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Minireview

Organization of the Locus Coeruleus-Norepinephrine System

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The release of the neurotransmitter norepinephrine throughout the mammalian brain is important for modulating attention, arousal, and cognition during many behaviors. Furthermore, disruption of norepinephrine-mediated signaling is strongly associated with several psychiatric and neurodegenerative disorders in humans, emphasizing the clinical importance of this system. Most of the norepinephrine released in the brain is supplied by a very small, bilateral nucleus in the brainstem called the locus coeruleus. The goal of this minireview is to emphasize the complexity of the locus coeruleus beyond its primary definition as a norepinephrine-producing nucleus. Several recent studies utilizing innovative technologies highlight how the locus coeruleus-norepinephrine system can now be targeted with increased accuracy and resolution, in order to better understand its role in modulating diverse behaviors.

Introduction

Throughout the day, our brains must constantly process a wide variety of stimuli from our environment to decide what requires our immediate attention. We may, however, only appreciate the consequences of this ongoing neural process when it reaches certain strengths; for instance, if we are suddenly startled, or experience a stressful situation. Impressively, this continuous modulation of our brain's alertness and acuity is in large part regulated by one of its smallest nuclei, the locus coeruleus (LC). Despite comprising only a few thousand neurons, the LC releases the neurotransmitter norepinephrine in many anatomically and functionally diverse brain regions. In mammals, the LC is involved in modulating numerous behaviors, including sleep/wake states, attention and memory during cognitive tasks, and stress response [1].

Despite these complex and diverse roles, several observations have led to the belief that the locus coeruleus-norepinephrine (LC-NE) system is largely homogeneous. The LC is comprised of a densely packed population of cells (~1600 cells per LC in the rodent) with a common embryonic origin, all of which produce norepinephrine [2]. In addition, populations of LC neurons have been reported to exhibit synchronous firing patterns and send extensive projections throughout the brain and spinal cord to release norepinephrine [3–5]. Though several decades of research have focused on understanding LC anatomy and function, a central question in the field remains: how does a small, seemingly homogeneous, structure respond to diverse sensory stimuli and modulate neuronal activity in distinct brain regions, with a variety of behavioral consequences?

A major reason for this lingering question comes from the structure of the LC: its small size and shape make it challenging to discretely target for ablation/inhibition studies without also affecting nearby structures. Furthermore, because of its extensive projection pattern, manipulations of the LC affect norepinephrine signaling in many brain regions. Finally, chronic loss of norepinephrine signaling, through LC ablation or transgenic knockout of genes crucial for norepinephrine synthesis, alters signaling in several neuromodulatory pathways [6–8]. Together, these limitations have made it challenging to address whether discrete differences in LC anatomy or activity may underlie its diverse roles in the mammalian brain.

Even with these limitations, heterogeneity within the LC-NE system has been uncovered. While intriguing, the functional relevance of these observations has been slow to develop. The goal of this review is to highlight specific examples of organization within this circuit that have been uncovered over the past 40 years to suggest its diversity beyond a single functioning unit. With the rapid advancement of technologies that allow us to characterize and target small populations of neurons in order to uncover their identity, organization, and function, the time has come to build on these findings to dissect the complexity of this small but powerful nucleus.

Molecular Organization within the LC

Norepinephrine was one of the first neurotransmitters to be identified, being discovered in the central nervous system by Swedish physiologist Ulf von Euler in the 1940s, but it was the experiments of Dahlström and Fuxe that identified the LC (group A6) as the main source of norepinephrine in the brain [9,10]. Use of immunolabeling techniques that were specific to dopamine-β-hydroxylase (Dbh), the enzyme responsible for converting dopamine to norepinephrine, allowed for precise detection of norepinephrine$^+$ neurons, including their extensive axonal projections [5]. These reagents also provided confirmation that the LC is composed solely of norepinephrine$^+$ neurons, supporting the idea that it is a homogeneous structure.

Several studies, however, have observed that, while all LC neurons contain norepinephrine, they have other distinct characteristics that may provide heterogeneity to their function. At least two types of norepinephrine$^+$ cells, the large multipolar cells (~35 μm) and smaller fusiform cells (~20 μm), have been observed within the LC [11,12] (Figure 1A). While both cell types are located throughout the LC, their distribution is biased, with

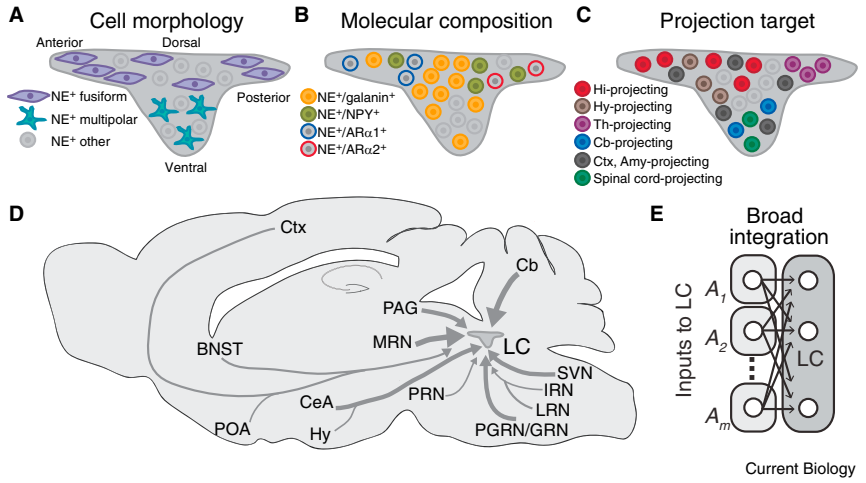

Figure 1. The LC-NE system has molecular heterogeneity and receives inputs from many brain areas to promote functional diversity.

(A) Within the LC, norepinephrine$^+$ cells with different morphologies have biased locations along the dorsal–ventral axis. These cells also have biased projections to different brain regions [11,26]. (B) While all LC neurons contain norepinephrine, some also express other molecules that may provide them with unique properties. Subsets of LC-NE neurons co-release other peptides, such as galanin and NPY, in addition to norepinephrine [13]. Subsets of LC-NE neurons also express different neurotransmitter receptors, which could alter the properties and conditions of their activation [18]. (C) LC-NE neurons projecting to different output sites have biased locations within the LC structure along the dorsal–ventral (for hippocampus-, cerebellum- and spinal cord-projecting) or anterior–posterior (for thalamus- and hypothalamus-projecting) axes. Cortex- and amygdala-projecting LC-NE neurons are located throughout the LC [26–28]. It is unknown to what extent the characteristics represented in (A–C) overlap within populations of LC-NE neurons. (D) A sagittal schematic illustrates the location of the LC in the brainstem (dark gray), and brain regions that provide the largest fraction of direct input to LC-NE neurons (arrows), determined by trans-synaptic rabies tracing [28]. The thickness of the arrows represents the relative fraction of input neurons contributed by each region. (E) Inputs from many brain regions converge onto individual LC-NE neurons [28]. Abbreviations: ARα1, adrenoceptor α1 subtype; ARα2, adrenoceptor α2 subtype; BNST, bed nucleus of the stria terminalis; Cb, cerebellum; CeA, central amygdala; Ctx, cortex; Hi, hippocampus; Hy, hypothalamus; IRN, intermediate reticular nucleus; LC, locus coeruleus; LRN, lateral reticular nucleus; MRN, midbrain reticular nucleus; PAG, periaqueductal gray; PGRN/GRN, paragigantocellular/gigantocelluar nucleus; POA, preoptic area; PRN, pontine reticular nucleus; SVN, spinal vestibular nucleus; Th, thalamus. Panels D and E reprinted by permission from Macmillan Publishers Ltd: Nature [28], copyright 2015.

more fusiform cells located in the dorsal LC, and more multipolar cells in the ventral LC [11].

In addition to morphological differences, several neuropeptides are expressed within subsets of LC neurons (Figure 1B). The most abundant example is galanin (Gal), which is expressed in up to 80% of LC neurons [13]. Release of Gal in the brain modulates many behaviors, such as wake/sleep states, nociception, feeding, and parental behavior [14]. Neurons co-expressing Gal and norepinephrine were found throughout the LC, but were most densely localized to the dorsal and central LC sub-regions. Gal expression in the LC has also been compared to Neuropeptide Y (NPY), which is co-expressed in a smaller population of LC neurons (~20%) restricted to the dorsal portion of LC [13]. While not directly confirmed, a small population of LC-NE neurons has been hypothesized to co-express Gal and NPY. The functional relevance of norepinephrine and neuropeptide co-release from LC neurons is virtually uncharacterized, as is the distribution of norepinephrine$^+$/Gal$^+$ and norepinephrine$^+$/NPY$^+$ axons throughout the brain. However, neuropeptide co-release could in principle modify the effect of norepinephrine release at specific output sites [15]. Many other neuropeptides have been detected in small subsets of neurons in the LC, though their role is unclear [16].

LC neurons also contain many neurotransmitter receptors, which, if variably expressed, could promote important differences in how individual LC neurons respond to similar inputs. For instance, the LC itself is responsive to norepinephrine release, and LC-NE neurons contain several adrenoceptor subtypes, most abundantly α2, with lower expression of α1 [17]. These receptors may be differentially localized within the LC, as α1 binding sites, detected by radioactive ligand, were more abundant in the anterior portion of the LC, while α2 binding sites were more dense in the posterior LC (Figure 1B) [18]. Also, these adrenoceptor sub-types are coupled with different G protein signaling pathways that could promote opposing actions on local LC activity, depending on which receptors were activated [19]. Nicotinic acetylcholine receptors (nAChRs) are also highly expressed within the LC, where application of acetylcholine and nicotine were shown to depolarize LC neurons and increase their firing rate [20]. Single cell RT-PCR of LC neurons suggested that nAChRs were differentially expressed in these cells, and could be classified into two groups: small cells, with higher levels of α3 and β4 mRNAs, and large cells, with more mRNA of the α6 and β3 subtypes. These two classes of LC neurons displayed different strength of responses upon nicotine application [21]. Other neurotransmitter and neuropeptide receptors, such as GABA, orexin/hypocretin, and opioid receptors, are present in LC neurons, but it is unclear if they are differentially expressed [22–24].

As these findings indicate, neurons within the LC are much more molecularly diverse than their primary definition as norepinephrine-producing cells. It is surprising, however, that while many of these molecules have been identified in the LC for decades, almost nothing is known regarding their roles there. With the development of public gene expression databases, such as the Allen Brain Atlas and Eurexpress, as well as technologies that allow high-resolution sequencing of discrete cell populations, our awareness of the LC's molecular diversity will only grow. Meanwhile, tools for exploring the function of these molecules are also rapidly advancing. For instance, the recent development of a transgenic mouse expressing Flp recombinase in norepinephrine$^+$ neurons now allows researchers to target molecularly diverse norepinephrine$^+$ cell populations in the brain using intersectional strategies, in order to precisely characterize their function [2].

Anatomical Organization of the LC-NE System

In addition to its molecular composition, the anatomical connectivity between the LC and the rest of the brain is crucial for proper

LC function during diverse behaviors. From the medulla to the olfactory bulb, nearly all brain regions contain Dbh$^+$ axons; the exceptions include the striatum, globus pallidus, nucleus accumbens, and substantia nigra, all of which are rich for dopamine neuron axonal projections or cell bodies. The lack of LC projections within these regions suggests a division of labor between the two types of catecholamine neurons [11]. Separate experiments, where radioisotopes were injected directly into the LC, confirmed that it sends extensive projections to the forebrain, cerebellum, brainstem, and spinal cord [25].

But while these studies demonstrated that the output projections of the LC are broad, they provided only limited information about the organization of these efferent projections, and did not address if LC neurons project homogeneously throughout the nervous system, or if sub-populations project to specific targets. The use of retrograde tracers was an important step in addressing these issues. By injecting the retrograde dye horseradish peroxidase (HRP) into brain regions receiving LC input, several groups observed a similar phenomenon: that certain cells within the LC are topographically organized based on their output target (Figure 1C). In general, LC neurons projecting to forebrain regions (such as hippocampus and septum) are located in dorsal LC, while cerebellum- and spinal cord-projecting LC neurons are located more ventrally. Output-specific organization was also observed along the anterior-posterior axis, with hypothalamic-projecting LC cells located anteriorly, thalamic-projecting cells located posteriorly, and cortical and amygdala-projecting cells scattered throughout the LC [26–28]. One group also noted that this output-dependent topography corresponded with different cell morphologies in the LC [26].

For the most part, it is not known how this topographic organization relates to LC function, but a recent study [29] highlights how we can begin to understand this relationship using viral reagents and optogenetic methods. To optically activate populations of LC-NE neurons, lentivirus expressing channelrhodopsin2 (ChR2) under the control of a catecholaminergic-specific promoter was injected into the LC of rats. Upon illumination of the LC, roughly half of the animals displayed an antinociceptive effect when thermal heat was applied to their paw, while the other half had a pronociceptive response. Post-hoc histological analysis revealed that animals with antinociceptive responses had more ChR2$^+$ LC-NE neurons located in the ventral LC, while pronociceptive animals had more ChR2$^+$ neurons in the dorsal LC [29]. Hence, activation of dorsal or ventral biased populations of LC-NE neurons can modulate distinct behaviors in the rodent. But the topography of norepinephrine$^+$ somata within the LC is only one component of its output organization: of equal importance is an understanding of how the LC axons themselves are organized; specifically, do populations of LC neurons contact discrete brain regions, or collateralize to simultaneously reach multiple targets?

Classic studies approached this question by injecting a pair of retrograde tracers into two LC projection sites, such as cortex and cerebellum, or hippocampus and thalamus. It was observed that a substantial portion of LC neurons were labeled by both tracers, indicating that they sent projections to both injection sites and supporting the idea that LC neurons collateralize over long distances throughout the brain [30–32]. Recent work, however, has found that LC collateralization is more segregated in cortical areas [33]. While these studies provide insight regarding LC output organization, they only assess collateralization of LC neurons between two brain regions, and different tracers may have different labeling efficiencies for LC neurons. To circumvent these limitations, a recent study utilized canine-adenovirus type 2 (CAV), which robustly infects neurons via their axon terminals, to visualize the output organization of LC-NE neuron populations projecting to many regions throughout the mouse brain [28]. LC output was found to be highly divergent, yet the density of labeled LC axons within certain brain regions was not completely homogeneous. Therefore, while overall the LC-NE system collateralizes broadly (an important characteristic with regard to its role in modulating entire brain states), small populations of LC neurons may innervate certain targets more selectively.

Deciphering the organization of inputs received by the LC is another crucial component in understanding how its activity is regulated during behaviors. By injecting retrograde tracers directly into the LC, several groups reported that the LC receives input from many brain regions, while others argued that its inputs were much more restricted [34,35]. These discrepancies are likely explained by two limitations of retrograde tracers: they can be taken up by axons-in-passage as well as axonal terminals, resulting in labeling of 'inputs' that are not directly connected to the LC; and uptake is restricted to the injection site at the LC cell bodies, so distant inputs onto dendritic arbors would be missed.

Only recently was the brain-wide synaptic input onto LC neurons more definitively determined through the use of trans-synaptic rabies viral tracing methods (Figure 1D) [28]. In contrast to classic studies that reported limited inputs, rabies tracing revealed that up to 111 different brain regions send direct input, with varying number of input neurons, to the LC. A simulation analysis of the tracing data suggested that individual LC-NE neurons receive input from 9–15 different brain regions as a lower bound, indicating that the inputs received by the LC are largely integrative (Figure 1E). This study also introduced a new method called 'tracing the relationship between input and output' (TRIO) and cell-type-specific TRIO (cTRIO), which restricts trans-synaptic tracing to subsets of neurons based on their cell-type and projection pattern. Using TRIO and cTRIO, it was found that LC-NE neurons projecting to diverse brain regions received mostly similar input. This result is consistent with the idea that the LC-NE system integrates and broadcasts information widely to modulate brain states. Discrete differences in input–output connectivity were observed, however, for certain LC-NE sub-circuits, which support previous observations that the LC is not entirely homogeneous [28].

These findings suggest that mechanisms exist beyond the anatomical organization of the LC-NE system that help it to generate diverse behavioral responses. In support of this, classic studies have shown that the firing rates of LC-NE neurons increase when the animal is aroused [4,36]. Furthermore, the frequency with which LC neurons fire is positively correlated with their synchrony [37]. But it was only recently, through the use of optogenetic methods, that the causal role of variable LC firing to mediate specific behaviors was directly demonstrated (Figure 2A) [38,39]. In one study [38], the authors closely controlled the firing rate of LC-NE neurons in awake-behaving mice to show that LC activation maintains the duration of wake

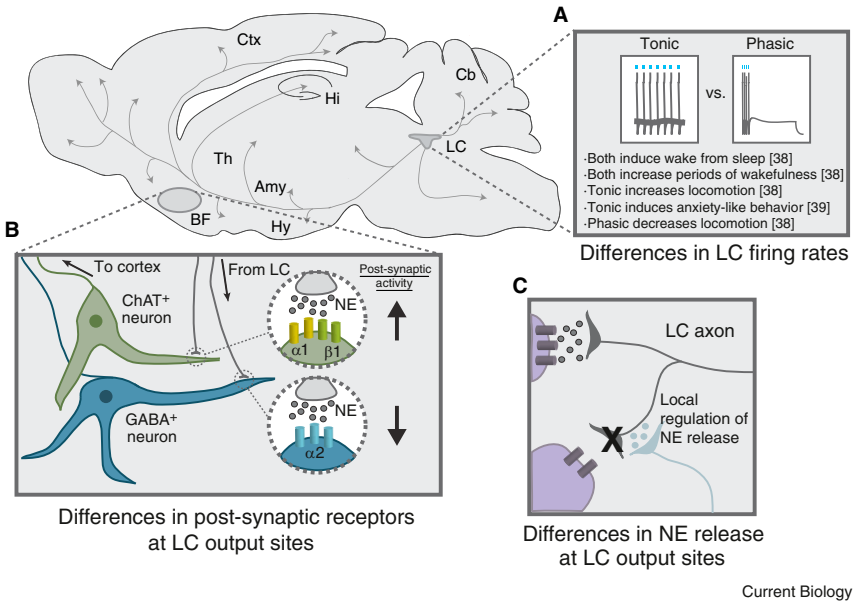

Figure 2. Variability in LC firing rates, and in the local environment where norepinephrine is released, promotes functional diversity.

A sagittal schematic illustrates projections from the LC to almost all brain regions (arrows) [11]. (A) Optogenetic methods have been used to demonstrate how differences in LC firing rates generate unique behaviors in the mouse. For instance, induction of tonic or phasic firing in the LC promotes wakefulness [38], while high-frequency tonic firing can induce anxiety-like and aversive behaviors in the mouse [39].

(B) Differences in post-synaptic receptor expression in neurons targeted by LC-NE axons allow target cells to be differentially activated by norepinephrine. Interspersed ChAT$^+$ and GABA$^+$ neurons in the basal forebrain receive input from the LC and project to the cortex. When norepinephrine is released, the ChAT$^+$ neurons, which express the α1 and β1 adrenoceptors that promote excitation, release more acetylcholine in the cortex to promote arousal. Simultaneously, release of norepinephrine suppresses the GABA$^+$ neurons, which express inhibitory α2 adrenoceptors. These neurons normally promote sleep by releasing GABA in the cortex [41–45]. (C) Differential presynaptic regulation of neurotransmitter release (for example, via axo-axonic synapses) can promote differences in downstream signaling from the same LC-NE neurons. It is unknown whether this phenomenon occurs within the LC-NE system, but examples can be found for pre-synaptic gating in other neuronal types in the cortex, hippocampus [46], and dorsal raphe [47]. Abbreviations: Amy, amygdala; BF, basal forebrain; Cb, cerebellum; ChAT, choline acetyltransferase; Ctx, cortex; GABA, γ-aminobutyric acid; Hi, hippocampus; Hy, hypothalamus; LC, locus coeruleus; NE, norepinephrine; Th, thalamus. Sagittal schematic reprinted by permission from Macmillan Publishers Ltd: Nature [28], copyright 2015.

episodes and promotes immediate sleep-to-wake transitions. They also observed differences in locomotor activity and behavioral arrest in the mice, depending on the pattern (tonic or phasic) and length of the optogenetic stimulation applied to the LC. A second study [39] also used optogenetics to manipulate LC activity during stress-induced behaviors. In this way, the authors demonstrated that high-frequency tonic, but not phasic, activation of the LC could elicit anxiety-like and aversive behaviors. They also found that chemogenetic inhibition of the LC during stressful stimuli prevented subsequent anxiety-like behavior, an important insight for developing effective treatments for stress-related disorders.

Together, these findings nicely highlight the importance of distinct LC firing patterns for the generation of different behaviors. The input connectivity that drives these differences is still largely unknown, but the generation of tools that now allow one to manipulate, record, and trace the connection of specific subsets of neurons within the LC should help clarify these outstanding issues.

Effects of LC-mediated Norepinephrine Release at Output Sites

Several characteristics, such as the molecular composition and topographical organization of the LC, and subtle differences in its anatomical connectivity, suggest that while the LC-NE system is largely integrative, it does not perform completely homogeneously. Even if LC-NE neurons are homogeneous in their axonal projections anatomically, however, there are several mechanisms by which they could differentially affect the physiology of their targets. While much remains to be investigated, the best characterized of these phenomena is the differential expression of post-synaptic adrenoceptors.

For instance, post-synaptic adrenoceptor diversity in the basal forebrain (including the medial septal nucleus, medial preoptic nucleus, and substantia innominata) allows the LC to promote both excitation and inhibition of target cells that are important for wakefulness (Figure 2B). The basal forebrain contains interspersed populations of excitatory/modulatory cholinergic neurons and inhibitory GABAergic neurons, which send parallel projections to the cortex to mediate arousal behaviors [40,41]. The cholinergic population is activated during arousal, while the GABAergic population is activated during sleep states [42,43]. Release of norepinephrine from LC terminals in the basal forebrain simultaneously activates the cholinergic neurons, which express α1 and β1 adrenoceptors, while inhibiting the GABAergic neurons, which express α2 adrenoceptors [44,45]. In this way, LC activation leads to norepinephrine release in the basal forebrain that modulates cholinergic and GABAergic neurons in opposing manners, which act in a concerted fashion to promote arousal.

Another potential mechanism in which similar LC-NE neurons could differentially affect their targets would be through local presynaptic regulation of norepinephrine release at target sites (Figure 2C). Such a phenomenon has yet to be uncovered for the LC-NE system, but other examples of axo-axonic regulation of neurotransmitter release have been described in the brain [46,47].

Implications for Brain Disorders

Though the findings summarized in this minireview focus on the basic biological mechanisms underlying LC organization and function, our increased understanding of this system also greatly impacts our progress in treating several neurological diseases. For instance, drugs that block the re-uptake of norepinephrine

are frequently prescribed to patients suffering from psychiatric disorders such as anxiety and depression, suggesting involvement of the LC-NE system in these conditions [48]. But the success of these treatments can vary and come with many negative side effects, likely due to their broad action on norepinephrine signaling in the nervous system. LC dysfunction is also strongly correlated with several neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis, where LC-NE neurons undergo selective and early degeneration [49,50]. That the LC is implicated in such a variety of psychiatric and neurological disorders emphasizes both its importance and complexity within the brain. While many studies over the past 40 years have contributed to our understanding of how the LC-NE system is organized in the brain, the recent development of genetic, viral, and optogenetic tools now allow us to more precisely determine the functional significance of these findings, and apply them to improve our understanding and treatment of these disorders.

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