An interaction between locus coeruleus activation modes and heterogeneous adrenoceptor expression in the basolateral amygdala for valence signaling

by

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Abstract

The locus coeruleus (LC) is a neuromodulatory brainstem nucleus which signals arousal via the release of norepinephrine (NE) throughout the central nervous system. Norepinephrine facilitates adaptive behavioural responding, perception, and learning and memory via enhancing the signal-to-noise ratio at downstream structures. The LC has recently been suggested to be involved in positive and negative valence signaling via two distinct activation modes, phasic and tonic. This effect has been shown to depend on adrenoceptor engagement in the basolateral amygdala (BLA). Here, we sought to determine whether phasic and tonic modes of LC activation differentially engage functionally distinct subpopulations of the BLA and whether naturally-produced valence recruits the same circuitry. Finally, we investigated the adrenoceptor profile of these subpopulations, as our valence effects may depend on their unique adrenoceptor expressions. Phasic and tonic LC photostimulation preferentially recruited nucleus accumbens (NAc)- and central amygdala (CeA)-projecting subpopulations of the BLA in the presence of an odor, respectively. Natural reward and aversive learning showed patterns of BLA activation similar to that of phasic and tonic LC photostimulation, respectively. Immunohistochemistry revealed differences in adrenoceptor expression across BLA subpopulations. These findings offer a mechanism underlying the differential valence effects of phasic and tonic LC activation.

General Summary

Rewarding and threatening situations demand action on behalf of the organism being rewarded or threatened. This action is both external, as in the case of physically moving toward rewards and away from threats, and internal, as in the case of the associated attentional shifts and encoding of relevant information into memory stores. In mammals, these actions are heavily dependent on a brain structure known as the locus coeruleus (LC), which has powerful influence over brain activity in times of stress or arousal. Phasic (bursting) and tonic (prolonged) LC activity lead to real-time and long-term pursuit of reward- and avoidance of threat-indicating stimuli, respectively. Here, we suggest that an interaction between LC activation mode and downstream receptor heterogeneity in the amygdala underlies these responses.

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List of Abbreviations

AAV adeno-associated virus

Antag antagonist

AP anterior-posterior

AR adrenoceptor

BLA basolateral amygdala

cAMP cyclic adenosine monophosphate

CeA central amygdala

ChR2 channelrhodopsin-2

COPT conditioned odor preference test

CTB cholera toxin sub-unit B

Ctrl control

DA dopamine

DG dentate gyrus

DV dorsal-ventral

GANE glutamate amplifies noradrenergic effects

ICSS intra-cranial self-stimulation

LC locus coeruleus

LTP long-term potentiation

ML medial-lateral

mPFC medial prefrontal cortex

NAc nucleus accumbens

NE norepinephrine/noradrenaline

O odor

PBS phosphate buffer saline

PC piriform cortex

PFA paraformaldehyde

PFC prefrontal cortex

PKA protein kinase A

PVN paraventricular nucleus

PVP polyvinylpyrrolidone

ROPT real-time odor preference test

SD Sprague Dawley

TH tyrosine hydroxylase

VTA ventral tegmental area

Co-authorship statement

A version of the work presented in this thesis has been published in Ghosh et al. (2021) (Fig. 1, 2, 3, 4, 5) and Omoluabi et al. (2022) (Fig. 6, 7). The authors have the copyright of this work, which is open-access and which may be reused with proper citation.

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1. Introduction

1.1. The locus coeruleus

In the mammalian brain, arousal is largely mediated by the release of norepinephrine (NE) from neurons of the brainstem locus coeruleus (LC) (Berridge & Waterhouse, 2003). The LC has long been assigned a role in arousal and vigilance (Foote & Morrison 1987; Aston-Jones, 1985), owing to the type of afferent information it receives (Aston-Jones et al., 1991), its brain-wide projection patterns (Foote et al., 1983; Aston-Jones & Cohen, 2005), and its responding to arousing stimuli (Foote et al., 1983). The LC responds to arousing stimuli, positive and negative in nature (Bouret & Sara, 2004; Chen & Sara, 2007), in turn releasing NE to virtually all areas of the central nervous system, including the amygdala, hippocampus, hypothalamus, cortex, and spinal cord (Loizou, 1969). Such wide-ranging efferents, in conjunction with predominant volume transmission of NE, allow the LC to ubiquitously modulate the activity of neurons and neuronal networks. Decades of research have implicated the LC-NE system in fulfilling the role of arousal via facilitation of attention (Robbins, 1984), sensory processing (Foote & Morrison 1987), adaptive behavioural responding (Aston-Jones & Cohen, 2005), as well as learning and memory (Lalumiere et al., 2017; Tully & Bolshakov, 2010).

Norepinephrine exerts its effects on target cells and networks by binding G-protein-coupled adrenoceptors, the main subtypes of which are α_1-, α_2-, β_1-, and β_2-adrenoceptors, which couple to G_s, G_q, and G_i proteins (Tully & Bolshakov, 2010). Beta (β)-adrenoceptors are coupled to G_s proteins, are predominately expressed post-synaptically, and generally increase cellular excitability and thus evoked-activity and network activity via an increase in intracellular cAMP and subsequent protein kinase A (PKA) activation and blocking of Ca2+-dependent K+ currents

(Berridge & Waterhouse, 2003; Ramos & Arnsten, 2007; Sara, 2009; Tully & Bolshakov 2010; Nomura et al., 2014). Alpha-1 (α_1)-adrenoceptors are coupled to G_q proteins, are predominately expressed post-synaptically, and generally increase cellular excitability by triggering phospholipase activation and the blocking of K+ channels (Berridge & Waterhouse, 2003; Ramos & Arnsten, 2007; Wang & McCormick, 1993). Lastly, α_2-adrenoceptors are coupled to G_i proteins, and are expressed pre- and post-synaptically, hampering NE release and reducing cellular excitability (Berridge & Waterhouse, 2003; Carter et al., 2010; Starke, 2001). Affinity for NE increases from lowest affinity β-adrenoceptors to intermediate-affinity α_1-adrenoceptors and highest affinity α_2-adrenoceptors (Arnsten, 1999; Ramos & Arnsten, 2007). As such, the overall effect of NE in a given target structure depends upon the concentration of NE and relative densities and distributions of adrenoceptor subtypes. These differential effects of NE on target structures depending on the subtype of adrenoceptor engaged allows NE to carry out one of its major functions—enhancing the signal-to-noise ratio of activation in relevant brain areas.

The widespread projection pattern of the LC and the postsynaptic effects of NE led to S. Kety's original hypothesis that '[norepinephrine] affects synapses throughout the nervous system, suppressing most, but permitting or even accentuating activity in those that are transmitting novel or significant stimuli' (Kety, 1970). Early work verified this claim, particularly in sensory areas. It has long been known that the predominant effect of NE, applied by iontophoresis or released via electrical stimulation of the LC is widespread inhibition of spontaneous neuronal activity (Szabadi, 1979; van Dongen, 1981; Olpe et al., 1980; Segal & Bloom, 1976). NE enhances the signal-to-noise ratio or gain of physiologically evoked activation in sensory regions. Initial work in the auditory cortex of monkeys (Foote et al., 1975) revealed that NE inhibited spontaneous activity to a greater degree than it did acoustic vocalization-

evoked activity. Subsequently, Waterhouse and Woodward (1980) showed a similar discrepancy between the inhibition of spontaneous- vs evoked-activity upon NE application in the rat somatosensory cortex. More recently, Polack et al. (2013) demonstrated that NE mediates the enhancement of signal-to-noise ratios in the visual cortex during arousal. It has been hypothesized that such facilitation of sensory processing may serve, in part, to heighten perception (Berridge & Waterhouse, 2003). This claim has since been supported by research on tasks of odor discrimination. For instance, Doucette et al. (2007) showed that combining α- and β-adrenoceptor antagonists impaired odor discrimination performance. Shakhawat et al. (2015) showed that LC-NE stabilizes small odor representations in the olfactory cortex underlying similar odor discrimination. In adult rats, adrenoceptor blockade is associated with impaired odor pattern separation (Shakhawat et al. 2015), whereas a short burst of electrical stimulation of the LC sharpens odor representations in the piriform cortex (PC) (Bouret & Sara 2002). Increased levels of NE are also known to increase signal-to-noise ratios (de Almeida et al. 2015) and lower thresholds for odor discrimination (Escanilla et al. 2010). Such a role for NE in perceptual acuity is also in line with observations that arousing stimuli are often detected quicker than low-arousal stimuli (Leclere & Kensinger, 2008). Aside from the proposed role of NE-induced signal-to-noise enhancements in perceptual acuity, other theories of LC function focus on its fulfillment of other functions of arousal such as adaptive behavioural responding (Aston-Jones & Cohen, 2005; Bouret & Sara, 2005) and learning and memory (Mather et al., 2016).

1.2. Theories of locus coeruleus function

1.2.1. Adaptive Gain Theory

The adaptive gain theory of LC function developed by Aston-Jones and Cohen (2005) instead places emphasis on the modulation of gain in cortical circuitry underlying task performance, suggesting that the LC primarily modulates behavioural responding rather than sensory processing. More precisely, this model emphasizes the roles of the two firing modes of the LC, phasic and tonic, in adaptive behavioural responding in a changing environment. Phasic activation of the LC is characterized by a brief burst of activity followed by a pause, while tonic activity refers to the baseline firing rate of the LC, which is lowest during low arousal and sleep and highest during periods of heightened arousal. The timing of the phasic LC response suggests that it is driven by the outcome of task-relevant decision processes, allowing it to augment effective responding for the exploitation of rewards. During accurate performance on a task of operant conditioning in which monkeys are trained to press and refrain from pressing a lever discriminately to obtain a juice reward, the phasic LC response is observed to follow presentation of task-relevant target stimuli, but not task-irrelevant distractors (Aston-Jones et al., 1994). Further work suggested that the phasic response was more related to behavioural output than sensory input. For instance, Clayton (2004) demonstrated that the phasic response is more tightly coupled to the behavioural response than the presentation of sensory stimuli. Furthermore, in reversal experiments in which utility of presented stimuli was reversed, adjustments of the phasic response to new target stimuli preceded changes in behavioural responding (Aston-Jones et al., 1997). A similar role for the phasic response has been supported by operant conditioning and reversal experiments in rats (Bouret & Sara, 2004), interpreted there as a reflection of reward anticipation. Importantly, LC activity influences activity of the frontal cortex in about 60-70msec (Aston-Jones, 1985) and precedes behavioural responding by about 200ms, making it possible for NE to modulate activity developing in the motor cortex related to the behavioural response.

However, the phasic response is not simply involved in the motor response, as it does not precede, for instance, spontaneous responses during inter-trial-intervals. Instead, authors inferred that the phasic response is driven by decision processes which link relevant stimuli to the appropriate behavioural response by increasing the gain of cortical representations responsible for selective behavioural responding. The high tonic response on the other hand, is observed during periods of uncertainty regarding task utility, and is proposed to adaptively facilitate the exploration of alternate sources of reward via more widespread increases of gain leading to distractibility and disengagement from the task at hand. Importantly, the authors propose that although the studies reviewed focused on the facilitation of behavioural responding, such modulation of gain should apply equally well to facilitation of other operations such as the encoding of information into long-term memory stores.

1.2.2. Network Reset Theory

Subsequent work from Bouret and Sara (2005) dispensed with interpretations of the phasic response as indicating reward anticipation or a decision process in their formulation of network reset theory, based on the observation that reward-directed behaviours, in the absence of conditioned stimuli, are not preceded by the phasic response. Instead, the phasic response may be in response to relatively unexpected signals which require attentional and behavioural shifts and might promote such shifts (Aston-Jones & Bloom, 1981). Because attentional states are supported by activity in specific functional networks (Buser & Rougeul-Buser, 1995), behavioural shifts require rapid modification of network activity (Buser & Rougeul-Buser, 1995), and the phasic response is observed to precede such shifts, the phasic response may be

better interpreted as reflecting a "reset" signal responsible for the reconfiguration of network activity for the sake of rapid behavioural adaptation. Under this model, the distractibility and task-disengagement associated with the tonic LC response would be a product of multiple competing shifts to task-irrelevant stimuli. Supporting this, simultaneous recording studies suggest that LC activation promotes changes in medial prefrontal cortex (mPFC) and CeA activity, structures which likely work in concert with the LC to carry out its functions (Bouret & Sara, 2005). LC activity modulates activity in these structures at similar intervals and in response to similar events, which could reflect a network state change.

More direct support for the network reset theory of LC function has come from Harley's group (e.g. Brown, 2005; Grella et al., 2019). In the context of spatial learning, hippocampal sequences are instrumental for an organism's understanding of space. Such maps not only incorporate spatial information, but non-spatial information such as that regarding motivation (Teyler & Rudy, 2007) to allow for purposeful navigation of the environment. For instance, a brief foot shock globally resets hippocampal sequences in CA1 (Moita, 2004), presumably to update understanding of a now threatening environment. Further, silencing the LC prevents formation of spatial maps in CA3 in a novel environment. Similarly, Grella et al. (2019) have shown that bilateral phasic, but not tonic, activation of the LC generates novel map formation in the dentate gyrus (DG), CA1, and CA3 in a familiar environment, while inactivation of the LC results in recall of a familiar map in the DGS, CA1, and CA3c when an animal is placed in a novel environment. Similarly, LC-hippocampal fiber activation promotes reward remapping among place cells (Kaufman et al. 2020). As such, similar to the proposed role it plays in biasing network activity towards adaptive behavioural responding (Bouret & Sara, 2005), the phasic LC response may also bias network activity towards episodic memory encoding and updating in the

hippocampus. In this way, the LC signal may compliment spatial information, ultimately facilitating adaptive behaviour at both shorter (Bouret & Sara, 2005) and longer time scales (Grella et al., 2019).

1.2.3. Glutamate Amplifies Noradrenergic Effects Theory

More recently, Mather et al. (2016) have proposed the Glutamate Amplifies Noradrenergic Effects (GANE) model of LC function, which emphasizes the role of adrenoceptor affinity in NE effects and provides mechanistic insight into increased selectivity of perception and memory under arousal. In brief, high-priority or important information (as determined by bottom-up and/or top-down processes) is reflected in the brain as areas of increased glutamatergic activity. At the local circuit level, spillover glutamate at such representations interacts with NMDA receptors on neighboring noradrenergic terminals (Mather et al., 2016). Under arousal, phasic activation of the LC axonal fibers provides the coincident activation for NMDA receptor activation, leading to terminal release of NE. The resultant elevated levels of NE at such "hotspots" has multiple effects, ultimately enhancing the throughput and processing of high-priority information. For instance, elevated NE can interact with low affinity β-adrenoceptors on glutamatergic terminals to enhance glutamate release, or at the postsynaptic membrane to increase sensitivity to glutamate. At the same time, areas conveying low-priority information, under arousal, are bathed in lower concentrations of NE which interacts with higher affinity, inhibitory adrenoceptors, attenuating neighboring activity. Strong glutamatergic and noradrenergic activity at hotspots also activates local GABAergic cells projecting to neighboring circuitry, while NE simultaneously disinhibits local glutamatergic

activity, all effects of which increase lateral inhibition and throughput of glutamate signaling to further enhance signal over noise. Such enhancements of signal-to-noise or gain in brain areas conveying high-priority information is proposed to underlie, in part, selective attention and perception in arousing situations.

Notably, and echoing S. Kety's original hypothesis of a permissive role for NE in both the throughput of information and the formation of faciliatory synaptic changes (Kety, 1970), such strengthening of glutamatergic signals would not only lead to real-time effects such as selective perception, but also longer lasting effects through resultant synaptic changes (Mather et al., 2016). As such, the LC-NE system allows the organism to both deal with the arousing situation at hand while at once preparing it for potential future encounters. In fact, attention has long been known to be prerequisite for memory in both rodents and humans (Kandel, 2001). In line with this, arousal not only enhances perception, but perceptual learning of important information (Lee et al., 2012). Norepinephrine enhancement of long-term potentiation (LTP) processes (Yuan et al. 2000; Yuan 2009; Morrison et al. 2013) is also likely to enhance discrimination learning. Furthermore, increased cellular excitability and activation of the cAMP-PKA signaling cascade, both effects of which have been attributed to β-adrenoceptor activation, increases the probability of a cell being incorporated into an engram (Han et al., 2007; Frankland & Josselyn, 2015; Zhou et al., 2009).

Norepinephrine before, during, or after learning contributes to selective memory consolidation. In this way, arousal not only assists an organism in immediately coping with arousing events, but enhances consolidation of precedent information to which they may be related in an important way. Supporting this, arousal strengthens recent memory traces of

important information (Sakaki et al., 2014). For instance, water reward given after high frequency stimulation of the DG enhances LTP (Seidenbecher et al., 1997), an effect not observed with propranolol pretreatment (Straube et al., 2003). Harley first proposed a role for NE in hippocampal LTP in 1983 (Neuman & Harely, 1983). Subsequent experiments provided more indirect evidence for a role of NE in LTP in the hippocampus and other areas such as the amygdala (Hopkins & Johnston, 1988; Huang & Kandel, 1996; Huang et al., 2000). More recent work ubiquitously supports the role of NE in memory consolidation (LaLumiere et al., 2017). Johansen's group has suggested that NE not only enhances, but may be prerequisite for associative learning. Hebbian processes alone may be insufficient to give rise to associative learning, and may rely upon an interplay with neuromodulators such as NE to give rise to lasting synaptic changes (Bailey et al., 2000; Johansen et al., 2014).

1.2.4. A modular locus coeruleus

Importantly, the aforementioned models of LC-NE functioning are compatible with a functionally homogeneous view of the LC. For the first 50 years following the discovery of noradrenergic neurons in the LC it was thought that the LC received broad afferents which activated the LC as a whole, the result of which was brain-wide release of NE. More recently, the possibility of a functionally modular LC has arisen (Poe et al., 2020; Uematsu et al., 2017; Chandler et al., 2019). Under this model, while some afferents, such as those conveying autonomic information, may activate the LC as a whole, others may activate discrete LC modules or subpopulations. Similarly, some LC neurons project to a wide variety of target structures, while some have preferred terminal sites. As such, the LC is now thought to be

capable of both widespread signaling and more nuanced conveying of information, which requires further refinements of our understanding of the LC in its relation to adaptive functioning. It should also be noted that these theories of LC function focus on its impact on brain arousal. The LC is also known to play a critical role in bodily arousal via its interaction with the sympathetic division of the autonomic nervous system. For instance, the LC sends projections to areas of the hypothalamus such as the paraventricular nucleus (PVN), and activation of α_1-adrenoceptors in the PVN have been associated with autonomic responses to stressors (Stone et al., 2006). Sympathetic activation may in turn influence brain arousal. For instance, sympathetic activation is known to communicate with the CNS by way of the vagus nerve (Carabotti et al., 2015). Vagus nerve activation is known to increase activity in the LC (Groves et al., 2005). As such, an organism may call upon global and modular LC activation, the influence of the LC on bodily arousal, as well as the interaction between brain- and bodily arousal to deal with arousing stimuli.

1.2.5. Locus coeruleus activation modes and valence signaling

As indicated by models of LC function such as the GANE hypothesis, the LC need not depend upon modularity for its enhancement of specific circuitry. Another way specific effects may be achieved is via alterations in LC activation modes and the interaction between resultant alterations in NE levels and heterogeneous adrenoceptor expression at downstream projection sites. For instance, Arnsten (2011) has suggested that, in the prefrontal cortex (PFC), phasic LC activation engages higher affinity α_2-adrenoceptors while tonic LC activation engages lower

affinity β-adrenoceptors to enhance and degrade working memory functioning respectively. Our lab has shown that phasic and tonic LC stimulation can produce real-time and conditioned odor preference and aversion respectively (see Fig. 1.), and furthermore that these effects are dependent on adrenoceptor engagement in the BLA (Ghosh et al. 2021 (see Fig. 2.)). The phasic photostimulation parameters used in this study were 10 Hz long phasic (10 sec light pulse every 30 sec) and 10 Hz brief phasic (300 msec light pulse every 2 sec). These stimulation parameters are consistent with recent studies regarding frequency range and duration (Carter et al., 2010; Kempadoo et al., 2016; Vazey et al., 2018). The 10 Hz brief phasic pattern in particular mimics LC activity in response to environmental stimuli (Aston-Jones & Bloom, 1981; Nakamura et al., 1987). The tonic photostimulation parameters used were continuous photostimulation at 10 Hz and 25 Hz, which correspond to output frequencies of 10-15 Hz (Ghosh et al., 2021). This study also demonstrated that only the 25 Hz tonic pattern was capable of producing anxiety-like behaviour.

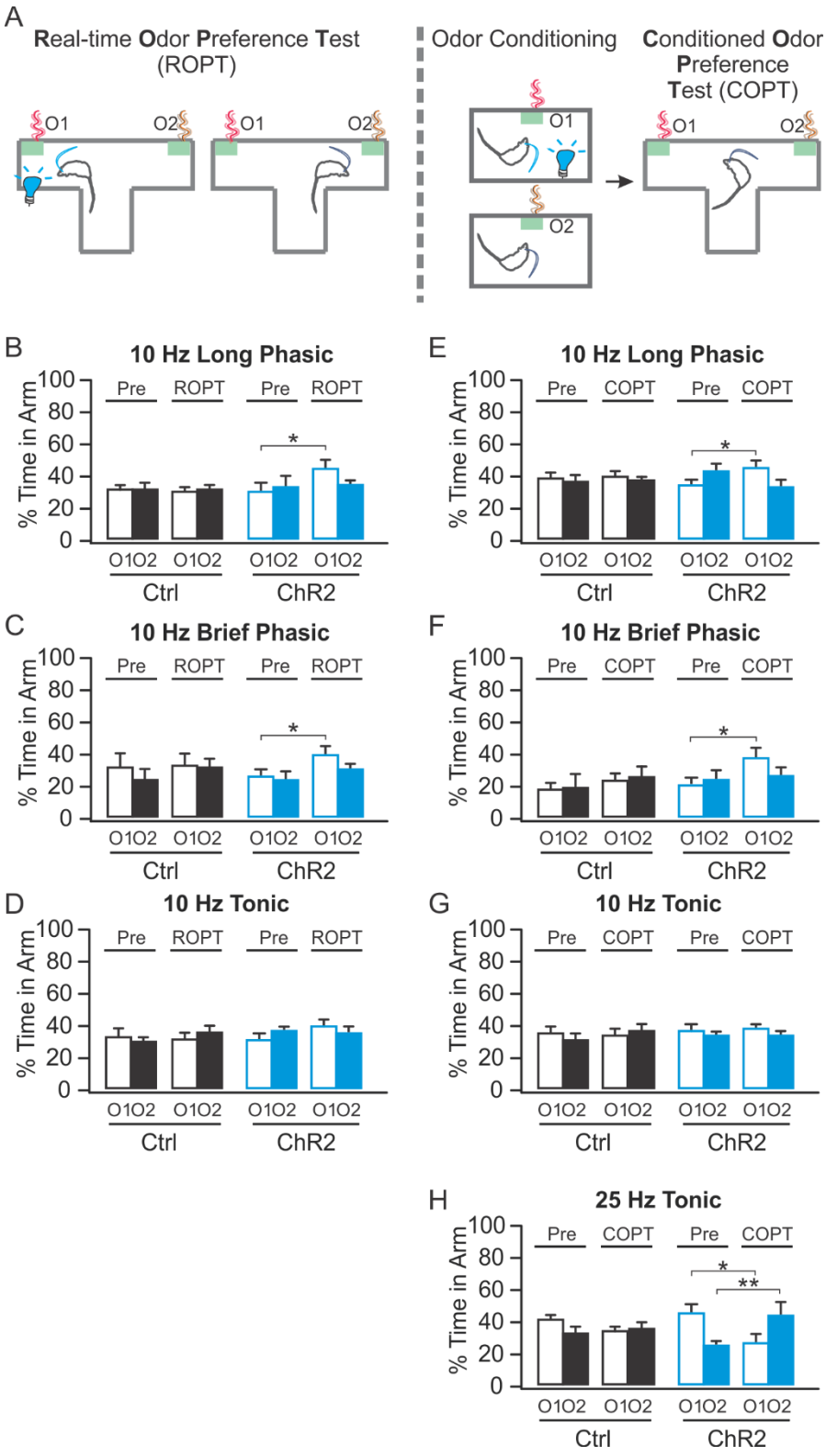

Figure 1. Twenty five hertz tonic LC activation leads to conditioned odor aversion while 10-Hz phasic LC photostimulation results in odor preference. (A) Schematic of real-time odor

preference test (ROPT) and conditioned odor preference test (COPT). (B) Percentage time spent in each odor in ROPT, with 10-Hz long phasic light paired with odor 1 (O1) and not odor 2 (O2) (n [ChR2/Control] = 9/8). (C) Percentage time spent in each odor in ROPT with 10-Hz brief phasic light (n [ChR2/Control] = 6/8). (D) Percentage time spent in each odor in ROPT with 10-Hz tonic light (n [ChR2/Control] = 10/7). (E) Percentage time spent in each odor in COPT, with 10-Hz long phasic light conditioned with O1 (n [ChR2/Control] = 11/12). (F) Percentage time spent in each odor in COPT, with 10-Hz brief phasic light (n [ChR2/Control] = 6/7). (G) Percentage time spent in each odor in COPT with 10-Hz tonic light (n [ChR2/Control] = 11/10). (H) COPT with 25-Hz tonic light (n [ChR2/Control] = 8/11). Ctrl: control. ChR2: experimental rats expressing channelrhodopsin-2 in the LC. Pre: pre-photostimulation. Panels B, C, and D show behaviour pre-photostimulation and during real-time photostimulation. Panels E, F, G, and H show behaviour pre-photostimulation and after conditioning with photostimulation. Two-way repeated ANOVAs followed by post-hoc Tukey tests were used to test significance. *$p < 0.05$. **$p < 0.01$. (adapted from Ghosh et al. 2021).

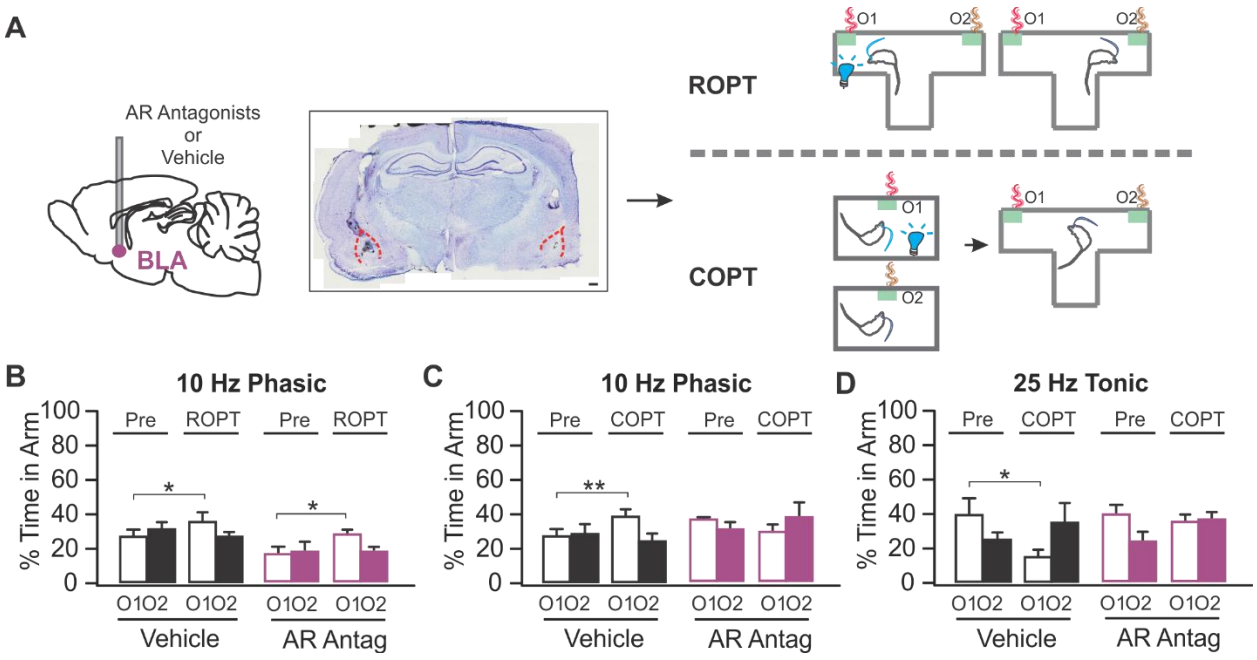

Figure 2. BLA ARs mediate conditioned preference and aversion in COPT. (*A*) Schematic of brain infusion, followed by ROPT and COPT. An example targeting image of BLA is shown in the middle. Scale bar, 500 μm. (*B*) Percentage time spent in each odors in ROPT, with 10-Hz phasic light paired with O1 (*n* [Vehicle/AR antagonists] = 7/7). (*C*) Percentage time spent in each odor in COPT, with 10-Hz phasic light conditioned with O1 (*n* [Vehicle/AR antagonists] = 8/6). (*D*) COPT with 25-Hz tonic light (*n* [Vehicle/AR antagonists] = 8/6). AR: adrenoceptor. Antag: antagonist. AR Antag: a mixture of α_1-adrenoceptor antagonist phentolamine and β-adrenoceptor antagonist alprenolol. Two-way repeated ANOVAs followed by post-hoc Tukey tests were used to test significance. *$p < 0.05$. **$p < 0.01$. (adapted from Ghosh et al. 2021).

These results are in line with the prescribed role of the LC in positive (Ritter & Stein 1973; Chen et al., 2021) and negative (McCall et al., 2015; Llorca-Torralba et al., 2019) valence signaling. Positive and negative valence are here being used in their motivational, and not necessarily emotional, senses. The term valence is used when referring to stimuli that elicit motivational behaviour: stimuli are said to be of positive valence if they elicit approach- or otherwise appetitive behaviours; stimuli of negative valence elicit avoidance- or otherwise defensive behaviours (e.g. Namburi et al., 2015; Beyeler et al., 2016). Motivated behaviours, however, do not imply subjective experience (LeDoux, 2012). In the 1970s, phasic electrical stimulation of LC was identified as having the effect of positive valence (Ritter & Stein 1973), but this association was criticized as electrical stimulation lacks specificity and a causal relationship was not established (Wise, 1978). Ghosh et al. (2021) address these deficiencies and reveal a positive valence associated with phasic LC activation. This work is also in line with work from Valentino and Van Bockstaele (2008) who have suggested that opioid inputs to the LC induce phasic LC activation for the attenuation of stress. Tonic LC patterns on the other hand, promote aversion and anxiety-like behaviour in mice (McCall et al. 2015, 2017) and rats (Hirschberg et al. 2017; Llorca-Torralba et al. 2019).

1.3. The basolateral amygdala

The BLA is a main input site of the amygdala, receiving input from sensory systems (Ledoux et al., 1990; Romanski et al., 1993; Fontanini et al., 2009). The majority of BLA output goes to the CeA, which receives sensory and pain information (Hasanein et al., 2008), allowing

for connections between the BLA and CeA to be a site for LTP-mediated threat learning (Solano-Castiella et al., 2010; Maren, 1999). The BLA also has projections to the NAc which are responsible for motivational salience in reward learning (LaLumiere, 2014; Nieh et al., 2013).

Beyeler et al. (2018) defined the anatomical arrangements of functionally distinct BLA neurons projecting to the NAc and CeA. They injected retrograde tracers conjugated to fluorescent cholera toxin subunit B (CTB) into the NAc and CeA. They identified that BLA-CeA neurons were preferentially located in the dorsal BLA in anterior and intermediate sections, as well as in the lateral BLA in more posterior sections. Conversely, BLA-NAc neurons were most dense in the medial BLA, a trend that was consistent across all anterior-posterior (AP) planes.

1.3.1. The basolateral amygdala to central amygdala for negative valence

Jimenez and Maren (2009) demonstrated that BLA-CeA projections are essential for anxiety-like behaviour. Rats underwent a standard fear conditioning paradigm in which an auditory stimulus was paired with a foot-shock. Conditioned fear, as indicated by freezing behaviour in response to the shock-paired auditory stimulus was similar among all rats. Rats then underwent surgery; lesions were made to the BLA in one hemisphere and the CeA in the contralateral hemisphere, effectively abolishing ipsilateral BLA-CeA connections bilaterally. One week post-operation, rats that received contralateral lesions froze significantly less to the conditioning context than did controls.

Building on the importance of BLA-CeA projections for anxiety-like behaviour, Beyeler et al. (2016) showed that BLA-CeA neurons respond preferentially to aversive outcome-predicting cues. A viral vector delivered Cre-dependant ChR2 to the BLA, and a second retrograde viral vector carrying Cre-recombinase was delivered to the CeA to allow for photolabelling of BLA-CeA neurons. Next, mice were trained to discriminate two auditory stimuli associated with a sucrose reward of positive valence and an aversive outcome (quinine) of negative valence. After the association was made between one tone and sucrose (as indicated by licking upon tone onset) the second association was introduced. When criteria was met for each association, recordings of BLA neurons during retrieval were performed. All BLA-CeA neurons responding only to the quinine-predictive cue were excited by it as opposed to 49% of BLA cells that only responded to the cue.

Furthermore, Namburi et al. (2015) have shown that BLA-CeA connections strengthened after a fear learning paradigm and that photostimulation of these projections was sufficient to signal negative valence. BLA-CeA neurons were retrogradely labelled with fluorescent beads. Mice were then subjected to fear conditioning where a tone was associated with a foot-shock. Synaptic strength between the BLA and CeA was then measured as AMPA/NMDAR ratio using whole-cell patch clamp recordings from BLA-CeA neurons. Synaptic strength increased after fear conditioning. They next expressed ChR2 in BLA-CeA neurons and mice freely explored in a place avoidance assay in which photostimulation of BLA-CeA neurons was paired with one of two chambers. Mice spent significantly less time in the photostimulation-paired chamber, demonstrating the importance of BLA-CeA projections for the signaling of negative valence.

1.3.2. The basolateral amygdala to nucleus accumbens for positive valence

Beyeler et al. (2016) showed that BLA-NAc neurons respond preferentially to reward-predicting cues using the same technique as described above for the BLA-CeA subpopulation. 77% of BLA-NAc neurons were excited by the sucrose-predictive cue as opposed to 51% in the entire BLA. Further, 100% of BLA-NAc neurons that responded selectively to the quinine-predictive cue as a conditioned stimulus were inhibited by it.

Furthermore, Namburi et al. (2015) also showed that BLA-NAc connections strengthened after a reward learning paradigm and that photostimulation of these projections was sufficient for positive valence. After undergoing a reward learning paradigm where a tone was associated with a sucrose reward, synaptic strength between the BLA and NAc increased. They next expressed ChR2 in BLA-NAc neurons and subjected mice to an intracranial self stimulation (ICSS) task. ICSS was observed upon photostimulation of BLA-NAc neurons, thus demonstrating their role in positive valence. These results demonstrate that valence encoding in the BLA is at least partially explained by anatomically distinct subpopulations.

1.4. An interaction between locus coeruleus activation modes and basolateral amygdala subpopulations

Given the role of both the LC and BLA in valence signaling, LC innervation of the BLA (McCall et al., 2015), and the role of BLA adrenoceptor engagement in the valence effects of LC photostimulation, it therefore seems plausible that the positive- and negative valence signaling

effects of the LC may be at least in part mediated by an interaction with the BLA subpopulations known to be involved in positive and negative valence. Using retrograde tracing and selective stimulation of LC-BLA terminals, McCall et al. (2017) have shown that the anxiogenic and negative valence effects of tonic LC activity are at least partially mediated by its interaction with the BLA-CeA subpopulation, and in a β-adrenoceptor-specific manner. Conditioned place aversion and increased anxiety-like behavior have been demonstrated by increasing tonic firing of BLA-projecting LC neurons (McCall et al. 2017; Llorca-Torralba et al. 2019).

1.5. The current study

Here, we primarily sought to determine whether different activation modes of the LC (phasic and tonic) would engage different BLA subpopulations (NAc- and CeA-projecting), therefore serving as a pathway mechanism for our observed valence effects of LC stimulation. To explore this, we used a combination of retrograde-CTB tracing in the BLA and optogenetic photostimulation in the LC. We hypothesized that phasic LC activation, which we have shown to signal positive valence, would bias activation towards the BLA-NAc subpopulation known to be involved in reward-seeking behaviour. We hypothesized that tonic LC activation, which we have shown to signal negative valence, would bias activation towards the BLA-CeA subpopulation known to be involved in avoidance behaviour. We also explored whether our LC activation modes lead to downstream differences in ventral tegmental area (VTA) activation. We then explored whether we could explain these effects in the BLA by examining adrenoceptor expression in the BLA using immunohistochemistry. Lastly, we explored whether natural

learning such as threat- and reward conditioning differentially engage the NAc-projecting subpopulation of the BLA in a manner consistent with differential engagement via phasic and tonic LC photostimulation, to see if similar engagement of the BLA occurs in both artificial and physiologic settings. Our results shed light on the mechanisms underlying the activation mode-specific effects of the LC.

2. Materials and Methods

2.1. Contribution Statement

All surgeries were carried out by myself, except for those for the no light control group for Fig. 3, which Camila Reinhardt assisted with. For cFos induction (Fig. 3), Tamunotonye Omoluabi and Abhinaba Ghosh carried out photostimulation on days three and four. Reward conditioning was carried out by myself. Aversive conditioning was carried out by myself and Tayebeh Sepahvand. Brain processing and immunohistochemistry was carried out by myself except for assistance with brain sectioning and immunohistochemistry from Tayebeh Sepahvand (Fig. 3) and assistance with brain sectioning from Tamunotonye Omoluabi (Fig. 7). All images were acquired by myself. All images were analyzed by myself, except for a subset of those used for Fig. 3 which was analyzed by Abhinaba Ghosh.

2.2. Animals and ethics statement

Tyrosine hydroxylase (TH)-CRE homozygous male breeders (Sage laboratories) were bred with Sprague–Dawley female breeder rats (Charles River) for TH-CRE heterozygous offspring that were used in this study. For natural learning, SD rats were used. Rats of both sexes were housed in a 12-h light/dark cycle (7am-7pm light) and had ad libitum access to food and water except for during food restriction (20 g food per day). All experimental protocols followed the guidelines of Canadian Council of Animal Care and were approved by the Memorial University Animal Care Committee.

2.3. Viral transduction

For experiments requiring optogenetic stimulation, an adeno-associated virus (AAVdj or AAV8) served as a vector to carry the genetic construct of channelrhodopsin-2 (ChR2) with a reporter gene for fluorescent proteins (EYFP or mCherry) under a double-floxed inverted open reading frame (DIO). Experimental constructs were AAVdj-EF1a-DIO-hChR2 (H134R)-mCherry or AAV8-Ef1a-DIO-eChR2 (H134R)-EYFP. The control construct was AAVdj-EF1a-DIO-mCherry. The Deisseroth Laboratory at Stanford University provided all AAVs.

2.4. Stereotaxic surgeries

2.4.1. Optogenetic surgeries

For experiments requiring optogenetic stimulation, three to 10-month-old adult TH-CRE rats received bilateral virus infusions (5E+12 vg/mL) in the LC under isofluorane anesthesia in a stereotaxic frame. Each hemisphere received two infusions, each of 0.7 μL (fluorescent beads: virus = 2:5) at the rate of 0.5 μL/min. The cannula was lowered at a 20◦ angle to avoid the transverse sinus. Infusion coordinates were 11.8–12.2-mm posterior, 1.2 and 1.4-mm bilateral, and 6.3-mm ventral with respect to bregma. At a minimum 1 month after infusion surgery, rats underwent optical fiber cannula (ferrule attached, containing optical fiber; Doric Lenses) implantation surgeries (11.8–12.2-mm posterior, 1.3-mm bilateral, and 6.3-mm ventral with respect to bregma), followed by one to four weeks of recovery before commencing behavioral tests.

2.4.2. Retrograde labeling

For experiments requiring Cholera Toxin B (CTB) infusions, surgeries were either done alone or combined with LC optical fiber cannula implantation and rats were allowed a 10-day recovery before carrying out experiments. CTB-594 and CTB-488 (1% w/v in phosphate buffer; Invitrogen) were infused by separate 32g beveled 1-μL Hamilton syringes (Neuros 7001 KH) attached to a vertical infusion pump (Pump 11 Elite; Harvard Apparatus; Dong et al. 2017; McCall et al. 2017) in NAc (200 nL; AP: 1-mm anterior, medial-lateral (ML): 1-mm bilateral, and dorsal-ventral (DV): 6.5 mm) and CeA (150 nL; AP: 2.1- mm posterior, ML: 4.2-mm bilateral, and DV: 7.5mm) respectively. Each infusion lasted 5 min, followed by a 5 min wait before withdrawing the syringe. Rats were allowed one to four weeks for recovery before the behavioral experiments or perfusion with 4% paraformaldehyde (PFA).

2.5. Behavioural tests

2.5.1. Optogenetic experiments

For cFos induction in Fig. 3, rats were habituated to the experimental environment for two days. On the mornings of the third and fourth days, rats were optically stimulated with either phasic or tonic patterns in their home cages, for 10 min/day, while being exposed to an odorized sponge (benzaldehyde 0.05%). Control rats were exposed to the odor only without light stimulation. Ninety min following the odor + light, or odor only stimulation on the fourth day, rats were anesthetized, perfused, and brains were collected.

2.5.2. Light stimulation for optogenetic experiments

Bilateral photostimulation at 450nm (20 mW/mm2 at fiber tip) was delivered by two laser light sources (LDFLS_450; Doric Lenses) through mono-fiberoptic patch cords. Current equivalence of power was 150 mA. For cFos activation in the BLA (Fig. 3), 10-Hz brief phasic (300 msec every 2 sec) was compared with 25-Hz tonic light stimulation. Different patterns of stimulation were controlled from Doric software.

2.5.3. Odor reward conditioning

In a modified food retrieval test (Ghosh et al., 2021), rats were food-restricted for 4-7 days before training and food restriction continued throughout the experiments. Following a 3–5-day habituation to the training chamber (a 60 cm x 60 cm x 40.5 cm Plexiglas box) and sponge with a food pellet (chocolate cereal), rats performed odor discrimination learning consisting of 16 trials/day for three days. Two sponges were infused with 60 µL of either odor 1 (O1) or odor 2 (O2). A retrievable chocolate cereal was placed in a 2 cm hole on the surface of the O1 sponge, while a non-retrievable cereal was placed in a hidden hole in the O2 sponge to control for the smell of chocolate cereal. During the trial, rats freely explored the box and sponges; the position of the sponges was changed in each trial. A trial ended when a nose poke was made in the sponge, irrespective of the sponge choice. A trial was termed correct response if a nose poke was made in the O1 sponge containing the retrievable cereal and the food was retrieved. Rats were confined to a corner in the test box with a barrier for 20 sec between the trials. The percentage of correct responses was calculated as the number of correct nose pokes over the total number of nose pokes. A trial ended if no nose poke occurred in 3 min and was excluded from analysis. In the control condition, the food pellet was paired with both O1 and O2 pseudo-randomly.

2.5.4. Odor aversive conditioning

Rats were habituated to a shock chamber (San Diego Instruments) for 30 min for three consecutive days. On the fourth day, rats were exposed to four trials of shock paired with an odor at the 5^{th}, 15^{th}, 20^{th}, and 30^{th} min during a 30 min training session. An odor was delivered to the

shock chamber by an olfactometer for 1 min at each time point, terminating with the shock (0.5 mA for 1 sec). On the fifth day (test day), rats were exposed to the shock chamber with no odor delivery for 5 min to measure baseline activity, followed by a 5 min exposure to the conditioned odor. The experiment was videotaped and the percentage of time freezing (freezing defined as no body movement except breathing) was calculated. Rats exposed to only odor were used as control.

For cFos induction following natural odor conditioning, rats were re-exposed to the conditioned odor in the home cage for 10 min, 24 hr following the odor preference test or freezing test, and perfused 90 min later with 4% PFA. Brains were extracted and stored in 4% PFA solution overnight and then transferred to PVP solution (1% polyvinylpyrrolidone, 30% sucrose, 30% ethylene glycol in 0.1M PBS) storage solution until used for immunohistochemistry.

2.5.5. Odorants used in behavioural experiments

For optogenetic experiments, benzaldehyde (0.005%; based on previous publication (Shakhawat et al., 2015)) was used. For odor food reward conditioning, almond (2%)/coconut (2%) or vanilla (2%)/peppermint (2%) pairs were used. For odor shock conditioning, either benzaldehyde (0.05%) or vanilla (2%) was used.

2.6. Immunohistochemistry

Rats underwent trans-cardiac perfusion with cold isotonic saline followed by 4% PFA. Brains were extracted and kept in 4% PFA. Brains were then sectioned using a vibratome (Leica VT 1000P; Leica Biosystems) or compresstome (Precision Instruments) in 50-μm thick coronal slices and saved in a PVP solution.

Fifty-μm free floating sections belonging to similar positions in the anterior–posterior axis of the brain as determined by unaided visual observation were chosen in an unbiased manner. The sections were washed in Tris buffer (0.1 M, pH 7.6) twice for 10 min each, followed by 10 min in Tris A (0.1% TritonX in Tris buffer), and Tris B (0.1% TritonX and 0.005% BSA in Tris buffer) before applying a blocking solution of 10% normal goat serum (Sigma-Aldrich) for 1 hr. This was followed by a 10-min wash each in Tris A and Tris B before incubating in a primary antibody solution prepared in Tris B at 4 ◦C (cFos, 1:2000, Cell Signaling). For the adrenoceptor expression experiment, sections were incubated with primary antibodies (α_1, 1:2000, Alomone; β_1, 1:2000, Abcam; β_2, 1:2000, Alomone) at 4°C for two nights. After two nights, sections were washed for 10 min each in Tris A and Tris B and incubated in a secondary antibody solution prepared in Tris B at 4 ◦C (anti-rabbit Alexa 647, 1:1000, Thermo Fisher Scientific; anti-rabbit Alexa 647, 1:1000, Invitrogen). This was followed by 10-min washes in Tris A, Tris D (0.1%Triton X and 0.005% BSA in 0.5 M Tris buffer), and Tris buffer, respectively. Finally, sections were mounted onto slides and cover-slipped with DAPI mounting media.

2.7. Image acquisition and analysis

Fluorescent images were acquired by an EVOS 5000 (Thermo Fisher Scientific). Images were acquired similarly for rats, keeping gain and exposure time the same throughout each experiment. Images were only analyzed in hemispheres with correct CTB targeting in the NAc and CeA. Images were analyzed using ImageJ software.

For optogenetic and natural learning experiments, cFos+ cells and double-labeled CTB cells were counted. For optogenetic experiments, images underwent background subtraction before manual cell counting.. Three to six images per animal were analyzed and values from both hemispheres were averaged. A subset of the images was analyzed blindly. For natural learning experiments, images underwent background subtraction before automatic cell counting using the Trainable Weka Segmentation plugin. For adrenoceptor distributions, images underwent background subtraction before manual cell counting. Three images per animal were analyzed.

2.8. Statistics

A One-way ANOVA followed by post hoc Tukey tests were used for Figure 3. Independent samples t-tests were used for Figure 4. A One-way ANOVA followed by post hoc Bonferonni tests were used for Figure 5. A Two-way repeated ANOVA was used to examine the overall patterns of adrenoceptor distribution in Fig 6A2-d3 and a paired t-test was used to compare percentages of NAc- and CeA-projecting cells that expressed adrenoceptors. Unpaired

t-tests were used in Figure 7. Data are presented in graphs as Mean +/- standard error of the mean (SEM). Statistics were performed using OriginPro 9.1.

3. Results

3.1. Locus coeruleus phasic and tonic patterns engage differential basolateral amygdala circuitry in odor valence learning

We tested whether tonic and phasic activation of the LC biases activation of the BLA ensembles projecting to CeA (aversive) and NAc (reward) circuitry respectively. We infused retro-tracing dyes linked to CTB in the CeA and NAc and examined the overlap of CeA or NAc projecting neurons with cFos+ cells in the BLA activated by odor only (no-light control), 10-Hz brief phasic, or 25-Hz tonic LC photostimulation. All experimental rats were photostimulated in the presence of an odor (Fig. 3).

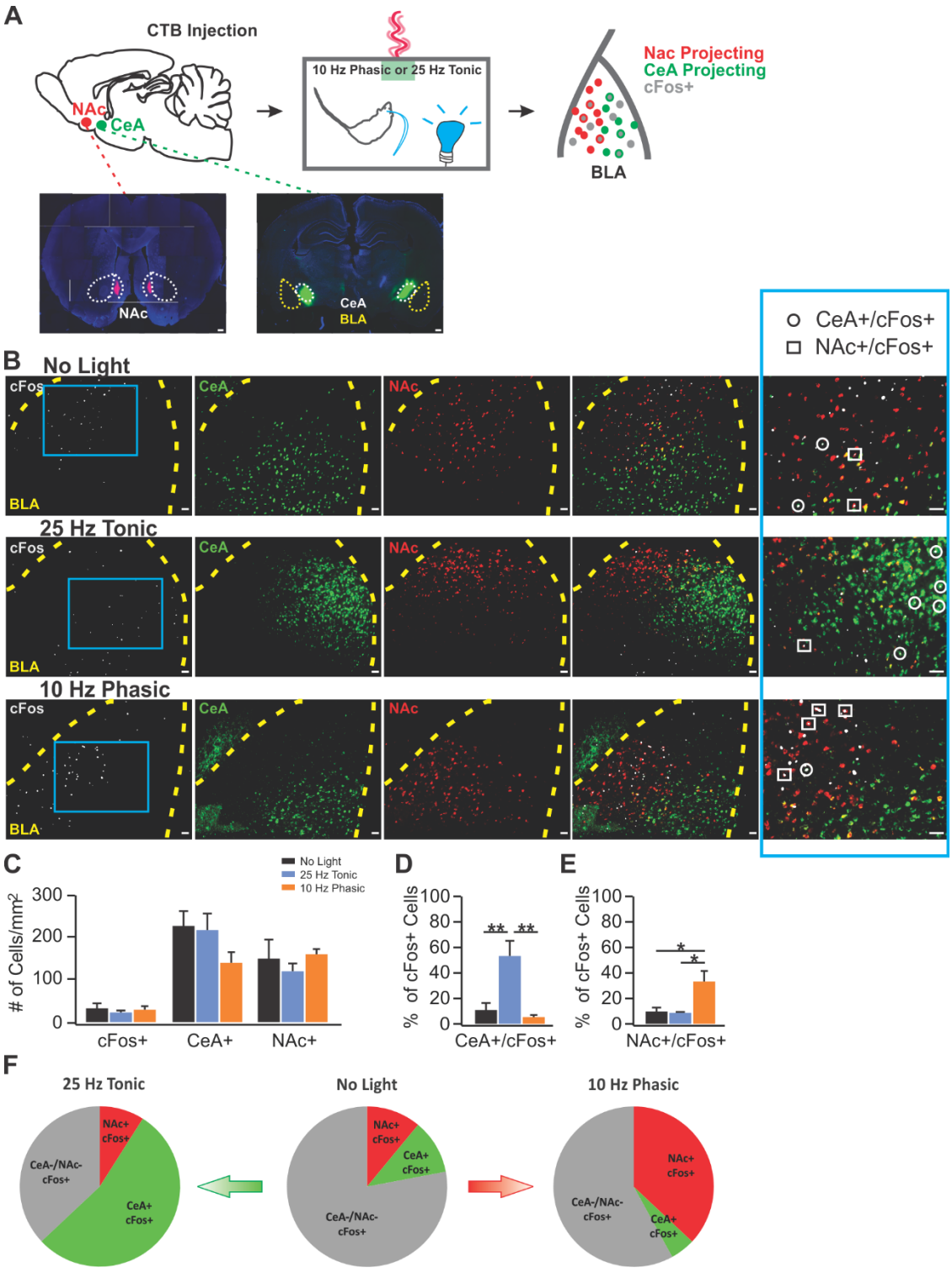

Figure 3. Ten-hertz phasic and 25-Hz tonic LC activation engage positive and negative projecting circuitry respectively in the BLA. (*A*) Schematic of measuring cFos activation in the BLA with CTB labeling NAc and CeA projecting neurons. (*B*) Examples images of cFos, CTB-

488 (labeling CeA projecting neurons) and CTB-594 (labeling NAc projecting neurons) in the BLA in no-light control (upper panels), activated by 25-Hz tonic (middle panels) and 10-Hz phasic light (lower panels). Last column shows enlargement from the blue squares in the cFos images of the first column. Scale bars, 50 μm. (*C*) Total cFos+, CeA+ and NAc+ cells (*n* [control/tonic/phasic] = 4/3/3). (*D*) Percentage CeA+/cFos+ cells over total cFos+ population. (*E*) Percentage NAc+/cFos+ cells over total cFos+ population. $*P<0.05$. (*F*) Distributions of cFos+ cells in the BLA in no light (middle), 25-Hz tonic light (left) and 10-Hz phasic light (right) conditions. A One-way ANOVA followed by post hoc Tukey tests were used to test significance. *p < 0.05. **p < 0.01.

The CTB labeled CeA ($F2,7 = 2.028$, $P = 0.202$) and NAc ($F2,7 = 0.340$, $P = 0.723$) projecting cell numbers were comparable in the three groups (n (control/tonic/phasic) = 4/3/3; Fig. 3*C*). Intriguingly, although the two LC light patterns activated similar numbers of cFos+ cells in the BLA compared with the control ($F2,7 = 0.888$, $P = 0.453$), the distribution patterns of cFos+ cells were dramatically different (see example images in Fig 3*B*). The proportion of CeA+ cells that were cFos+ cells was significantly higher in the 25-Hz tonic group ($F2,7 = 14.232$, $P = 0.003$) compared with either the nonlight control ($t = 4.539$, $P = 0.008$) or the 10-Hz brief phasic light ($t = 4.796$, $P = 0.006$). On the other hand, the proportion of NAc+ cells in cFos+ cells was significantly higher in the 10-Hz phasic light group ($F2,7 = 10.648$, $P = 0.008$) compared with either non-light controls ($t = 4.033$, $P = 0.015$) or the 25-Hz tonic light ($t = 4.052$, $P = 0.015$). The differential distributions of cFos+ cells in different groups are displayed in the pie charts (Fig. 3*F*). In no light controls, equal amounts of cFos+ cells (11%) were NAc and CeA projecting cells, whereas 37% were NAc projecting and 5% were CeA projecting with the 10-Hz brief phasic light, and 54% were CeA projecting and 9% were NAc projecting with the 10-Hz tonic light. A small portion of projecting cells (4.4%) expressed both CTBs, however, the activation of the double-CTB-labeled cells was very low (0.72%).

Additionally, phasic and tonic light activations in the absence of an odor did not lead to a difference in cFos activation patterns (Fig. 4), consistent with the role of NE in the modulation of ongoing activity. In this experiment, CTB labeled CeA ($t = 0.395$, $P = 0.719$), NAc ($t = 1.044$, $P = 0.373$) projecting cell numbers and cFos+ ($t = 1.936$, $P = 0.148$) cell numbers were comparable in the two groups (Fig. 4*C*). Distribution patterns of cFos+ cells were also comparable across groups (see example images in Fig 4*B*). The proportion of CeA+ cells that were cFos+ was not

significantly different across groups ($t = 0.102$, $P = 0.924$). The proportion of NAc+ cells that were cFos+ was not significantly different across groups ($t = 0.007$, $P = 0.996$).

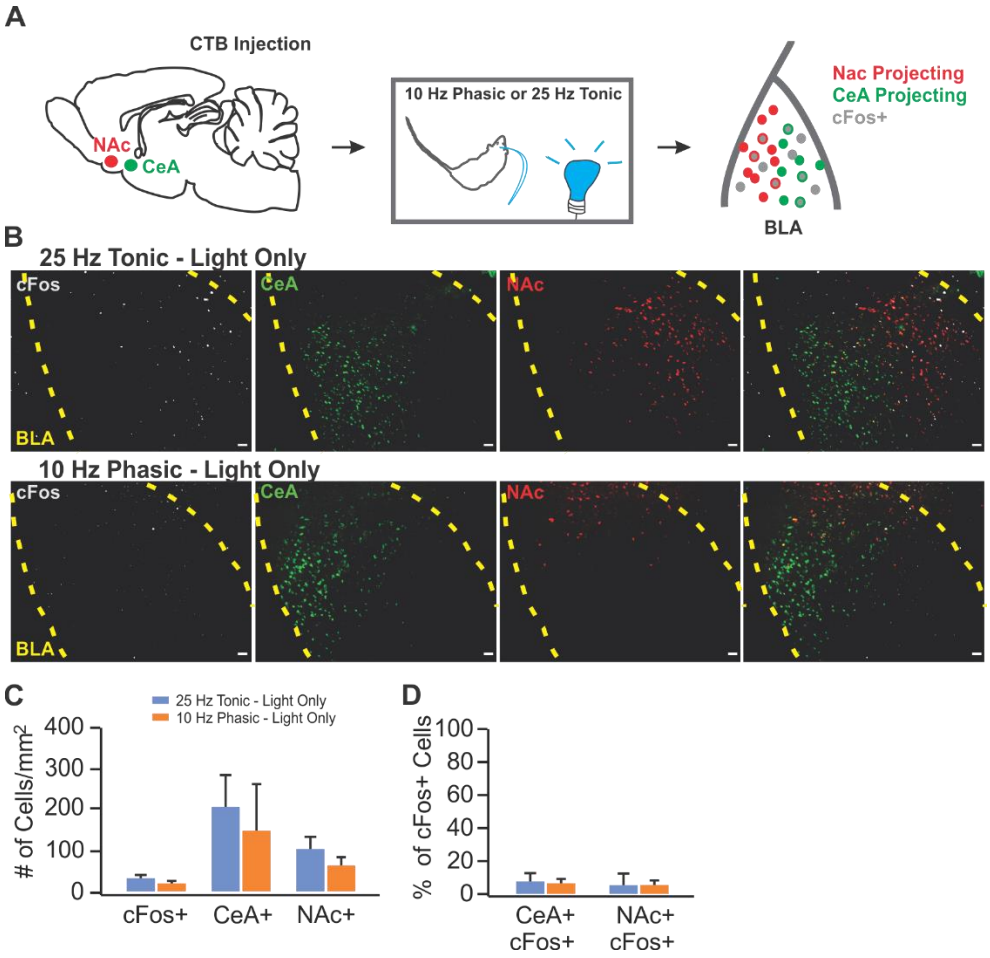

Figure 4. Ten-Hz phasic and 25-Hz tonic LC activations do not induce distinct cFos activation patterns in the BLA in the absence of an odor. *(A)* Schematic of measuring cFos activation in the BLA with CTB labeling NAc and CeA projecting neurons. *(B)* Examples images of cFos, CTB-488 (labeling CeA projecting neurons) and CTB-594 (labeling NAc projecting neurons) in the BLA with 25-Hz light only (upper panels), and 10-Hz phasic light only (lower panels). Scale bars, 50 μm. *(C)* Total cFos+, CeA+ and NAc+ cells activated by tonic and phasic lights (*n* [tonic/phasic] = 3/3). *(D)* Percentage CeA+/cFos+ and NAc+/cFos+ cells over total cFos+ population. Independent samples t-tests were used to test significance.

Phasic and tonic modes of LC activations re-distributed neuronal ensembles activated by an odorant in the BLA. Selective activation of one valence encoding ensemble may inhibit the ensemble of the opposite valence in the BLA (Namburi et al. 2015). Taken together, the 10-Hz LC phasic pattern preferentially activates NAc projecting neurons in the BLA, whereas the 25-Hz tonic LC activation preferentially engages CeA projecting neurons.

3.2. Locus coeruleus phasic and tonic patterns differentially engage ventral tegmental area to nucleus accumbens circuitry in odor valence learning

VTA projections to the NAc facilitate BLA–NAc circuitry in promoting a reward-seeking response to sensory cues (Yun et al. 2004; Ambroggi et al. 2008). Here, 10-Hz phasic LC activation engaged the VTA–NAc pathway more efficiently compared with a 25-Hz tonic pattern (Fig. 5). We infused retro-tracing dye linked to CTB in the NAc and examined the overlap of NAc projecting neurons with cFos+ cells in the VTA activated by odor only (no-light control), 10-Hz brief phasic or 25-Hz tonic LC lights (Fig. 5*A* and *B*).Similar numbers of NAc projectors were observed in all groups ($F2,8 = 0.436$, $P = 0.661$). More cFos+ cells were observed with 10-Hz phasic LC activation ($F2,8 = 5.476$, $P = 0.032$). Of cFos+ cells, an equal portion were NAc projectors across groups ($F2,8 = 2.044$, $P = 0.192$. A greater portion of NAc projectors were cFos+ in phasic compared to other groups ($F2,8 = 11.386$, $P = 0.005$).

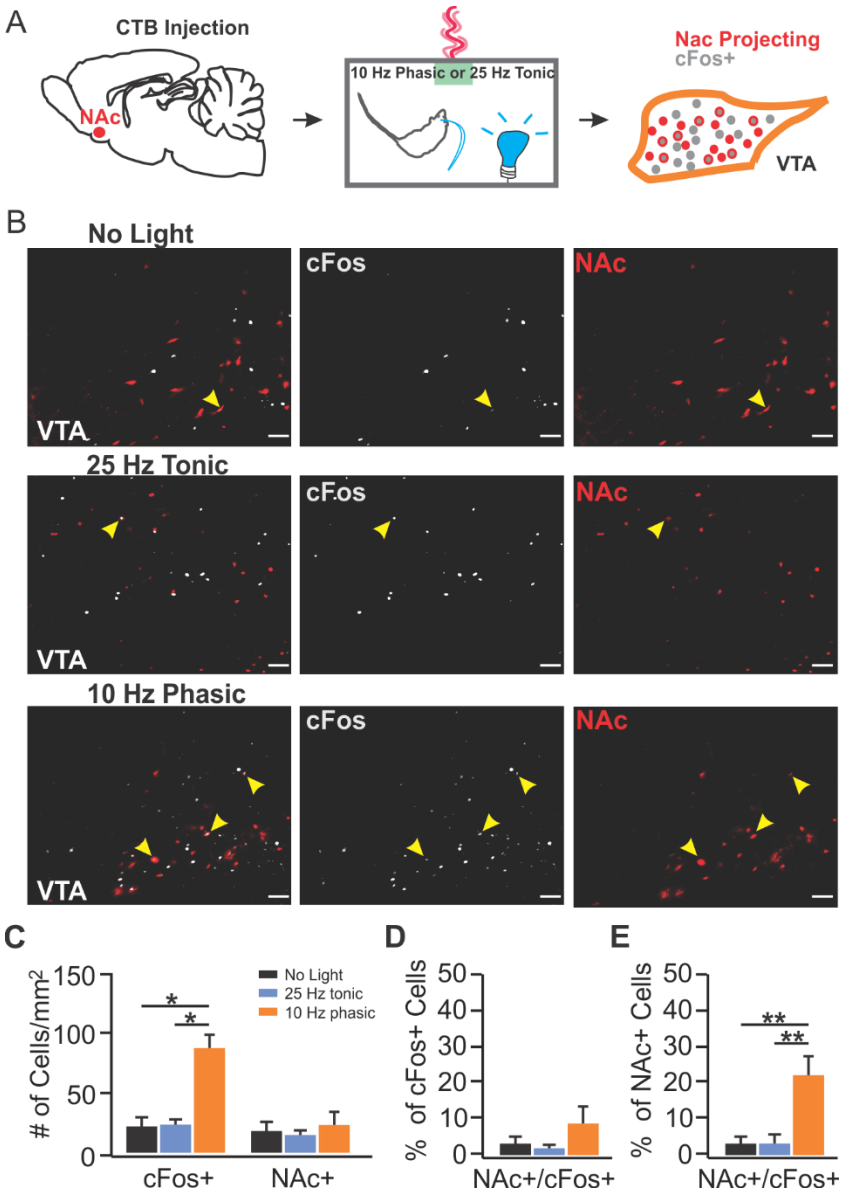

Figure 5. Ten-Hz phasic, but not 25-Hz tonic, LC activation engages NAc projecting neurons in the VTA. *(A)* Schematic of measuring cFos activation in the VTA with CTB labeling NAc-projecting neurons. *(B)* Examples images of cFos and CTB-594 (labeling NAc-projecting neurons) in no-light control (upper panels), activated by 25-Hz tonic (middle panels) and 10-Hz brief phasic light (300 msec every 2 sec; lower panels). Scale bars, 50 µm. *(C)* total cFos+ and NAc+ cells activated in different groups (n (control/tonic/phasic) = 4/4/3). *(D)* Percentage of cFos+ cells that are NAc+. *(E)* Percentage of NAc+ cells that are cFos+. Arrows indicate double-

labeled cells. A One-way ANOVA followed by post hoc Bonferonni tests were used to test significance. *$p < 0.05$. **$p < 0.01$.

3.3. Adrenoceptor subtype distribution in the basolateral amygdala

We next explored the expression patterns of adrenoceptor subtypes (α_1, β_1, β_2, β_3) in the BLA using immunohistochemistry. Despite the lack of literature on brain-expression of the β_3-adrenoceptor, evidence suggests it is expressed in the central nervous systems of rats and humans (Rodriguez et al., 1995; Summers et al., 1995). As such, the β_3-adrenoceptor was included in the analysis. Co-labeling with NAc- and CeA-projectors was measured. We compared the numbers of the projector cells that expressed an adrenoceptor (co-labeled with an adrenoceptor) and those without adrenoceptor labeling (Fig 6).

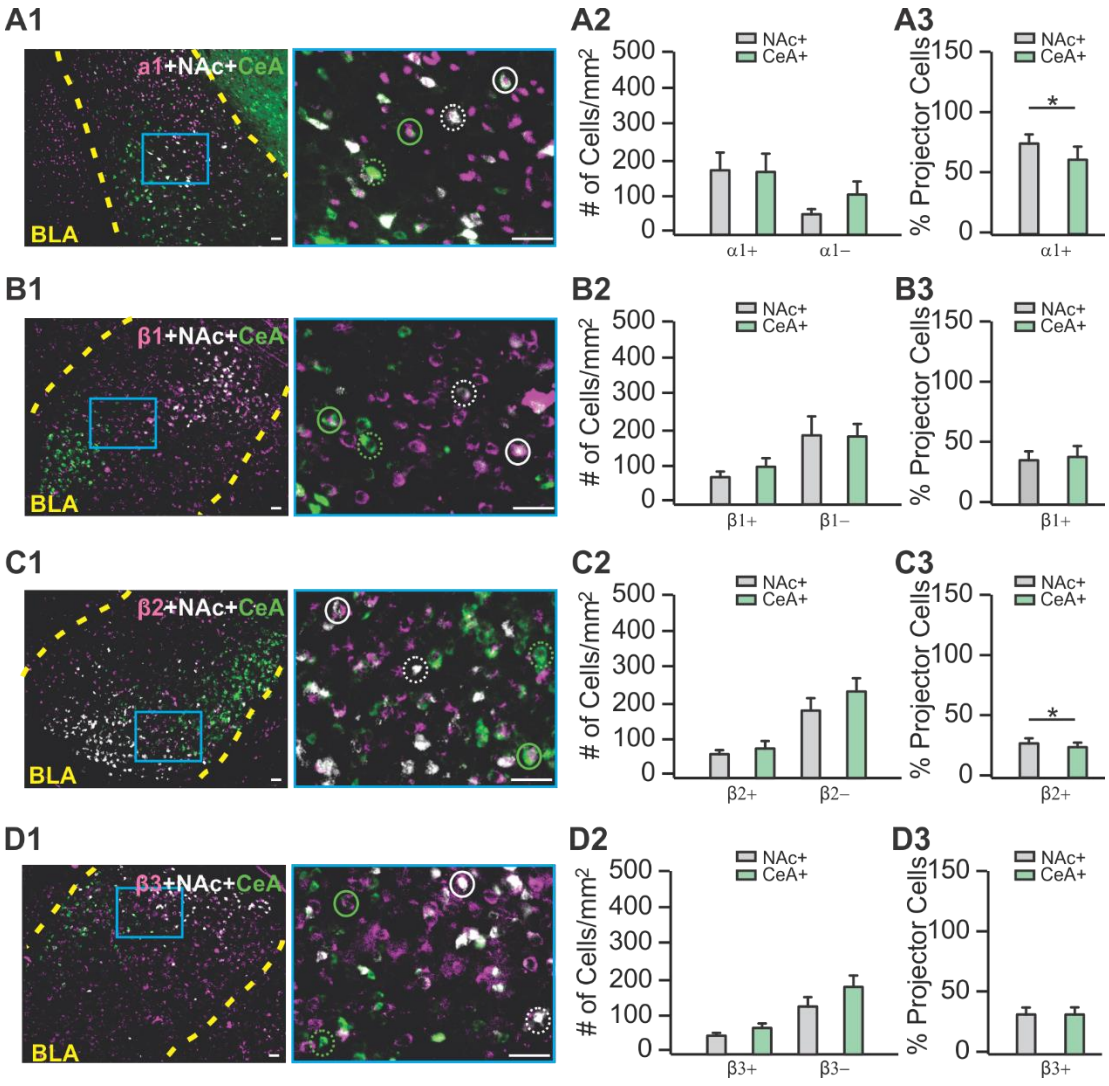

Figure 6. Adrenoceptor subtype expressions in the basolateral amygdala. *(A1-A3)* Expression patterns of α1-adrenoceptors in the BLA and co-labeling with nucleus accumbens (NAc) or central amygdala (CeA) projecting cells. *(A1)* example image of α1-adrenoceptor staining (magenta), NAc (white) and CeA (green) projecting cells are indexed by CTB. Image on the right is the zoom in image of the area indicated by the blue square on the left image. Solid circles, α_1^+ projecting cells. Dashed circles, α_1^- projecting cells. *(A2)* numbers of α_1^+ and α_1^- NAc and CeA projecting cells. *(A3)* percentage of α_1^+ projecting neurons over the total projecting neurons. *(B1-B3)* Expression patterns of β_1-adrenoceptors in the BLA and co-labeling with NAc

or CeA projecting cells. Solid circles, β_1^+ projecting cells. Dashed circles, β_1^- projecting cells. *(C1-C3)* Expression patterns of β_2-adrenoceptors in the BLA and co-labeling with NAc or CeA projecting cells. Solid circles, β_2^+ projecting cells. Dashed circles, β_2^- projecting cells. *(D1-D3)* Expression patterns of β_3-adrenoceptors in the BLA and co-labeling with NAc or CeA projecting cells. Solid circles, β_3^+ projecting cells. Dashed circles, β_3^- projecting cells. (n [α_1/β_1/ β_2/ β_3] = 5/5/5/5). Scale bars: 50 µm. A Two-way repeated ANOVA was used to examine the overall patterns of adrenoceptor distribution in Fig 6A2-d3 and a paired t-test was used to compare percentages of NAc- and CeA-projecting cells that expressed adrenoceptors. *$p < 0.05$.

For the α_1-adrenoceptor, no difference in overall distribution was observed by 2-way repeated ANOVAs ($F1,4 = 2.318$, $P = 0.203$). However, NAc-projectors have a higher proportion of α_1^+ cells compared to CeA-projectors ($t = 3.627$, $P = 0.022$). For β_1-adrenoceptor, there was no difference in the distribution ($F1,4 = 5.009$, $P = 0.089$) and the proportion of β_1^+ cells in the projectors ($t = 0.704$, $P = 0.520$). β_2-adrenoceptors showed no difference in overall pattern ($F1,4 = 3.029$, $P = 0.157$), however, a larger proportion of β_2^+ cells was observed in NAc-projectors ($t = 3.539$, $P = 0.024$). For the β_3-adrenoceptor, there was no difference in the distribution ($F1,4 = 0.878$, $P = 0.402$) and the proportion of β_3+ cells in the projectors ($t = 0.005$, $P = 0.996$).

3.4. Natural odor reward- and aversive conditioning engage differential basolateral amygdala circuitry

We then compared odor-evoked cFos activation in the BLA following natural odor conditioning with either food or shock as the unconditioned stimulus (Fig. 7). BLA neurons engaged in fear learning are re-activated during memory retrieval (Reijmers et al., 2007). As such, patterns of cFos activation upon retrieval can be taken to reflect patterns of activation upon learning. Following successful conditioning, the shock-paired rats showed significantly more freezing to the conditioned odor compared to controls ($t = 2.596$, $P = 0.032$), whereas the food rewarded rats showed higher percentage of correct nose poke towards the food-rewarded odor sponge ($t = 8.227$, $P < 0.001$). Similar to odor-photostimulation conditioning, total numbers of $cFos^+$ cells were comparable in aversive and reward learning groups ($t = 0.744$, $P = 0.481$). However, rats that underwent reward learning showed a higher percentage of NAc-projector

activation upon odor re-exposure compared to the rats that experienced shock training (t = 2.007, P = 0.042).

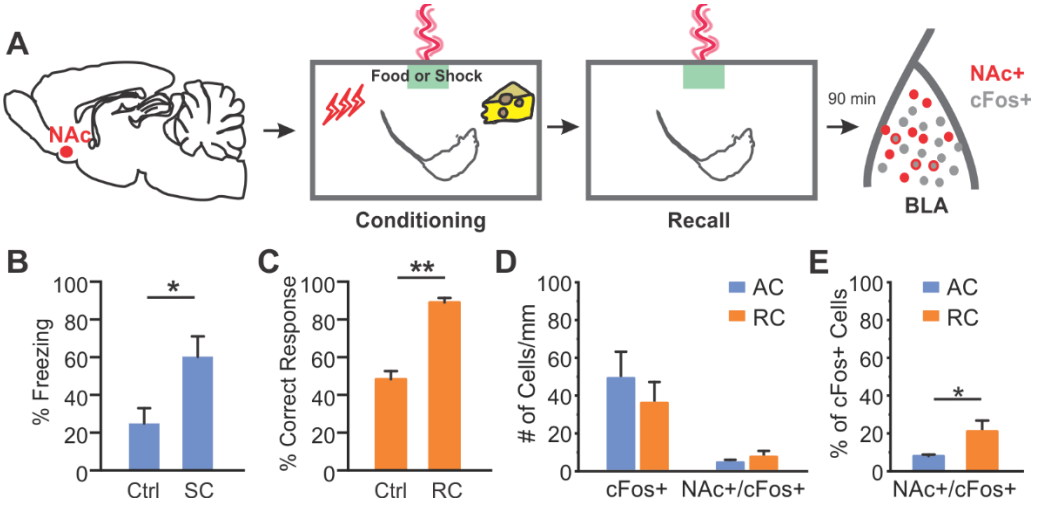

Figure 7. Different activation of nucleus accumbens projecting neurons in the basolateral amygdala by odor valence learning. *(A)* Schematics of the odor conditioning with natural stimuli (food or shock). *(B)* Percentage time spent freezing upon the odor exposure during the testing in odor conditioned rats with shock (n [shock/control] = 5/5). *(C)* Percentage correct response towards the rewarded odor during the testing in odor conditioned rats with food reward (n [reward/control] = 7/7). *(D)* Numbers of $cFos^+$ cells and NAc projecting $cFos^+$ cells per mm^2 following different valence odor conditioning (n [AC/RC] = 4/5). *(E)* Percentage NAc projecting cells in total active ($cFos^+$) cells (n [AC/RC] = 4/5). Ctrl: control. SC/AC: shock conditioning/aversive conditioning. RC: reward conditioning. Unpaired t-tests were used to test significance. *$p < 0.05$. **$p < 0.01$.

4. Discussion

The LC has long been implicated in adaptive responding during arousal. Various frameworks of LC function place emphasis on different facets of its facilitation of adaptive responding, both internally (e.g. Grella et al., 2019; Mather et al., 2016) and externally (e.g. Aston-Jones & Cohen, 2005; Bouret & Sara 2005). More recently, these theories have been extended by the notion of a functionally modular LC (Poe et al., 2020; Chandler et al., 2019; Uematsu et al., 2017) capable of point-to-point communication. Interestingly, we have previously shown that non-specifically applied LC photostimulation can produce differential effects, such as positive and negative valence signaling, through distinct activation modes (see Fig. 1) (Ghosh et al., 2021). Furthermore, we have demonstrated the requirement of BLA β-adrenoceptor activation for negative valence and BLA β- and α_1-adrenoceptor activation for positive valence, whether artificially- or physiologically produced (Omoluabi et al., 2022). Here, we show that these effects may involve activation mode-specific recruitment of the BLA, a structure known to be involved in positive and negative valence signaling. More specifically, 10 Hz phasic and 25 Hz tonic photostimulation of the LC recruited NAc- and CeA projecting subpopulations of the BLA, respectively (Fig. 3). Importantly, no bias in activation pattern was observed with photostimulation alone (without odor), which is to be expected given the role of NE as a neuromodulator of ongoing activity (Fig. 4). Similarly, 10 Hz phasic photostimulation activated more NAc projecting cells of the VTA than did tonic photostimulation, suggesting parallel routing of positive valence signaling with phasic LC photostimulation (Fig. 5). We further suggest that the effects in the BLA are made possible by differential adrenoceptor

expression on BLA subpopulations (Fig. 6). Lastly, we have shown that natural reward- and threat conditioning differentially engage the BLA in a manner consistent with phasic and tonic LC photostimulation, respectively (Fig. 7).

It is possible that phasic and tonic activation modes, by adjusting the concentration of NE at downstream projection sites, bias activation of differential circuitry through an interaction with heterogeneously distributed adrenoceptors to give rise to differential valence effects. Supporting this hypothesis of the importance of downstream adrenoceptor heterogeneity in LC functioning, a brain-wide MRI study has suggested that chemogenetic LC activation increases connectivity in salience- and amygdala networks in a manner related to the density of α_1- and β_1-adrenoceptors (Zerbi et al. 2019). Downstream adrenoceptor heterogeneity offers another way by which the LC may facilitate adaptive responding during positive and negative arousal. More moderately arousing stimuli, reflected as phasic LC-NE release, may encourage exploratory behaviours through the engagement of circuitry such as the BLA- and VTA-NAc pathways. Glutamate released onto VTA–dopamine (DA) neurons is enhanced by pre-synaptic α_1-adrenoceptors (Velasquez-Martinez et al. 2012), which likely occurs in our study where phasic LC light facilitates VTA neuron activation during reward conditioning. Highly arousing stimuli on the other hand may lead to avoidance behaviours through circuitry such as the BLA-CeA pathway. DA is known to increase in the BLA following foot shock, and post-training BLA infusions of DA is known to enhance consolidation of aversive memories (Coco et al., 1992; LaLumiere et al., 2004). Furthermore, as mentioned, VTA-NAc projections facilitate BLA–NAc circuitry in promoting reward-seeking (Yun et al. 2004; Ambroggi et al. 2008). Therefore, DA likely works together with NE to facilitate both aversive and reward learning.

The finding of differential BLA activation using the same stimulation patterns shown to induce positive and negative valence signaling is in line with the functions traditionally prescribed to the NAc and CeA (Namburi et al., 2015; Kim et al., 2016). However, these structures are also capable of differential valence signaling. Kim et al. (2017) has identified CeA subpopulations involved in positive valence signaling. A recent paper (Soares-Cunha et al., 2020) reported that NAc neurons signal both reward and aversion depending on photostimulation patterns. A brief phasic pattern induces reward signaling, whereas prolonged tonic-like stimulation leads to aversion. As such, details pertaining to the transmission of activity through these downstream structures during physiologically- and artificially produced valence signaling remain to be elucidated.

Our results suggest that physiologically- and artificially produced valence signaling draw upon the same LC-inter-BLA circuitry. We are currently examining whether our natural learning paradigms differentially engage the CeA-projectors of the BLA as does our LC photostimulation. We suggest that this will be the case, given previous findings (McCall et al., 2017, Llorca-Torralba et al., 2019) and that our photostimulation- and natural learning-induced negative valence have the same BLA adrenoceptor requirements. Our findings are in line with the known role of BLA β-adrenoceptors in aversive memory formation (McGaugh et al., 1996; Johansen et a., 2014; Bush et al., 2010). The involvement of α-adrenoceptors in aversive conditioning is less clear. A facilitating effect of BLA α_1-adrenoceptor on β-adrenoceptor-mediated inhibitory avoidance learning is thought to involve enhanced cAMP production (Ferry et al., 1999a,1999b), while antagonizing BLA α_1-adrenoceptors alone does not affect auditory fear conditioning but

facilitates fear extinction (Lucas et al., 2019). Terazosin, an α_1-adrenoceptor antagonist, facilitates fear conditioning and long-term potentiation *via* its effect on inhibitory neurons (Lazzaro et al., 2010). Opposing roles of β- and α_2-adrenoceptors in NE and LC stimulation-induced BLA neuronal responses have been reported, with strong inhibitory effects on neuronal firing mediated by α_2-, and milder excitatory effects mediated by β-adrenoceptors (Buffalari & Grace, 2007). Consistent with an inhibitory role, blocking α_2-adrenoceptors in the BLA enhances avoidance memory retention (Ferry & McGaugh, 2008). The roles of adrenoceptors in reward/appetitive learning are much less understood. The hedonic value of LC-NE was first proposed in the '70s (Ritter & Stein 1973) and has been supported by recent evidence (Ghosh et al., 2021; Chen et al., 2021). Our work provides some of the first evidence of specific BLA adrenoceptor involvement in positive valence formation.

The modes and amount of NE release associated with LC activation patterns may be critical in target structure neuronal recruitment such as in the BLA. It has been demonstrated that phasic LC stimulation releases higher amounts of NE in the prefrontal cortex than tonic stimulation, when stimulation pulse numbers are matched (Florin-Lechner et al., 1996). However, if the numbers of pulses are not matched and tonic stimulation yields a higher number of pulses per unit time (in our case for example), tonic stimulation likely causes higher NE release. One recent report using pupillometry (measuring changes in pupil diameter as a proxy for NE release) showed that continuous tonic stimulation for 10 sec dilates pupils more than a short burst at a higher frequency (Privitera et al., 2020). We assume that our physiological and artificial negative valence signaling leads to greater levels of NE in the BLA compared to our positive valence signaling, however, measurement of NE output during behaviour and

photostimulation is needed to determine this. While the complex relationship between firing pattern and NE release needs to be established in the future, our results nevertheless suggests that differential NE release by LC activation patterns is involved in distinct sub-population recruitment in the BLA. It is also worth noting that although periodic optogenetic activation is an imperfect tool for mimicking natural LC patterns, phasic photostimulation of the LC has been shown to mimic the effect of physiologic phasic LC activation in terms of spatial memory enhancement (Takeuchi, 2016), suggesting the physiologic relevance of photostimulating the LC.

Our previous findings that artificial and physiologic positive and negative valence signaling have the same BLA adrenoceptor engagement requirements, together with our supporting immunohistochemistry presented here suggest that our observed valence effects with LC photostimulation rely on a heterogeneous distribution of adrenoceptors in the BLA. However, technical limitations elude precise measurement of adrenoceptor expression levels in BLA subpopulations. Fluorescence-activated cell sorting of specific neuronal populations (e.g. NAc-projecting *vs.* CeA-projecting) and qualitative protein or mRNA measurement, together with opto- or chemogenetic antagonism or activation of specific adrenoceptors in selective neuronal populations (Airan et al., 2009) may shed further light on the relationship between LC activation pattern, NE release, and adrenoceptor recruitment in the BLA. Furthermore, an alternative possibility is that our different learning paradigms and photostimulation patterns recruit distinct LC modules, thereby separating their downstream effects. To verify or exclude this possibility, simultaneous recording of the LC during behaviour and photostimulation is needed. Further work may also study whether our photostimulation patterns can cancel out

natural learning-induced valence and BLA recruitment. That is, we can now explore whether the positive and negative valence, as well as the associated patterns of activation in the BLA, produced by reward- and aversive conditioning, can be attenuated by tonic and phasic photostimulation, respectively. This would further support our findings of segregation of activity in the BLA in response to our photostimulation patterns, as activation of BLA subpopulations has been shown to be mutually exclusive via reciprocal inhibitory connections (Kim et al., 2016).

In summary, our work extends current models of LC functioning by suggesting the role of downstream receptor heterogeneity in LC functioning, particularly in the BLA for differential valence effects. That our natural learning experiments are replicating results from our photostimulation of the LC suggests that our photostimulation captures physiologic properties of the LC, and may thereby be useful for our understanding of LC function in normal and disordered states.

5. References

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Appendix 1

Animal protocol approval

MEMORIAL UNIVERSITY

Dear: Dr. Qi Yuan, Faculty of Medicine\Division of BioMedical Sciences

Researcher Portal File No.: 20220211
Animal Care File:
Entitled: Locus coeruleus norepinephrine modulation in learning and Alzheimer's Disease
Status: Active
Related Awards:

Awards File No	Title	Status	
20190269	Material Transfer Agreement - Not Publishable	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20191784	Locus coeruleus NE modulation in learning and Alzheimer's disease	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20200652	MTA for cis p-tau antibody from Harvard University	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20200817	Understanding what pretangle tau does to neurons and testing potential damage control by an anti-cis-ptau treatment	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses

Ethics Clearance Terminated: May 01, 2024

Your three-year renewal application was reviewed by the ACC on Tuesday June 1, 2021, and the committee approved the renewal, however they did note to please write lay summaries in simpler terms in the future.

This ethics clearance includes the following Team Members: Dr. Qi Yuan (Principal Investigator)
Dr. Xihua Chen (Co-Investigator)
Dr. Susan Walling (Co-Investigator)
Dr. Carolyn Harley (Co-Investigator)

This ethics clearance includes the following Sponsors: [[AllSponsorAgencyNames]]
This ethics clearance includes the following related awards:

Awards File No	Title	Status	
20190269	Material Transfer Agreement - Not Publishable	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20191784	Locus coeruleus NE modulation in learning and Alzheimer's disease	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20200652	MTA for cis p-tau antibody from Harvard University	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20200817	Understanding what pretangle tau does to neurons and testing potential damage control by an anti-cis-ptau treatment	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses

An Event [Annual Report] will be required following each year of protocol activity.

Should you encounter an unexpected incident that negatively affects animal welfare or the research project relating to animal use, please submit an Event [Incident Report].

Any alterations to the protocol requires prior submission and approval of an Event [Amendment].

NOTE: You can access a copy of this email at any time under the "Shared Communications" section of the Logs tab of your file in the Memorial Researcher Portal.

Sincerely,

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