		DIV 7–10	↑	—	[40]
		DIV <10	↑	—	[41]
		DIV 11–13	↑	—	[42]
		DIV 14	↑	—	[43]
		DIV >18	↑	↑	[41]
Hippocampus (Hpc)	TTX	DIV 7	↑	—	[44]
		DIV 10	↑	—	[41]
		DIV 14	↑	↑	[45]
		DIV 14	↑	↑	[44]
		DIV 14	↑	—	[35]
		DIV 14	↑	—	[46]
		DIV 14	↑	N/A	[47]
		DIV 18	↑	↑	[41]
		DIV 21-22	↑	—	[48]
		DIV 21	N/A	"↑" *	[29]
		DIV 27–40	↑	—	[49]
Hpc	TTX + APV	DIV 14	↑	—	[35]
		DIV 14-15	↑	—	[50], [46]
Hpc	TTX + CNQX	DIV 14	↑	—	[46]
Hpc	TTX + NBQX	DIV 27–40	↑	—	[49]
Hpc	NBQX	DIV 14–16	↑	↑	[36]
		DIV 17	↑	↑	[51]
		DIV 17	↑	↑	[24]
		DIV 21	N/A	"↑" *	[29]
		DIV 27–40	↑	↑	[49]
Hpc	CNQX	DIV 14	↑	—	[46]
		DIV 21	↑	↑	[46]
		DIV 21–38	↑	↑	[49]
Hpc	Kir2.1 expression	DIV 14-15	—	↑	[45]
		DIV 15–24	↑	N/A	[52]

Table 1: Continued.

Cell type	Inactivity paradigm	Days in vitro (DIV) or postnatal day (P)	Amp.	Freq.	Reference
		In vitro (organotypic slice, all from P6-8 cultures)			
Hpc	TTX	DIV 8 (CA3)	↑	↑	[53]
		DIV15 (CA3)	↑	↑	[53]
		DIV 21–25 (MF-CA3)	—	↑	[54]
		DIV 21–25 (CA3-CA3)	—	↓	[54]
		DIV 21–25 (CA3-CA1)	—	—	[54]
Hpc	TTX + APV	DIV 5–7 (CA1)	↑	—	[55]
		DIV 6–8 (CA1)	↑	—	[50]
		Ex vivo (acute slice)			
Hpc	TTX ex vivo incubation	P4 (CA3)	↑	↑	[53]
		P8 (CA3)	—	—	[53]
		P21–28 (CA1)	—	—	[35]
Hpc	TTX in vivo implantation	P15 (CA1)	↑	↑	[56]
		P30 (CA1)	—	↑	[56]
Hpc	TTX + APV ex vivo	P21–28 (CA1)	↑	—	[35]
Visual cortex	Intraocular TTX	P21	↑	—	[27]
	Monocular deprivation	P21	↓	↓	[27]
	Binocular deprivation	P23	↑	—	[57, 58]

For example, polo-like kinase Plk2 transcription is tightly regulated by neuronal activity and, upon induction, downregulates excitatory synapses and dendritic spines [68–72]. Thus, the amount or balance of these factors could establish the length of time and/or extent of deviation from the desired set point.

4.2. Reduced versus Abolished Postsynaptic Activity: Global versus Local HSP. Various activity sensors and homeostatic mechanisms can be pharmacologically dissected using antagonists of specific ion channels. TTX initiates slow compensatory responses in AMPAR mEPSC amplitude on the scale of 12–48 hrs in developing hippocampal neurons (e.g., [38, 45]; see Table 1). The time course of adaptation can be rapidly accelerated to 4 hours or less by blockade of glutamatergic synaptic transmission with antagonists of AMPARs [49] or concurrent application of TTX with NMDAR antagonist APV [35, 49]. Interestingly, NMDAR blockade alone did not appear to induce a homeostatic AMPAR response in at longer time points in developing cortical neurons [38, 39, 46].

Not only the timecourse but the compensatory response varies between inactivity paradigms. AMPAR blockade alone induces an increase in both mEPSC frequency and amplitude (e.g., [24, 46, 49]; see Table 1 for others), suggesting concerted pre- and postsynaptic adaptations to inactivity. TTX by itself generally induces an increase only in mEPSC amplitude (e.g., [38, 46, 49]; see Table 1 for others), suggesting a predominantly postsynaptic response. Furthermore, treatment of mature hippocampal neurons with TTX together with the selective AMPAR blocker NBQX has been found to be actually *subtractive* [46, 49]. TTX appeared to block the NBQX-induced changes in frequency [46, 49], supporting the notion that the coordination of presynaptic function with postsynaptic status requires ongoing AP firing [31, 73, 74], possibly due to the state-dependent interaction of presynaptic terminals with inactivity-released dendritic BDNF [49].

A drawback to the use of bath application of drugs is that these manipulations are not particularly "clean," in that TTX and NBQX will both reduce synaptic input and action potential firing either directly or indirectly. Nevertheless, the combined pharmacological manipulations reveal that the inactivity induced with TTX is not equal to the inactivity induced with glutamatergic receptor blockade, suggesting that somatic and synaptic activity may be differentially regulated. Indeed local TTX blockade of somatic activity is capable of inducing neuron-wide scaling [40], while local TTX blockade of dendritic activity does not induce upregulation. To our knowledge, neuron-wide scaling in response to the over- or underactivity of a subpopulation of synapses (as might result from input-specific Hebbian modifications) has not been reported.

What is the biological significance of these two mechanisms? Decreased AP firing (due to TTX treatment) can be interpreted by a receptive neuron as a deficiency of postsynaptic function, thus resulting in a slow upregulation of AMPAR synaptic content. The silencing of AMPAR transmission (due to NBQX treatment) could therefore represent the most extreme end of this postsynaptic deficit spectrum. The magnitude of input "error" resulting from complete AMPAR blockade would be considerably larger than that from TTX

Table 2: Inactivity paradigms: consequences and responses. Inactivity paradigms are grouped by scope: network-wide, cell autonomous, or synapse specific. Each inactivity paradigm is evaluated based on its type: presynaptic (Pre) or postsynaptic (Post) mode of action, and reduction (↓) or elimination (X) of activity.

Paradigm type			Synaptic/cellular consequences	Perceived situation	Cell autonomous response
Network-wide inactivity					
TTX	Pre	↓	*Developing network*: fewer presynaptic inputs; no emergence of AP firing to constrain synapses	Participation in a sparsely connected network	Calibration of synaptic strength to higher level [26, 38, 59] via constitutive insertion of somatically synthesized GluA1/2 AMPARs [34]
			Established network: Sudden decrease in output with concurrent decrease in presynaptic inputs	Change in network activity state	Compensation via insertion of somatically synthesized GluA1/2 AMPARs [34] with possible coordination of presynaptic properties (↑ release probability or # synaptic vesicles) or potential ↑ # synaptic sites
APV	Post	↓	Diminished Ca^{2+} influx at synapses	Disrupted synaptic Ca^{2+} homeostasis	Minimal effect at AMPARs [38]
TTX+ APV	Post	↓↓	Sudden decrease in output with concurrent decrease in presynaptic inputs, and diminished synaptic Ca^{2+}	Change in network activity state, disrupted synaptic Ca^{2+} homeostasis	Homeostatic compensation via rapid insertion of locally synthesized Ca^{2+} permeable homomeric GluA1 AMPARs [35]
NBQX	Post	X	Sudden decrease in postsynaptic efficacy at an otherwise functional synapse	Disrupted synaptic function and synaptic Ca^{2+} homeostasis	Homeostatic compensation via increase in presynaptic release probability and rapid insertion of locally synthesized Ca^{2+} permeable homomeric GluA1 AMPARs [24, 51]
Cell-autonomous inactivity					
Kir2.1	Post	↓	*Developing network*: less action potential firing than neighbors; less activity-dependent strengthening of synaptic connections	Participation in an "irrelevant" circuit	Inability to compete for synaptic connections in an activity-dependent fashion; lower levels of AMPAR input; lower frequency of inputs (note: this "competition" effect is reversed by global TTX which equalizes activity across the network [45])
			Established network: gradual decrease in output without decrease in presynaptic inputs	Decreased postsynaptic efficacy	Homeostatic compensation via increase in presynaptic release probability [45]
Synapse-specific inactivity					
Kir2.1	Pre	↓	Diminished presynaptic input in a normally functioning network	Decreased presynaptic efficacy	Homeostatic compensation via insertion of GluA1 AMPARs [47]
TeTx	Pre	X	Absent presynaptic input in a normally functioning network	Nonfunctional presynaptic terminal	Lack of activity-induced maintenance of GluR1 via diffusional trapping [75]; loss of GluR1 but not GluR2/3 or synaptic proteins [76]

Inactivity paradigms: AP blockade (TTX); NMDAR blockade (APV); AMPAR blockade (NBQX); hyperpolarization (via transfection of Kir2.1 potassium channel); presynaptic release inhibition (via transfection of tetanus toxin, TeTx).

blockade, leading to a correspondingly faster rate of the response. The existence of a minimum postsynaptic activity threshold (e.g., calcium) could explain why APV and TTX together are able to induce rapid responses, while neither do so alone.

However, it seems that the two responses to TTX- and NBQX-induced inactivity have different underlying compensatory mechanisms and likely achieve separate physiological goals. The rapid HSP induced by glutamatergic receptor blockade appears to specifically involve enhanced GluA1 synthesis and synaptic incorporation of Ca^{2+}-permeable GluA2-*lacking* AMPARs [24, 33, 35, 36], whereas the slow HSP induced by TTX generally increases both GluA1 and GluA2 subunits [23, 34, 35] and in fact selectively requires the GluA2 C-terminal tail [77]. Since the homeostatic responses differ in these two activity paradigms, it is possible that distinct mechanisms are recruited in the fast and slow forms of HSP. We note that complete cessation of AMPAR- or NMDAR-mediated transmission is not a physiological response under normal circumstances. Perhaps such inactivity occurs when existing synapses become damaged, defective, or otherwise nonfunctional, and the rapid response to these manipulations could therefore represent emergency synaptic "repair" mechanisms. A bang-bang control strategy would

be ideal for implementing such pathways. Glutamatergic receptor blockade has been shown to induce dendritic translation of retinoic acid [46, 50] and the multifunctional neurotrophin BDNF [49], both of which have been shown to play roles in HSP. These dendritically synthesized proteins potentially function in a form of bang-bang control of local synaptic strength, in which dendritic protein synthesis is turned on once local Ca^{2+} levels have dropped below a certain threshold and is turned off once newly inserted Ca^{2+} permeable AMPARs allow for sufficient Ca^{2+}-influx. In contrast, somatic Ca2+ levels may be monitored continually on slower timescales by a somatically deployed PI control mechanism.

4.3. Abolished Presynaptic Activity? Existing global inactivity paradigms reduce or block postsynaptic activity (Section 4.2), and reduce presynaptic activity (Section 4.1). Global cessation of presynaptic input has not been reported, but could potentially be achieved by infecting cultured neurons at sufficiently high titer of viruses expressing tetanus toxin to inactivate all presynaptic vesicular release in the culture. This manipulation might be useful to dissect the effects of presynaptic activity versus presynaptic neurotrophic support.

5. Cell Autonomous Inactivity: Synaptic Competition versus HSP

In contrast to the global amplitude effects observed in developing networks treated with TTX, a different outcome is observed when the excitability of a single neuron is reduced by transfection of hyperpolarizing potassium channel Kir2.1 [45]. Expression of the channel in cultured hippocampal neurons prior to extensive synaptogenesis did not induce homeostatic upregulation, instead causing a reduction in the number of functional excitatory synapses onto the transfected cell and smaller presynaptic boutons, with no change in mEPSC amplitude. This non-homeostatic effect appeared to be due to developmental competition among neurons for inputs, as this imbalance in synapse formation was eliminated if all cells were inhibited with TTX. Interestingly, expression of the Kir2.1 channel *after* the bulk of synapse formation initiated a homeostatic upregulation of presynaptic function (increased AMPAR-mEPSC frequency due to a larger vesicle pool and presynaptic release probability), with no change in synapse number or mEPSC amplitude. The presynaptic homeostatic adjustment appears to fully compensate for the initial reduction in postsynaptic activity, as the firing rate of Kir2.1-transfected cells eventually returns to control values. In this scenario, the functional deficit induced by Kir2.1 can be viewed as decreased postsynaptic efficacy with normal presynaptic function. Why then does the inhibited neuron not initiate a global synaptic scaling of AMPAR-mEPSC amplitudes, as observed with TTX? It is possible that the effect of Kir2.1 is less severe than TTX and does not reduce somatic calcium sufficiently to induce a scaling response. Another possibility is that the severe decrease in presynaptic release due to TTX treatment results in compensatory boosting of the properly functioning postsynaptic side, whereas the postsynaptic impairment from Kir2.1 hyperpolarization is combated via compensatory upregulation of the unperturbed presynaptic apparatus.

6. Synapse-Specific Inactivity

6.1. Reduced Presynaptic Input: Synapse-Specific HSP. A prediction from synaptic scaling is that activity changes at any given synapse do not initiate global homeostatic compensation, as the neuron is somatically monitoring the sum of all synaptic activity and coordinating any necessary homeostatic adaptation among all ~10,000 synapses of a typical neuron. This prediction is borne out by several studies that show that local synaptic inactivation does not cause global scaling [40, 75, 76]. However, modulation of single synapses does yield input-specific effects. The activity of individual presynaptic terminals can be decreased due to presynaptic neuronal hyperpolarization via sparse transfection with the rectifying potassium channel Kir2.1 [47, 52]. In young hippocampal neurons, the rare postsynaptic targets of the selectively depressed presynaptic neuron's terminals homeostatically upregulated their AMPAR content and strength, though neighboring synapses did not, in a process involving GluA2-lacking receptors and Arc [47, 52].

What might be the functional importance of this synapse-specific homeostatic control? We suggest that scaling and synapse-specific HSP are dual mechanisms that operate in tandem in developing neurons to establish proper network and synaptic functionality. During synapse formation, one may imagine that it would be useful to employ a program of synaptic quality control during the construction of an appropriately functioning synaptic tree. Scaling may be responsible for globally establishing and maintaining an appropriate set point (or rather a set range) for synaptic strengths, based on the total innervation pattern and firing rate of the cell. Meanwhile, synapse-specific HSP may represent the means of adjusting individual synaptic strengths to values within the globally established range that are most appropriate based on the activity of the corresponding pre/postsynaptic terminal and on that of neighboring synapses. The AMPAR content of excitatory synapses appears to consist of both "stable" and "labile" populations [78]. The labile population may be a more dynamic, heterogeneous set of receptors that can be mobilized by Hebbian or synapse-specific homeostatic plasticity, whereas the size of the core stable AMPAR population may be established during development in a relatively standardized way throughout the dendritic tree.

6.2. Abolished Presynaptic Input. Interestingly, completely abolishing presynaptic vesicular release does not merely exaggerate the response seen with diminished presynaptic release. Instead, seemingly opposite effects are observed if presynaptic vesicular release is abolished (using tetanus toxin) rather than diminished (using presynaptic Kir2.1). If a similar presynaptic manipulation is performed as in Section 6.1, using instead tetanus toxin to completely inactivate presynaptic terminals, no change in AMPAR-mediated currents is observed [79], only a specific reduction in GluA1 (and not GluA2/3) AMPAR subunits [76], likely

involving increased diffusional exchange of this AMPAR subunit [75]. As in the postsynaptic scenario discussed in Section 5, input blockade may not represent merely a more extreme portion of the signaling spectrum. The absence of any activity emanating from the presynaptic terminal may be a qualitatively different activity signal than a simply a decrease in presynaptic release. Indeed, abolished (not diminished) presynaptic activity may indicate a nonfunctional presynaptic terminal, in which case postsynaptic homeostatic compensation would be futile. The loss of GluA1 in this context would therefore not represent a homeostatic response, but a lack of activity-dependent GluA1 trapping [75]. It is conceivable that in this situation mechanisms are initiated to upregulate or "repair" presynaptic activity but are obscured by the inability of the system to overcome the inhibition of the tetanus toxin.

6.3. Reduced Postsynaptic Responsiveness. Although local dendritic application of TTX alone did not cause homeostatic responses [40], dendritic application of TTX with the NMDAR antagonist APV induced robust upregulation of surface AMPAR levels in the deprived area [35]. Taken together, these findings suggest that glutamate receptor activity serves as a local signal regulating the strength of individual synapses in an autonomous fashion.

Analogous to the role of somatic calcium in global responses, calcium entry into spines is also likely to play an important role in local synapse-specific regulation. Indeed, the response to AMPA receptor blockade has frequently been detected as the selective insertion of GluR2-lacking AMPA receptors which are Ca^{2+} permeable [24, 33, 35, 36]. These findings suggest that the local synapse-specific responses may be an attempt to restore local Ca^{2+} levels. Local synaptic activity has been heavily implicated in the regulation of dendritic protein synthesis [35, 80]. In fact, miniature synaptic currents have been shown to negatively constrain dendritic protein synthesis, making it possible that the default state of the neuron is to produce proteins for synaptic integration. Postsynaptic activity (in the presence of a functioning presynaptic terminal) may therefore negatively constrain a default program of local homeostatic "upregulation."

6.4. Other Activity Paradigms. While global hyperactivity paradigms have been shown to induce global decreases in mEPSC amplitude and/or frequency [23, 38, 51, 70], to date, no experiments have examined the effect of synapse-specific overactivation. Chronically increasing presynaptic activity at a single synapse could be accomplished with sustained optogenetic activation of a channelrhodopsin-expressing presynaptic neuron. Homeostatic adaptation to increased activity of a single postsynaptic site has also not yet been reported but may be possible with chronic local uncaging of glutamatergic agonists.

7. Nonuniform HSP of Mature Neurons

An appealing theoretical aspect of global multiplicative synaptic scaling is the preservation of the pattern of relative differences in synaptic weights established by Hebbian forms of synaptic plasticity that is postulated to encode information [9]. However, while uniform synaptic scaling has been reproducibly observed in young neurons under appropriate conditions, older neurons (here defined as those beyond the period of bulk synaptogenesis, for example, >DIV21 or in the adult animal) from a variety of preparations do not show scaling, even with global activity manipulations [27, 53, 56, 57]. The occurrence of multiplicative scaling only during the period of peak synaptogenesis (and not in older neurons) suggests that this mechanism may actually be more relevant to synapse formation rather than information processing per se.

Instead, TTX applied to older neurons elicits nonmultiplicative increases in mEPSC amplitudes [56], as well as elevated mEPSC frequency (e.g., [41, 44, 45, 53, 56]; see Table 1 for others). A perplexing question that then arises is that if synapse strength is affected in a nonuniform way, how can homeostatic adjustments coexist with Hebbian information encoding? One proposal for allowing the coexistence of Hebbian and homeostatic mechanisms is if the former is implemented by dynamically moving the set point of the latter [8, 17], in much the same way that a thermostat can be turned up or down, but still remains under feedback control. However, this mechanism does not explain the nonmultiplicative HSP in older neurons. The basis of this HSP in mature neurons remains unknown, but by definition a nonmultiplicative process implies that certain synapses are affected differentially, and in mature neurons HSP has indeed been shown to influence larger synapses disproportionately [24]. The implication of these results is that, in older neurons, some synapses retain higher capacity to generate strong homeostatic responses, while others may become relatively insensitive to chronic changes in activity. We note that the latter population would be ideally suited to durable and persistent information encoding. We speculate that this hypothetical division of plasticity labor would nicely allow homeostatic adjustment without interference with Hebbian plasticity, but such a mechanism remains to be identified and described.

Consistent with the notion that older neurons have populations of synapses that may be resistant to homeostatic adjustment, blocking presynaptic neurotransmitter release at single synapses with tetanus toxin transfection in mature hippocampal neurons did not cause changes in AMPAR-mediated currents at contacting postsynaptic sites but did cause changes in NMDAR subunit composition in an interesting form of metaplasticity or the "plasticity of plasticity" [79]. In older neurons, metaplasticity may provide an attractive alternative (or additional) strategy for restraining the capacity of Hebbian plasticity without interfering with synaptic weighting [7]. Alternatively, changes in presynaptic release probability may allow for homeostatic adjustments without altering postsynaptic information encoding. Indeed, in the intact adult hippocampus, CA1 synapses do not show mEPSC amplitude changes in response to TTX but only increased frequency [56].

In vivo, network stability may also arise as a consequence of the specific arrangement of connectivity and not merely the individual synaptic strengths. For instance, chronic inactivity in mature organotypic hippocampal slices induced upregulation of synaptic efficacy in a manner which reflected

the underlying computations of the network. Within the hippocampal trisynaptic circuit, CA3 "throughput" synapses were upregulated in response to inactivity, while recurrent synapses were downregulated [54]. It is therefore possible that, in functional circuits, certain synaptic interfaces are a designated homeostatic locus. Similar synapse-specific adaptations have been detected in the visual system, and interestingly the locus of the homeostatic adaptation appeared to change with development. Visual deprivation induced selective homeostatic adaptation in layer II/III neurons in adult visual cortex, while inducing selective layer IV adaptation in developing neurons [57]. These results suggest not only that multiple HSP mechanisms exist in vivo [27] but also that specific cell types may differentially mediate HSP and that the computations of the network at different developmental time points can alter the locus of homeostatic adaptation.

8. Culture Clash: Experimental Preparations

Unlike LTP of hippocampal CA1 synapses, the most well-studied form of plasticity, no standard preparation exists for studies of HSP, leading to experimental variability as noted previously [5]. The problem is particularly acute for cultured cortical or hippocampal neurons, popular but notoriously variable systems for the in vitro study of HSP. Technical aspects of the culture procedures (media preparation, growth substrate, time of culture, age of animals used, culture density or size, etc.) can all influence basal culture properties including synaptic connectivity and strength [26]. The same treatment or combination of treatments can produce different effects in different labs even in what appears to be the same preparation (Table 1).

It should therefore be pointed out that dissociated cultures are not homogenous pools of interchangeable neurons, but are instead highly heterogeneous populations consisting of multiple neuronal types (pyramidal neurons, interneuron subtypes, granule cells, etc.) which vary in proportion depending on the preparation. Rarely do studies attempt to distinguish which cell types are analyzed. Even the balance of glial cells versus neurons can affect synaptic properties and HSP responses [40], since astrocyte- and glial-derived factors regulate scaling of synaptic activity [81, 82]. We therefore emphasize the importance of such variables with the idea that these differences are not simply technical inconveniences but are actually meaningful and can inform our ideas about the functions being supplied under particular circumstances.

9. Conclusions and Perspectives

A great deal of progress has been made in identifying HSP mechanisms and the molecules involved. However, a more careful consideration of the experimental variables of network size, age, and cell type is necessary to clearly parse out the rich and fascinating diversity of homeostatic neuronal adaptations. In developing neurons, the primary goal may be to generate synapses and networks with fidelity and stability, involving neuron-wide regulation of synaptic strength and number. In mature neurons, HSP may be restricted to certain subsets of synapses or cells in an effort to more efficiently respect information encoded in synaptic weights. Thus, framing HSP in biological functions will help understand what goals are sought and hence what underlying mechanisms need to be recruited. Instead of referring by HSP as a monolithic entity, several independent subclasses will likely need to be recognized that operate in different ways. But HSP, by any other name, would be as exciting and interesting an avenue for continued research in the years to come.

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Review Article

AMPA Receptor Trafficking in Homeostatic Synaptic Plasticity: Functional Molecules and Signaling Cascades

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Homeostatic synaptic plasticity is a negative-feedback response employed to compensate for functional disturbances in the nervous system. Typically, synaptic activity is strengthened when neuronal firing is chronically suppressed or weakened when neuronal activity is chronically elevated. At both the whole cell and entire network levels, activity manipulation leads to a global up- or downscaling of the transmission efficacy of all synapses. However, the homeostatic response can also be induced locally at subcellular regions or individual synapses. Homeostatic synaptic scaling is expressed mainly via the regulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) trafficking and synaptic expression. Here we review the recently identified functional molecules and signaling pathways that are involved in homeostatic plasticity, especially the homeostatic regulation of AMPAR localization at excitatory synapses.

1. Introduction

The brain has the amazing ability to adapt through its capability to change in response to experience and use. This fundamental property of plasticity serves to learn and remember complex tasks, obtain rewards, or even recover after injury. Surprisingly, with constant dynamic changes occurring in the brain, neuronal activity remains stable over an entire lifespan. Our brains appear to be constructed in such a manner that the mechanisms involved in learning and memory can be balanced by another distinct form of neuronal modulation, homeostatic plasticity. These two forms of plasticity coexist to adapt to the changing sensory world while maintaining a balance of neural activity within a physiological range.

Hebbian synaptic plasticity is associative and input specific, which strengthens or weakens the transmission efficacy of individual synapses. Long-term potentiation (LTP) and depression (LTD), the two best studied forms of Hebbian plasticity, are widely considered to be the cellular mechanisms for learning and memory. However, given the positive-feedback nature of Hebbian plasticity, this form of synaptic modulation could potentially result in synapses of either functional saturation or silence, driving the whole network into an unstable state if left unchecked. Hebbian synaptic plasticity therefore necessitates distinct homeostatic mechanisms that can stabilize a network in the face of constant dynamic changes in synaptic strength. Indeed, neuronal networks use an array of homeostatic negative-feedback mechanisms that allow neurons to assess their activity and adjust accordingly so as to restrain their activity within a physiological range [1–3].

2. Expression of Homeostatic Regulation via Scaling Synaptic Strength and AMPAR Abundance

At the neuronal level, homeostatic plasticity aims to maintain a stable firing rate of action potentials. This can be achieved through adjustments in the strength of synaptic inputs, neuronal excitability, neuronal connectivity, or the balance between excitation and inhibition. Among these possibilities, regulation of synaptic strength has been the most extensively studied and is believed to be the most crucial measure in homeostatic regulation. This form of regulation, known as homeostatic synaptic plasticity or synaptic scaling [4, 5], is expressed mainly by an alteration in AMPAR synaptic

accumulation [1, 6–9]. During homeostatic regulation, AMPAR numbers at the postsynaptic surface are accordingly scaled up- or downwardly in response to activity deprivation or overexcitation, respectively, presumably via changing AMPAR trafficking processes including receptor insertion and internalization (Figures 1 and 2).

In central neurons, the most studied model of homeostatic synaptic plasticity is activity deprivation by a sodium channel blocker, tetrodotoxin (TTX). When cultured cortical neurons are incubated with TTX to chronically abolish action potentials and thus silence network activity, the synapse responds in a compensatory manner, resulting in an increase in the strength of synaptic transmission [4, 10, 11]. By measuring AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs), it has been shown that chronic suppression of network activity results in an upscaling of synaptic activity [4, 12, 13]. Conversely, chronic network hyperactivation, commonly induced by bath application of bicuculline, an antagonist of the inhibitory gamma-aminobutyric acid A ($GABA_A$) receptors, results in a homeostatic downscaling in mEPSCs [4].

3. Global and Local Synaptic Homeostatic Plasticity

Homeostatic plasticity has traditionally been considered a response that globally affects the entire synapse population of a neural network or a whole neuron, in which AMPAR accumulation at every synapse is up- or downwardly scaled according to activity manipulation [6, 14]. In practice, global homeostatic regulation is usually induced by bath application of reagents that affect the activity of the whole network, in which mEPSC amplitude and AMPAR synaptic amounts at each cell in the network are affected in a compensatory manner [4]. In addition to a network scale, global homeostatic regulation has also been studied at a level of individual neurons. By overexpression of inward-rectifier potassium channels, Kir 2.1, Burrone et al. show that single neuron inhibition results in a homeostatic upregulation in mEPSCs [15]. Ibata et al. show that inhibition of single neuron activity by local perfusion of TTX at the somatic area induces homeostatic regulation in mEPSC and AMPAR synaptic expression [16]. In a recent study, individual neurons expressing light-sensitive channels are selectively activated for 24 hrs [17]. Using this paradigm, Goold and Nicoll demonstrate that chronic activation of single neurons lead to a homeostatic reduction in AMPAR-mediated synaptic transmission [17].

In contrast to global regulation, recent studies indicate that homeostatic synaptic responses could also occur locally at subcellular regions [8, 14, 17, 18]. In cultured hippocampal neurons, synapses in a small region of dendrites are suppressed by microperfusion of TTX together with the N-methyl-D-aspartate (NMDA) receptor antagonist, APV. Using this method, Sutton et al. demonstrate that rapid synaptic scaling of AMPARs is induced locally at the silenced region [19]. However, in another study, local application of TTX on dendrites fails to alter synaptic AMPAR expression [16]. This discrepancy may be due to the difference between activity manipulation paradigms (with or without NMDA receptor inhibition).

Although findings indicate that the traditionally considered global homeostatic plasticity can be induced at local dendritic areas, whether it occurs at the level of individual synapses, an important question regarding synapse functional stability, has not been studied until recently. Our work shows for the first time that the homeostatic response is indeed employed locally at the single synapse level [18, 20]. In cultured hippocampal neurons, overexpression of Kir2.1 inward-rectifier potassium channels to selectively inhibit the activity of individual presynaptic terminals results in a homeostatic increase in corresponding postsynaptic AMPAR expression [20]. To study the opposite paradigm, Hou et al. selectively activate single synapses by employing a light-gated glutamate receptor [18, 21]. We demonstrate that the level of AMPARs at excited synapses is selectively downregulated via receptor internalization and proteasomal degradation [18]. These findings suggest the existence and autonomous execution of homeostatic mechanisms at individual synapses. Although AMPAR trafficking is likely shared by global and single synaptic homeostatic responses, it remains unclear whether similar or distinct signaling cascades and molecular components are adopted in global versus single-synaptic homeostatic regulation.

4. Signaling Molecules and Pathways Regulating AMPAR Trafficking in Homeostatic Plasticity

Recent studies have shown that different functional molecules and signaling cascades are involved in the expression of homeostatic up- or downregulation of synaptic activity and AMPAR expression. For instance, TNFα, the PI3K-Akt pathway, integrin, GluA2-lacking AMPARs (Cp-AMPARs), and retinoic acid have been implicated in inactivity-induced homeostatic upscaling, whereas PICK1, a postsynaptic scaffolding protein, is involved in overexcitation-induced downscaling. Other molecules, such as CaMKs, Arc/Arg3.1, and certain cell adhesion molecules, have been implicated in both directions of homeostatic regulation. Given that AMPARs are the substrate for the expression of homeostatic plasticity, it is not surprising that most of these molecules and cascades are known to play an important role in AMPAR trafficking and synaptic accumulation. This paper summarizes recent findings on the signaling molecules and cascades that regulate AMPAR trafficking in homeostatic plasticity (Figure 3).

4.1. TNFα. Tumor necrosis factor-alpha (TNFα) is an inflammatory cytokine that is involved in inflammation, immune activation, cell death, and degradation [22, 23]. In addition to its important functions in immune responses, TNFα is also important in maintaining the neural network stability [24, 25]. TNFα has been found to mediate the global homeostatic upscaling of mEPSCs and postsynaptic AMPARs induced by prolonged TTX treatment [24]. When

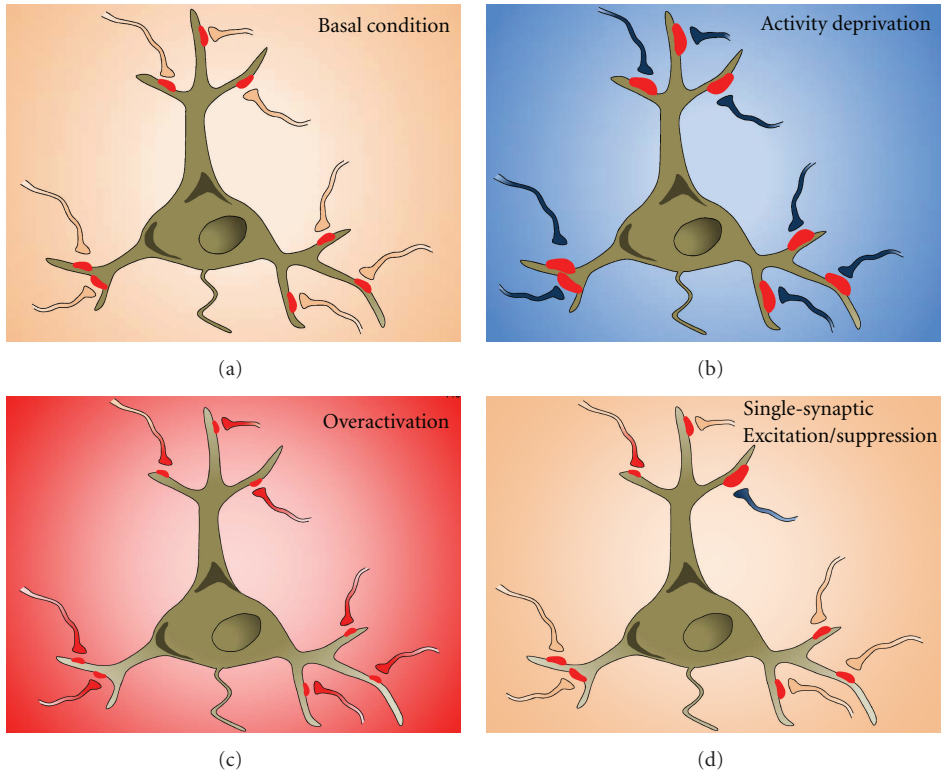

Figure 1: Global and local homeostatic synaptic plasticity. Under basal condition, neurons have a relative stable level of activity as a result of basal synaptic inputs mediated by AMPARs (red clusters) at the postsynaptic domains (a). When the activity of a neuron or a network is chronically suppressed such as by TTX incubation, the strength of all synapses is elevated proportionally via an increase in AMPAR abundance (b). In contrast, an overall reduction in synaptic AMPAR accumulation is induced by lasting overactivation of neuronal activity (c). At individual synapses, long-term synaptic inactivity (terminal in blue) leads to a homeostatic increase in AMPAR amount, whereas lasting synaptic excitation causes selective AMPAR reduction at the stimulated synapse (terminal in red), without affecting the neighboring normal synapses (d).

incubated with medium obtained from TTX-treated neural cell cultures, cultured hippocampal neurons globally scale up mEPSC amplitudes and postsynaptic AMPAR numbers. This effect can be abolished by applying exogenous high-affinity TNFα receptors to scavenge TNFα from the medium, indicating a critical role of free TNFα released to the extracellular environment to mediate synaptic upscaling during activity deprivation. To further support TNFα signaling in homeostatic plasticity, TTX-induced global upscaling in mEPSC is completely abolished in cultured hippocampal neurons or brain slices from TNFα knockout mice [24]. Interestingly, this abolished scaling can be rescued by coculture of TNFα knockout neurons with wild-type glial cells. However, although neurons produce TNFα by themselves, coculture of wild-type neurons with TNFα knockout glial cells still abolishes the upscaling in wild-type neurons [24]. Therefore, TNFα released from glia, but not neurons, plays a crucial role in the induction of global, slow homeostatic synaptic upregulation.

TNFα seems to mediate TTX-induced synaptic upscaling by regulating the trafficking and synaptic accumulation of AMPARs. Studies have shown that application of TNFα in cultured hippocampal neurons induces a rapid translocation of AMPARs (within 15 min) to the postsynaptic domain in a subunit-specific manner [26, 27]. After TNFα treatment, the delivery of AMPARs to postsynaptic surface is enhanced significantly, through downstream activation of PI3K pathway [28]. It also has been found that the effects of TNFα on AMPAR trafficking are mainly through TNFR1 but not TNFR2 [29]. Of note, the enhanced delivery of GluA1 subunit occurs faster than GluA2, leading to the generation of GluA2-lacking AMPARs. Calcium influx through these special type of AMPARs is believed to have an important role in the initiation of homeostatic response [30] (see Section 4.6 on Cp-AMPARs). In addition to enhancing AMPAR synaptic delivery, TNFα also decreases the trafficking of $GABA_A$ receptors to the synapses [27]. Thus, TNFα-mediated synaptic homeostatic regulation of neuronal activity could be achieved via rebalancing excitation and inhibition.

4.2. CaMKs. Calcium (Ca^{2+}) is one of the most important signaling molecules in the nervous system. In neurons, Ca^{2+} transients from ligand- and voltage-gated calcium channels and intracellular calcium stores activate a family of Ser/Thr protein kinases known as Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) to execute signaling functions [31, 32]. Among all CaMKs, CaMKII and CaMKIV are mostly known

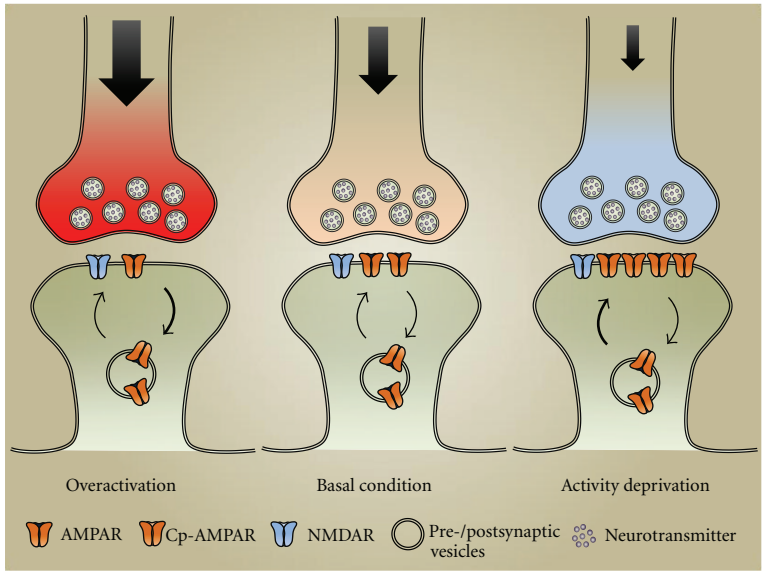

Figure 2: Activity-dependent homeostatic regulation of AMPAR synaptic localization. Under basal conditions, a stable level of AMPARs at postsynaptic surface is maintained by balanced trafficking processes of receptor insertion and internalization (middle). High synaptic activity can be detected by neurons, leading to enhanced receptor internalization and reduced surface receptor localization (left). Conversely, when neurons are treated by long-term activity deprivation, higher levels of AMPARs are expressed at postsynaptic surface via enhanced receptor insertion, including both GluA2-containing and GluA2-lacking receptors (Cp-AMPAR) (right). During homeostatic regulation, the number of NMDARs at postsynaptic surface is not significantly altered under either condition.

to be closely involved in synaptic transmission and different forms of synaptic plasticity by regulating receptor synthesis and trafficking [32, 33], through their ability to either directly phosphorylate AMPARs (CaMKII) or to regulate Ca^{2+}-stimulated gene expression [34–36]. Although the important role of CaMKs in Hebbian synaptic plasticity has been extensively studied [32, 37], their involvement in homeostatic regulation has not been studied until recently. In a cultured cortical neuron, Ibata et al. inhibit single neuron firing by local perfusion of TTX on the cell body. They show that 4 hr selective inhibition of somatic activity induces a rapid, global upscaling in mEPSC amplitude and postsynaptic AMPARs in the inhibited neuron [16]. The quantity of surface AMPARs is imaged and measured by live imaging of EYFP-tagged GluA2 subunits expressed in dissociated rat cortical neurons. This homeostatic response results from a drop in somatic Ca^{2+} level and reduced CaMKIV activation. Coexpression of a dominant-negative form of CaMKIV (dnCaMKIV) with EYFP-GluA2 for 24 hrs mimics the TTX effect on mEPSCs [16]. In another study, Goold and Nicoll employ an optogenetic technique to activate individual neurons. Channelrhodopsin (ChR2) is expressed in hippocampal CA1 neurons in brain slices for 2-3 days, and a light train at 3 Hz is applied to stimulate the transfected neurons for 24 hrs. This lasting activation leads to a homeostatic depression of both AMPAR- and NMDAR-mediated currents, and a reduction in surface expression of AMPARs and NMDARs [17]. CaMKIV, activated by Ca^{2+} influx through the L-type voltage-gated calcium channels, plays a key role in the light activation-induced responses; expression of a dominant-negative CaMKIV blocks the homeostatic downregulation in AMPARs and NMDARs [17]. Together, these studies indicate an important role for CaMKIV in cell-autonomous bidirectional homeostatic plasticity.

In addition to CaMKIV, other CaMK members, like CaMKII, may also participate in homeostatic regulation. Thiagarajan et al. show that α- and βCaMKII are inversely regulated by neuronal activity and they also have opposite effects on mEPSCs [38]. In cultured hippocampal neurons, activity deprivation by 24-hour TTX treatment significantly decreases αCaMKII but increases βCaMKII expression. Conversely, chronic hyperactivity by 24-hour bicuculline treatment significantly increases αCaMKII and decreased βCaMKII levels. Overexpression of αCaMKII in hippocampal neurons significantly decreases, while βCaMKII increases, the amplitude of mEPSCs after 20–30 hrs [38]. Recently, another study shows that knockdown of βCaMKII in hippocampal neuron blocks NBQX-induced homeostatic increase in synaptic AMPAR expression whereas βCaMKII overexpression increases synaptic AMPAR levels [39]. These findings suggest an important role of CaMKII in the expression of homeostatic synaptic response.

4.3. BDNF. Brain-derived neurotrophic factor (BDNF) is broadly involved in many physiological processes in the developing or mature nervous system. BDNF is released from neurons in an activity- and calcium-dependent manner [40]. Once released, BDNF binds to the TrKB receptors to trigger a series of downstream signaling pathways [41]. Given its positive role in neuroprotection, BDNF is considered valuable in the management of several neurological diseases such as Huntington's disease, epilepsy, and Alzheimer's Disease [42].

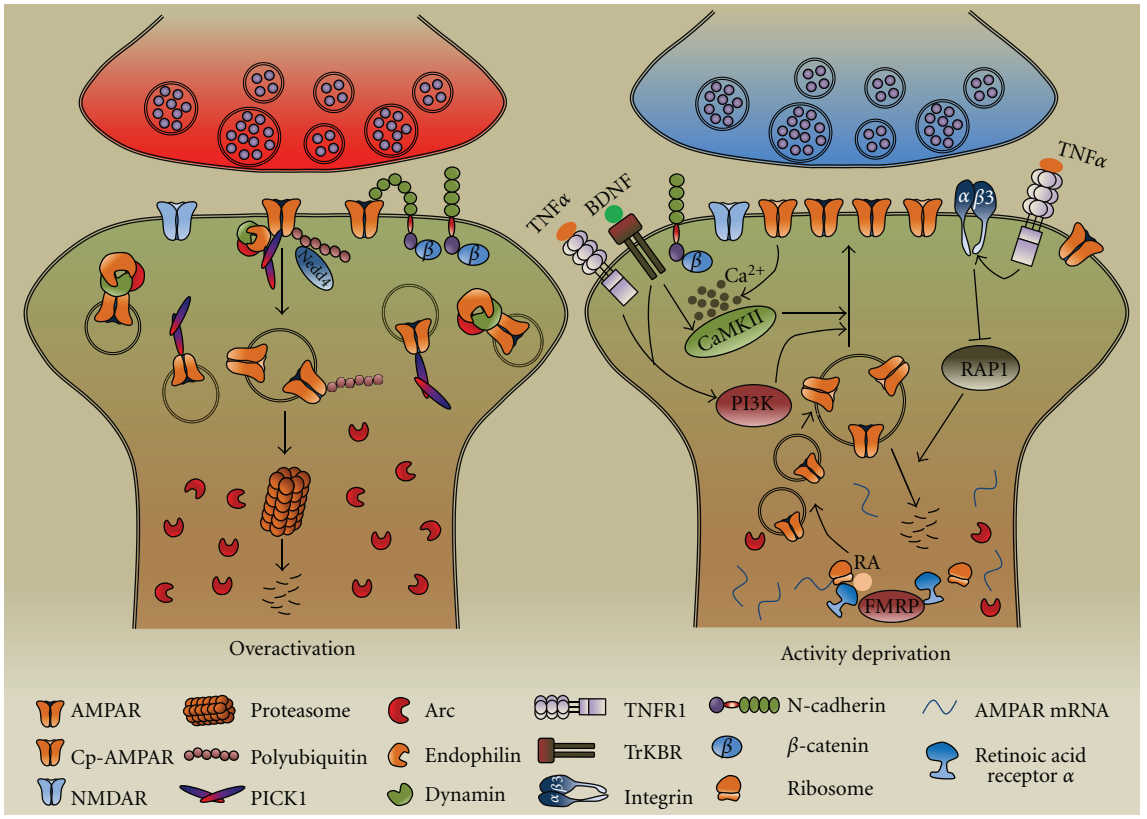

Figure 3: Regulation of homeostatic AMPAR trafficking and turnover. Multiple functional molecules and signaling cascades are involved in the homeostatic up- or downregulation induced by prolonged activitydeprivation or overactivation, respectively. Elevated synaptic activation stimulates the expression of an immediate-early gene product, Arc, which, together with endophilin and dynamin, promotes AMPAR internalization. This process also requires the association of AMPARs with PICK1, and Nedd4-mediated receptor ubiquitination and proteasomal degradation (left panel). Under activity deprivation, varied signaling cascades are implicated in AMPAR trafficking and synaptic localization. Activation of TNFR1 by glia-derived TNFα, or PI3K pathway by BDNF/TrKBR and TNFR1 signaling, significantly enhances the postsynaptic AMPAR levels. β3 integrin signaling inhibits the activity of RAP1, which is known to enhance AMPAR degradation. Also, calcium influx through the inactivity-induced GluA2-lacking AMPARs (Cp-AMPAR) activates the CaMKII pathway, causing AMPAR phosphorylation and insertion. In addition, retinoic acid receptor α (RARα) and FMRP mediate AMPAR local synthesis in postsynaptic domain during activity deprivation-induced upscaling (right panel).

The chronic presence of BDNF enhances synaptogenesis [41] and controls synaptic transmission and plasticity at both glutamatergic and GABAergic synapses [43]. In cultured cortical neurons derived from rat visual cortex, BDNF is first found to mediate homeostatic downregulation of mEPSCs [44]. Application of exogenous BDNF blocks activity deprivation-induced homeostatic upscaling, whereas BDNF depletion by high-affinity TrKB receptors mimics inactivity-induced upward homeostatic change in mEPSCs [44]. BDNF has been found to affect mEPSCs differentially in pyramidal neurons and interneurons [44]. In cultured rat visual cortical neurons, BDNF attenuates the TTX-induced homeostatic increase in mEPSC amplitudes at synapses formed onto pyramidal neurons, but enhances the TTX effect at synapses formed onto interneurons [44]. Intriguingly, in cultured hippocampal neurons, BDNF treatment enhances mEPSCs in excitatory synapses, likely due to enhanced AMPAR trafficking [45, 46], which is inconsistent with its role in homeostatic downregulation. Recently, several studies show that BDNF treatment enhances AMPAR trafficking to glutamatergic synapses, both *in vitro* and *in vivo* [47, 48]. In cultured hippocampal neurons, Caldeira et al. show that elevated AMPAR trafficking by BDNF is receptor subunit specific. Surface expression of GluA1 is preferentially increased in the first 30 min of BDNF incubation, leading to the formation of calcium-permeable, GluA1 homomeric AMPARs. At a later stage, BDNF incubation enhances the delivery of GluA2 and GluA3 subunits [47]. Enhanced AMPAR synaptic delivery requires the activation of TrKB receptors and the PI3K-Akt pathway [49], most likely through phosphorylation of the GluA1 C-terminal at S831 [47].

In addition to its role in AMPAR trafficking, BDNF has also been shown to function as a retrograde messenger released from the postsynaptic site to alter presynaptic activity in an activity-dependent manner [50, 51]. Inhibition of AMPAR activity by NBQX for 24 hrs or CNQX for 3 hrs homeostatically increases the frequency and amplitude of mEPSCs. The increase of mEPSC frequency is directly mediated by the retrograde signaling of BDNF. Removal of BDNF by adding high-affinity TrKB receptors or BDNF

antibodies and pharmacologically blocking of the downstream function of BDNF all lead to an abolishment of the enhanced presynaptic response without affecting postsynaptic activity. Consistently, application of BDNF itself induces similar presynaptic changes as produced by AMPAR inhibition [51].

4.4. PI3K-Akt Pathway. The phosphoinositide 3-kinase-(PI3K-) Akt pathway is wellknown for its involvement in AMPAR trafficking, synaptic plasticity, and memory consolidation both *in vivo* and *in vitro* [52, 53]. For instance, protein synthesis and AMPAR insertion in late-phase LTP requires PI3K pathway activation [53–55]. *In vivo*, activation of the PI3K pathway is also required for the maintenance of LTP in hippocampal CA1 neurons [53]. Consistent with its important role in LTP, PI3K is implicated in memory consolidation as well [52]. However, in contrast to abundant evidence about its role in Hebbian synaptic plasticity, involvement of PI3K signaling in homeostatic synaptic plasticity remains unclear. The PI3K cascade is potentially a good mediator in homeostatic regulation. First, the PI3K-Akt pathway is known to induce protein synthesis [55] and AMPAR membrane insertion [54, 56], processes known to occur in homeostatic AMPAR upregulation [19]. Second, both TNFα and BDNF, the two major factors that mediate homeostatic plasticity, activate the PI3K-Akt pathway and increase AMPAR expression at the postsynaptic surface [28, 41, 49]. Indeed, work by Hou et al. first revealed a requirement of PI3K in homeostatic response. In cultured hippocampal neurons, inhibition of presynaptic activity by overexpression of an inward-rectifier potassium channel Kir2.1 results in a significant increase in postsynaptic AMPARs. This homeostatic upregulation of AMPARs is abolished by application of a PI3K inhibitor wortmannin [20]. Consistent with this finding, a recent study also demonstrates the PI3K-Akt pathway as a mediator of global homeostatic plasticity. Presenilin 1 (PS1) is an integral component of γ-secretase that is closely linked to Alzheimer's disease [57, 58]. PS1 activates the PI3K-Akt pathway by promoting the formation of Akt-activating cadherin/PI3K complexes [59], Pratt et al. have found that the global homeostatic upscaling of mEPSCs induced by prolonged TTX treatment is impaired in cultured hippocampal neurons derived from PS1 knockout mice, suggesting a possible involvement of PI3K cascade [60]. In support of this possibility, overexpression of constitutively active Akt rescues impaired global synaptic scaling in PS1 knock-out neurons without affecting mEPSC amplitude in wild-type neurons [60]. Together these studies indicate the necessity of the PI3K-Akt pathway in homeostatic upregulation.

4.5. Cell Adhesion Molecules. Integrins and neuronal (N)-cadherin/β-catenin are cell adhesion molecules (CAMs) that are expressed at synapses and possess important functions in synapse formation, differentiation, and maturation, as well as synaptic plasticity [61, 62]. Malfunction of CAMs can produce severe problems in the nervous system including abnormal spine morphology, decreased synapses quantity, and impaired cognitive function [61, 63–66]. N-cadherin is a Ca^{2+}-dependent homophilic adhesion protein that is present at both pre- and postsynaptic membranes [67]. It signals through Rho-family GTPases, via catenins, to control dendritic spine morphology and motility [68]. N-cadherin forms a complex with β-catenin in the synapse. The N-cadherin/β-catenin complex regulates the surface expression and intracellular trafficking of AMPARs by directly interacting with AMPARs [69–71]. The N-terminal domain of N-cadherin physically interacts with the GluA2 subunit of AMPARs at the extracellular space, promoting spine growth and synaptic transmission [71]. Nuriya and Huganir have shown that overexpression of wild-type N-cadherin in cultured hippocampal neurons can specifically increase the postsynaptic AMPAR expression [69]. By targeting AMPARs during endocytosis and exocytosis at the postsynaptic surface, the N-cadherin/β-catenin complexes also regulate the bidirectional homeostatic responses of neural activity. Okuda et al. show over-expression of a dominant-negative mutant of N-cadherin compromises the chronic up-scaling of quantal AMPAR responses induced by TTX treatment, whereas selective deletion of β-catenin in cultured hippocampal neurons eliminates the global homeostatic regulation [72, 73]. In addition to their roles in conventional postsynaptic homeostatic regulation, a recent study has demonstrated a role for N-cadherin/β-catenin in the compensatory adaptation of presynaptic release to chronic activity deprivation by TTX treatment. Vitureira et al. have found overexpression of dominant-negative N-cadherin (DN-NCad) in cultured hippocampal neurons significantly reduces the basal presynaptic release probability, but does not change the homeostatic upregulation of presynaptic release [73]. In contrast, ablation of β-catenin has no effect on basal presynaptic release but completely abolishes the homeostatic increase in presynaptic release induced by chronic activity deprivation by TTX treatment [73].

Integrins are heterodimeric transmembrane molecules of which many subunits are expressed in the central nervous system. Previous studies have revealed multiple roles for integrins in synaptogenesis, synaptic transmission, and plasticity, as well as memory formation [65, 74]. β3 integrin regulates synaptic strength by modulating the surface expression of AMPARs in a subunit-specific manner [75]. Over-expression of β3 integrin in dissociated hippocampal neurons enhances AMPAR surface expression via suppression of RAP1 [75], a pathway known to negatively control AMPAR trafficking during synaptic plasticity by enhancing endocytosis of GluA2 subunits [76]. Because TNFα, an important molecule in homeostatic plasticity, causes an elevated cell-surface expression of β3 integrin, it suggests a possible involvement of β3 integrin in homeostatic response by regulating AMPAR trafficking. Consistent with this hypothesis, a recent study shows that 24 hr TTX or bicuculline treatment causes a corresponding increase or decrease of surface β3 integrin without affecting β1 integrin [75]. Prolonged TTX treatment enhances the surface β3 integrin to a level similar to that by TNFα treatment. More directly, overexpression of dominant-negative β3 integrin, CTβ3, completely eliminates the TTX-induced global homeostatic up-scaling of mEPSCs and synaptic AMPAR expression [75].

4.6. GluA2-Lacking, Calcium-Permeable AMPARs (Cp-AMPARs). Under physiological conditions most AMPARs contain at least one GluA2 subunit and allow only sodium influx to depolarize membrane potential during synaptic activation. When AMPARs are composed without GluA2 subunits, the receptor channel will permeate calcium in addition to sodium. Due to a channel blockade by intracellular polyamine at positive membrane potential, currents from Cp-AMPARs show a signature feature of inward rectification in the current-voltage relationship. It has been found that the expression of GluA2-lacking receptors is regulated by development, synaptic activity, or pathological challenges such as ischemia or amyotrophic lateral sclerosis (ALS) [77]. In the early postnatal ages, cortical pyramidal neurons have higher rectification in AMPAR-mediated synaptic currents, which diminishes in more mature animals, indicating a developmental switch in AMPAR composition and calcium permeability [78]. By providing an unconventional source of calcium other than NMDAR or calcium channels, GluA2-lacking AMPARs may play an important role in synaptic plasticity. Indeed, in the expression of hippocampal LTP, GluA2-lacking AMPARs are first incorporated into the synapse, which will then be replaced with GluA2-containing receptors [79]. In cerebellar stellate cells which normally contain GluA2-lacking AMPARs, high-frequency presynaptic activity induces a calcium-dependent increase in synaptic insertion of GluA2-containing AMPARs [80]. These findings strongly indicate the presence of a self-regulating mechanism by which Cp-AMPAR-mediated calcium flux triggers recruitment of normal GluA2-containing AMPARs to synapses.

Activity deprivation has been shown to induce inward rectification in the current-voltage relationship of AMPAR-mediated current, suggesting the formation of GluA2-lacking, calcium-permeable AMPARs [19, 81–83]. Consistently, following treatment paradigms for homeostatic plasticity, AMPAR-mediated currents become sensitive to Cp-AMPAR-selective antagonists philanthotoxin-433 (PhTx) or Naspm [19, 20, 81–83]. Interestingly, multiple signaling molecules that are involved in homeostatic synaptic plasticity including TNFα, retinoic acid, Arc/Arg3.1, and β3 integrin are capable of causing imbalanced GluA1 and GluA2 regulation and Cp-AMPAR expression. The homeostatic factor TNFα is known to cause rapid membrane insertion of GluA2-lacking AMPARs [28, 84]. In retinoic acid-mediated synaptic scaling, the increase in AMPAR surface expression is GluA1 specific, and the homeostatic response in mEPSCs is abolished by suppression of Cp-AMPARs [82]. An unbalanced regulation in AMPAR subunits is also observed in Arc/Arg3.1-mediated homeostatic regulation. Knockout of Arc/Arg3.1 results in a typical synaptic scaling of AMPAR-mediated mEPSCs. Interestingly, Arc/Arg3.1 knockout neurons reveal a significant increase in GluA1 surface expression, whereas surface GluA2 shows no change [85], implicating membrane addition of GluA2-lacking AMPARs. In addition, disruption of β3 integrin induces internalization of GluA2, but not GluA1 subunits, resulting in GluA2-lacking AMPARs at the cell surface. In our own study, we find that homeostatic regulation by single synaptic suppression is abolished by the application of PhTx, indicating the requirement of Cp-AMPAR signaling [20]. Interestingly, the blockade of homeostatic plasticity is observed only when PhTx is applied at the early stage of activity deprivation [20], indicating that Cp-AMPARs are needed for the initiation, but not maintenance, of homeostatic synaptic regulation.

4.7. Arc/Arg3.1. Arc/Arg3.1 is an immediate-early gene product whose abundance at synapses is strictly coupled with neural activity level. Strong synaptic activation will dramatically enhance the expression of Arc/Arg3.1 in dendrites and spines while synaptic suppression decreases its expression [86, 87]. Arc/Arg3.1 is broadly involved in different forms of synaptic plasticity including both Hebbian plasticity and homeostatic regulation [88]. Rial Verde et al. find that Arc/Arg3.1 controls synaptic transmission strength by negatively regulating the surface expression of AMPAR expression at the post-synaptic surface. Over-expression of Arc/Arg3.1 in hippocampal neurons promotes the endocytosis of GluA2/3 containing AMPARs as well as the reduction of AMPAR-mediated synaptic current amplitude. Importantly, knockdown of Arc/Arg3.1 by siRNA abolishes this effect [87]. In Arc/Arg3.1 knockout mice, AMPARs show markedly reduced endocytosis and enhanced steady-state surface expression [85, 89]. Shepherd et al. also find that Arc/Arg3.1 is involved in bidirectional homeostatic regulation of neural activity via regulating AMPAR internalization and endocytosis [85]. In primary neuronal culture, synaptic expression of Arc/Arg3.1 is enhanced after chronic activity deprivation, while over-expression of Arc/Arg3.1 blocks the global synaptic up-scaling of mEPSCs and AMPARs induced by chronic activity deprivation. Conversely, in cultured hippocampal neurons from Arc/Arg3.1 knockout (KO) mice, either global up- or down-homeostatic scaling induced by prolonged TTX or bicuculline treatment is impaired [85]. Together these studies demonstrate strong evidence about the involvement of Arc/Arg3.1 in the bi-directional homeostatic regulation of neural activity through regulation of the trafficking of AMPARs. It has also been shown that Arc/Arg3.1 directly interacts with endophilin and dynamin to form post-synaptic endosomes which facilitate the endocytosis of AMPARs [89]. In addition to global regulation, Arc/Arg3.1 also mediates local homeostatic plasticity at individual synapses. Using Kir2.1 paradigm, Béïque et al. show that homeostatic upregulation of mEPSCs is abolished in cultured cortical pyramidal neurons derived from Arc knock-out mice [90].

4.8. Retinoic Acid and FMRP. Retinoic acid (RA), also known as Vitamin A, is best known for its role in regulating the development of the nervous system, including neurogenesis and neuronal differentiation [91, 92]. In a recent study, Aoto et al. show that 24 hr TTX + APV treatment, a homeostatic paradigm that induces global synaptic upscaling of mEPSCs and AMPARs [19], significantly enhances the synthesis of RA in both cultured hippocampal neurons and brain slices [82]. Application of RA rapidly increases the strength of synaptic

transmission mainly through an increase in surface expression of AMPARs [82]. Effects of RA are translation, but not transcription dependent [93], and are occluded by TTX + APV treatment, indicating an involvement of RA signaling in homeostatic plasticity. In addition, AMPAR upregulation by APV + TTX treatment is subunit specific, with a preferential increase in GluA1 over GluA2, leading to the production of GluA2-lacking, calcium-permeable AMPARs [19]. A recent study from the same group indicates a role of the fragile-X mental retardation protein (FMRP) in RA-induced GluA1 local translation [94]. FMRP is a dendritic RNA-binding protein encoded by the Fmr1 gene that is involved in the down-regulation of local mRNA translation and protein synthesis [95, 96]. In Fmr1 knockout mice, both TTX + APV-induced AMPAR homeostatic upregulation and RA-induced local AMPAR synthesis are impaired. Over-expression of WT-FMRP but not mutant FMRP in Fmr1 knockout neurons restores the impaired homeostatic upscaling of mEPSCs and AMPARs [94]. Therefore, via the effect of FMRP, homeostatic regulation may be implicated in the neurodysfunction in fragile X syndrome.

4.9. PICK1. Protein interacting with C-kinase 1 (PICK1) is a PDZ domain-containing protein that directly interacts with the GluA2 subunit of AMPARs [97, 98]. PICK1 also directly interacts with the AMPAR adaptor protein, ABP/GRIP to regulate AMPAR trafficking [99]. The importance of PICK1 in Hebbian synaptic plasticity, especially in LTD, has been well documented both *in vivo* and *in vitro* [100, 101]. PICK1 influences synaptic plasticity by stimulating AMPAR internalization [102, 103]. For instance, in PICK1 knockout mice, NMDA-induced LTD is abolished in hippocampal neurons due to disrupted internalization, recycling, and retention of GluA2-containing AMPARs [100, 101]. By interacting with the GluA2 subunit, PICK1 plays a key role in the plasticity involving the calcium-permeable, GluA2-lacking AMPARs [104]. In a specific type of LTP induced by cocaine exposure at the glutamatergic synapses of dopaminergic neurons in the ventral tegmental area, PICK1 directly mediates the switch of GluA2-containing to GluA2-lacking AMPARs [105]. Since calcium-permeable AMPARs serve as an important signal in homeostatic scaling [30], these findings imply a regulatory role for PICK1 in homeostatic regulation. Indeed, a recent study using cultured cortical neurons showed that PICK1 specifically mediates the TTX-induced global up-scaling of mEPSCs and synaptic AMPARs, without affecting bicuculline-induced downward homeostatic regulation [106]. In cultured cortical pyramidal neurons derived from PICK1 knockout mice, while the number of synaptic AMPARs are increased, TTX-induced mEPSC up-scaling is occluded due to altered AMPAR subunit composition and aberrant receptor trafficking [106].

4.10. Ubiquitin-Proteasome System. The ubiquitin-proteasome system (UPS) is a crucial proteolytic mechanism. UPS uses a small protein of 76 amino acids, namely, ubiquitin, to mark proteins destined for degradation. Following ubiquitination, a polyubiquitin chain is attached to the lysine residues of the target protein so that it can be recognized by the degradation machinery proteasome [107]. UPS components are widely distributed in a neuron from the soma to dendrites and synapses [107, 108]. It regulates many important synaptic functions including synapse development, maturation, and synaptic plasticity [109]. Given the importance of proper protein turnover in cells, UPS dysfunction is implicated in the pathogenesis of many neurodegenerative diseases [110].

UPS function is closely related to the neural activity levels [108, 111] to control the post-synaptic proteins composition including PSD-95 [112, 113] and AMPAR-associating protein GRIP [114]. AMPARs are directly subjected to ubiquitination, leading to their internalization and degradation [115–117]. The degradation of NMDARs is also regulated by the UPS [118]. Several recent studies have shown the UPS activity is also involved in the regulation of both global and local homeostatic plasticity. Jakawich et al. show that application of a proteasome inhibitor, lactacystin, for 24 hrs in cultured hippocampal neurons causes a global up-scaling of mEPSCs and AMPAR expression, which mimics and occludes the TTX-induced homeostatic synaptic response [119]. In addition to the pharmacological study, expression of a mutant ubiquitin which inhibits protein ubiquitination by blocking ubiquitin chain elongation produces similar effects as prolonged lactacystin treatment [119]. In another recent study, Hou et al. utilize light-controlled glutamate receptor (LiGluR) to selectively activate individual synapses in cultured hippocampal neurons. Single synaptic activation leads to homeostatic downregulation of postsynaptic AMPAR abundance as a consequence of enhanced AMPAR internalization and degradation [18]. This activity-dependent homeostatic AMPAR alteration is accompanied by a recruitment of polyubiquitinated proteins and AMPAR E3 ligase Nedd4 [115, 116, 120] and is blocked by the application of proteasome inhibitors, strongly indicating a key role of the UPS in hyperactivity-induced homeostatic plasticity [18].

5. Conclusion

Homeostatic synaptic regulation is one of the fundamental forms of plasticity serving to maintain the functional stability of the nervous system from a single synapse to an entire neural network. At a cellular level, homeostatic response will keep the firing rate within a physiological range, and at individual synapses, it prevents synaptic activity from running away to the extremes. Although a change in AMPAR trafficking and synaptic accumulation has been considered the primary mechanism, homeostatic regulation of neuronal activity can also be expressed via regulating other cellular components such as presynaptic transmitter release, cell intrinsic excitability, synaptic connectivity, and the relative balance between excitation and inhibition. Potentially, dysfunction of homeostatic regulation will lead to a shift in basal neuronal and synaptic activity, and altered sensitivity to stimuli. Consequently, super up-scaling could cause epileptic activity and excitotoxic cell death, whereas abnormal

downscaling would result in neural suppression and impaired cognitive function. Therefore, homeostatic plasticity may play a crucial role in the pathogenesis of multiple neurological disorders including neurodegenerative diseases, which, we expect, will continue to be a topic of investigation for the foreseeable future.

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Review Article

Time to Be SHY? Some Comments on Sleep and Synaptic Homeostasis

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Sleep must serve an essential, universal function, one that offsets the risk of being disconnected from the environment. The synaptic homeostasis hypothesis (SHY) is an attempt to identify this essential function. Its core claim is that sleep is needed to reestablish synaptic homeostasis, which is challenged by the remarkable plasticity of the brain. In other words, sleep is "the price we pay for plasticity." In this issue, M. G. Frank reviewed several aspects of the hypothesis and raised several issues. The comments below provide a brief summary of the motivations underlying SHY and clarify that SHY is a hypothesis not about specific mechanisms, but about a universal, essential function of sleep. This function is the preservation of synaptic homeostasis in the face of a systematic bias toward a net increase in synaptic strength—a challenge that is posed by learning during adult wake, and by massive synaptogenesis during development.

1. Introduction

In "Erasing synapses in sleep: is it time to be SHY?" (this issue), Marcos Frank provides an up-to-date evaluation of several aspects of the synaptic homeostasis hypothesis (SHY) of sleep function ([1, 2] and subsequent work). While this is not the place for a comprehensive discussion of the ideas and evidence behind SHY (Tononi and Cirelli, in preparation), Frank's commentary offers a welcome opportunity to address some of the experimental evidence about synaptic plasticity in wake and sleep, and to reconsider the involvement of additional factors affecting synaptic function, such as brain temperature and glucocorticoids. However, the way SHY is presented in the commentary suggests that it may be just as important to clarify what the hypothesis actually claims and what it does not. As acknowledged by Frank, SHY is eminently falsifiable, but one must make sure that what is put to the test are indeed SHY's tenets. With this in mind, it is useful to provide a brief summary of the motivations underlying SHY.

2. The Logic of SHY

Sleep is a behavior characterized by a reversible disconnection from the environment (when asleep we are "off-line") and usually, but not always, by immobility. Sleep is present in all species studied so far (from fruit flies to humans), occurs from early development to old age, occupies a large fraction of the day, is tightly regulated (sleep homeostasis) and irresistible (it cannot be postponed indefinitely), and its loss leads to negative consequences, especially on cognitive functions [3]. These features strongly suggest that sleep must serve an essential, universal function, one that offsets the risk of being disconnected from the environment and the opportunity cost of not engaging in other behaviors. SHY is an attempt to identify this essential function. Its core claim is that sleep is needed to reestablish synaptic homeostasis, which is challenged by the remarkable plasticity of the brain. In other words, sleep is "the price we pay for plasticity [2]."

Briefly, the logic behind SHY is as follows. (i) The brain is extraordinarily plastic—changes in the number and efficacy of synapses, in intrinsic excitability, and in several

other neuronal and glial parameters are the rule rather than the exception. Plasticity, of course, is essential for the development of neural circuitry and the adaptation to a changing environment. These noncontroversial premises are supported by overwhelming evidence. (ii) During wake, plastic changes are biased toward potentiation—for example, a net strengthening of synaptic efficacy and/or a net increase in the number of synapses. This is a novel claim, based on the premise that neurons usually signal important inputs by spiking more, rather than less. It follows that, to ensure that such signals percolate to other neurons deep inside the brain, connections conveying the signals should be strengthened, rather than weakened. A net increase in synaptic strength after wake is a key prediction of SHY and much effort has gone into testing it in different species using various experimental paradigms. (iii) A net increase in synaptic strength cannot be sustained indefinitely. This is because stronger synapses consume more energy, occupy more space, require more cellular supplies, saturate the capacity to learn and decrease signal-to-noise ratios (if more and more inputs are strengthened, neurons become progressively more excitable, and it becomes difficult to distinguish between signals that are important and ones that are not). (iv) Therefore, synaptic strength must be regulated and returned to a sustainable level, restoring synaptic homeostasis. In this way, the costs in terms of energy, space, supplies, signal-to-noise ratios, and learning capacity are restored to baseline. (v) Synaptic homeostasis is best achieved during sleep, a time when there is no demand for learning and neurons can sample most of their inputs in an unbiased manner through off-line spontaneous activity. By contrast, during wake neurons preferentially sample the particular subsets of inputs determined by interactions with the environment, and they are required to learn on-line. (vi) A similar need for synaptic homeostasis, hence for sleep, may exist during development. In many species, there is an initial overproduction of synapses, followed by net pruning down to adult levels [4]. Sleep would seem to be an ideal time for the selection of which synapses should remain and which should be pruned, through the unbiased, off-line sampling of a neuron's inputs. In summary, the core claim of SHY (and the reason it is called SHY) is that the universal, essential function of sleep is the restoration of synaptic homeostasis. If that turns out to be incorrect, so is SHY. For this reason, much effort has been devoted to evaluating structural, molecular, and physiological indices of synaptic efficacy before and after sleep. So far, evidence obtained using a variety of experimental approaches have been supportive in flies, rodents, and humans [5–14].

In addition to its core claim, SHY proposes some corollaries that are specific to animals showing slow wave activity (SWA) during sleep, such as mammals and birds. One corollary is based on the idea that synaptic number and strength influence the amplitude and slope of sleep slow waves. This is because stronger synapses increase neuronal synchrony, which in turn is reflected in larger and steeper slow waves in the EEG. Indeed, converging evidence indicates that the amplitude and slope of EEG slow waves is related to the number of neurons that enter an up state or a down state near-synchronously, and that synchrony is directly related to the number and strength of synaptic connections among them [15–17]. To the extent that this is correct, SHY entails, for example, that sleep SWA should be higher after wake and that it should decrease after sleep, in accord with evidence in many species of mammals and birds [18–21]. Moreover, SHY predicts that SWA should be locally regulated [1, 2]. For example, if a particular brain region undergoes a high amount of learning/synaptic potentiation in wake, that region should show a local increase in slow waves during subsequent sleep. This prediction has been confirmed by several studies, both in rodents and humans (e.g., [22, 23]).

A second corollary of SHY is that sleep slow waves may not simply reflect the number/strength of synapses, but they may be causally involved in synaptic homeostasis. Intriguingly, slow waves occur on average once a second or so, a frequency that is often associated with synaptic depression [12, 24–30]. The alternation of depolarized up states and hyperpolarized down states may also favor depression, and through spike-timing-dependent plasticity mechanisms, so would the increased synchrony caused by high synaptic strength [30, 31]. Finally, the neuromodulatory milieu in sleep, unlike that in wake, may also dampen potentiation and enhance depression [32, 33]. Irrespective of the particular mechanisms, the appealing feature underlying this corollary is that a positive link between synaptic strength and slow waves, coupled to a positive link between slow waves and synaptic depression, instantiates an elegant control mechanism that automatically regulates synaptic strength toward a baseline value [1, 2]. As shown through large-scale simulations [15, 31], the higher synaptic strength, the higher neuronal activity, and synchrony, yielding larger/steeper slow waves. On the other hand, the larger/steeper the slow waves, the more they produce synaptic depression. Moreover, when synaptic strength has been downregulated to a sustainable, baseline level, neurons are less synchronous, slow waves are small, and synaptic depression stops, avoiding the risk of run-away depression and possible memory loss. While this second corollary is certainly compatible with the mechanisms of synaptic depression mentioned above, as well as with some experimental findings involving manipulations of sleep SWA (e.g., [5, 34, 35]), so far the evidence supporting it remains limited and indirect.

3. Misunderstandings and Clarifications

This brief summary of the logic, core claim, and corollaries of SHY provides some context that should help, first, to clarify some misunderstandings that run through Frank's commentary and, second, to address some of the specific issues raised.

3.1. Function versus Mechanisms. The most important misunderstanding in Frank's commentary is the conflation between mechanisms and function. As briefly outlined above, SHY is first and foremost a hypothesis about the universal, essential function of sleep, not about which specific mechanisms mediate that function. From the start,

SHY assumed that the proposed function of sleep—synaptic homeostasis—might be carried out through different mechanisms in brain structures with different sleep rhythms, such as the hippocampus, and in species with a very different brain, such as *Drosophila* [1, 2]. How exactly *Drosophila* neurons might achieve synaptic homeostasis is an interesting mechanistic question—as pointed out by Frank, *Drosophila* neurons may not undergo slow oscillations—but it has no bearing on whether the core claim of SHY is true or false. Given that *Drosophila* does sleep [36, 37], the central issue for SHY is whether its neurons need synaptic homeostasis, and whether synaptic homeostasis requires sleep. So far, the evidence is positive [7, 8, 10, 11].

The focus on mechanisms rather than function may explain why, on several occasions, Frank is troubled by the perceived "vagueness" of SHY concerning the particular molecular pathways that may underlie synaptic homeostasis. In fact, SHY is purposely liberal about specific mechanisms not out of vagueness, ignorance (though still substantial), or a desire to eschew falsification, but because it is the proposed function of sleep that is universal, not the particular mechanisms, especially in view of the extraordinary variety of cellular and molecular pathways involved in plasticity. Indeed, if synaptic homeostasis turns out to be implemented differently in different species and brain structures, the hypothesis would be strengthened, not weakened: as with convergent evolution, if the same function is achieved with different means in different species, that function is probably fundamental.

3.2. Synaptic Homeostasis versus Activity-Dependent Homeostatic Plasticity. A second misunderstanding is closely related to the first: Frank often portrays SHY as if it were a hypothesis about sleep implementing a particular mechanism, *synaptic scaling*, first observed *in vitro* after massive manipulations of neuronal activity [38], rather than a core functional effect, the "generalized depression or downscaling of synapses" [1]. In other words, Frank equates synaptic homeostasis—the proposed function of sleep—with a specific mechanism of activity-dependent homeostatic plasticity. Homeostatic plasticity refers to an array of phenomena whose goal is to maintain a key parameter, neuronal activity, around some set-point value [38]. Synaptic scaling is the best characterized mechanism underlying homeostatic plasticity and, as Frank discusses at length, it allows neurons to counteract excessive or insufficient activity by down- or up-scaling all their synapses by the same factor [38]. Homeostatic plasticity is typically contrasted with synapse-specific, associative "Hebbian" plasticity both conceptually and in terms of the molecular mechanisms involved, although experimentally the distinction has become more complicated and nuanced [38]. SHY noted that scaling principles of the kind observed with homeostatic plasticity might be involved in synaptic homeostasis during sleep. Indeed, scaling is an attractive mechanism because it can produce a net reduction in synaptic strength while preserving the relative strength of synapses. On the other hand, SHY clearly stated that the primary variable regulated by synaptic homeostasis is synaptic strength, rather than the average neuronal firing rate, as in homeostatic plasticity [38]. Moreover, other mechanisms, including activity-dependent long-term depression, are compatible with SHY as long as depression is generalized to the majority of synapses, thanks to the unbiased off-line activity of sleep. For example, in large-scale simulations, synaptic homeostasis was implemented through generalized synapse-specific depression, with the amount of depression inversely proportional to synaptic strength [31]. In this way, signal-to-noise ratios increased and performance improved. Furthermore, SHY explicitly considered the possibility that some synapses may be strengthened during sleep [2], thereby further enhancing competition, as long as the net effect was an overall reduction of synaptic efficacy. Finally, SHY emphasized that the specific mechanisms involved in synaptic homeostasis can vary—borrowing from homeostatic plasticity, long-term depression, depotentiation, and so on—as long as the end result was a net depression of synapses: "Whichever the specific mechanism, the hypothesis is that a generalized synaptic downscaling during sleep, including possibly the downselection or pruning of certain synapses, serves to ensure the maintenance of balanced synaptic input to cortical neurons" [1, 2].

Retrospectively, the conflation of "synaptic homeostasis" and "homeostatic plasticity" may be attributed to the confusion generated by the shared concept of "homeostasis." SHY pointedly refers to homeostasis to emphasize that sleep serves a fundamental regulatory function—maintain an appropriate level of a key biological parameter, namely, synaptic strength—in the face of variations imposed by learning and development. Homeostatic plasticity is called so because global synaptic scaling is used to regulate another biologically relevant parameter—the level of neuronal activity [38]. Considering that scaling mechanisms involved in homeostatic plasticity may also be involved in maintaining synaptic strength around stable levels, and that activity and plasticity are linked, a certain amount of confusion was perhaps inevitable. This confusion was compounded because SHY loosely referred to the postulated net decrease in synaptic strength as "downscaling." However, SHY never necessarily implied either precise proportionality (all synapses scaled down by the same factor) or a specific molecular mechanism. For this reason, later publications have employed the more neutral term synaptic "renormalization" to describe how synaptic homeostasis is reestablished [6–9, 39, 40].

3.3. Wake and Long-Term Potentiation (LTP). In a similar vein, SHY does not assert that plasticity during wake should be exclusively equated with homosynaptic, associative, "Hebbian" long-term potentiation. Again, the core claim is that wake is associated with a net increase in synaptic strength, irrespective of the particular mechanisms involved, and notwithstanding the possibility that synaptic depression may also occur [1, 2]. As briefly explained above, the prediction that learning during wake should lead to a net increase in synaptic strength is based on the idea that neurons should signal important events to the rest of the brain through increased rather than reduced firing, implying that learning,

too, should be biased toward potentiation. For this reason, the most important evidence for SHY is the demonstration, using structural, electrophysiological, and molecular tools in different species, that net synaptic strength increases with wake and decreases with sleep. By contrast, much of the evidence that Frank considers problematic for SHY has to do with instances in which some molecules that may be implicated, say, in synaptic depression, may occasionally be highly expressed in wake; or in which a particular molecule that is highly expressed in wake, say BDNF or Arc, can be involved in both potentiation and depression. Once again, given the extraordinary complexity of plasticity mechanisms, the large number of possible molecular and electrophysiological interactions, the differences between brain structures and species, and the complicated influence of neuromodulators, one would not expect a simple mapping between synaptic homeostasis and particular molecular or electrophysiological mechanisms. Thus, when Frank argues that the "simplistic" idea that wake and sleep are dominated by net synaptic strengthening and weakening, respectively, is based on a "very narrow view of brain plasticity" (which is certainly multifarious) or that SHY is "oddly disconnected from our rapidly evolving view of synaptic plasticity" (which is becoming increasingly complex), he has it exactly backwards. The appeal of SHY is precisely that it proposes a universal function for sleep—synaptic homeostasis—in the face of the variety and complexity of plasticity mechanisms across different brain circuits, species, developmental phases, and behavioral contexts. What matters for function is the end result, irrespective of the particular molecular interactions involved, and the particular role of specific molecules. SHY predicts that wake will result in a net increase in synaptic strength, and that sleep is needed for its renormalization. If the data show eventually that such net changes do not occur, SHY is wrong. But if such net changes do occur, they most likely involve multiple, complicated, and interacting mechanisms, and different ones in different species and brain structures.

3.4. Cellular Consequences of Synaptic Homeostasis. Finally, in his commentary, Frank restricts his discussion of the potential benefits of synaptic homeostasis to the increase in signal-to-noise ratios, which would help memory consolidation. SHY certainly proposes that sleep-dependent synaptic renormalization should increase signal-to-noise ratios and thereby enhance performance, as suggested both by computational and experimental work [22, 31, 35, 41]. However, SHY has always ascribed to sleep-dependent synaptic homeostasis a much broader function in counteracting the accumulation of synaptic strength [1, 2]. In addition to a reduction in signal-to-noise ratios, high synaptic strength has other costs, including higher energy consumption (synaptic signaling accounts for most of the brain's energy, and stronger synapses consume more energy [42, 43]); decreased space available for further growth (stronger synapses are usually also larger [44]); increased need of cellular supplies, because synaptic plasticity enhances the turnover of proteins and various cellular constituents, requiring a substantial involvement of transport processes, energy delivery processes, and endoplasmic reticulum functions including protein folding [45–48]. Moreover, a net increase in synaptic strength may lead to saturation of the capacity to learn, which can occur quite rapidly in cortex and hippocampus [49–53]. For example, a recent experiment found that synaptic potentiation by direct electrical stimulation of the cortex was difficult to induce after wake but easy after sleep, again suggesting that several hours of wake are enough to bring cortical synapses close to their level of saturation [5]. In short, the benefit that sleep provides for memory consolidation, while important, is certainly not the sole reason underlying the need for synaptic homeostasis.

4. Specific Issues

Having clarified the core claim and corollaries of SHY, it is helpful to consider specific issues raised by Frank's commentary one-by-one. Some of these issues offer excellent points of discussion, highlight areas of current ignorance, and suggest relevant experiments for the future.

4.1. On the Mechanisms of Plasticity in Sleep.

> *"(SHY) argues that learning is largely mediated by LTP. . . .Learning is a deceptively simple term for a complex set of neural events . . . while some forms of learning may be associated with LTP, others are not or involve a mixture of LTP and LTD-like synaptic changes . . . The potentiation hypothesized to occur in wakefulness is considered Hebbian . . ."*

SHY claims that learning—an enduring modification of brain circuits as a result of perception, cognition, and action that occurs throughout wakefulness, is inherently biased towards a net increase in synaptic strength. The postulated net increase in synaptic strength after wake was termed "LTP-like" with reference to the most studied experimental paradigms for producing long increases in synaptic efficacy, to indicate that it was an increase (potentiation) and that it was enduring (long term). In reviewing the available evidence for or against SHY when the hypothesis was first proposed, it was pointed out that there were many correlative, indirect data, such as gene expression changes, which were consistent with a predominance of LTP-like changes during wake, and moreover that LTP-like changes underlie the majority of learning paradigms that have been studied from a cellular perspective. This was before direct experimental tests inspired by the hypothesis could be performed, including the demonstration of wake-associated increases in evoked responses, in miniature synaptic potentials, in AMPA receptor density at the synapse, and in the number of synapses themselves [5–14].

As always, however, SHY did not endorse a particular mechanism of plasticity (hence LTP-like), only the end result. It never stated that wake-related potentiation is accounted for by a single mechanism such as classic "Hebbian" homosynaptic plasticity (occurring only at the stimulated synapse),

and in fact terms such as "Hebbian" or "homosynaptic" were never mentioned [1, 2]. Indeed, it is fair to ask what "homosynaptic" truly means, in light of the current evidence for synaptic tagging and capture, and recent data showing that the primary functional unit for long-term synaptic potentiation may be a dendritic branch, not an individual synapse [54].

Similarly, SHY never claimed that learning during wake only occurs via synaptic potentiation—just that the overall net result is biased towards potentiation. Indeed, there are several well-characterized forms of learning "by depression" that certainly occur during wake. These include reversal learning in the hippocampus, fear extinction in the amygdala, familiarity recognition in perirhynal cortex, and other forms of "behavioral flexibility" that involve either decreasing the response to a familiar stimulus or forgetting old strategies, objects, or spaces [55]. Fittingly, it appears that enduring synaptic depression is associated more with forgetting what was previously known, than with acquiring new knowledge. Consistent with this notion, acute stress impairs hippocampus-dependent memory retrieval, and hippocampal synaptic depression seems to play a role in this effect [55].

Of note, Frank quotes the work of Manahan-Vaughan and colleagues to make the point that learning includes both synaptic potentiation and depression. He fails to mention, however, that these authors, in discussing the overall implications of their results, noticed that exposure to a novel environment induces synaptic strengthening in all the four types of hippocampal synapses studied, while synaptic depression was not as universal. In their most recent paper, Manahan-Vaughan and colleagues concluded that synaptic potentiation "may represent a fundamental coding response to changes to the environment," whereas synaptic depression may add "a more qualitative component" ([56] page 2446 and Figure 10). Also, a study quoted by Frank in the section "Learning and LTP" as evidence for depression [57] is actually noncommittal about the mechanisms of learning but rather shows that long-term consolidation of spatial memory, 24 hours after learning, may require synaptic depression.

As anybody studying brain mechanisms of plasticity knows all too well, many forms of LTP-like paradigms have been described in different species, brain structures, and developmental times. Controversies have raged as to whether classic LTP/LTD paradigms induce changes resembling those occurring physiologically, whether changes are primarily postsynaptic, presynaptic, or both; whether changes are strictly "Hebbian" or not; whether *in vivo* synaptic changes are driven by mean firing rates, by spike-timing-dependent plasticity, or some other combination of mechanisms; whether changes are strictly confined to individual synapses or to a larger volume of neuropil; as to the involvement of glia, the role of neuromodulators, the participation of mitochondria and energy constraints, and of course to the particular molecular pathways and scores of molecular mechanisms that seem to be involved under different conditions. In short, the mechanisms of learning and plasticity are extraordinarily complex, involving at least dozens of different synaptic mechanisms and hundreds if not thousands of molecules, many still unknown or incompletely understood and run the gamut from short-term, medium-term, and long-term potentiation, to depression, depotentiation, spike-timing-dependent plasticity, scaling, intrinsic plasticity, structural plasticity, metaplasticity, and so on. SHY fully acknowledges this extreme biological complexity, but proposes that, (literally) at the end of the day, energy and information constraints on the brain—the fact that strong firing must be reserved for important signals that need to percolate among long chains of neurons—necessarily bias learning toward increasing overall synaptic strength.

> *"… surprisingly, many … findings cited in support of SHY are inconsistent with net synaptic downscaling…"*

Again, one needs to distinguish between the end result and the specific mechanism. As already stated, the evidence for a net decrease in synaptic strength or number after sleep is strong. If this were not the case, SHY would have to be abandoned. On the other hand, just as SHY did not commit to a specific mechanism resulting in net potentiation after wake, it also did not endorse a particular mechanism for bringing about net synaptic depression during sleep. While SHY referred to the recently discovered mechanisms of global synaptic scaling as a possible means for proportionally depressing synapses without compromising their relative strength, it did not endorse the specific mechanism. Indeed, an earlier paper already pointed out in 2001, as Frank does now in his commentary, that the available evidence was not supportive: "BDNF, which plays a critical role in synaptic scaling *in vitro*, is expressed at higher levels in the waking rather than in the sleeping brain" [58]. Moreover, SHY pointed out explicitly that synaptic scaling was meant to "ensure that neurons maintain a regulated firing level in the face of uncontrollable changes in their input," whereas synaptic homeostasis in sleep was meant to "ensure primarily the homeostatic control of synaptic weight, and only indirectly of neuronal firing levels" ([2], page 53). This point was further addressed in 2009, in a study that showed that cortical firing rates increase in the course of wake and decrease during sleep [17]. Since these sleep/wake changes in cortical firing are small, of the order of a few Hz, it was deemed unlikely that they could trigger the same homeostatic changes observed by Turrigiano and colleagues after extreme changes in firing rates ([17], page 874).

> *"What seems more likely is that sleep is characterized by multiple forms of synaptic plasticity, including classic Hebbian LTP and LTD, as well as downscaling and upscaling. This may explain why the evidence for "net" downscaling after sleep critically depends on what is measured (*e.g., *neuromodulin versus BDNF) …"*

Once more, it is virtually certain that multiple forms of plasticity can occur both in wake and in sleep—what matter for SHY is only whether wake inevitably tends toward net potentiation, and sleep is needed to restore homeostasis.

Before more direct tests of net synaptic strength could be performed, the overall picture provided by gene expression studies in the waking and sleeping brain [59–61], though imperfect and indirect, was at least broadly compatible with the core claim of SHY. However, it is clear that the expression level of any single molecule, be it BDNF, Arc, Homer, neuromodulin, or any other, is not substitute for a direct assessment of net synaptic strength. This is indeed what needs to be measured, and this is why, after SHY was first proposed, many experiments were performed to try and assess synaptic strength directly, using as many experimental approaches as possible: first molecular and electrophysiological markers *in vivo* (AMPA receptors density in synaptoneurosomes and slope of evoked responses), then electrophysiological markers *ex vivo* (minis), and finally structural markers (synapse size and number).

Incidentally, while as Frank suggests the results of early studies of gene expression in wake and sleep may have inspired the idea of a bias toward potentiation in wake, the central motivation for SHY was the search for a function for sleep that should be carried out off-line rather than on-line, and one that could have universal significance. Many ideas about the function of sleep start from the notion that wake may result in the accumulation of some "toxin" and that sleep may be necessary to restore the brain to a healthier state. If one adds the insight that one needs to explain the apparent need for such restoration to occur off-line, despite the considerable risk imposed by the disconnection from the environment, one could say that the "toxin" that necessarily accumulates in wake may be synaptic strength itself.

> *"… the term "net" is somewhat nebulous…" … "This broad description of the mechanisms of downscaling has the advantage that any evidence of synaptic weakening after sleep … can be cited in support of the theory. It is disadvantageous in that no single, clear mechanism is presented for careful and in depth investigation… One important future direction is to delve more deeply into the underlying mechanisms of SHY. To date, this has received less attention than studies aimed at collecting supportive findings."*

After evaluating the evidence for SHY as if the hypothesis were not about function but about the occurrence during sleep of a specific mechanism—activity-dependent scaling—and finding the evidence wanting, Frank then remarkably goes on to chastise SHY because it does not single out a single, clear mechanism. Once more, Frank seems to value whatever SHY may or may not say about mechanism much more than what it says about function. SHY aims at identifying a universal, essential function for sleep—a function that must necessarily transcend specific mechanisms if it is to apply to many species, brain circuits, and developmental periods in the face of an extraordinary biological diversity. This does not mean, however, that SHY ignores mechanisms. A case in point is the suggestion that, in mammals and birds, sleep slow waves may constitute an advantageous mechanism both for sampling in an unbiased manner the overall synaptic strength impinging on a neuron, and for renormalizing it in a controlled, self-limiting way [1, 2]. But of course, whether and how, exactly, slow waves may do so (with or without the contribution of other features of sleep, such as spindles), whether synaptic strength decreases in a proportional manner or enforces a competition between stronger and weaker synapses, older and newer memories, and so on, are questions that are as important as they are difficult to address experimentally.

Incidentally, characterizing the experimental work conducted so far as aimed at "collecting supportive findings" is puzzling because it suggests that evidence was collected selectively. The core claim of SHY, according to which wake led to a net increase in synaptic strength and sleep to a net decrease, had never been considered or tested before. *A priori*, the results of many different experiments conducted in different species and with different approaches could easily have been negative—most of the plasticity literature implicitly assumes that a balance between potentiation and depression is a given—or it might have turned out that sleep leads to a net potentiation. Collecting evidence that turned out to be supportive (so far) is not the same as "collecting supportive evidence."

4.2. On the Role of Slow Wave Activity in Synaptic Weakening. Sleep SWA features in two prominent corollaries of SHY, in the first as a possible sensor of synaptic weight, and in the second as a possible effector of sleep-dependent synaptic renormalization. Frank discusses these two corollaries at some length, and it is important to maintain a clear distinction between these two postulated roles for sleep SWA.

The evidence for SWA as an index of synaptic strength rests on the fact that the sleep slow waves recorded from the scalp by the EEG are a reflection of near-synchronous transitions between up and down states in large populations of cortical neurons [62, 63]. Both theoretical considerations [64], large-scale simulations [15] and empirical studies, [16, 17] indicate that the amplitude and slope of sleep slow waves are related to the number of neurons that enter an up state or a down state near-synchronously, and that synchrony is directly related to the number and strength of synaptic connections among them. More specifically, the data showing that SWA can be used as a "proxy" of synaptic strength come from studies in humans using high-density EEG and, in animals, from experimental approaches that can reveal the local aspect of sleep regulation. For instance, in humans, SWA increases locally over parietal cortex following learning of a visuomotor task [22], while arm immobilization during the day, which leads to a decrease in motor performance and sensory evoked responses, consistent with synaptic depression, is followed by reduced SWA over the contralateral sensorimotor cortex [65]. Cortical potentiation and depression triggered in humans by paired-associative stimulation also result in increase and decrease in SWA, respectively [66]. In rats, training on a reaching task known to induce long-term synaptic potentiation results in a local increase in SWA in the activated motor region [23]. Cortical infusion of BDNF, whose local brain application *in vivo* is sufficient to induce synaptic potentiation, results in an

increase in SWA only in the injected cortex [67]. Moreover, in the rat cortex, the wake-related increase in the slope of local field potentials discussed above correlates with the increase in SWA: the steeper the slope at the end of wake, the higher SWA at sleep onset [5].

There are also developmental studies that link SWA to synaptic strength. In both mice and cats, visual deprivation during the critical period, which is associated with synaptic depression [68], results in a 40% decrease in SWA [69]. Moreover, recent and growing evidence in humans suggests that the well-documented inverted U curve of SWA during development, with an early progressive increase during childhood, followed by a rapid decline, may reflect the equally well-documented early cortical increase in synaptic density, followed by synaptic pruning during adolescence [70–74]. In summary, it seems that the evidence for SWA as a sensor of synaptic strength is quite strong. Instead, the role of SWA as an effector of sleep-related synaptic renormalization remains hypothetical: SWA may not be an effector at all or may be just one of the mechanisms to achieve synaptic downregulation, and perhaps only in some animal species [2, 6, 8, 31].

> *"if decreases in SWA directly reflect decreases in synaptic strength . . . . . physiological markers of synaptic weakening should be detectable when SWA first declines . . . .The few studies . . . .have produced very mixed results . . . . "*

Frank rightly notices that more studies are needed to show that the time course of synaptic renormalization is linked to that of sleep. However, some evidence that markers of synaptic strength decline in proportion to sleep already exists. In flies, spine pruning after an enriched experience occurs only if they are allowed to sleep, but not if they remain awake [8]. Crucially, spine density was negatively correlated with the amount of sleep during the last 7 hours, as well as with the maximal duration of sleep bouts [8]. Turning to electrophysiological markers in rodents, local field potentials were recorded from left frontal cortex after electrical stimulation of the right frontal cortex. After transcallosal stimulation, the slope of the first negative component of cortical evoked responses—a monosynaptic response—increased after wake and decreased after sleep [5]. Importantly, changes in slope were correlated with the duration of prior wake or NREM sleep. In relation to this study, Frank points out a "discrepancy" with another study in the visual cortex, in which evoked responses declined in amplitude during the active phase [75]. In fact, the two studies were designed to ask very different questions, and thus differed in a crucial experimental detail. The Vyazovskiy study was designed to assess how sleep/wake history affects cortical strength, and, therefore, the behavioral state was kept constant (quiet wake) at the time the evoked responses were collected. By contrast, Tsanov and colleagues compared responses collected during sleep with those collected during wake. This is crucial because independent of sleep/wake history, evoked responses are much larger during sleep than during wake. Thus, inferences about 24-hour changes in cortical strength are simply impossible to make if one does not control for behavioral state. A recent study in humans also found that human cortical evoked responses, reflected in the immediate (0–20 ms) electroencephalographic reaction to transcranial magnetic stimulation, progressively increased with time awake, from morning to evening and after one night of sleep deprivation, and decreased after recovery sleep [14]. This study, as the rat study, collected evoked responses during the same behavioral state (wake), and after controlling for drowsiness [14].

4.3. On the Evidence for SHY, in Mammals and in Insects.

> *". . . SHY is supported by an impressive number of findings . . . mostly reported by the same group . . . "*

Over the past several years, the core claim of SHY has been put to direct test using several experimental approaches aimed at estimating synaptic efficacy, in different species, *in vivo* as well as *ex vivo*. These include molecular studies in rats (changes in AMPA receptors; [5]) and flies [7]; electrophysiological studies in rodents and humans, including changes in the slope of evoked responses *in vivo* [5], changes in cortical excitability as assessed by transcranial magnetic stimulation [14], and changes in frequency and amplitude of minis *in vitro* [6]; as well as morphological studies showing changes in the number of synapses in flies [7, 8] and mice [9]. None of these approaches, taken in isolation, can offer an exhaustive, unambiguous view of synaptic efficacy: morphological changes in the number or size of synapses are not necessarily accompanied by changes in their efficacy; changes in the number of AMPA receptors in synaptic fractions cannot tell how functional those receptors may be; changes in spontaneous miniature synaptic potentials (minis) measured *ex vivo* may not accurately reflect the efficacy of synapses when neural activity is high *in vivo*; and changes in field evoked responses after electrical or magnetic stimulation cannot easily distinguish between changes in synaptic strength and changes in neuronal excitability due to other causes. Nevertheless, taken together, these various sources of evidence complement each other. While it is true that the results mentioned above were obtained from the same laboratory (or through collaborations), several of the findings have already received independent support from three different laboratories [10–13]. Obviously, further direct tests of the main tenets of SHY—in different species, brain structures, and developmental periods—will be important to establish if and to what extent the predictions of the hypothesis can be generalized.

> *". . . the most dramatic evidence of SHY is found in ectothermic insects . . . "*

Frank sees the structural changes in flies as the most dramatic examples of sleep/wake effects on synaptic strength. While this may indeed be the case, trying to compare effect size across studies done in different species and using very different methods is tricky. At this point, it is not obvious that when comparing wake to sleep, the 100% increase in the frequency of miniature postsynaptic currents observed in rodent cortex [6], or the 30% increase in the number of

synaptic AMPA receptors across the entire rat cortex [5], reflects a less significant change in synaptic strength than the 2-fold increase in size of presynaptic terminals or the 30% increase in spine density seen in the fly brain [8]. What matters most, of course, is that all of these findings go in the same direction.

> "*It also appears that SWA cannot be a common mechanism for downscaling in mammals and insects.*"

It is currently unknown whether neurons in the fly brain (or in the brain of other insects) undergo slow oscillations in membrane potential or alternate between firing and silence during sleep. Do they instead stop firing altogether, as is reportedly the case in some part of the mammalian brainstem? It is also unknown whether sleep and wake are accompanied by systematic changes in the levels of neuromodulators. On the other hand, since the demonstration that fruit flies sleep more than 10 years ago [36, 37], it has become especially relevant to try and identify a universal function for sleep that might apply also to invertebrates. For this reason, it seemed important to establish whether in flies, too, sleep would renormalize synapses. We now know that, at least in *Drosophila*, there is a major reduction in the number of synapses and in the expression of both pre- and postsynaptic proteins after sleep. How this renormalization happens—especially if it happens using very different mechanisms from those employed in mammalian cortex—is an intriguing question for the future.

4.4. On the Role of Temperature and Glucocorticoids. An important issue brought up by Frank concerns alternative mechanisms that could account for the observed changes in synaptic strength across sleep and wake: specifically, changes in brain temperature and changes in glucocorticoids levels. In principle, these mechanisms could complement the others discussed in SHY, such as the switch between tonic and burst firing that accompanies the transition from wake to sleep, and the changes in the levels of neuromodulators. As discussed below, however, the evidence supporting a role for temperature and glucocorticoids is far from compelling.

In relation to changes in brain temperature, Frank notices that the sleep/wake changes in dendritic branching and spine number that were observed in flies (e.g., a ~30% change in spine density in visual neurons) are similar to those seen in hibernators. However, during hibernation, core temperature drops by 20–30°C, and the work quoted by Frank [76] shows that there is a linear relationship between temperature and spine density: a ~30°C drop in core temperature during hibernation leads to a ~30% decrease in spine density, while a ~20°C drop results in a ~20% spine decrease. In the *Drosophila* studies, on the other hand, flies were kept inside environmental chambers whose temperature was carefully maintained at 20°C at all times. Moreover, presynaptic structural changes occurred in flies kept in small glass tubes that allow for little movement, so it is unlikely that locomotor activity could cause major changes in core temperature—of the order of 15–30°C—that are necessary to trigger massive dendritic and spine remodeling. In relation to the results obtained in rodent cerebral cortex, Frank also refers to *in vivo* studies showing that locomotor activity can enhance synaptic currents in the hippocampus by increasing hippocampal temperature by 2-3°C [77]. However, as described in detail in the original publication, molecular results (e.g., AMPA receptors changes) were obtained from rats whose cortical temperature increased by 0.3-0.4°C in wake relative to sleep [5]. Moreover, the cortical evoked responses in the two experimental conditions—"after sleep" and "after wake"—were collected in the same behavioral state, quiet wake.

Glucocorticoids can both enhance and suppress synaptic plasticity. Frank quotes evidence for their role in enhancing glutamatergic transmission and AMPA receptor trafficking, but there is strong evidence also for the opposite: stress-induced glucocorticoids also reduce synaptic efficacy in cortex [78], affect AMPA receptor trafficking in a way conducive to synaptic depression [79], and lead to long-lasting net spine elimination in cortex [80]. This last study is especially relevant because it was performed in the same mouse strain, of the same age (~1 month old), using the same method (*in vivo* repeated two-photon imaging), and focusing on the same cortical area (barrel cortex) as the study of synaptogenesis and pruning as a function of sleep and wake [9]. Maret, Faraguna, and colleagues found that spine growth and loss occur at all times, but growth prevails over loss during wake, while the opposite occurs in sleep. Liston and Gan found instead that acute and chronic corticosteroid treatment increases both spine formation and elimination, but the latter more than the former, resulting in a net decrease in spine density in the long run. Therefore, at least in the adolescent mouse cortex, it seems unlikely that the net effects of sleep/wake on spine turnover can be ascribed to glucocorticoids.

More generally, not unlike catecholamines, glucocorticoids are important for optimal performance and behavioral adaptation. Thus, it is reasonable to assume that mildly elevated levels of glucocorticoids and catecholamines during wake may both contribute to the net increase in synaptic strength observed in this behavioral state [81]. Direct evidence for this is lacking, however, and in fact the only available data suggest a very different picture. Specifically, lesion studies show that the induction of plasticity-related genes such as *BDNF* (for which a role in synaptic potentiation is overwhelming) and the associated buildup of sleep pressure are related to the activation of the noradrenergic system [59, 82, 83], while corticosterone affects neither the induction of these genes nor the homeostatic regulation of sleep [84]. Moreover, there is some evidence that low levels of catecholamines and of BDNF [32, 33, 85] may promote synaptic depression, at least *in vitro*, while no such evidence is available for low levels of glucocorticoids.

4.5. On the Meaning of Synaptic Homeostasis in Development

> "*... sleep amounts are maximal during periods of heightened synaptogenesis including in utero when waking experience is negligible. It seems*

highly unlikely that a fundamental purpose of sleep is to principally weaken synapses during these developmental periods."

When SHY was initially proposed, it emphasized predictions that could be tested in adult mammals, where sleep would be essential in rebalancing net synaptic strength that is biased towards an increase during wake. Many of these predictions have since been corroborated. However, it was clear from the start that if sleep serves an essential function, and if that function is synaptic homeostasis, then it should apply even more to development, a time when sleep is an even more prominent part of life. Indeed, the main reason why sleep need, and thereby the need for synaptic homeostasis, would be paramount during development is fairly obvious, and it has little to do with the amount of wake: it is well known that neurodevelopment is characterized by an early phase of net synaptogenesis, followed by net pruning [4]. The increase in the number of synapses during early development is explosive, and it is bound to pose even greater challenges to neurons (and glia) than the increase in synaptic strength that occurs during wake in adult mammals: it is hard to imagine that such a massive, fast-paced formation of new synapses can be perfectly regulated, precisely titrating the total amount of synaptic weight impinging on each neuron. It is much more likely that, as a rule, during synaptogenesis neurons may undergo a substantial synaptic overload, such that proper function requires an equally substantial restoration of synaptic homeostasis. For the reasons discussed in the previous sections, such rebalancing is best achieved off-line, when a neuron can sample most of its inputs in an unbiased manner and make the necessary adjustments. The very first studies investigating the occurrence of sleep-dependent synaptic homeostasis during neurodevelopment (adolescent mice) once again support the idea that sleep is associated with a net decrease in the number of synapses [9, 13], thus possibly helping to maintain synaptic homeostasis in the face of ongoing synaptogenesis. Whether sleep plays a similar role in earlier developmental stages, the role played by different kinds of sleep, and the consequences of sleep deprivation at such critical periods on appropriate pruning and refinement of neural circuits are key questions that await investigation.

4.6. On "Why Stronger Synapses Should Make One Sleepy." Frank correctly points out that it is not clear why stronger synapses, if that is indeed the price we pay for plasticity, should produce sleepiness. It is worth remembering, however, why according to SHY renormalization is necessary: a net increase in synaptic strength comes at a substantial price to nerve cells, most relevantly in this context a price in terms of energy metabolism, since stronger synapses consume more energy [42, 43]; and in terms of cellular supplies, since stronger synapses are likely to require more building blocks and may stress the supply-and-demand requirements of neurons. After all, neurons are unique in their need to sustain thousands of synapses distributed along an extraordinary large axonal and dendritic tree, and if these synapses become on average stronger, so do their demands on the cell. There is indeed evidence that markers of cellular stress, such as BiP, are higher after wake [86]. A tendency for increased adenosine—a marker of energetic stress—has also been observed in several brain structures after extended wake [87].

Recently, it was reported that, the longer a rat stays awake, the more cortical neurons show brief periods of silence in their firing that are essentially indistinguishable from the OFF periods observed during slow oscillations in a sleeping animal [88]. These OFF periods are local, in that they may occur at different times in different brain regions, and when they occur in the wrong region at the wrong time, they can produce performance deficits. Preliminary results indicate that such OFF periods become more frequent after the induction of LTP, as well as after intense learning. While it is unknown what drives the occurrence of OFF periods at the cellular level, it is conceivable that net synaptic strengthening may have something to do with it, for example, due to increased metabolic demand. If indeed a progressively larger fraction of neurons in the brain begins to undergo local sleep, and especially if neurons in hypothalamic and brainstem areas that exert a central control on wake and arousal also suffer from synaptic overload and respond by going briefly off line, it would not be surprising if sleepiness would also increase. In fact, whether and how a net increase in synaptic strength may translate into an increased drive for hyperpolarization and an increased occurrence of OFF periods is an experimental question motivated by SHY that seems ideally suited for mechanistic investigations.

5. Conclusion

Frank's detailed commentary provides a helpful, critical review of the evidence concerning the mechanisms that may bring about an imbalance of synaptic homeostasis during wake and its restoration by sleep. As this response hopefully shows, it is important to distinguish between the particular mechanisms of plasticity that are engaged in the waking and sleeping brain and the universal, essential function that SHY attributes to sleep—the reestablishment of synaptic homeostasis. So far, structural, molecular, and electrophysiological studies support the notion that sleep leads to the renormalization of synaptic strength in several species. Instead, the specific mechanisms involved in the constant battle between upregulation and rebalancing of synaptic strength are bound to be many, not mutually exclusive, and different in different species, brain structures, and developmental periods. While SHY offers several corollary claims, such as the significance of sleep slow waves in mammals and birds, its core claim remains that sleep is universally needed to combat, through off-line renormalization, the neural costs of increasing synaptic strength: energy, space, cellular supplies, signal-to-noise ratios, and saturation of the ability to learn.

Abbreviations

LTD: Long-term depression
LTP: Long-term potentiation
SHY: Synaptic homeostasis hypothesis
SWA: Slow wave activity.

Authors' Contribution

Both authors wrote the manuscript.

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Review Article

Erasing Synapses in Sleep: Is It Time to Be SHY?

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Converging lines of evidence strongly support a role for sleep in brain plasticity. An elegant idea that may explain how sleep accomplishes this role is the "synaptic homeostasis hypothesis (SHY)." According to SHY, sleep promotes net synaptic weakening which offsets net synaptic strengthening that occurs during wakefulness. SHY is intuitively appealing because it relates the homeostatic regulation of sleep to an important function (synaptic plasticity). SHY has also received important experimental support from recent studies in *Drosophila melanogaster*. There remain, however, a number of unanswered questions about SHY. What is the cellular mechanism governing SHY? How does it fit with what we know about plasticity mechanisms in the brain? In this review, I discuss the evidence and theory of SHY in the context of what is known about Hebbian and non-Hebbian synaptic plasticity. I conclude that while SHY remains an elegant idea, the underlying mechanisms are mysterious and its functional significance unknown.

1. Introduction

A preponderance of evidence supports the view that sleep promotes brain plasticity. For example, a large number of studies in humans and animals show that sleep enhances and stabilizes memory (i.e., consolidation, reviewed in [1–3]). What remain more mysterious are the underlying cellular mechanisms that promote plastic changes in the sleeping brain. Until quite recently, the synaptic mechanisms were generally considered to be Hebbian. That is, scientists conceptualized and investigated the problem in terms of what was known about long-term synaptic potentiation (LTP) and depression (LTD) (reviewed in [4, 5]). These forms of plasticity are considered Hebbian because they involve changes in specific synapses mediated by coordinated activity in pre- and postsynaptic neurons (Figure 1). In the late 1990s, a non-Hebbian type of plasticity was described that adjusted all synapses in a neuron or network of neurons upward or downward in response to global changes in activity (reviewed in [6–8]). This type of plasticity was dubbed "synaptic scaling" or "homeostatic synaptic plasticity" (Figure 2), and as a concept was incorporated into the synaptic homeostasis hypothesis (SHY) [9–11].

According to SHY, sleep promotes global, or "net" synaptic downscaling which offsets global or "net" synaptic potentiation produced by wakefulness [9, 10]. The idea that sleep weakens synapses is not novel [4, 12, 13], but SHY has a number of unique aspects. First, it attempts to connect the homeostatic regulation of sleep to a putative function of sleep (plasticity). Sleep homeostasis refers to an enigmatic process that makes animals sleep longer (or more intensely) as a function of prior time awake [14]. It is logical that the regulation of sleep is linked to its core function [15], but the nature of this linkage has proven elusive [16]. Second, SHY is supported by an impressive number of findings in insects [17–19], rodents [20–22], and humans [23], mostly reported by the same group. These experiments use an equally impressive variety of tools including sophisticated molecular, cellular, electrophysiological, and computational techniques.

Despite the elegance of SHY and the arsenal of resources employed in its pursuit, there remain important unanswered questions about its core concepts, and the significance of its supportive findings. In this review, I take a closer look at SHY. I begin by briefly reviewing the basic concepts of synaptic scaling (see [6–8, 24] for more extensive discussion).

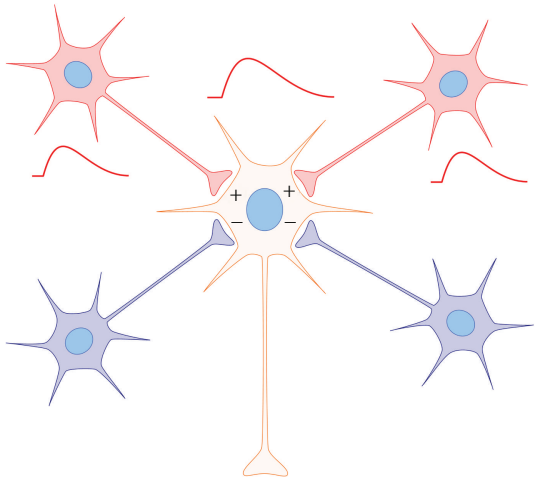

Figure 1: Hebbian plasticity. Classic Hebbian plasticity includes homosynaptic long-term synaptic potentiation (LTP) and long-term synaptic depression (LTD). Coincident activation in pre-synaptic neuronal inputs and the post-synaptic neuron strengthens specific synapses (shown in red). Inactive pre-synaptic inputs (or inputs out of phase with post-synaptic depolarization) are not potentiated and/or are depressed (shown in blue). The term "homosynaptic" refers to the fact that plasticity only occurs at the stimulated synapse.

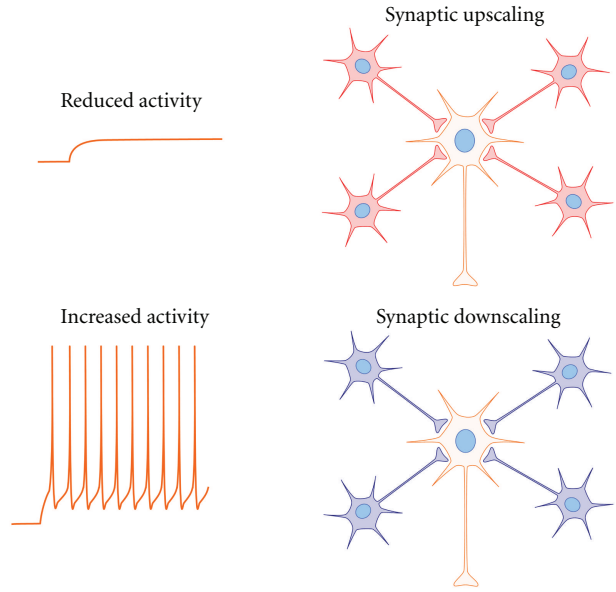

Figure 2: Synaptic scaling. Synaptic scaling involves global adjustments of all synapses in a neuron in response to global changes in neuronal activity. *Decreases* in neuronal activity lead to global increases in synaptic efficacy in the target neuron (upscaling). *Increases* in neuronal activity lead to global decreases in synaptic efficacy in the target neuron (downscaling). This form of plasticity is considered "non-Hebbian" because it involves global adjustments of synapses, rather than input-specific changes in discrete synapses.

I then review the theory of SHY and its empirical supports. I then address the proposed mechanisms governing SHY and the extent to which they agree with our current understanding of synaptic plasticity. In cases where they do not agree, alternative mechanisms are considered. I then discuss unanswered questions and future experiments that may provide strong tests of SHY.

2. Synaptic Scaling

Synaptic scaling refers to global adjustments of all synapses in a neuron or a neuronal network in response to global changes in activity. These adjustments manifest either as changes in synapse number or in post synaptic electrical currents (defined here as "synaptic efficacy") [6–8, 24]. They are considered homeostatic because they restore total synaptic inputs to a specific range while maintaining the *relative* strength of all synapses. Synapses can be "downscaled" and "upscaled" which is thought to offset Hebbian changes that if left unchecked would quickly saturate synaptic strength in a network [6–8]. Because synaptic scaling involves global changes in synapses, rather than input-specific change at a given synapse, it is considered non-Hebbian. More recent work suggests that synaptic scaling can also occur regionally (i.e., "local" scaling) but since this is less understood, it is not discussed further here [6–8, 24]. In addition, there is increasing evidence that synaptic scaling also occurs in inhibitory circuits [6–8]. However, as inhibitory synapses do not factor prominently in SHY [9, 10], inhibitory scaling is also not discussed.

The central principle of synaptic scaling is quite simple: *decreases* in neuronal or network activity *upscale* synapses while *increases* in neuronal or network activity *downscale* synapses. This principle is key to our later discussion of the mechanisms of SHY. The first demonstration of synaptic scaling was made in cell culture where drugs that inhibited neuronal activity (e.g., tetrodotoxin) led to upscaling while drugs that increased neuronal activity (e.g., bicuculine) led to downscaling [6–8]. The effects of synaptic scaling manifested as changes in the frequency or amplitude of miniature excitatory postsynaptic currents (mEPSCs). More recent studies suggest that synaptic scaling also occurs *in vivo* under more naturalistic manipulations. For example, sensory deprivation *in vivo* leads to compensatory synaptic upscaling (reviewed in [6]) as measured by changes in dendrite spine morphology [25] and cortical mEPSCs [26]. In addition, although early *in vitro* studies suggested that scaling was a slow process (occurring over 24–48 hours [27]), more recent findings demonstrate that it can occur much more rapidly (over minutes [28]).

2.1. The Cellular Mechanisms of Synaptic Scaling. A very exciting area of neuroscience is the search for what distinguishes scaling mechanistically from classic Hebbian forms of plasticity like LTP. This understanding remains far from complete, but several important clues have been discovered. Calcium flux appears central to the initiation of synaptic scaling [6, 7]. In cortical neurons, decreases in intracellular calcium lead to upscaling, while increases lead to downscaling. These events are in turn mediated by calcium-/calmodulin- dependent kinases which remove or insert

Table 1: Scaling factors and the sleep-wake cycle.

	Wake	Sleep	Promotes
BDNF	↑↑	↓↓	Synaptic downscaling [7, 30–33]
Arc	↑↑	↓↓	Synaptic downscaling [30, 31, 34–37]
Homer1A	↑↑	↓↓	Synaptic downscaling [34, 37]
Tnfα	↓↓	↑↑	Synaptic upscaling [38–40]
Retinoic acid	??	??	Synaptic upscaling* [41, 42]

The expression of scaling factors is inconsistent with net downscaling during sleep.
*Retinoic acid is linked to SWA generation, but sleep/wake expression patterns are unknown.

AMPA receptors into the plasma membrane [29]. Although Hebbian LTP and LTD also involve the trafficking of AMPAR this requires different calcium dynamics (opposite to those mediate upscaling and downscaling, resp.). Other signaling molecules linked to synaptic scaling are shown in Table 1. Some of these molecules and signaling pathways have also been examined across sleep and wakefulness (more detailed description of these mechanisms can be found in [7, 24]). Their pattern of expression will also be key to our discussion of SHY.

3. The Synaptic Homeostasis Hypothesis (SHY)

The central principle of SHY is also refreshingly simple as it embodies the basic concept of synaptic scaling and its importance into the sleep/wake cycle. Wakefulness is associated with net synaptic potentiation while sleep is associated with net synaptic downscaling. Although the term "net" is somewhat nebulous, this construction is useful because it divides the scaling problem neatly into two parts. The potentiation hypothesized to occur in wakefulness is considered Hebbian, because in the original description of SHY, analogies are drawn between molecular and cellular correlates of wakefulness and LTP. For example, as stated by Tononi:

> "…the first part of the hypothesis states that wakefulness is generally accompanied by LTP-like changes in the brain…" and that molecular correlates of LTP are "restricted to wakefulness" ([9, page 144]). This proposition is retained in a second theoretical paper by Tononi:
>
> "During wakefulness we interact with the environment and acquire information about it…the neuromodulatory milieu (e.g., high levels of noradrenaline, NA) favors the storage of information, which occurs largely through long-term potentiation of synaptic strength" ([10, page 50]).

With respect to synaptic downscaling, this is hypothesized to be driven by slow-wave electroencephalogram (EEG) activity (SWA) in non-REM sleep:

> "According to the hypothesis, slow waves occurring in the cortex during sleep would actively promote a generalized depression or downscaling of synapses." ([9, page 145]).
>
> And "…slow waves are not just an epiphenomenon of the increased synaptic strength, but have a role to play. The repeated sequences of depolarization-hyperpolarization cause the downscaling of the synapses impinging on each neuron…" ([10, page 53]).

In contrast to the proposed Hebbian-like synaptic changes in wakefulness, how SWA downscales synapses is less precisely defined:

> "…we hypothesize that downscaling is likely to use many of the same molecular mechanisms involved in depression/deprotentiation and activity-dependent scaling" ([10, page 54]).

This broad description of the mechanisms of downscaling has the advantage that *any* evidence of synaptic weakening after sleep, however measured, can be cited in support of the theory. It is disadvantageous in that no single, clear mechanism is presented for careful and in depth investigation. This is a limitation of SHY because it leaves supportive findings open to alternative explanations that may be unrelated to sleep.

4. The Theory of SHY Re-Examined

Let us consider the principal claims of SHY. First, it is argued that learning (a waking phenomenon) is largely mediated by LTP. Second, it is argued that the neurochemical and molecular milieu of wakefulness preferentially favors synaptic strengthening while sleep favors synaptic weakening.

4.1. Learning and LTP. Learning is a deceptively simple term for a complex set of neural events, often involving multiple brain areas and signaling pathways [43–45]. Perhaps not surprisingly, while some forms of learning may be associated with LTP [46, 47], others are not or involve a mixture of LTP- and LTD-like synaptic changes [48–51]. For example, extinction is a form of learning with obvious survival value to animals [52] as it allows them to change prior learned behaviors in light of new information. Several forms of extinction involve synaptic weakening, either through NMDA receptor mediated LTD [53] or endocannabinoid-mediated LTD [54]. A related phenomenon, the ability to reverse learning in order to adapt to new information (behavioral flexibility) requires LTD-like mechanisms (e.g., AMPA receptor internalization) [55]. Spatial memory also appears to require LTD and AMPAR internalization [56]. Exposure to novel environments induces (or involves) LTP-like synaptic changes in the hippocampus, *and* changes similar to LTD [48–50]. In perirhinal cortex, visual experience weakens responses to familiar visual stimuli, a phenomenon that may contribute to visual recognition memory. More specifically, LTD is prominent in perirhinal cortex, and

peptides that block AMPA receptor internalization block both LTD and visual recognition memory [57, 58]. A requirement for LTD in some forms of learning is not restricted to vertebrates. Associative olfactory learning in the honey bee mushroom body involves a dampening of responses in PE1 neurons—which in turn may involve LTD or changes in inhibitory input [59]. To summarize, there are many forms of learning that require different types of synaptic plasticity. It is thus improbable that sleep need—to the extent this is determined by learning—is determined solely by Hebbian LTP (or any other single form of synaptic strengthening).

4.2. Sleep, Synaptic Strengthening, and Synaptic Weakening. SHY appears to have its origins in two molecular studies from the Tononi and Cirelli laboratory [11]. It was shown that a number of mRNAs implicated in LTP such as *bdnf*, *arc,* and *narp* were upregulated in the neocortex in animals sacrificed after the end of the normal wake period, or after an additional 6 hour sleep deprivation period which extended into the rest period. For *bdnf* and *arc*, the cortical expression of transcript and protein required noradrenaline (NA) as NA depletion reduced the expression of these molecules during wakefulness [30, 31]. It was then proposed that the comparatively low levels of NA during sleep ensured that "synaptic activity is not followed by synaptic potentiation" ([10, page 51]).

Before addressing this issue, two caveats should be discussed. First, there are relatively few plasticity-related molecules with single effects on synaptic efficacy. Molecules reported at higher levels in the neocortex after wakefulness relative to sleep mediate not only LTP, but also LTD, non-Hebbian scaling (e.g., Arc [30, 60]), and the synthesis of GABA in inhibitory interneurons (i.e., BDNF [61, 62]). Other molecules cited as evidence of synaptic weakening during sleep (e.g., CaMKIV [30]) are also required for some forms of LTP [63]. Therefore, the mere appearance of these molecules—especially if only measured at the transcript level—does not tell you if synapses are weakening or strengthening. Second, neuromodulators such as NA have complex and diverse effects on synaptic plasticity. NA, acetylcholine, and serotonin can promote LTP or LTD depending on brain location (or cortical lamina) and receptor subtypes [64–71]. Thus, the type and valence of plastic change cannot be predicted solely by the relative concentrations of these neuromodulators.

The cerebellum provides an illustrative example of this problem. The cerebellum exhibits state-dependent changes in mRNA transcript levels that are strikingly similar to those in the neocortex [30]. In the neocortex *and* cerebellum mRNA transcript levels of *bdnf* and *narp* are higher when measured after wakefulness (relative to sleep). Learning and plasticity in the neocortex *and* cerebellum, are also both strongly influenced by NA [72, 73]. Learning and plasticity in the cerebellum, however, appear to be governed by LTD, and to a lesser extent, forms of LTP that are quite distinct from those observed in the neocortex [21]. With these caveats in mind, let us consider whether molecular and neurochemical changes conducive for synaptic potentiation also occur during sleep.

There is some molecular evidence for "net" synaptic potentiation during sleep. This includes the observation that nonREM sleep promotes neural protein synthesis—an essential step in persistent forms of synaptic potentiation [74–77]. The complete identities of these proteins are unknown, but some are involved in LTP. For example, a proteomic study in adult rats [77] identified two of these cortical proteins as actin and neuromodulin which play important roles in pre- and postsynaptic modifications, respectively, in LTP [78, 79]. Sleep deprivation also reduces forebrain concentrations of several proteins implicated in LTP (e.g., snap25b, NSF, neuromodulin, neurogranin) [80–83]. The latter experiments suggest that these proteins are normally synthesized during sleep. Collectively, these findings are consistent with net synaptic potentiation as they are based on overall changes within large areas of the brain (e.g., cortical or forebrain)—as opposed to discrete synapses.

Sleep can also promote molecular events conducive for synaptic potentiation within specific regions of the cerebral cortex. During a critical period of development, if vision in one eye is occluded (monocular deprivation: MD), most cortical neurons lose their ability to respond to the deprived eye [84, 85]. This is followed by a strengthening of response to the nondeprived eye [86–88] and anatomical rearrangements of thalamocortical and intracortical circuitry in favor of the intact visual pathway [89, 90]. This form of plasticity, known as ocular dominance plasticity, is considered a canonical model of synaptic plasticity *in vivo* [91, 92].

In the cat, ocular dominance plasticity is consolidated by sleep and this involves synaptic weakening *and* strengthening of cortical circuits [93, 94]. For example, when cortical responses are measured after a period of MD and sleep, responses to the deprived are weaker and responses to the open eye are stronger [94]. The sleep-dependent strengthening of cortical responses is best explained as an increase in glutamatergic synaptic strength. First, consistent with results from LTP protocols, after 1-2 hours of post-MD sleep cortical AMPAR, glur1 subunits are phosphorylated at two sites [94] known to lead to trafficking and insertion of AMPAR in the postsynaptic membrane [95, 96]. The first few hours of sleep are also accompanied by cortical activation of two kinases implicated in LTP at glutamatergic synapses (ERK and CaMKII) [94] and by heightened mTOR-dependent protein synthesis, and increased cortical expression of proteins implicated in LTP (e.g., BDNF and PSD-95) [97]. In rodents, the enhanced response to the open eye is dependent upon Tnfα—which promotes glutamatergic synaptic upscaling [98] and is at its highest brain concentrations during sleep [99]. The role of Tnfα in feline ODP is unknown, but collectively these findings indicate that the enhanced response to the nondeprived eye involves glutamatergic synaptic potentiation. Interestingly, the effects of MD on nondeprived visual pathways (synaptic potentiation) are retained into adulthood [87, 88]. Given the necessity of sleep for this type of plasticity, this suggests that sleep might have similar effects on cortical circuits throughout the lifespan.

Molecular changes in sleep conducive for synaptic potentiation also occur in adult animals. Sleep-dependent consolidation of two-way active avoidance learning in adult rats is correlated with hippocampal immediate-early gene expression and protein phosphorylation in the first few hours following training [100, 101]. Increased hippocampal expression of the LTP-related gene *zif268* has been reported in adult rats during rapid eye movement (REM) sleep following exposure to novel, enriched environments [102]. A similar expression of *zif268* is also reported in the hippocampus and cortex during REM sleep following LTP protocols *in vivo* [103]. REM sleep deprivation reduces several molecular markers/mediators of LTP and the ability to induce LTP in the hippocampus; events that are reversed when animals are allowed recovery sleep [104].

The neurochemical milieu of the sleeping brain may also promote synaptic potentiation under certain conditions. REM sleep, for example is well suited for synaptic potentiation as it is characterized by waking levels of membrane depolarization combined with elevated cortical levels of acetylcholine. Indeed, LTP can be reliably induced during this state (reviewed in [4, 5]). Recent studies also show that non-REM sleep can be accompanied by increases in neuromodulators that, according to SHY, mediate synaptic potentiation [105, 106]. Chronic recordings of NA neurons in the principle forebrain source of NA (the locus coeruleus (LC)) show that LC neurons increase their activity during the first 2 hours of post-learning sleep [106]. Intriguingly, the activity of LC neurons is time locked to the cortical slow oscillation of non-REM sleep. LC neurons increase their activity on the rising limb of the cortical upstate [105]; a sequence of events that theoretically could potentiate synapses. An important functional role for such nonREM LC activation is suggested by two recent studies in humans that have shown that manipulating NA during sleep alters olfactory-based, and hippocampal-amygdalar-based learning [107, 108].

5. Evidence in Support of SHY

An impressive number of studies provide evidence consistent with SHY. I restrict my discussion to what I consider the most compelling studies. The first are studies in rodents which, in addition to the molecular studies already discussed, report changes in proteins, synaptic efficacy, and dendrite morphology consistent with predictions of SHY [20–22]. Briefly, they show that markers of synaptic potentiation (e.g., changes in AMPAR subunit number or phosphorylation) are elevated in the brains of adult rats sacrificed at the end of the active phase (or after sleep deprivation), relative to animals sacrificed at the end of the rest phase [20]. Similar results are reported for measures of synaptic efficacy (electrically evoked cortical potentials and mEPSCs), which are also elevated at the end of the active phase (or after sleep deprivation) relative to sleep [20, 22]. Two recent imaging studies of cortical dendrite spine morphology showed that the ratio of spines eliminated versus those formed was greater after a period of sleep than a period of wakefulness [21, 109]. Interestingly, these results were restricted to stages of development when there is an overall pruning of synapses and were entirely absent in adult mice [21]. The second are experiments in *Drosophila melanogaster* which show changes in synaptic proteins or morphology consistent with SHY [17–19]. In Drosophila, pre- and postsynaptic proteins and proteins involved in neurotransmitter release are elevated in the brain after extended waking periods or sleep deprivation (relative to sleep) [17]. A second study showed that presynaptic structures, axonal arbors, and postsynaptic spines in Drosophila neurons expanded after extended waking periods (or sleep deprivation); a process also reversed by extended periods of sleep [18]. Similar results were observed in a separate study from Donlea et al. [19].

6. The Mechanisms of SHY

The underlying cellular mechanisms governing SHY have not been pursued with equal vigor. Consequently, it is not clear what mechanism, or collection of mechanisms, uniformly explains the synaptic changes reported in insects and mammals. We begin by examining whether those few mechanisms proposed in SHY can explain synaptic changes reported in these species. We then address whether these changes fit with what we know about synaptic scaling, as originally defined and further elaborated by scientists like Turrigiano [7]. This is a reasonable line of inquiry because synaptic scaling features prominently in SHY [9, 10] and is a mechanism for global adjustments of synaptic strength. Lastly, we can consider the role of physiological processes other than sleep that have yet to be excluded as causal factors in the aforementioned findings.

6.1. Synaptic Strengthening and Sleep Homeostasis. One important aspect of SHY is that it attempts to link the homeostatic accumulation and discharge of sleep need to synaptic plasticity. According to SHY, synaptic potentiation in wakefulness leads to enhanced sleep need, at least as measured by increased SWA in non-REM sleep. SWA is a reliable index of sleep need in mammals, as it increases in proportion to wake time and decreases during nonREM sleep [14]. A linkage between LTP and SWA is supported by computational models that show that stronger synaptic connections produce higher SWA *in silico* [110, 111]. It has been argued that the neurotrophin BDNF mediates similar events *in vivo* [112, 113]. Brain concentrations of BDNF are highest during waking [30, 31], intracortical infusion of BDNF increases SWA, and intracortical infusion of anti-BDNF antibodies or a BDNF TrkB receptor antagonist decreases SWA [112]. However, it is not clear if these BDNF-mediated changes in SWA are caused by changes in excitatory synaptic strength. While BDNF can promote glutamatergic synaptic potentiation [114], it also promotes GABAergic neurotransmission [62, 115]. Given that GABAergic neurons may also influence SWA [116, 117], it is possible that these results instead reflect changes in inhibitory circuits. The results of intracortical TrkB antagonism are equally difficult to interpret. Removing BDNF (via anti-BDNF antibodies) would affect inhibitory *and* excitatory neurons and the

antagonist used (K252A) has non-specific effects on several other kinases [118]. Even if BDNF release during waking leads to net cortical synaptic strengthening, it is not clear why stronger synapses should make one sleepy. Heightened sleep need manifests in several ways, including reduced latencies to sleep and increases in sleep continuity and efficiency. These behavioral aspects of sleep need are not easily explained by simply increasing or decreasing cortical synaptic strength.

6.2. SWA and Synaptic Weakening. A principle claim of SHY is that the activity of the sleeping brain—specifically, non-REM SWA—mediates synaptic downscaling [9, 10]. It is suggested, for example, that the periodic appearance of downstates or the frequency of firing during slow oscillations (0.5–4 Hz) could be involved. The idea that SWA promotes synaptic weakening is supported by classic hippocampal LTD protocols, which involve stimulus trains of about 1 Hz. More recent studies *in vitro* and *in situ* indicate that depolarization trains (or intracellular current injections) of about 1 Hz also lead to LTD and the removal of calcium-permeable AMPAR [119, 120], supporting the hypothesis that slow oscillations *in vivo* weaken synapses. While these findings are intriguing, they do not tell the entire story. 1 Hz stimulation that more naturally approximates *in vivo* slow oscillations does not reliably induce LTD in cortical neurons *in situ* [121]. In addition, 1 Hz stimulus protocols that reliably produce LTD *in situ* fail to do so in cortical neurons *in vivo* [122]. These conflicting results may in part reflect limitations inherent in brain preparations *in situ*. These include nonphysiological conditions (e.g., removal of intracortical inhibition and long-range excitatory and neuromodulator inputs), and tissue obtained at ages when sleep regulatory mechanisms are immature [123]. On the other hand, they could also mean that SWA has more than one effect on synaptic strength.

One way that SWA could weaken *or* strengthen synapses is by promoting spike-timing-dependent-plasticity (STDP) [124]. STDP refers to bidirectional changes in synaptic strength that arise from small differences in the timing of presynaptic input relative to postsynaptic depolarization. STDP has been observed under naturalistic conditions in the cortex and the hippocampus *in vivo* [124]. It is thus conceivable that alterations in phase relationship between synaptic inputs and endogenous oscillation during natural brain states (like non-REM sleep) promote synaptic weakening (− phase; postsynaptic firing before presynaptic input) or strengthening (+ phase; pre before post), for additional discussion, see [4, 125]. A strengthening function for SWA is supported by the following findings. First, after learning LC activity precedes the rising edge of cortical upstates, thus providing NA inputs to depolarizing neurons. Second, endogenous busting of neurons within the classic SWA range promotes circuit formation in early life [126, 127] and stimulation protocols that fall within the classic SWA range can also produce LTP [128, 129]. Therefore, the appearance of heightened SWA in sleep after learning [23] (or synaptic potentiation) does not *a priori* mean that synapses are downscaling.

Although SWA theoretically could influence synaptic plasticity, there is little direct evidence for this during natural sleep (for further discussion, see [117]). Computational models show *in silico* that SWA is maximally expressed when cortical synapses are strong and then is reduced when synapses are weakened [110, 111]. One would therefore predict that physiological markers of synaptic potentiation *in vivo* would mirror changes in SWA. For example, if decreases in SWA directly reflect decreases in synaptic strength, then this must involve large, widespread synaptic changes to be detectable at the macrolevel of the EEG. It, therefore, follows that physiological markers of synaptic weakening should be detectable when SWA first declines. In adult rodents, this corresponds approximately to the second or third hour of the rest phase. Most studies, however, measure changes after 6–12 hours of sleep, well after SWA has neared (or obtained) its minima [20, 30]. The few studies that have examined shorter periods of sleep have produced very mixed results [32, 94]. In adult rat frontal cortex, electrically evoked field responses (a measure of cortical potentiation) climb throughout the active phase and modestly decline after the first 2 hours of the rest phase [20]. In the visual cortex, however, evoked field responses progressively decline during the active phase, and increase 2-3 hours after the onset of the rest phase [130]. Interestingly in the latter study, an increase in SWA *preceded* increases in potentiation; findings which are difficult to reconcile with a purely downscaling function for SWA. Similar discrepancies exist for molecular markers of synaptic potentiation. In adult rats, cortical spinophilin—a protein implicated in LTP—is elevated in animals sacrificed 2 hours after the beginning of the rest phase [131]. Another study in adult rats showed that 1 hour of sleep reduces cortical cFOS, but has no effect (relative to wakefulness) on Arc expression [32].

It also appears that SWA cannot be a common mechanism for downscaling in mammals and insects. This is because there is no evidence that the sleeping insect brain displays SWA (or up- and downstates) comparable to birds and mammals. Field recordings in *Drosophila melanogaster* central neurons show that resting states are accompanied by a general reduction of electrophysiological activity [132]. Neural activity in other invertebrate rest states bears little resemblance to mammalian non-REM sleep [133]. In the aquatic invertebrate crayfish, "slow-waves" are reported during reststates, but these waves are not within the typical slow-wave range typical of mammals (>15 Hz).

6.3. Synaptic Scaling and SHY. The evidence for SWA-mediated synaptic downscaling is, at best, equivocal, but are other events in the sleeping brain conducive for the global downscaling described by SHY? For example, do the long periods of neuronal silence during downstates, or the neurochemical/molecular changes reported after extended periods of sleep cause synaptic downscaling [9, 10]? We can try to answer these questions by comparing these phenomena with what is actually known about synaptic scaling.

Surprisingly, many of the molecular and electrophysiological findings cited in support of SHY are inconsistent with net synaptic downscaling during sleep (Table 1). The basic principle of synaptic scaling is that decreases in neuronal

activity upscale synapses, while increases in neuronal activity downscale synapses (Figure 2). Consequently, downstates in sleep—when vast numbers of cortical neurons are silent—should upscale, not downscale, synapses. Similarly, the neural expression of scaling factors (BDNF, Arc, Homer 1a and Tnfα) across the sleep-wake cycle is inconsistent with downscaling during sleep. The low cortical expression of BDNF during mammalian sleep [30–32] should upscale synapses, because reducing BDNF upscales synaptic strength [7, 33]. The low cortical expression of Arc and Homer1a, during sleep [30, 31, 34] should have similar effects because both molecules normally promote synaptic downscaling via AMPAR endocytosis [35–37]. Tnfα is released at higher concentrations during sleep [38, 39], has a permissive role in synaptic scaling [134], and promotes synaptic strengthening *in situ* [40] and *in vivo* [98]. Therefore, heightened brain levels of Tnfα combined with low levels of Arc, BDNF, and Homer1a during sleep are more conducive for net upscaling rather than net downscaling. With respect to neuromodulators, it has been suggested that sleep-related decreases in the insect analog to NA (octopamine) and NA in mammals might represent a common trigger for downscaling [18]. However, as discussed above, NA has multiple effects on synaptic plasticity and there is no evidence NA must be reduced for downscaling to occur.

An additional unresolved issue is the time course of synaptic scaling and SHY. If downscaling predominantly occurs during sleep, then it follows there is a delay between the induction signal during waking and downscaling. This delay, or arresting of the scaling process, must be hours long (in animals with consolidated wake periods) in order for downscaling to occur in tandem with sleep, but is there any evidence that synaptic scaling must wait for sleep? Although early *in vitro* work indicated that synaptic scaling was a slow process [27], perhaps reflecting a slow accumulation of a scaling signal, more recent studies indicate that scaling can occur very rapidly. At the Drosophila neuromuscular junction, a form of presynaptic scaling can occur within minutes [28]. A rapid form of synaptic scaling is also reported in rodent cortical pyramidal neurons *in vitro* (1 hour) [29]. These studies examined upscaling (not downscaling) and the timecourse of synaptic scaling *in vivo* has not been as finely measured. It is also possible that populations of neurons exhibit "microsleep," which might promote more rapid scaling [135]. Nevertheless, these studies suggest that downscaling might occur concurrently with Hebbian plasticity and without sleep.

7. Alternative Mechanisms

Given the uncertainty surrounding SHY mechanisms, it is important to consider physiological processes other than sleep that have yet to be excluded as factors. These include changes in brain temperature and glucocorticoids (i.e., corticosterone/cortisol). Both brain temperature and glucocorticoid release are strongly regulated by the circadian system. Brain temperature and glucocorticoid release are maximal during the active phase, both reach their nadir during the rest phase, but both are elevated by sleep deprivation (in rodents) [136–140]. Temperature and glucocorticoids also modulate neuronal function and synaptic efficacy in ways that resemble changes reported as evidence of SHY. Consequently, differences in synaptic efficacy or proteins analyzed at different circadian times may be due to differences in brain temperature or glucocorticoids, rather than vigilance state. The normal control for circadian effects (i.e., sleep deprivation in the rest phase) may be inadequate, as sleep deprivation increases brain temperature and glucocorticoids in rodents.

7.1. Brain Temperature. A role for brain temperature is suggested by the fact that the most dramatic evidence of SHY is found in ectothermic insects [17–19]. In contrast to birds and mammals, ectotherms do not internally regulate their core/brain temperature. Temperature is instead behaviorally regulated, either by selecting warmer environments or through activity [141]. As discussed above, long periods of sleep in *Drosophila melanogaster* massively prune back synaptic proteins and structures [17–19]. In rodents, changes in similar structures (e.g., dendritic spines) after sleep are much more modest and restricted to a narrow window of development [21, 109]. Interestingly, the only mammals which display large-scale synaptic changes during sleep-like states comparable to *Drosophila melanogaster* are hibernators [142, 143]. During hibernation (which is entered through sleep), brain temperature precipitously declines and there is a massive retraction of dendrites and synapses. This is followed by a rapid expansion of these structures during arousal and euthermia [142, 143]. These changes are strikingly similar to changes reported in sleep and wake in *Drosophila melanogaster* [17–19]. This raises the possibility that the results reported in ectotherms are not related to wakefulness or sleep *per se*, but to accompanying changes in core/brain temperature. Indeed, warm ambient temperatures lead to several changes in adult and larval Drosophila neurons that resemble those reported after long periods of wake (relative to sleep). These include increased axonal arborization in mushroom body neurons [144] and motor nerve terminals *in vivo* [145] and neurite extension *in vitro* [144]. Intriguingly, these temperature effects are mediated by signaling pathways shared by activity-dependent synaptic plasticity (e.g., cAMP) [144]. Whether similar temperature gradients exist across insect wake and sleep is unknown as this has yet to be measured. However, given that core temperature tracks motor activity in small terrestrial insects [141], sleep and wake may be accompanied by significant changes in brain temperature. A strong temperature effect in terrestrial insects may also explain the very faint effects of sleep on synaptic proteins in the zebrafish *Danio rerio* [146]. Zebrafish are also ectotherms, but are well adapted to fluctuations in surrounding temperature [147] and unlikely to experience large temperature gradients under experimental conditions [146].

Strong temperature effects in endothermic mammalian neurons are also reported under certain conditions. Hippocampal dendritic spines *in situ* are highly sensitive to changes in temperature, rapidly shrinking then reexpanding with cooler and warmer temperatures [148]. Similar temperature effects are observed in proteins that make up

the postsynaptic density [148]. Cooling the hippocampus *in situ* reversibly reduces excitatory postsynaptic field potentials (EPSPs), and reverses (de-potentiates) LTP [149]. Conversely, transient warming of hippocampal slices has biphasic effects, an initial depression, then prolonged enhancement of EPSPs [150]. Temperature effects are not restricted to the hippocampus, as the rate of mEPSCs in rodent cortical neurons is surprisingly temperature sensitive (Q10 of 8.9) [151]. One must be cautious in extrapolating from studies *in situ* or *in vitro*, which use large temperature gradients, to the situation *in vivo*. However, strong effects of naturally occurring brain temperature gradients on EPSPs are reported in freely behaving rodents [152]. As shown by Moser et al. [152], motor activity increases hippocampal temperature and EPSPs. This EPSP enhancement is unrelated to learning-related plasticity. It is instead caused by the normal rise in brain temperature associated with waking movement and dissipates as the brain naturally cools.

7.2. Glucocorticoids. In rodents, corticosterone also rises and falls in parallel with wake and sleep and has profound effects on synaptic efficacy and plasticity molecules. As is true for neuromodulators like NA (which is activated synergistically with corticosterone), these effects are diverse and dependent upon different classes of receptors [153]. They have also been chiefly explored in the hippocampus rather than the neocortex. Nevertheless, circadian increases in corticosterone (i.e., during the normal waking period), or after sleep deprivation may generally promote glutamatergic neurotransmission and neuronal excitability (relative to sleep) [153]. Acute increases in corticosterone (or stress) increase the frequency [154] and amplitude of mEPSCs in the hippocampus [155], strengthen glutamatergic synapses onto dopamine neurons [156], and increase glutamatergic release/calcium mobilization in cortical synaptoneurosomes [157]. Acute increases in corticosterone also promote AMPAR synaptic transmission, AMPAR trafficking and insertion into cortical and hippocampal synapses, and cortical dendritic spine turnover [158–161].

In conjunction with circadian rhythms in brain temperature, the cumulative effects of increased (wake) or decreased (sleep) corticosterone release may explain a number of findings in rodents ascribed by SHY to sleep and wake. These include differences in evoked field potentials [20] and mEPSCs [22] in animals examined at circadian times of low and high corticosterone release (or after sleep deprivation). They might also contribute to relative (sleep versus wake) differences in synaptic proteins, plasticity molecules, and dendritic spine morphology obtained from rodents sacrificed after long periods of waking and sleep [20, 21, 31]. A strong circadian component to SHY may also explain why mammals with weak circadian rhythms [94] do not show the same sleep-related decreases in "LTP" molecules and AMPAR phosphorylation as rodents [20].

8. Discussion

A scientific theory can be evaluated by several criteria. Does it attempt to explain and predict, better than other theories, empirical findings? Does it stimulate other scientists to challenge prior assumptions and perform new experiments? Does it address a problem of broad scientific interest and importance? In many new and exciting ways, SHY satisfies these criteria and thus represents a valuable contribution to the study of sleep, but, as with any new scientific theory, much more work is needed before its true importance can be gauged.

The core theoretical concepts of SHY are puzzling in several respects. The idea that waking and sleep are dominated by net synaptic potentiation and weakening, respectively, requires a very narrow view of brain plasticity. The waking brain is typified by many forms of learning, each likely employing complex combinations of Hebbian and non-Hebbian plasticity. If these waking forms of plasticity require secondary sleep-dependent processes, it is not clear why the latter should primarily manifest as (or sum to) "net" synaptic weakening. What seems more likely is that sleep is characterized by multiple forms of synaptic plasticity, including classic Hebbian LTP and LTD [4], as well as downscaling *and* upscaling. This may explain why the evidence for "net" downscaling after sleep critically depends on what is measured (e.g., neuromodulin versus BDNF) and when those measurements are made (e.g., early or late in the rest phase). What determines the types of plasticity engaged during sleep is unknown, but in addition to waking experience ontogenetic factors are likely important. For example, sleep amounts are maximal during periods of heightened synaptogenesis including *in utero* when waking experience is negligible [162, 163]. It seems highly unlikely that a fundamental purpose of sleep is to principally weaken synapses during these developmental periods.

A second unresolved issue is the function of sleep-dependent downscaling. It is theorized that downscaling in sleep improves signal-to-noise, which would benefit memory consolidation, or allow for new learning to occur during subsequent waking [9, 10]. This is because, according to SHY, functional synapses are preserved while nonfunctional ones are eliminated. Indeed, from a purely theoretical view, such precise scaling during sleep would be highly adaptive. Computational models support this idea [111], but this is largely untested *in vivo*. There is no evidence that the changes in neural protein phosphorylation [20], Arc or BDNF [30, 31], or dendrite morphology [164] reported after rodent sleep contribute to cognition or other adaptive behavior. In *Drosophila melanogaster*, sleep is required for new learning to occur, and this sleep is accompanied by a reduction in synapses [19]. However, it is unknown if this change in synapses and not some other process during sleep is the causal factor.

One important future direction is to delve more deeply into the underlying mechanisms of SHY. To date, this has received less attention than studies aimed at collecting supportive findings. As a consequence, it is not clear if the observed phenomena are due to sleep *per se*, or other physiological processes that coincide with sleep. One way to address this issue is to design experiments that address these factors. For example, does brain cooling in insects replicate (and brain warming prevent) the effects of sleep on

neuronal morphology and synapses? Do the same changes in mammalian synaptic efficacy, proteins, and dendrites observed after wake and sleep occur when corticosterone is experimentally clamped? This can be accomplished with adrenalectomy combined with hormone replacement—as recently demonstrated by Mongrain et al. [138]. A second way is to perform strong tests of SHY using modern molecular tools *in vivo*. According to SHY, cortical synaptic potentiation in wakefulness is a causal factor in sleep homeostasis (as measured by SWA) [112]. Therefore, one would predict that transgenic mice with deficits in cortical synaptic potentiation should also show reductions in sleep need. It would be interesting, for example, to examine sleep homeostasis in (CaM) KII^{T286A} point mutation [96, 165] and PKA RIIα null mutant $(-/-)$ mice [166], which exhibit large reductions in cortical LTP. There are also several techniques for inducible (as opposed to constitutive) deletion of floxed genes—which would allow one to examine sleep homeostasis after deletion of molecules necessary for cortical LTP (e.g., BDNF). Third, given that mechanisms involved in synaptic scaling are increasingly well understood, what is their expression pattern in the awake and sleeping brain? If their expression pattern is inconsistent with SHY, which so far appears to be the case, then what specific plasticity mechanism is responsible? Some promising work comes from Lanté et al., who showed that AMPAR internalization *in vitro* involves phosphatase and protein kinase C activity [119]. Do these signaling pathways play an equally important role in sleep-mediated plasticity *in vivo?*

A second important future direction is to integrate synaptic changes related to SHY with other sleep-dependent forms of plasticity. As discussed above, plasticity in the visual cortex is consolidated by sleep and this includes changes that are best explained by synaptic potentiation [94]. Sleep in mammals is also accompanied by hippocampal bursts of activity that "replay" patterns present during experience. This replay occurs during high-frequency firing ("ripples" and "sharp waves") that are well suited for events like LTP [167]. Thalamocortical spindles may also mediate various forms of synaptic strengthening during mammalian sleep [168, 169]. In *Drosophila melanogaster*, sleep not only scales back synapses presumably allowing new learning to occur, but sleep *after* learning is needed to make long-term memory; a process that requires the formation of new synapses [19]. In developing mice, cortical dendritic spines are not only eliminated, but also formed during sleep [21, 109]. Therefore, it is conceivable that sleep promotes a generalized synaptic downscaling, accompanied with Hebbian or non-Hebbian synaptic potentiation in select circuits [170–172]. However, this presupposes that net downscaling is directly sleep-dependent and as discussed above, this has not been conclusively shown.

9. Concluding Remarks

Over the last 100 years, numerous grand or unifying theories of sleep function have been proposed [16]. None, however, have adequately explained the presence of sleep across the animal kingdom, its unusual electrophysiological, neurochemical and molecular events, and its dramatic changes across the lifespan [16]. As new experiments accumulated, their predictive power failed, and they became little theories that only explained—often imperfectly—single sleep phenomena [16]. It is too soon to say where SHY fits in this story. SHY is a seminal theory, bold in its scope and challenging in its implications, but it seems oddly disconnected from our rapidly evolving views of synaptic plasticity. The proponents of SHY have also amassed an impressive set of supportive findings, but these have yet to be pursued in depth. These are not trivial matters. In the absence of a clearly proposed mechanism (informed by current views on synaptic plasticity), the empirical supports of SHY are hard to interpret. Therefore, the significance of SHY—and what it may one day reveal about sleep and synaptic plasticity—remains elusive.

Abbreviations

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
Arc: Activity-regulated cytoskeleton-associated protein
BDNF: Brain derived neurotrophic factor
CAMK: Calmodulin-dependent kinase
EPSP: Excitatory post-synaptic potential
ERK: Extracellular regulated kinase
GABA: Gamma-aminobutyric acid
LC: Locus coeruleus
LTD: Long-term depression
LTP: Long-term potentiation
mEPSC: Miniature excitatory post-synaptic current
NA: Noradrenaline
Narp: Neuronal activity-regulated pentraxin
NMDA: n-methyl-d-aspartic acid
NSF: N-ethylmaleimide-sensitive factor
PKA: Protein kinase A
Q10: The rate of change of a biological or chemical system following a 10°C change in temperature
REM: Rapid eye movement
SHY: Synaptic homeostasis hypothesis
Snap25b: Synaptosomal-associated protein, 25 k
STDP: Spike-timing dependent plasticity
SWA: Slow wave activity
Tnfα: Tumor necrosis factor-alpha
TrkB: Tyrosine receptor kinase B
zif268: Zinc finger protein 225 or NGFI-A (nerve growth factor-induced protein A).

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Review Article

GABA Metabolism and Transport: Effects on Synaptic Efficacy

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GABAergic inhibition is an important regulator of excitability in neuronal networks. In addition, inhibitory synaptic signals contribute crucially to the organization of spatiotemporal patterns of network activity, especially during coherent oscillations. In order to maintain stable network states, the release of GABA by interneurons must be plastic in timing and amount. This homeostatic regulation is achieved by several pre- and postsynaptic mechanisms and is triggered by various activity-dependent local signals such as excitatory input or ambient levels of neurotransmitters. Here, we review findings on the availability of GABA for release at presynaptic terminals of interneurons. Presynaptic GABA content seems to be an important determinant of inhibitory efficacy and can be differentially regulated by changing synthesis, transport, and degradation of GABA or related molecules. We will discuss the functional impact of such regulations on neuronal network patterns and, finally, point towards pharmacological approaches targeting these processes.

1. Introduction

Activity within neuronal networks is contained between the extremes of complete silence and exceeding neuronal discharges. This general statement may seem intuitively right but has severe and nontrivial consequences for the function of neuronal networks. Several theoretical arguments and experimental findings support the notion that specific mechanisms secure a limited mean level of activity. Information content within neuronal networks is maximal under conditions of sparse coding, which means that only a minority of all local neurons is activated above threshold [1]. Furthermore, neurons are severely damaged by both extremes, that is, prolonged inactivity [2–5] or severe hyperactivity during epileptic seizures [6].

Many different mechanisms contribute to regulation of overall neuronal activity, including intrinsic neuronal properties [7, 8] and energy metabolism [9, 10]. At the core of homeostasis, however, is the interplay between synaptic excitation and inhibition (Figure 1). All neuronal circuits of higher animals contain excitatory and inhibitory transmitter systems forming intense feed-forward and feedback connections [11, 12]. The functional architecture of such networks can already explain homeostatic regulation of activity to a certain degree, and excitatory feedback loops tend to build up activity, which is counterbalanced by dampening actions of inhibitory feedback connections. A further element of cortical and subcortical microcircuits is inhibition of inhibitory neurons, resulting in a net excitation of downstream target cells. This mechanism may serve further functions in synchronizing neuronal activity and can be mediated by specialized interneurons [13]. In contrast, interactions between inhibitory neurons may also desynchronize neurons as, for example, Renshaw cells in the spinal cord [14, 15]. This mechanism may serve to reduce fatigue of muscle fibers.

It should be noted that inhibitory neurons are not only important for balancing excitation. In several circuits, inhibitory neurons function as projection cells, rather than interneurons. For example, major projections within the basal ganglia and reticular nucleus of the thalamus and of the cerebellum are formed by GABAergic neurons [16–18]. In such networks, activity-dependent modulation of inhibition may have specific effects beyond balancing excitation, for example, the generation of specific physiological or pathological oscillation pattern [19].

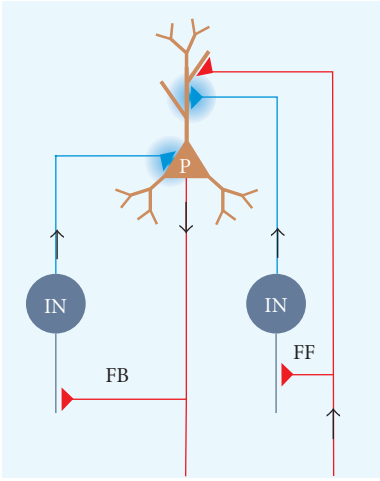

Figure 1: Local inhibitory connections of cortical networks. Note the efferent and afferent connections indicated by arrows. In red, connections indicate glutamatergic excitation and blue connections GABAergic inhibition. Brown soma indicates an excitatory pyramidal cell (P), and blue-grey somata show inhibitory interneurons (INs). The left interneuron is integrated into a feedback inhibition loop, (FB) while the right interneuron shows feed-forward inhibition (FF). Differential targeting by the interneurons to the soma or dendrite points towards possible layer-specific actions of inhibition. Note that GABA released at the right synapse may, eventually, spill over to the neighbouring glutamatergic synapse. The global light blue staining indicates background GABA concentration that mediates tonic inhibition depending on local synaptic activity.

Homeostasis between excitation and inhibition cannot be reduced to a simple rule of network wiring. Recent evidence shows that inhibition has multiple specific functions within neuronal networks, far beyond a simple "break" [20, 21]. Moreover, inhibitory strength is not constant but must adapt to dynamically changing patterns and degrees of network activity. It does therefore not come as a surprise that recent work has elucidated multiple mechanisms of plasticity at inhibitory synapses [4, 22–24]. An important subset of these mechanisms mediates homeostatic plasticity, that is, adaptation of inhibitory efficacy to the overall activity within a local network. Indeed, several lines of evidence suggest that GABAergic efficacy is upregulated in hyperactive networks [25–29] and downregulated under conditions of reduced activity [5, 30, 31].

Such homeostatic reactions can, in principle, be mediated by multiple pre- and postsynaptic mechanisms. A particularly important regulatory system, however, is the concentration of the main mammalian inhibitory transmitter GABA (γ-aminobutyric acid). This paper shall summarize the molecular elements and functional mechanisms involved in regulation of GABA concentration within vesicles, cells, and in the extracellular space. We will quote experimental evidence indicating that GABA is homeostatically regulated during physiological and pathological changes of network activity. Finally, we will consider how molecular determinants of GABA concentration can be targeted by drugs for pharmacological therapy of neurological or psychiatric diseases.

2. Organization of GABAergic Synapses

In the mammalian CNS, inhibition is mediated by the amino acids GABA and glycine. The GABAergic system has been intensely explored during recent years and will therefore be the main focus of this paper. As a starting point, we will briefly summarize the main functional and structural elements of GABAergic synapses.

GABA binds to two different types of receptors-ion channels and metabotropic receptors. GABA-gated ion channels are selectively permeable for chloride and bicarbonate and have reversal potentials close to Cl^- equilibrium (E_{Cl}). These channels are mostly termed $GABA_A$ receptors, but a molecularly and pharmacologically distinguishable subset has also been termed $GABA_C$ receptors until recently, as discussed by Olsen and Seighart [32]. In most cases, the increase in chloride (and bicarbonate) conductance resulting from activation of ionotropic GABA receptors causes inhibition of the respective neuron, that is, decreased probability of action potential generation. This is easy to understand in cases where E_{Cl} is more negative than the membrane potential, such that opening of $GABA_A$ receptors causes hyperpolarisation and enhances the distance between membrane potential and action potential threshold. However, inhibition can also be mediated by more complex biophysical mechanisms, for example, shunting of the local membrane resistance, which can also counteract excitatory inputs. Even depolarizing actions of GABA can, in certain cases, be inhibitory [33–35]. Conversely, excitatory actions of GABA may occur in specific situations, including early developmental stages [36–39] and maladaptive processes, for example, in chronic epilepsy [40, 41]. The occurrence of depolarizing GABA responses under physiological conditions is presently subject to some controversy [39, 42]. $GABA_B$ receptors, in contrast, are members of the family of G-protein-coupled proteins [43] and react to GABA binding by dimerisation [44] and activation of downstream signal cascades. These include decreased probability of transmitter release and increase in pre- and postsynaptic K^+ conductance [45, 46].

A complete survey of GABAergic mechanisms at the molecular, cellular, and network level is far beyond the scope of this paper. Rather, we will highlight three principles of organization of GABA-mediated inhibition that are particularly important for understanding how GABA regulates network activity. The molecular constituents involved in regulation of inhibitory strength are detailed below.

(i) GABA regulates excitability on different temporal and spatial scales. One important mechanism is tonic inhibition, which results from diffusely distributed GABA within the extracellular space of networks, thereby reducing excitability of all local neurons (Figure 1). Recent evidence has shown that tonic inhibition is of major importance for reducing firing probability of defined types of neurons within cortical networks [24, 47–49]. In some cells, this

mechanism accounts for more than 50% of GABA-induced chloride conductance [50]. Background levels of GABA in neuronal tissue have been estimated to reach high-nanomolar to low-micromolar concentrations [51, 52]. In good accordance with this relatively low concentration, extrasynaptic GABA receptors have particularly high agonist affinity [47, 53, 54]. At the other extreme, phasic inhibition is mediated by locally and temporally restricted release of GABA from synaptic terminals. This action causes a short, exponentially rising and falling of the postsynaptic chloride conductance which can last from few to tens of milliseconds [50, 55]. Most GABAergic neurons seem to form such specific synaptic sites for phasic inhibition, but recent evidence indicates that there are also specialized interneurons which release GABA for tonic inhibition [56–60]. Tonic inhibition depends on special GABA receptors, which can be selectively modulated by drugs, for example, neurosteroids. These specific receptor isoforms may be important in the pathophysiology of depression [61] and withdrawal symptoms [62]. Such examples of receptor heterogeneity may well open new therapeutic chances.

(ii) GABAergic interneurons are diverse. Work on different networks has revealed an unprecedented multitude of different GABAergic neurons which are classified by their somatic location, dendritic branching, axonal projection, afferent synaptic integration, intrinsic membrane properties, and expression of molecular markers, especially neuromodulatory peptides and calcium-binding proteins. Extensive classification systems have been established for different circuits, for example, for the rodent neocortex [63, 64] and the hippocampus [13, 65]. Moreover, introducing the juxtacellular recording technique has enabled recordings from individual interneurons in behaving animals and subsequent in-depth structural analysis [66]. These data have shown that different types of interneurons are specialized to organize different patterns of network activity [67].

(iii) In accordance with the heterogeneity and functional specialization of different cell types, experiments and computer modelling have revealed important functions of "inhibitory" interneurons in networks beyond merely dampening excitation. Interneurons turned out to play a key role in organizing the spatiotemporal activity of local networks, especially during synchronous network oscillations [68–73]. Complementary neuroanatomical work has highlighted the structural basis for this function: interneurons have highly divergent axonal projections, cell type-specific afferent and efferent connectivity, and synchronizing mutual connections. All these properties favour synchronous rhythmic inhibition of large populations of principal cells [13, 69, 71, 74–76]. It should be noted that the connections between excitatory projection cells and inhibitory interneurons provide an automatic homeostatic mechanisms at the network level. Feed forward or feedback inhibition is driven by excitatory inputs or outputs, respectively, from remote or local excitatory neurons. This mechanism does automatically recruit inhibitory neurons in an activity-dependent manner and, hence, balance local activity (Figure 1).

3. Key Molecules for GABAergic Signalling

The molecular organization of synapses is highly complex, and a complete review would be beyond the scope of this paper. We will restrict our remarks to some families of molecules that are crucial for understanding homeostatic regulation of GABA concentration (Figure 2).

Like many other neurotransmitters, GABA acts on ionotropic as well as metabotropic ion channels. $GABA_A$ receptors are pentameric ion channels composed out of a large variety of 19 homologous subunits [32, 77, 78]. Work during the past decades has elucidated numerous functional differences between molecular subtypes of $GABA_AR$, including different expression patterns, differential modulation by benzodiazepines, neurosteroids and Zn^{2+}, different compartmentalization within neurons, and different agonist affinity [32, 54, 79]. The latter properties are of special interest with respect to GABA concentration. $GABA_ARs$ with low agonist affinity appear to be clustered at postsynaptic sites, whereas receptors with high affinity are mostly found extrasynaptically [47, 48]. The underlying sorting mechanisms are partially known and involve specific subsynaptic sorting signals within the gamma subunit and interactions with postsynaptic scaffolding proteins like gephyrin and collybistin [80–83]. Extrasynaptic receptors, in contrast, are formed by subunits mediating high agonist affinity including $\alpha 4$, $\alpha 6$, and δ subunits [32, 47]. This distinction reflects the different concentrations of GABA at both sites: whereas synaptically released GABA may reach transient concentrations of $\sim$1.5–3 mM [84, 85], extrasynaptic transmitter concentration has been estimated to lie in the low micromolar range of about 0.2–2.5 μM [47, 51, 52, 86]. As mentioned above, these apparently low "background" concentrations of GABA may be very efficient in regulating excitability [47–50]. An additional distinct location of $GABA_A$ receptors is the presynaptic terminal itself. GABAergic auto- or heteroreceptors have been described at the axon terminals of various neurons, including spinal cord afferents [87], hippocampal mossy fibres [88], Schaffer collaterals [89], cerebellar interneurons [90], and pituitary terminals [91]. The effects of such receptors are diverse. Depending on the GABA-induced change in membrane potential and local membrane resistance, presynaptic $GABA_A$ receptors may increase or decrease transmitter release [92].

$GABA_B$ receptors, on the other hand, are G-protein-coupled transmembrane molecules which are activated by low concentrations of GABA and form dimers which then trigger secondary signalling cascades [43–45]. At presynaptic terminals, activation of $GABA_BRs$ reduces GABA release, forming the typical negative feedback loop of autoreceptor-mediated synaptic gain control. $GABA_B$ receptors are also

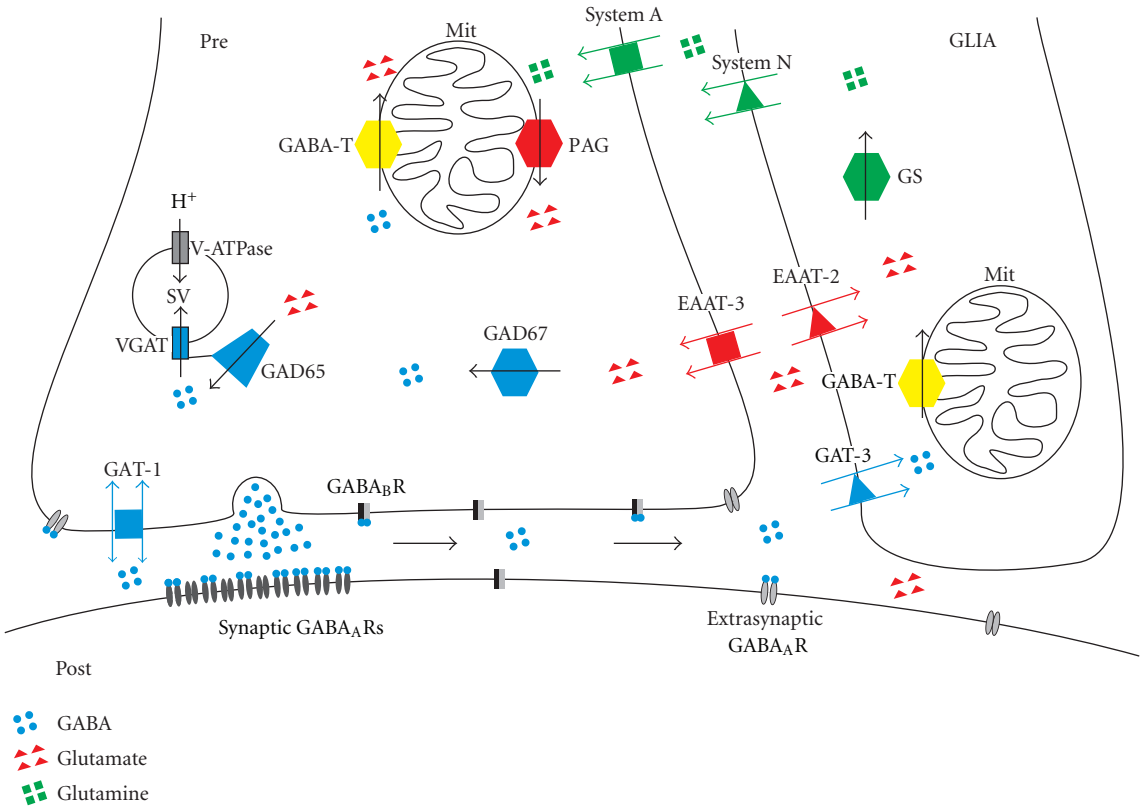

Figure 2: Schematic drawing of transmitter release, transport, and synthesis at a GABAergic synaptic terminal. The axonal ending of an inhibitory interneuron (PRE) is drawn on the left, a glial cell (GLIA) on the right. Bottom structure indicates postsynaptic membrane of a target cell (POST), for example, a pyramidal neuron. Transporters are marked by flanking arrows, and synthesizing or degrading enzymes are marked by a centred arrow. Transporters are colour matched to substrates: GABA is shown as blue particles, glutamate in red, and glutamine in green. GS: glutamine synthetase, Mit: mitochondrion, PAG: phosphate-activated glutaminase, SV: synaptic vesicle, and V-ATPase: vacuolar-type H^+-ATPase. For other abbreviations, see the main text.

present at glutamatergic terminals, pointing towards regular spillover of GABA from inhibitory to excitatory synapses (Figure 1, [47, 93–95]). Postsynaptically, $GABA_B$Rs hyperpolarize and inhibit neurons by activating inwardly rectifying K_{IR} channels, giving rise to the "slow" or "late" phase of inhibition that follows fast, $GABA_A$R-mediated effects and lasts several hundred milliseconds [96]. Furthermore, $GABA_B$ receptors can also mediate tonic inhibition, exerting negative control on overall network activity [97].

4. GABA Transport and Synthesis

While GABA receptors act as "detectors" of local GABA concentration, the regulation of GABA itself is achieved by several specialized molecular mechanisms mediating transport, sequestration, synthesis, and the degradation of GABA. We will briefly address each class of molecules involved in these processes.

Membrane-bound GABA transporters move GABA across the cell membrane (Figure 2). The direction and efficacy of this Na^+-coupled transport results from the driving electrochemical gradient and is directed inwardly in most situations [98, 99]. However, upon strong depolarization or altered ion homeostasis, GABA transporters can also reverse direction. This mechanism leads to nonvesicular release of GABA which may be of special importance in pathophysiological situations [60, 100, 101]. GABA transporters appear in four different isoforms with affinities around 7 μM for rat GAT-1, 8 μM for rat GAT-2, 12 μM for rat GAT-3, and 93 μM for rat BGT-1 [102–106]. Terminology of GABA transporters is not fully compatible between rats and mice [107]. In the following, we use the abbreviations for rat GABA transporters where ratGAT-1 = mouseGAT1; ratGAT-2 = mouse GAT3; ratGAT-3 = mouseGAT4; ratBGT-1 = mouse GAT2. GABA transporters are differentially expressed in the CNS. As a global rule, GAT-1 is the prevailing neuronal isoform in the rodent brain, and GAT-3 is strongly expressed in glial cells [108–110]. Expression of different GAT isoforms is, however, overlapping, so that selective modulation of one isoform will always affect more than one cell type. It might therefore turn out impossible to achieve a strictly selective block of glial or neuronal GABA uptake with conventional pharmacological tools.

An alternative pathway for enriching GABA in presynaptic terminals is transmitter synthesis from glutamate. Similar to GAT-1/3, there are membrane-bound glutamate transporter molecules at presynaptic terminals of inhibitory interneurons, namely EAAC1 (also called EAAT3)

[111–113]. Moreover, neurons can synthesize glutamate from glutamine which can also be taken up by specialized transporters (see below) [114, 115]. GABAergic neurons express both mature isoforms of glutamate decarboxylase, GAD65 and GAD67 [116, 117], that convert the excitatory amino acid into GABA. The smaller isoform GAD65 is directly associated to presynaptic vesicles, indicating that glutamate, once present in the presynaptic cytosol, can be rapidly used for vesicular enrichment of GABA. Indeed, there are direct protein interactions between GAD65 and the vesicular GABA transporter VGAT (= VIAAT, vesicular inhibitory amino acid transporter), suggesting that conversion of glutamate into GABA and subsequent vesicular uptake of the transmitter may be strongly coupled processes [118].

More recently, glutamine has gained interest as an alternative source of GABA. The amino acid glutamine has long been known as the immediate precursor for glutamate. In the extracellular space, glutamine may reach concentrations of hundreds of μM [119, 120]. Enrichment of glutamate in excitatory central neurons involves uptake through specific glutamate transporters by glia cells, conversion into glutamine, export via "system N" glutamine transporters, uptake into neurons by "system A" glutamine transporters, and conversion into glutamate [121, 122]. There is increasing evidence for a similar role of this glutamate/glutamine cycle in GABA synthesis. Indeed, inhibitory interneurons in the hippocampus express the system A transporter SNAT1 [115], but not SNAT2 [123]. Recordings of epileptiform activity in rodent brain slices *in vitro* have revealed functional evidence for boosting of inhibition by glutamine via this mechanism [124–127]. Using high-resolution recordings of miniature IPSCs in conjunction with pharmacological manipulation of glutamine levels and glutamine transport, these studies showed that glutamine can serve as a source for GABA, especially under conditions of increased synaptic activity. More recent evidence from rat hippocampal slices showed that the contribution of glutamine to vesicular GABA content is more pronounced in immature tissue, and that glutamine forms a constitutive source of vesicular GABA in immature hippocampal synapses on CA1 pyramidal cells. At later stages, the functional importance seems to be restricted to periods of enhanced synaptic activity [128]. This loss of function for constitutive GABA release under resting conditions goes along with an age-dependent decline in expression of SNAT1, both absolutely and in relation to the GABA-synthesizing enzyme GAD65.

5. Sequestration and Degradation of GABA

Within presynaptic terminals of GABAergic neurons, GABA is enriched in vesicles by the vesicular inhibitory amino acid transporter (VGAT = VIAAT). This protein is embedded in the vesicular membrane and uses the electrochemical gradient for H^+ to shuffle GABA into small synaptic vesicles [129–133]. Additionally, chloride gradients between vesicle lumen and presynaptic cytosol may contribute to the vesicular loading of GABA [129, 131]. Interestingly, VGAT processes both major mammalian inhibitory transmitters, GABA and glycine. This is a prerequisite for the observed GABAergic/glycinergic cotransmission by single vesicles in the spinal cord [134]. Modelling studies and biochemical data suggest that vesicular GABA uptake may achieve an ~1000-fold increase of the transmitter in vesicles as compared to the presynaptic cytosol [135]. On the other side, recent evidence suggests that GABAergic synaptic vesicles are leaky, implying generation of a dynamic equilibrium between accumulation and loss of GABA, given that there is enough time to reach such a steady state [132, 136]. Taking this bidirectional transport into account, the "leaky bathtub" model of synaptic vesicles comes to rather low estimates of concentration gradients between cytosol and the inner vesicle space [132, 135, 137].

Finally, GABA and α-ketoglutarate can be transaminated, producing succinic semialdehyde and glutamate. The reaction is catalysed by GABA transaminase (GABA-T) which is present in mitochondria of glial cells and neurons [138–140]. It is estimated that more than 90% of all GABA in the mammalian CNS is degraded in this way and contributes to energy metabolism in the tricarbonic acid cycle.

In summary, there are several different molecular pathways and compartments for enrichment, synthesis, and degradation of GABA (Figure 2). The resulting concentration of GABA in synaptic vesicles and in the extracellular space depends on the equilibrium between these mechanisms. It should be clearly stated that the absolute concentrations of GABA in the presynaptic cytosol, in vesicles, and in the extrasynaptic space are not known. The affinity constants of extrasynaptic GABA receptors may serve as a rough estimate of background concentrations (0.2–2.5 μM) [86]. Direct measurements from rat cerebrospinal fluid yielded similar or slightly higher values which may be lower in humans [141].

The highly dynamic time course of transmitter concentration in the synaptic cleft, on the other hand, has been estimated based on experimental and theoretical work in different types of neurons. Peak concentrations may be as high as 0.3 to 3 mM [85, 142–147]. The cytosolic GABA concentration is most difficult to estimate or measure, especially since most of the neuronal GABA pool is used for energy metabolism rather than for synaptic inhibition.

It should be explicitly stated that none of the above-given numbers has been directly measured. Indeed, our knowledge on local GABA concentrations in different compartments is far from sufficient. This is even more concerning when we take into account the enormous heterogeneity of neurons [20, 63, 65], the different microarchitecture of different local circuits, and activity-dependent changes in GABA release and ionic homeostasis. A major challenge is the lack of quantitative data about key molecules and structures: How many GABA-uptake molecules are present at a given inhibitory synapse? What is their distribution with respect to the site of release? What is the precise extracellular volume at the synaptic cleft? How much GABA does go into glia cells and neurons, respectively? An important example for progress in this quantitative molecular approach to subcellular structure and function is the recent work on the vesicular proteasome by Takamori and colleagues [136].

6. Regulation of (GABA) in Physiology and Pathophysiology

Different lines of evidence support the view that the cellular and molecular mechanisms mentioned above make important contributions to homeostatic synaptic plasticity. This term covers changes of intrinsic and synaptic neuronal properties, which maintain the mean network activity within a determined range [4, 28]. Taking into consideration that network states change rapidly with changes in vigilance and behavioural state [148, 149], this is a nontrivial task. Individual neurons can change their activity at least by a factor of ~6 in different network patterns [150]. Nevertheless, under normal conditions, networks do neither fall into complete silence, nor into pathological hyperactivity.

Inhibition plays a critical role in network homeostasis. Most circuits contain specialized inhibitory cells which are activated by external afferent excitatory inputs (feedforward inhibition) or by collaterals from efferent excitatory axons (feedback inhibition) as illustrated in Figure 1 [11]. These inhibitory control loops ensure that excitatory neurons are inhibited in an activity-dependent manner. It should be noted, however, that inhibitory interneurons are much more than a "brake" or "gain control." Recent evidence has revealed many other functions for these heterogeneous neurons: they are critical for organizing the complex spatial and temporal patterns of network oscillations [70, 71], selective gating of defined inputs or outputs [20], suppression of background activity [151], and precise timing of action potentials [67]. Corresponding with these specific functions, we are gaining increasing insight into the complexity of GABAergic signalling, diversity of interneurons, and plasticity of inhibitory synapses.

Notwithstanding these recent findings, however, inhibition does still have its traditional function, that is, limitation of neuronal activity. With respect to network homeostasis, this control function must adapt to changing degrees of activity in the local network. Several lines of evidence indicate that modulating GABA content of inhibitory interneurons is a key mechanism in this regulation process. For example, repetitive hyperactivity in the hippocampus of chronically epileptic rats causes upregulation of GADs, the key enzymes for production of GABA [29]. Conversely, the partial deafferentation of somatosensory cortex resulting from partial limb amputations leads to a downregulation of GABA, but not of GADs [30, 152]. These findings indicate that GABA levels are increased or decreased, respectively, in response to increasing or decreasing network activity. The underlying mechanisms are diverse with respect to time course and source of GABA.

Long-term changes in excitability, such as described above, require regulation of protein expression. Multiple studies from excitatory synapses show that changes in synaptic activity do indeed include lasting effects on protein synthesis and synaptic protein content [153, 154]. The underlying mechanisms involve calcium signalling in dendrites and nuclei [30, 155]. Much less is known about similar mechanisms in inhibitory interneurons. It would be of special importance to understand the activity-dependent regulation of key proteins such as GAD, VGAT, and others. Interestingly, BDNF (brain-derived neurotrophic factor) increases expression of GAD, indicating that neurotrophins are involved in inhibitory homeostatic plasticity. This would be well compatible with the general role of these molecules in activity-dependent plasticity [156]. Surprisingly, genes for inhibitory transmission can also be upregulated in excitatory, glutamatergic neurons following periods of enhanced activity. This intriguing finding suggests that excitatory neurons can adopt an active role in synaptic inhibition in certain situations. Such a "dual phenotype" has been clearly demonstrated in dentate granule cells, a major excitatory input cell type in the rodent hippocampus [157–159]. The axons of granule cells, called mossy fibres, form strong glutamatergic synapses on proximal dendrites of CA3 pyramidal cells and do also contact inhibitory interneurons in this region (an example of feedforward inhibition). Upon strong repetitive stimulation or following epileptic seizures, mossy fibres start expressing proteins needed for the production and vesicular storage of GABA. Electrophysiological measurements show that this GABAergic phenotype is indeed functional, giving rise to mixed excitatory and inhibitory potentials in CA3 pyramids. The GABAergic phenotype of mossy fibres seems to be more pronounced in the juvenile brain [157], consistent with the general principle of enhanced plasticity in immature neurons. While the dual phenotype of granule cells may be an extreme example, several observations indicate that similar activity-dependent changes in expression of GABAergic molecules affect the vesicular pool of GABA in typical inhibitory interneurons. For example, expression of VGAT is altered following ischemia or excitotoxic stimulation [160–162]. These changes go along with altered composition of the vesicular proteome, indicative of altered supply or release of GABAergic vesicles [163].

At a shorter time scale, GABA levels might be regulated by activity-dependent uptake of transmitter molecules. Experimental evidence for such changes came from direct injection of glutamate [164] or glutamine [124, 125] into hippocampal slices. Both approaches increased the amplitudes of miniature inhibitory postsynaptic currents (mIPSCs), indicating that the precursors had indeed been used to fuel the vesicular transmitter pool. Consistent with these findings, blocking membrane-bound transporters for glutamine, GABA, or glutamate can reduce the size of IPSCs [128, 162, 165, 166]. The relative contribution of GABA, glutamate, or glutamine uptake to the vesicular GABA pool remains, however, unknown. It can be expected that the contribution of different transmitter transporters differs among neuronal subtypes, brain regions, and developmental stages [128, 167]. However, due to the fast action of uptake molecules, it is well possible that homeostatic adaptations of intravesicular GABA concentration occur at time scales of few seconds. Strong activation of axons in the CA1 area of mouse hippocampal slices results in a rapid increase of mIPSC amplitudes, with onset time below 20 s. This increase is dependent on uptake of glutamate and GABA, indicating that increased extracellular transmitter concentrations in active neuronal networks automatically provide more "fuel"

to the pool of releasable GABA, thereby constituting a negative feedback loop [165].

We have already discussed that tonic activation of GABA receptors by ambient transmitter concentrations provides a major mechanism for regulation of excitability [47, 48, 50]. It may, therefore, well be that changes in GABA uptake, production, and release cause altered tonic inhibition, possibly mediated by specialized subtypes of interneurons [56]. Quantitative knowledge about the contribution of these mechanisms is still lacking. It is also unclear how much nonvesicular release of GABA by reverse transport contributes to ambient GABA concentration. Situations of hyperactivity may favour such release mechanisms by sustained depolarization and altered local ion homeostasis [59, 60, 100, 168].

7. Pharmacological Use

Enhancing GABAergic inhibition is useful for the treatment of several pathological situations, including chronic pain, sleep disorders, anxiety, and—most importantly—epilepsy. In accordance with the principles outlined above, several drugs have been developed which alter presynaptic GABA content. One approach is blocking GABA degradation by GABA transaminase (GABA-T), using the suicide inhibitor γ-vinyl-GABA (GVG). Indeed, this drug does increase GABA levels in the brain [169, 170] and has anticonvulsant efficacy [171, 172]. Studies at the single cell level show that GVG increases miniature IPSC amplitude, consistent with a dynamic regulation of vesicular GABA concentration by the equilibrium between synthesis and degradation [173, 174]. Clinical use of GVG is, however, limited due to pathological changes of retinal cells and resulting scotoma [175].

An alternative approach suited to enhance synaptic GABA levels is the redirection of GABA uptake from glia to neurons. In glial cells, most GABA is degraded and fed into energy metabolism [176]. In contrast, neuronal GABA uptake can recycle the amino acid for use as a transmitter. It would therefore be ideal to have glia-specific GABA uptake inhibitors. Unfortunately, the molecular distinction between glial and neuronal GABA uptake is not strict, although there is some bias for GAT-1 in neurons and GAT-3 in glia [108–110].

In summary, there is no doubt that changes in GABA concentration contribute significantly to network homeostasis in health and disease. More quantitative information about sources, compartmentalization, and local concentration of GABA is urgently needed, not at least in order to develop more specific drugs for reconstituting excitation-inhibition balance in pathological situations.

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Research Article

Modulation of CREB in the Dorsal Lateral Geniculate Nucleus of Dark-Reared Mice

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The cAMP-response element-binding protein (CREB) plays an important role in visual cortical plasticity that follows the disruption of sensory activity, as induced by dark rearing (DR). Recent findings indicate that the dorsal lateral geniculate nucleus (dLGN) of thalamus is also sensitive to altered sensory activity. DR disrupts retinogeniculate synaptic strength and pruning in mice, but only when DR starts one week after eye opening (delayed DR, DDR) and not after chronic DR (CDR) from birth. While DR upregulates CREB in visual cortex, whether it also modulates this pathway in dLGN remains unknown. Here we investigate the role of CREB in the dLGN of mice that were CDR or DDR using western blot and immunofluorescence. Similar to findings in visual cortex, CREB is upregulated in dLGN after CDR and DDR. These findings are consistent with the proposal that DR up-regulates the CREB pathway in response to decreased visual drive.

1. Introduction

Long-lasting changes in neuronal circuitry are known to require nuclear gene expression and protein synthesis [1]. A critical component is the cAMP-response element (CRE)/CREB transcriptional pathway [2]. CREB is regulated by phosphorylation in response to neuronal activity [3], and its function in long-term plasticity, memory formation, and circuit development has been demonstrated in several neuronal systems [4–7]. In particular, studies in the developing visual cortex reveal a role for CREB in the rearrangement of connections in response to altered sensory input [8–11].

One of the key protocols for examining such experience-dependent plasticity in the visual cortex is raising animals in complete darkness. Dark rearing (DR) has been shown to increase the synaptic strength of excitatory intracortical synapses [12], lower dendritic spine density of pyramidal neurons [13], and enhance long-term potentiation and decrease long-term depression of layer III visual cortical neurons [14]. These changes have been associated with the upregulation of genes subserving synaptic transmission and neural activity [10]. For example, CREB transcription and translation are significantly increased in the visual cortex of mice reared in complete darkness from birth till postnatal day (P) 27, the peak of cortical plasticity in mice [15]. These changes have been attributed to synaptic compensatory mechanisms that are triggered by a reduction in afferent drive brought about by DR [10].

While CREB has been implicated in the refinement of subcortical visual connections, such as the remodeling of retinal projections from the two eyes to the dorsal lateral geniculate nucleus (dLGN), these changes take place before the onset of patterned visually evoked activity [16]. However, it has been recently shown that DR can disrupt retinogeniculate connectivity by altering the synaptic strength and pruning of dLGN retinal connections [17, 18]. These changes prevail only when animals are put in the dark one week after eye opening and not when chronically deprived from

birth. Interestingly, this form of dLGN visual experience-dependent plasticity occurs around the peak of visual cortical plasticity [15]. Whether CREB expression is upregulated in the dLGN, as it is in the visual cortex after DR, remains to be explored. To address this issue, we examined the effects of DR on CREB expression and phosphorylation in mouse dLGN using two DR protocols: chronic DR from birth (CDR) and DR following one week of vision (delayed DR, DDR).

2. Methods

2.1. Subjects. All procedures were performed in compliance with the Institutional Animal Care and Use Committee at Virginia Commonwealth University. C57BL/6 mice (Taconic Farms, Hudson, NY, USA) ranging in age from postnatal day (P) 0 to P27 were used in this study. The mice resided in colonies at the Virginia Commonwealth University Medical Center. Under normal light-rearing conditions (NR), mice were raised in a 12 hour light/12 hour dark cycle.

2.2. Dark Rearing (DR). Dark-reared pups and their mothers were housed inside light-tight containers in a dark room. There were four DR conditions (Figure 1(a)): chronic DR, delayed DR, chronic DR + light, and delayed DR + light. For CDR, absolute light deprivation began at birth (P0) and lasted for 27 days. For DDR, animals were reared in normal light-dark cycle until P20 and then placed in the dark for 1 week. For CDR + L and DDR + L conditions, DR was followed by 2 hours of light exposure. All animals were sacrificed at P27. For CDR and DDR, mice were decapitated in complete darkness [19]. Daily care (changes of food, water, and litter) was performed in the dark using infrared viewing goggles.

2.3. dLGN Tissue Extraction and Sample Preparation. Mice were deeply anesthetized with isoflurane and decapitated. The brain was quickly removed and placed in a 4°C solution (in mM) of 2.5 KCl, 10 glucose, 126 NaCl, 1.25 NaH_2PO_4, 2 $MgCl_2$, 2 $CaCl_2$. 400 μm thick sections were cut in the coronal plane with a vibratome (Leica VT1000S, Wetzlar, Germany). Tissue was extracted from sections through the middle of dLGN, then placed in a sucrose buffer (320 mM with 2% Complete Protease Inhibitor, Roche Diagnostics, Indianapolis, IN, USA) and stored at −80°C. Each sample contained the dLGN of both hemispheres from 3 to 4 animals. Whole tissue was homogenized in a sucrose buffer (320 mM with 2% Complete Protease Inhibitor and 1% PhosSTOP Phosphatase Inhibitor, Roche Diagnostics), and total protein concentration was determined using the Quick Start Bradford protein assay kit (Bio-Rad, Hercules, CA, USA).

2.4. Western Blotting Using Odyssey Infrared Imaging. For gel electrophoresis, 20–30 μg of protein for each sample was loaded onto a Criterion 10% Tris-HCl polyacrylamide gel (Bio-Rad). Then the protein was transferred to a nitrocellulose membrane (Bio-Rad). Membranes were blocked with the Odyssey blocking buffer (LI-COR, Lincoln, NE, USA). Rabbit anti-phospho-CREB (1 : 200; Cell Signaling, Danvers, MA, USA) and mouse anti-CREB (1 : 400; Cell Signaling) were diluted in Tris-buffered saline with Tween 20 (TBST) and 5% bovine serum albumin (BSA) and incubated overnight at 4°C. Rabbit anti-GAPDH (1 : 100,000; Cell Signaling) was used as a loading control. Then the membranes were treated with secondary antibodies conjugated to infrared (IR) fluorophores, IRDye 800CW goat anti-rabbit (1 : 10,000; LI-COR), and IRDye 680 goat anti-mouse (1 : 10,000; LI-COR), added to the blocking buffer for 1.5 hours at room temperature.

2.5. Western Blotting Using Chemiluminescence Detection. Gel electrophoresis and transfer were performed as described above. Membranes were blocked with TBST and 5% powdered nonfat milk, then incubated with either rabbit anti-phospho-CREB (1 : 500; Cell Signaling) in TBST and 5% BSA or rabbit anti-CREB (1 : 500; Cell Signaling) in blocking buffer overnight at 4°C. Rabbit anti-GAPDH (1 : 100,000; Cell Signaling) was used as a loading control. The membranes were then treated with peroxidase goat anti-rabbit (1 : 2000; Jackson ImmunoResearch, West Grove, PA, USA), in blocking buffer for 1.5 hours at room temperature. SuperSignal West Pico Chemiluminescent Detection Reagent (Pierce, Rockford, IL, USA) was used to visualize immunoreactivity.

Analysis for western blotting using IR imaging was performed using the Odyssey Application Software 3.0 (LI-COR). Analysis for western blotting using chemiluminescence detection was performed using Kodak 1D Image Analysis Software (Kodak, Rochester, NY, USA). Five separate measurements were taken for each band and the median value was used for estimating the protein levels. To minimize variability between blots, we assured that each blot had at least one sample from each group, so that relative changes to control could be examined within the same blot. Data were submitted to one-way analysis of variance (ANOVA) followed by post hoc comparisons to ascertain group differences. The least significant difference t-test (LSD) was used when equal variances were assumed and Tamhane's test when they were not. Significant differences between groups were defined as $P < 0.05$ (two tailed).

2.6. Immunofluorescence. Animals were perfused directly with 4% paraformaldehyde to preserve the phosphorylated state of CREB [8, 16]. Forty-micron-thick sections were then cut with a vibratome and blocked for 1 hour at room temperature using a solution of 2.5% BSA, 0.03% Triton X-100, and 5% normal goat serum in PBS. The primary antibodies were diluted in blocking solution, and sections were incubated in either rabbit anti-CREB (1 : 2000; Cell Signaling) or rabbit anti-phospho-CREB (1 : 800; Cell Signaling) overnight at 4°C. Sections were then incubated with secondary antibody (Alexa Fluor 488 goat anti-rabbit; 1 : 250; Invitrogen) in blocking solution for 2 hours at room temperature. Sections were placed on slides and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen).

To quantify cellular labeling of total CREB and phosphorylated CREB (pCREB), we measured the mean pixel

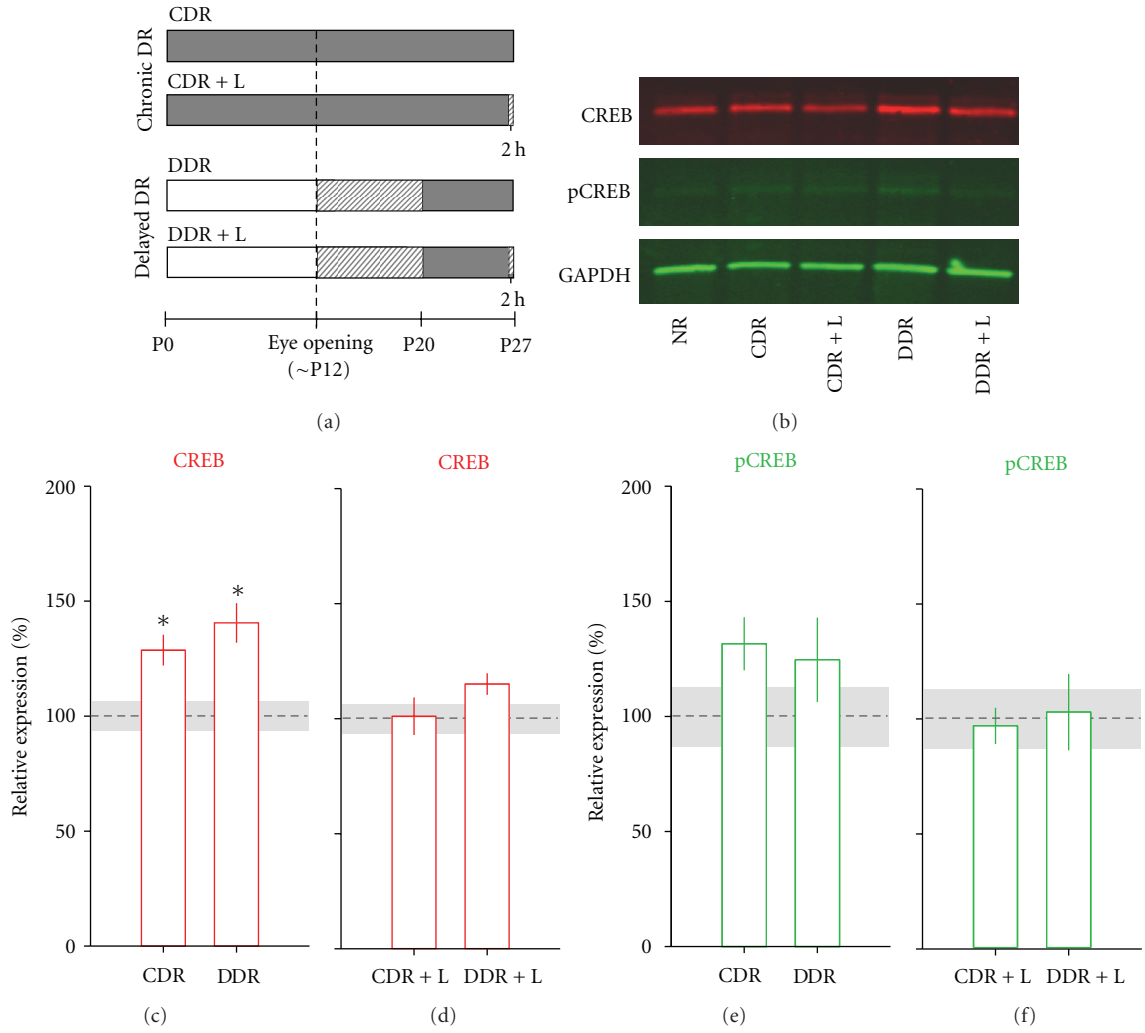

Figure 1: Dark rearing (DR) upregulates total CREB levels in the mouse dLGN. (a) Schematic showing the different DR conditions. The timeline depicts postnatal age in relation to the periods of DR (dark gray) and patterned visual experience (light gray). CDR and DDR; chronic and delayed DR; CDR + L and DDR + L; chronic and delayed DR followed by 2 hours of normal visual experience. Dotted line indicates the time of natural eye opening. For all conditions, tissue was harvested at postnatal day (P) 27. (b) Representative fluorescent immunoblot showing CREB (red) and pCREB (green) protein levels. GAPDH (green) was used as a loading control. CREB levels were higher in CDR and DDR compared to age-matched controls (normal-light reared, NR), and levels were restored after 2 hours of visual experience (CDR + L and DDR + L). pCREB levels were low and barely detectable (see the Results section for details). ((c)–(f)) Quantification of immunoblots ($n = 5$) for CREB ((c) and (d)) and pCREB ((e) and (f)) levels for DR ((c) and (e)) and DR + L conditions ((d) and (f)). The bars depict the relative averaged optical densities for each of the DR conditions ($n = 6$–8 dLGNs for each group per blot). Error bars represent ±SEM. The dotted line and corresponding shaded area reflect the averaged and ±SEM of CREB or pCREB protein levels obtained from age-matched controls. Total CREB levels for both DR conditions were significantly higher than control ((c), LSD post hoc test, $^*P < 0.05$ for both conditions).

intensity of fluorescence found in individual cells located within three to four 100 μm × 100 μm squares in dLGN. Measurements were conducted blind using Image J software and restricted to somata. Only those cells with a well-delineated continuous membrane within a given focal plane were included in the analysis. Using Metamorph software, a threshold was set so that there was a clear distinction between signal and residual background fluorescence [20]. Data were submitted to the Mann-Whitney tests to ascertain group differences. Significant differences between groups were defined as $P < 0.05$ (two tailed).

3. Results

We examined CREB expression in the dLGN of mice that were dark reared using western blot and immunofluorescence. Figure 1(a) provides a schematic delineating the different DR conditions. We first investigated the role of CREB in the dLGN of CDR (P0 to P27) and DDR (P20 to P27) P27 mice using infrared imaging detection. As shown in Figures 1(b) and 1(c), quantification of immunoblot optical densities indicated that both CDR and DDR led to a significant increase in total CREB levels (30–40%) relative to

values obtained in age-matched controls (one-way ANOVA, $F = 6.18$, $P < 0.05$). However, CDR and DDR were not significantly different from each other (LSD post-hoc test, $P = 0.25$). These results are consistent with observations made in the mouse visual cortex indicating that DR per se leads to an increase in CREB-mediated transcription [10]. Interestingly, while our results show that both CDR and DDR lead to a comparable upregulation of CREB, DDR has a much greater impact on the retinogeniculate circuitry [17, 18].

We also investigated whether the upregulation of CREB observed after DR could be restored to control levels by returning animals to the light. Figures 1(b) and 1(d) show that 2 hours of light exposure brings CREB back to normal levels for both DR groups (CDR+L and DDR+L). These findings are in agreement with results showing that DR-induced synaptic disruptions in dLGN circuitry recover when animals were returned to a normally lit environment [18].

Quantification of immunoblot optical densities for pCREB protein levels revealed that, similar to total CREB, pCREB was upregulated after CDR and DDR and that these elevations returned to normal levels after 2 hours of light (CDR+L and DDR+L, Figures 1(b), 1(e), and 1(f)). However, the observed increase in pCREB levels after CDR and DDR was not significantly different from control (one-way ANOVA, $F = 1.26$, $P = 0.32$). The latter was likely due to the higher variability observed for pCREB optical density values (compare the ±SEMs of Figures 1(c) and 1(d) versus Figures 1(e) and 1(f)). Such increased variability could in part be explained by the fact that pCREB protein content is greatly reduced at these late postnatal ages [16] and represents only a fraction of the total CREB protein levels for all groups. Therefore, the strength of the optical signal for pCREB is relatively weak compared to background levels. Although always higher than background, averaged pCREB optical density measured across all conditions was only 3% (±0.52) higher than background levels. By contrast averaged total CREB was 21% (±2.04) higher than background.

To verify whether pCREB levels are downregulated early in development, we investigated CREB expression during the first postnatal weeks of the mouse dLGN (Figure 2). As shown in Figures 2(a) and 2(c), quantification of immunoblot optical densities confirmed that pCREB levels were high during the first postnatal week, then significantly decreased by P14 and continued to remain low at P21 (one-way ANOVA, $F = 89.59$, $P < 0.001$). A similar result was observed for total CREB levels (Figures 2(a) and 2(b); one-way ANOVA, $F = 19.86$, $P < 0.001$). In fact, similar results were obtained when the same experiment was carried out using chemiluminescence techniques (Figures 2(d)–2(f); one-way ANOVAs, $F = 113.37$ for pCREB, $F = 9.18$ for CREB, $P < 0.05$ for both cases). Thus, these findings indicate that CREB protein levels are developmentally regulated and greatly reduced after the third postnatal week. As a result, low protein levels may become problematic when averaging the relative optical densities of multiple blots and should be considered in future studies that make use of infrared imaging techniques.

To examine the pattern of neuronal CREB expression in CDR and DDR mice, we used immunofluorescence labeling techniques on fixed slices of dLGN (Figures 3(a)–3(f)). For both CREB and pCREB, cellular labeling was present throughout dLGN. High-power views of dLGN indicated that staining was highly enriched in somata (Figures 3(a)–3(f), insets). For CREB, labeling was also apparent in the surrounding neuropil (Figures 3(a)–3(c), insets). Interestingly, such extrasomatic staining seemed confined to dLGN and adjacent visual nuclei (e.g., intrageniculate leaflet and ventral lateral geniculate nucleus, not shown) but was absent from other nonvisual structures such as the hippocampus (Figures 3(a)–3(c)).

Estimates of soma area ($\sim 100\,\mu m^2$) suggest that CREB and pCREB labeling was limited to relay neurons [20]. Similar to immunoblot results, both CREB and pCREB were upregulated after CDR and DDR. Interestingly, quantification of fluorescence signals within somata revealed significant increases in pixel intensity for DR groups compared to normal-light-reared (NR) animals (Figures 3(g) and 3(h)). This increase was true for total CREB levels (Kruskal-Wallis, $\chi^2 = 49.94$, $P < 0.0001$) as well as for pCREB (Kruskal-Wallis, $\chi^2 = 126.65$, $P < 0.0001$). Thus, our results are consistent with the proposal that DR upregulates the CREB pathway in response to decreased visual drive.

4. Discussion

Experiments in dark-reared mice suggest that normal-patterned vision is needed for the continued pruning of retinal connections onto dLGN cells as well as the synaptic strengthening of remaining ones [17, 18]. Here we show that a decrease in visual drive brought about by DR also upregulates total CREB protein levels in the dLGN. While we observed a concomitant increase in pCREB after DR, this change was not statistically different from protein levels obtained in controls. Because these measurements were obtained at late postnatal ages, a time when pCREB is extremely low compared to early postnatal ages, it could have made western blot quantification more difficult. Nonetheless, our immunofluorescence results indicate that both total CREB and pCREB are upregulated by DR. Thus, the changes in CREB reported here after DR suggests that this signaling pathway could contribute to plastic changes in retinogeniculate connectivity brought about decreased visual drive [17, 18]. Finally, it is important to note that, even though CREB expression was increased by DR, the degree of change was far below levels one sees at early postnatal ages, a time when retinal waves prevail and retinogeniculate projections are segregating into eye-specific domains [21].

Our results are consistent with observations made in visual cortex showing that DR upregulates the CREB transcription pathway [10]. However, in apparent contradiction to our findings, Pham et al. [8] showed that CRE-mediated transcription in dLGN is unaffected by visual deprivation. Perhaps this discrepancy is due to differences in deprivation paradigms, since Pham and colleagues [8] used monocular eyelid suture rather than DR. Indeed, monocular deprivation leads to unbalanced activity between the two eyes, whereas DR promotes an overall reduction of neuronal activity. In

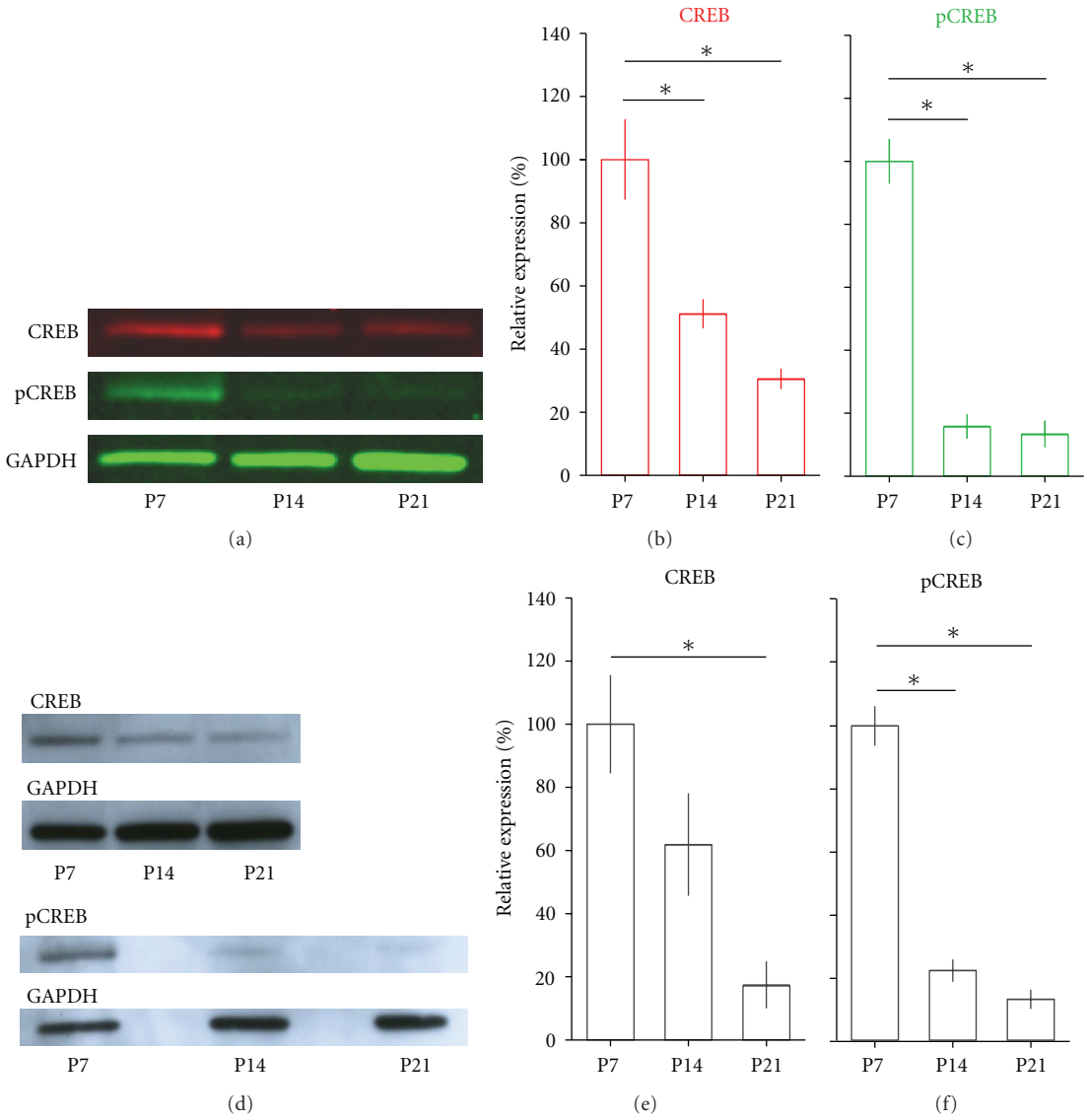

Figure 2: CREB and pCREB levels during early postnatal development in mouse dLGN. (a) Representative western blots of CREB and pCREB protein levels measured in dLGN at P7, P14, and P21 using infrared imaging detection. Red represents CREB levels and green pCREB and GAPDH levels. Both CREB and pCREB levels decreased with age. ((b) and (c)) Quantification of immunoblots for CREB (c) and pCREB (d) using infrared imaging. The bars depict the relative averaged optical densities measured at P7, P14, and P21. CREB and pCREB levels were significantly higher at P7 than at P14 and P21 (LSD post-hoc tests, $^{*}P < 0.01$). (d) Representative western blots of CREB and pCREB protein levels measured in dLGN at the same postnatal ages as in (a) using chemiluminescence detection. ((e) and (f)) Quantification of immunoblots for CREB (e) and pCREB (f) using chemiluminescence. Similar to infrared imaging, CREB and pCREB levels significantly decreased with age ((e) LSD post-hoc test and (f) Tamhane's post-hoc test; $^{*}P < 0.01$). Error bars represent ±SEM. Infrared imaging detection, $n = 5$ blots; chemiluminescence, $n = 3$-4 blots; $n = 6$–8 dLGNs in each age group per blot for both techniques.

the developing visual cortex, these manipulations lead to different structural and functional outcomes [22–24] and also activate differentially a number of molecular pathways involved in activity-dependent plasticity, including CREB [10].

What are the possible mechanisms underlying the activation of the CREB pathway during DR? A common feature for many identified neuronal networks is the ability to adjust activity and cellular signaling in the face of decreased drive in an attempt to maintain an optimal balance between excitation and inhibition [25, 26]. It has been suggested that neurons are able to detect decreased firing rates through calcium-dependent mechanisms that regulate glutamate receptor trafficking [27]. Indeed, recent findings have implicated the CREB pathway in this process. For example, in mouse cultured cortical neurons, chronic activity deprivation activates cAMP signaling pathways, which in turn promote CREB-mediated transcription that subsequently leads to the synthesis of proteins necessary for AMPA receptor trafficking [28]. Interestingly in dLGN, decreased

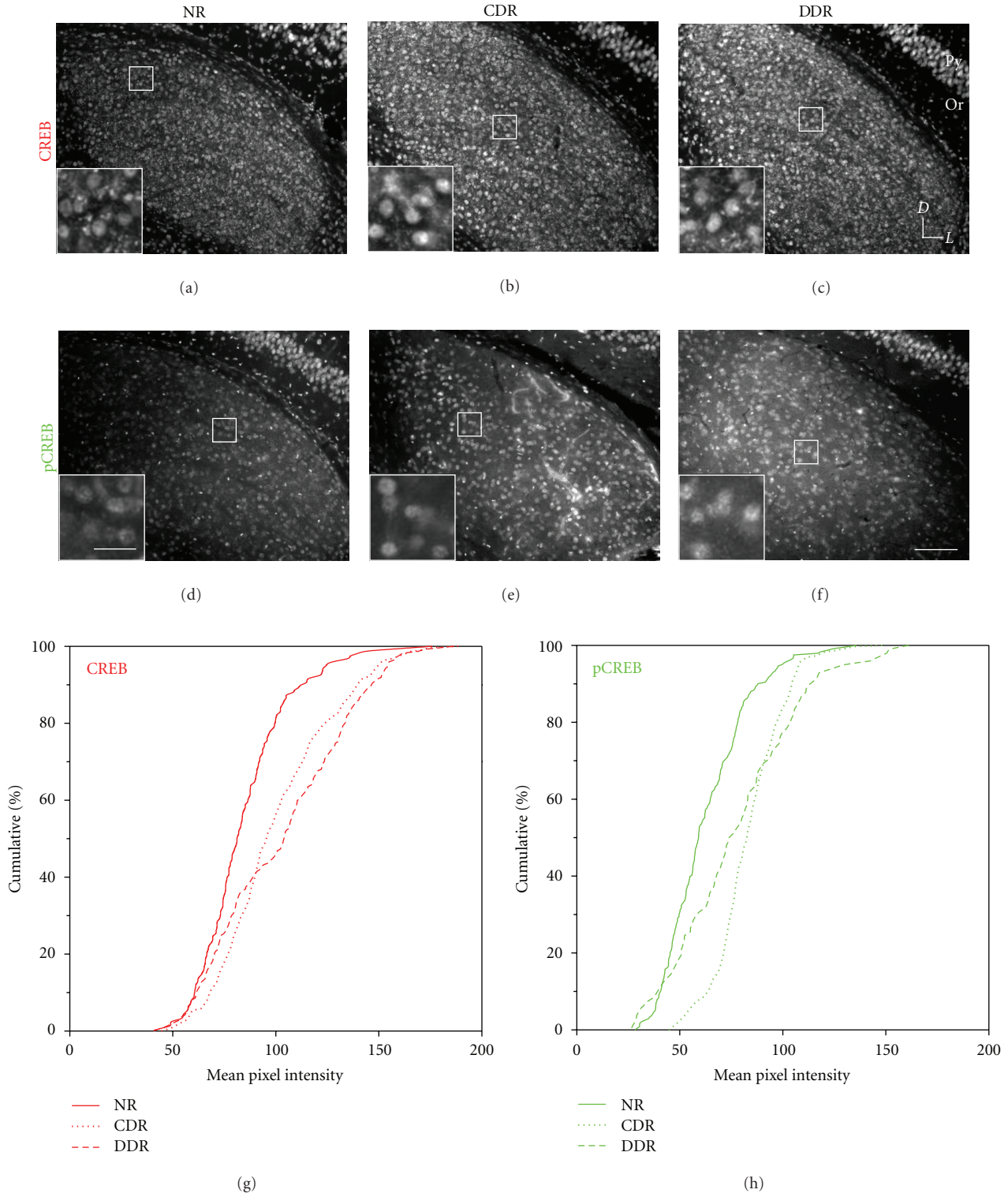

Figure 3: Modulation of CREB immunofluorescence in the dLGN of dark-reared mice. ((a)–(f)) Representative coronal sections of dLGN showing the labeling of total CREB ((a)–(c)) and pCREB ((d)–(f)), in NR ((a) and (d)), CDR ((b) and (e)), and DDR ((c) and (f)) mice. Corresponding high-magnification insets show that staining was prominent in somata. Py, pyramidal cell layer of hippocampus; Or, oriens layer of hippocampus; D, dorsal; L, lateral. Scale bars, 100 μm and 25 μm. ((g) and (h)) Cumulative histograms of cellular fluorescence intensity measured in somata in NR, CDR, and DDR for total CREB (g) and pCREB (h). Note that, for both total CREB and pCREB, CDR and DDR significantly shifted the distribution of fluorescence intensity toward larger values when compared to NR (the Mann-Whitney tests, $P < 0.0001$ for all comparisons). For CREB; NR $n = 159$ cells, CDR $n = 267$, DDR $n = 224$; for pCREB; NR $n = 242$ cells, CDR $n = 276$, DDR $n = 144$; from 2–6 dLGNs per group.

visual drive brought about by DR leads to a modification in glutamatergic synaptic transmission [17]. Whether the upregulation of CREB noted after DR is responsible for these changes or involves the activation of other molecular pathways in conjunction with CREB remains to be explored. For instance, manipulations that decrease neural activity affect a number of CREB-related signaling cascades including ones that involve brain-derived neurotrophic factor (BDNF) [29], the cytokine tumor necrosis factor α (TNFα) [30, 31], and the calcium/calmodulin-dependent protein kinase (CaMK) family [32–34].

It is important to note that CREB was upregulated whether DR was implemented from birth (CDR) or delayed (DDR) for one week or so after eye opening. Although both manipulations modify glutamatergic synaptic currents in dLGN, DDR appears to have a greater impact on retinogeniculate circuitry, weakening synaptic connections and altering the degree of retinal convergence [17, 18]. How then can we account for the fact that both CDR and DDR show comparable levels of CREB? One possibility is that the upregulation of CREB after DR reflects synaptic modifications other than those involving retinal inputs since dLGN receives afferent input from many sources, most notably excitatory feedback from the visual cortex [35, 36]. In fact, we have shown that corticogeniculate feedback can modulate the frequency and amplitude of miniature EPSCs recorded from dLGN cells after visual deprivation [37]. Another and perhaps more important possibility is that the *induction* of the reported modifications at the retinogeniculate synapse is driven by molecular pathways that are activated independently of CREB. However, our results suggest that, regardless of the molecular pathways underlying the induction of such changes, their *maintenance* seems to involve CREB. In fact, when visual activity is brought back to normal levels by exposing dark reared mice to the light, the upregulation of CREB is terminated.

In summary, our results reveal that the CREB pathway, while developmentally regulated in dLGN, can also be modulated by early visual experience. The latter extends the observations made in the visual cortex and suggests that the CREB pathway may represent a more generalized mechanism by which the developing visual system adapts to decreased visual drive.

Acknowledgments

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