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Neuromodulatory Mechanisms Of Neuropeptides In The Amygdala

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NEUROMODULATORY MECHANISMS OF NEUROPEPTIDES IN THE AMYGDALA

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ABSTRACT

The amygdala is a limbic structure critical for emotional processing as well as roles in learning and memory. Disruption of the normal activity of the amygdala has been implicated in several disorders including phobia disorders, anxiety-like disorders, and substance abuse. The function of the amygdala and its role in various disorders is directly related to its underlying cellular activity. The amygdala is both a cortical- and striatal-like structure containing mainly glutamatergic and GABAergic neurons, respectively. This dissertation has three aims related to the neuropeptide modulation of these cell types in the basolateral (BLA) and central amygdala (CeA). The first aim addresses how the neuropeptide, neuromedin B (NMB), alters excitability within the CeA and contributes to cardiac output and fear responses. The second aim addresses how NMB modulates neuronal activity in the BLA and anxiety-like behaviors. Finally, the third aim studies the roles and mechanisms of neurokinin B-mediated modulation of neuronal excitability and fear responses.

For Study 1, bath application of NMB excited CeL neurons *in vitro*. NMB selectively activated bombesin 1 receptors (BB1Rs) increasing action potential firing that was dependent on the activity of PLC and PKC, but excitation was independent of Ca^{2+} flux. NMB enhanced CeL neuronal excitation by inhibiting inwardly rectifying K^+ channels. Direct application of NMB into the CeA reduced mean arterial pressure and heart rate and reduced fear potentiated startle responses.

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For Study 2, activation of BB1Rs excited BLA neurons and enhanced glutamatergic transmission. NMB-mediated excitation of BLA neurons was mediated through the activation of non-selective cation channels and depression of inwardly rectifying K^+ channels. NMB enhanced spontaneous glutamatergic transmission but inhibited evoked glutamate responses through TRPV1 channels. Direct application of NMB into the BLA produced anxiogenic phenotypes in the Vogel Conflict Test.

For Study 3, selective activation of neurokinin 3 receptors (NK3Rs) with senktide or the endogenous ligand neurokinin B enhanced neuronal excitability of BLA neurons. NK3R-elicited excitation of BLA neurons is mediated by the activation of non-selective cation channels and depression of inwardly rectifying K^+ channels. The NK3R-mediated excitation of BLA neurons required the function of PLC and cleavage of membrane phosphatidylinositol 4,5-bisphosphate. Direct application of senktide into the BLA augmented fear potentiated startle responses. TRPC4/5 and GIRK channels are involved in senktide-induced augmentation of fear potentiated startle responses.

PREFACE

The topic of this dissertation is focused on neuropeptide modulation within the amygdala, an amalgam of nuclei comprised of the basolateral and central amygdala (BLA and CeA, respectively). As a component of the limbic system, the amygdala is critical in the interpretation and processing of sensory stimuli as well as encoding of emotional memory. To improve our understanding of the function of the amygdala and its role in disease, it is important to better understand the neuromodulatory mechanisms that alter amygdala activity.

The principal neurotransmitter of the CeA is γ-aminobutyric acid (GABA), whereas in the BLA the principal neurotransmitter is glutamate. The purpose of this dissertation will be to address some gaps in our knowledge regarding the modulation of GABAergic neurons in the CeA and glutamatergic neurons in the BLA by neuropeptides. A fundamental understanding of the amygdala neuroanatomy, neuronal circuitry, and both physiological and pathophysiological functionality will be needed to provide the reader with the needed background information framing the work presented herein.

For this dissertation, three separate studies were conducted. The first two concern the neuropeptide NMB and NMB-mediated modulation of neuronal activity in the CeA and BLA using *in vitro* and *in vivo* methods. The third study concerns the modulation of BLA principal neurons by the tachykinin neuropeptides. Specifically, examining neurokinin B-mediated modulation of glutamatergic neurons in the BLA using *in vitro* and *in vivo* technique.

CHAPTER 1

INTRODUCTION TO THE AMYGDALA AND NEUROPEPTIDES

Study of the Limbic System

Early Theories of Emotion

Early theorists believed distinct brain regions are involved in the circuitry and behavioral responses underlying emotion. The anatomical basis of emotion was first proposed by Dr. James Papez as a collection of interconnected structures within the mammalian brain [1]. His theory is based on clinical findings that excitatory activity in the cingulate gyrus, hippocampus, and hypothalamus could alter a patient's personality and affective state. Extending the work of Papez, Dr. Paul MacLean defined the 'visceral brain', comprising the hippocampus, thalamus, basal ganglia, cingulate gyrus, and amygdala. Together, their connections to the hypothalamus and the association areas of the temporal lobe were thought to control the expression of emotion [2]. Maclean referred to this circuitry between the visceral brain and hypothalamus as the limbic system [2, 3]. As a corollary to this idea, the visceral brain directs efferent and afferent signals between the hypothalamus to simultaneously generate the physiological actions of emotion, in agreement with the Cannon-Bard Theory of Emotion [4, 5]. Additional studies by Brown and Shäfer , and Klüver and Bucy, who received the most recognition, demonstrates that uni- or bilateral temporal lobe lesions could alter behavior in rhesus monkeys including hypersexuality, inappropriate emotional responses to novel or salient stimuli, and cognitive deficits [6, 7]. Although these brain regions and their associated

functions have been further characterized, these early studies were the first to identify the involvement of the temporal lobe with the affective behavioral response of emotion.

Lesion Studies of the Medial Temporal Lobe and the Amygdala

Therapeutic brain lesions of the medial temporal lobe provide early insights into the behaviors associated with the amygdala. In 1957, patient H.M. underwent bilateral excision of the medial temporal lobe, removing portions of the hippocampus and amygdala. As a result, patient H.M. presented with partial retrograde amnesia and complete anterograde long-term memory deficits. These observations suggest the medial temporal lobe is critical in memory consolidation [8]. Five additional patients underwent bilateral amygdalotomy, without resultant damage to the surrounding cortical and hippocampal tissue [8]. These patients displayed only minor associative and retrograde memory deficits, and their phenotypic psychosis and aggressive paroxysms were largely eliminated [8]. Bilateral lesions of the amygdala are reported to cause associative memory deficits and poor performance in word and visual association tests [9]. Later investigations of more selective amygdalotomy showed that removal diminished aggressive behaviors without the loss of other neurological functions [10]. Mammalian models have also demonstrated similar results as observed in these patients. Rhesus monkeys with selective lesions targeting the amygdala lost the ability to associate shock with predictive cues [11]. Lesions of the amygdala in rodents produced reduced stress responses, motivation, and associative memory deficits [12, 13]. Together, these reports of medial temporal lesions indicate that the amygdala has a role in processing sensory input and responsive behavioral outputs, including aggression, whereas the hippocampus is more intricately involved in memory [7].

Early Structural and Functional Investigations of the Amygdala

The term amygdala is Greek in origin, denoting an almond-shaped collection of gray matter found in the medial temporal lobe below the uncus and adjacent to the hippocampus of the mammalian brain. While the amygdala was first recognized by Dr. Karl Burdach in 1822 [14], an improved characterization of amygdala function has grown over the last 35 years [15]. J. Johnston pioneered the theory of functionally distinct nuclei within the amygdala based on comparative neuroanatomy [16]. He proposed that the amygdala is subdivided into distinct structures based on their phylogenetic relationship to sensory systems; an old division with striate-like neurons associated with the olfactory system (i.e., the central amygdala) and a new division of nuclei with cortical-like neurons (i.e., the basal and lateral amygdala) [16]. Further studies built upon this theory and discovered a complex neuronal network intertwined with the amygdala. In studies of rhesus monkeys, afferent neurons from the orbitofrontal cortex, insular cortex, cingulate, subcallosal gyri and temporal association areas project to amygdala nuclei, which suggests that multisensory information may converge at the amygdala [17]. Indeed, lesions of afferent fibers projecting to the amygdala can alter motivated and affective behaviors as well as produced alterations in autonomic and endocrine function [18]. Further reports in cats and dogs demonstrated that damage to fibers traversing lesions in subcortical brain structures can degenerate downstream efferent neurons in the amygdala. Overall, afferent neurons bring sensory information to the amygdala for emotional processing then these are transmitted to the peripheral nervous system to initiate a behavioral response. Taken together the amygdala is central within this network to transmit sensory information into action, and damage at either point within the pathway can disrupt sensory processing.

The combinatorial use of electrophysiological and neurohistological fiber tracing has further characterized the interconnectivity of the amygdala efferent network [19-21]. Electrical stimulation of the amygdala initiates monosynaptic and polysynaptic efferent projections to sensory association areas in subcortical nuclei, as well as throughout the neocortex [21]. Microelectrode recordings demonstrated that cerebellar [22] and medullary synaptic contacts [23] also converge at the amygdala to relay multisensory information about the proprioceptive and internal physiological state of the animal. Together, these reports indicate that the amygdala is anatomically and functionally positioned to incorporate multisensory information received from cortical and subcortical brain regions. In addition, the efferent fibers responsible for carrying signals from the amygdala to brainstem effector regions can coordinate the behavioral response to sensory information and coordinate behavioral responses via its efferent brainstem connections. Conservation of the amygdala, its distinct nuclei, and their connectivity suggests that findings in one species can inform our understanding of others. Studies of the amygdala in rodent models has enhanced our understanding of anatomical and functional aspects of the amygdala in human brains. The following sections describe how rodent models have provided key insight and knowledge of the amygdala, its connections, and functions.

Connectivity and Neuroanatomy of the Rodent Amygdala

Neuroanatomy of the Rodent Amygdala Nuclei

Anterograde and retrograde tracing techniques have provided a more detailed understanding of the intra- and inter-connectivity of the rodent amygdala. The colloquial almond structure refers to the basal portion of the amygdala, which comprises the lateral, basal, and basomedial nuclei of the amygdala (LA, BA, and BM, respectively; collectively referred to as BLA) [24]. The BLA complex has the most highly agreed upon

interconnectivity between species; however, many subdivisions have been described for each main division (e.g., ventral, dorsal, and lateral portions). Each subdivision is differentiated by its neuronal microarchitectures and peptide-expressing circuits [25]. The central amygdala (CeA) complex is comprised of ~8 structures, but data acquired with the use of Nissl, Golgi, histochemical, and immunohistochemical staining agree on three major divisions; a striatum-like cap that encases the central amygdala proper; a lateral division of the central amygdala (hereafter referred to as CeL); a ventral pallium-like medial nucleus of the CeA (CeM)(Figure 1, a) [26-28]. Collections of intercalated GABAergic neurons encase the BLA, acting as anatomically and functionally distinct units of the amygdala [29]. These studies lay the groundwork for understanding the flow of information into and out of the amygdala nuclei.

Cell Types of the Amygdala

Numerous morphological and functional investigations of the amygdala identified two neuronal populations; Class I projection neurons expressing vesicular glutamate transporter 1 [30] and Class II local inhibitory GABAergic interneurons [31-34]. Golgi stain indicates that the Class I projection neurons represent the major population of neurons in the human, primate, cat and rodent BLA [32, 35, 36] described morphologically as pyramidal or stellate, each with distinct electrophysiological properties [37]. Pyramidal neurons are characterized by spiny apical and basal dendrites with dense collateral arborizations in the vicinity of the parent soma and prominent AP-firing accommodation [24, 37, 38], whereas the stellate cells have smaller dendritic arbors whose axons pass between amygdala nuclei [37, 38]. Intracellular recordings from BLA Class I neurons suggest a heterogeneity of firing patterns that correlate with characteristically high input resistance and variable membrane time constants. For example, BLA projection neurons can fire an initial burst of APs in response to

depolarizing current injection before spike accommodation, fire near the end of the depolarizing step, or display a bursting pattern [38]. These patterns parallel firing patterns observed elsewhere in the neocortex. Regardless of the firing pattern, accommodation of action potential firing is mediated by a prolonged afterhyperpolarization potential (AHP) [38], likely due to differences in the density and distribution of calcium-activated potassium channels [39].

Local interneurons neurons are a minor population representing around 20% of the neurons found within the BLA and are the primary neuronal type of the CeA. These Class II neurons have highly variable electrophysiological and functional properties [34, 40], presenting with or without action potential (AP)-firing accommodation, medium to short duration AHPs, and can be further characterized by their AP firing patterns [33, 41, 42]. Like interneurons in the hippocampus and striatal regions, amygdala GABAergic neurons possess dense dendritic webs to provide both feedforward and feedback inhibition both locally between interneurons and on projection neurons [43-49]. Amygdala interneurons express GAD67/65 [33] and can be further grouped according to their expression of calcium-binding proteins and neuropeptide expression [33, 50]. Specific neuropeptide expressing interneuron circuits include, but are not limited to, parvalbumin (PV+), somatostatin (SOM+), cholecystokinin, calbindin, and calretinin expressing circuits [33]. These interneurons robustly regulate the activity of neuronal activity during synaptic plasticity and fear learning [51, 52] and dysregulation of the excitatory-inhibitory balance in the amygdala is a hallmark of numerous pathologies [53].

Intercalated cells (ITCs) are GABAergic neurons encircling the BLA and CeA that develop from the dorsolateral ganglionic eminence, unlike the GABAergic interneurons of the BLA and CeA arising from the medial ganglionic eminence and ventrolateral ganglionic eminence, respectively [54]. The majority of ITCs are medium spiny neurons although larger ITCs with aspiny dendritic processes have been reported [29]. ITCs have

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a large input resistance (520-860 mΩ) and small capacitance (25-70 pF) correlating to their small size, hyperpolarized resting membrane potentials (-76 ~ - 90 mV), and prominent AHPs [55]. These clusters of neurons are theorized to provide feedforward inhibition to normalize amygdala output in response to varying levels of excitatory input, increasing the signal to noise ratio [56]. ITCs possess a slowly de-inactivating K^+ channel that is inactivated by cell firing but slowly de-inactivates producing after-depolarization in response to excitatory inputs [57]. Thus, these K^+ channels allow for a self-renewing excitability in the ITCs increasing the feedforward inhibition and narrowing the output from downstream targets. Continued investigations into ITCs are underway to clarify their role in amygdala function and emotional processing.

Connectivity of the Amygdala

Broadly speaking, the generalized view of information transfer through the amygdala is as follows: the LA is the main entry point into the amygdala receiving sensory input, whereas the CeM is the output nucleus of the amygdala that sends projections to behavioral effector regions [58]. The principal neurons in the LA make glutamatergic synapses onto glutamatergic neurons in the BLA and GABAergic neurons in the medial ITCs [59] as well as the CeL. The BLA then sends glutamatergic projections to the CeL, the CeM [60-62] and the ITCs which further innervate CeA neurons [63]. The CeL and CeM also receive GABAergic afferents from other structures [64] and contain local GABAergic interneurons to inhibit each other via axon collaterals [65] and GABAergic projection neurons to relay information out of the amygdala [66, 67]. The CeL projects to the CeM, with no reciprocal projection from the CeM to the CeL [68]. The CeM is the major output nucleus of the amygdala and projects to the structures involved in emotional [68-70] and autonomic control [71-74], although the CeL sends GABAergic projections to behavioral and physiologic effector regions as well [75].

The amygdala is a site of sensory synaptic integration from the thalamic and sensory cortices. Injection of an anterograde tracer (*Phaseolus vulgaris)* into specific regions of the prefrontal cortex (PFC) revealed that deep layer projections neurons topographically innervate the amygdala [76]. Quantitatively, approximately 12% of the prelimbic projection neurons, 21% of the infralimbic neurons, and 10% of the projection neurons from the cingulate cortex send efferent projections to the BLA with increasing density at more distal sites [77, 78], indicating that the sensory cortices send projections about cognitive and polysensory information as well as aspects of reward associations directly to the amygdala. A neuroanatomic comparison of amphibian and mammalian cortico-amygdala interconnectivity supports the phylogenetic conservation of sensory and motor projections, whereas the ontogenetic divergence of the sensory association area in rats may represent mammalian specific circuits, for example suckling behavior and lactation [28]. In support of this developmental evolution of the amygdala, olfactory and gustatory information reaches the amygdala at earlier stages of cortical processing compared to visual, auditory, and somatosensory information which relay first at the level of the thalamus, where modality-specific projections then selectively innervate amygdala nuclei [28, 79, 80]. For example, projects from posterior thalamic nuclei strongly label afferents in the LA and CeL, whereas central medial thalamic projections densely labeled terminals in the BLA and not the CeA [80]. Injection into the medial geniculate nucleus, the principal thalamic relay station of auditory information between the auditory cortex and inferior colliculus, labeled axons that traveled through the thalamic peduncle with terminals found in the BLA and ITCs, with minor localization in the nuclei of CeA [80]. Initial reports of direct projections carrying visual stimuli information to the amygdala were thought to be scarce likely due to the convergence of olfactory and visual tracts [80], whereas other findings indicated visual projections of the posterior allocortex or retinal ganglia indirectly reach the LA or CeA [28, 81]. Subsequent

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work employing more sensitive tract tracing dyes suggested that thalamic visual nuclei (i.e. lateral posterior and posterior limitans nuclei) target the anterior LA as well as posterior BLA [82]. The findings that lesions to visual cortex in rats did not eliminate visually conditioned fear expression provide evidence of direct visuo-thalamic projections to the amygdala [82].

Following the convergence and integration of sensory input at the amygdala, the amygdala sends projections to behavioral and physiologic effector regions. Typically, BLA and CeA send projections to different brain regions [83]. The majority of projections to the brainstem nuclei generating behavioral output originate from the CeA, whereas BLA projections typically terminate in the striatum, thalamus, and cerebral cortex [84]. CeA contributes projections involved in autonomic adaptation via projections to the solitary tract nucleus [85] as well as receives information on sympathetic activity and respiratory responses [86]. CeM efferents for somatosensory information (i.e., pain, itch), reward and ingestive behaviors, as well as signals for hypervigilance reach distinct brain regions, including periaqueductal grey, parabrachial nucleus, and substantia nigra [87-91]. Additional amygdala outputs target various hypothalamic nuclei and may regulate the excitability of the entire forebrain through projections to other neuromodulatory cell groups in the forebrain and brainstem (i.e. acetylcholine, noradrenaline, dopamine, and serotonin) [58], though direct projections from the BLA to brainstem nuclei have not yet been fully characterized. It is most likely that sensory information integrated within the BLA projects first to the CeA before reaching brainstem nuclei [92, 93]. However, brainstem monoaminergic afferents from the solitary tract, locus coeruleus, periaqueductal grey area robustly innervate the BLA to modulate its output to the CeA [94-96]. The cortico-amygdala and thalamo-amygdala pathways together with bidirectional synaptic contacts from the brainstem center the amygdala as important

brain structure in processing sensory information to coordinate appropriate responsive behaviors.

Amygdala and Hippocampal Connections

Temporal lobe lesions targeting the amygdala and hippocampus generated deficits in emotional processing and memory, suggesting a functional interaction between the amygdala and hippocampus [6-8]. The hippocampal formation includes the dentate gyrus, areas CA1, CA2, and CA3 (CA4 in humans), and the subiculum as well as the entorhinal and perirhinal cortex in the adjacent parahippocampal area. Together, these areas are responsible for integrating highly processed sensory information from all modalities into complex representations of context (i.e., memory). Information processing within the hippocampus proceeds through a well characterized series of connections known as the tri-synaptic circuit. Briefly, excitatory connections from the entorhinal cortex to the dentate gyrus relay cortical information to the hippocampus. Information is then transmitted via the mossy fibers from the dentate gyrus to CA3. The Schaffer collateral pathway connects CA3 to CA1, which then has projections out of the hippocampus to the septum, hypothalamus and contralateral hippocampus [97]. The structures of this circuit are critical for memory formation and constitute the medial temporal lobe memory system. Together with its interconnectivity between the amygdala, this circuit is important for emotional memory.

The subiculum, entorhinal and perirhinal cortex robustly innervate the amygdala [78]. Subiculuar projections targeting the BLA form excitatory synapses on pyramidal neurons but may also contact dendritic branches of inhibitory interneurons to provide feedforward inhibitory signaling [98]. These projections directly or indirectly with projections from the prelimbic cortex convey contextual cues mediating fear extinction [99]. Parahippocampal projections targeting the BLA arise mainly from the entorhinal

cortex providing excitatory input onto the spines and dendritic shafts of projection neurons [100], whereas projections originating from the perirhinal cortex differential target populations of BLA interneurons. Efferents of the BLA and CeA reciprocally innervate these hippocampal structures as well as target the temporal regions of CA2 and CA3 [25]. The ventral hippocampal structures receive excitatory contact from all regions of the BLA thought to convey behaviorally relevant information [101, 102]. LA and BLA projections target parahippocampal structures providing excitatory inputs on dendritic spines of principal neurons and their dendritic arbors [67]. The large populations of interconnected neurons between the BLA and hippocampus have synchronized rhythmic firing (i.e., oscillations) that reflect brain states [69]. Disruption of these oscillatory patterns produces behavioral deficits in fear and stress responses and spatial navigation, indicating that these are important for emotional memory [69, 103].

Pathophysiology of the Amygdala

Amygdala dysfunction is implicated in various pathophysiological conditions. The amygdala is implicated in neurodevelopmental disorders, such as Autism Spectrum Disorder [104, 105], and neuropsychiatric disorders, such as anxiety [106, 107], depression [108, 109], and schizophrenia [110, 111]. However, a detailed discussion of all these disorders is beyond the scope of this dissertation. Instead, this section provides a more detailed examination of fear and anxiety-related disorders, with an emphasis on the contributions of the amygdala.

Fear vs. Anxiety

Fear is an emotional response of increased arousal to a specific stimulus and produces internal states of increased vigilance to perceived threats [112]. Fear serves as a protective function to enable an animal to perceive potential threats from their environment and prepare appropriate behavioral responses [113, 114]. This internal

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state, or the physiological fear response, leads to abrupt changes in behavior that include active or passive coping strategies used to palliate the physiological fear response. This active coping strategies result in sympathetic activation and a fight-or-flight response, activating the hypothalamic-pituitary axis to release neuroendocrine hormones, resulting in an increase in glucocorticoids and cardiovascular output [115]. Escape is the goal during active coping states, whereas passive coping strategies perceive escape as unlikely. Passive coping strategies are observed in rodent models as immobility or freezing behavior, which acts as measurable endpoints for quantifying the fear response.

Fear conditioning paradigms are effective tools for understanding the neural basis of fear responses. In fear conditioning, a neutral conditioned stimulus (i.e., a light or tone) is paired with an emotionally salient stimuli, (i.e., aversive electric shock) during the conditioning phase to form a learned association of these stimuli. This acquisition of a fear response is then assessed by measuring the conditioned responses, ranging from a startle, or a complete lack of movement, produced by the conditioned stimuli without the unconditioned aversive stimuli. This paradigm models real-life experiences, where threatening stimuli experienced with harm, stress, or pain are learned to prepare the animal for future situations.

Like fear, anxiety is an innate emotional response to threatening stimuli and produces internal states of increased vigilance encompassing physiological, affective and cognitive changes [116], however; unlike fear, anxiety is an internal state in response to non-specific stimuli from the environment. Mild anxiety is believed to increase performance, but chronic or severe levels of anxiety will lead to anxiety-related behaviors and disorders. Behavioral paradigms used to measure anxiety-like behaviors in rodents include, but are not limited to, the elevated plus maze, light-dark box, open-field, and conflict tests. The first three paradigms measure anxiety by quantifying the

amount of time a rodent spends in an 'anxious' or vulnerable location, including the open arms of the plus-shaped maze, center of an open box vs. near the walls, and illuminated vs. dark chambers. The conflict tests differ in that the animal is presented with a choice between a physiological drive (i.e., water, food) and safety. These paradigms do not present a specific threat per se, but rodents must perceive their environment and decide between their innate drive to explore or purse safety. Optimizing behavioral paradigms for rodent models has advanced our understanding of learning memory, and anxiety. Furthermore, manipulating the neurological pathways involved in fear- and anxiety-driven responses will reveal the components that prevail in fear- and anxiety-like behaviors.

Anxiety and Depression

The neuropsychiatric disorders of anxiety and depression present with a spectrum of symptoms that are characterized by negative emotional states. The primary symptoms and comorbidities of depression include, but are not limited to, social withdrawal, sadness, apathy, problems with memory or attention, and thoughts of suicide [117]. The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, Text Revision (DSM-5TR) recognizes several forms of depression with major depressive disorder being the most prevalent affecting an estimated 20% of people during their lifetime [118, 119]. Likewise, symptoms of anxiety and anxiety-related disorders include persistent and excessive worry, irritability, insomnia, weight loss, and difficulty concentrating. The DSM-5TR recognizes several distinct anxiety-related disorders based on symptoms and comorbidities, including post-traumatic stress disorder (PTSD), agoraphobia, and social anxiety disorder. The prevalence of anxiety disorders in the general population varies, but the lifetime prevalence of any anxiety disorder is estimated to be around 15-20%, with higher rates of anxiety among women [120].

Anxiety and depression can arise from the interplay of psychological, genetic, epigenetic, and social factors, with an estimated 50% comorbidity rate of major depressive disorder and some forms of anxiety [121], whereas chronic stress is likely the most important risk factor [108, 122]. Chronic stress can produce structural and functional alterations in brain regions involved in discriminating positively and negatively valenced stimuli [123]. As the involvement of the limbic system in emotion regulation is well established [93, 124], there has been continued interest in the role of the amygdala in anxiety and depression.

The amygdala is an essential brain structure in the circuit of anxiety. Patients with bilateral loss of the amygdala struggle to assess fear in face expression paradigms and have diminished startle responses to threatening simulations [125-127]. Conscious awareness is a significant aspect of fear responses in humans and is related to the level of activation in the PFC, a brain region involved in higher cognitive functions such as attention and the regulation of subcortical systems [128]. As such, higher self-reported anxiety levels in both children and adults are associated with increases in gray matter volume of the amygdala [129, 130]. In patients with PTSD, fMRI-imaging studies have shown exaggerated amygdala activation in response to fearful faces and a simultaneous decrease in PFC activity [131]. However, this exaggerated amygdala activation was not observed in patients with social phobia [132]. As the amygdala regulates multiple components of sensory association [127], the different amygdala activation found in anxious patients may represent failures in amygdala habituation or sensitization to salient stimuli [133]. Collectively, these findings support a role for amygdala activity in depression and anxiety disorders.

Fear conditioning paradigms have revealed specific circuits of the amygdala mediate environmental processing and process the cued associations during fear responses [93, 134]. Exposure to anxiogenic contexts, including open spaces, predatory

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odors, or aversive shocks, drives activity-dependent immediate-early gene expression, markers of neuronal activation, in the BLA and CeA [135]. In contrast, disruption of function in the BLA or CeA diminishes fear or anxiety-like behaviors in rats and mice [123, 136]. Single-unit recordings of BLA or CeA neurons demonstrated that single-unit activity of these neurons increases in response to conditioned, both positively and negatively valenced, stimuli and silencing of these neurons during acquisition results in deficits in behavioral output [96, 137, 138]. Indeed, diverse populations of BLA neurons are reciprocally active during exploratory or defensive behaviors such that neurons active in exploration are only active when defensive neurons are inactive [139]. Likewise, improved fluorescent labeling has identified two distinct cell types within the CeL that regulate anxiety---CeL$_{ON}$ neurons which are rapidly activated by the conditioned stimuli and CeL$_{OFF}$ neurons which are inhibited by the conditioned stimuli and express PKC∂ [140, 141]. Both CeL$_{ON}$ and CeL$_{OFF}$ neurons send projections to CeM neurons and function through an inhibitory loop within the CeL [140, 141]. These finding indicate that fear- and anxiety-like behaviors occur by activation of CeL$_{ON}$ neurons, and simultaneous downregulation of CeL$_{OFF}$ inhibitory projections to CeM neurons. Additionally, a SOM+-expressing subset of GABAergic neurons, largely non-overlapping with PKC∂-expressing CeL$_{OFF}$ neurons, receive excitatory inputs from LA projection neurons, producing experience-dependent alteration in synaptic strength to promote anxiety-like behaviors [142]. Anxiety and depression are associated with widespread changes in the excitability of amygdala neurons and these studies suggest anxiety/depression likely involve distributed and cell-type specific alterations in the excitatory-inhibitory balance within the amygdala as well as interconnected structures [139]. Moreover, an improved understanding in the stress-induced alterations in the neuronal ensembles stemming from the amygdala will highlight how animals cope and subsequently, succumb to anxiety and/or depression.

Specific circuits within the amygdala are believed to regulate anxiety-like behaviors in rodents. Recent advances in tract tracing techniques enable manipulation of specific circuits within the amygdala and identification of synaptic targets during anxiety-related behaviors. Optogenetic activation of glutamatergic neurons in the BLA produces anxiogenic behaviors; however, selective photoactivation of BLA-CeL terminals reduces the behavioral expression of anxiety in mice [143]. BLA neurons labeled with activity-dependent fluorescent tags increased projections to the PFC and hippocampus after fear conditioning [144], suggesting temporal plasticity within these circuits. Whereas PFC-BLA fluorescent intensity increases during specific stimulus extinction, there is a strengthening of synaptic contact between the BLA and ventral hippocampus in context-dependent paradigms and silencing these projections attenuates anxiety-like behavior [102]. These indicate that specific and non-specific negatively valenced stimuli differentially recruit efferent amygdala projections. Similarly, afferent projections to the amygdala may contribute more strongly to phenotypes of one disorder than the other. Projections from the solitary tract and locus coeruleus differentially contribute to positive memory formation in the BLA [96], whereas ventral tegmental projections to the amygdala are required for anxiety-like phenotypes in mice, but not depressive-like phenotypes [145]. These studies highlight the complexity in circuitry and distinct cell types within the amygdala that contribute to anxiety-related disorders and emphasize the shortcomings in current therapeutic options for these disorders.

Therapeutic Strategies

The neurological causes of anxiety and depression are not fully understood. An initial hypothesis proposed these disorders arise from a decreased function of certain monoaminergic neurotransmitters, including norepinephrine (NE), 5-hydroxytryptophan (5-HT or serotonin), and dopamine (DA). This theory was supported by clinical and

animal studies that used reserpine, a drug used to treat hypertension that results in symptoms associated with depression [146]. However, the monoamine hypothesis has some limitations. For example, quantification of NE, 5-HT, or the levels of their metabolites in post-mortem tissue, cerebrospinal fluid, or urine are largely inconsistent [147-149]. Likewise, the activity levels of the rate-limiting enzymes for DA and NE synthesis, as well as the activity of the catabolic enzyme monoamine oxidase, cannot explain changes in DA or NE neurotransmission [148]. Selective depletion of NE or 5-HT does not produce anxiety or depression-like symptoms in healthy controls, but can exacerbates symptoms in patients with major depressive disorder [150]. These observations suggest that these disorders are likely not caused by problems with a single neurotransmitter system, but rather suggest these disorders result from dysfunction of multiple interconnective pathways across different regions of the brain including the cortex, subcortex, and limbic regions, as well as associated neurotransmitter systems [151]. In the context of limbic-cortical system dysfunction, current pharmacological approaches used to treat anxiety and depression aim to bring about adaptive chemical and molecular changes to re-establish a normal mood state [122].

Treatments for anxiety and depression focus on the manipulation of monoamines, specifically 5-HT, DA, NE, and to a lesser extent, GABAergic transmission. Medications, including selective serotonin reuptake inhibitors (SSRIs), selective norepinephrine reuptake inhibitors (SNRIs), and tricyclic antidepressants target transport proteins that regulate the availability of monoamines in the synaptic cleft, reducing the effects of the neurotransmitter at pre- and post-synaptic sites. In contrast, benzodiazepines are positive allosteric modulators of GABAergic transmission [152, 153]. SSRIs and SNRI are potent neuromodulators of amygdala activity. The midbrain raphe nucleus, which is responsible for releasing serotonin into the BLA neurons in response to aversive stimuli

[154], innervates the amygdala [155] and is implicated in anxiety and fear learning [156, 157]. SSRI treatment also modulates neuronal function within the amygdala, depending on the duration of treatment and method of administration in both humans and rodents. In non-depressed patients, acute intravenous citalopram produces a dose-dependent increase in bilateral amygdala activation in response to fearful faces [158], but acute oral administration does not cause amygdala activation in response to a similar facial recognition paradigm [159]. In patients with major depressive disorder, fMRI-imaging revealed significantly higher amygdala activation in response to fearful faces compared to healthy controls, but this difference normalized after 7 days of oral citalopram treatment [160]. The dichotomous relationship between the acute and chronic application of SSRIs in humans has been mirrored by studies conducted in rodents. In rats, acute systemic administration of the SSRI citalopram produces marked increases in 5-HT release in the CeA, which can augment the acquisition of fear memory [161, 162], whereas chronic administration of citalopram induces a reduction in time spent frozen after fear conditioning, indicating an anxiolytic effect [162]. Although a conclusive answer to the delay in SSRI therapeutic action is yet to be determined, SSRI application produces systemic adaptive changes to serotonergic transmission and may also produce alterations in structural rearrangement and synaptic plasticity within the amygdala [163, 164], unfortunately a large number of patients see minimal to no therapeutic benefit from these treatments.

Benzodiazepines are a class of anxiolytic drugs commonly used to treat anxiety. While these drugs can be effective in treating anxiety, benzodiazepines are often used acutely as second-line or adjunct therapy prior to the onset of SSRI/SNRI because of their potential for tolerance and abuse [165]. They function by enhancing fast GABAergic inhibitory signaling within the brain by binding to $GABA_A$ complexes. $GABA_A$ complexes are pentameric chloride channels built from heteropentameric combinations of α-, ß-, γ-,

δ-, ε-, θ-, π-, and ρ-subunits, which contribute to unique electrical and pharmacological properties [166]. The most prevalent form of GABA$_A$ complex within the amygdala is composed of two α-subunits, two ß-subunits, and one γ-subunit. The mechanism by which benzodiazepines treat anxiety stems from their interaction with a binding pocket located between the α- and γ-subunits [166]. The γ$_2$ subunit, which is robustly expressed in the nuclei of the amygdala in humans [167] and rats [168], plays an important role in anxiety and its etiology. A mutant mouse model with a 35% reduction in the γ$_2$-subunit displayed increased anxiety-like behaviors in the elevated plus maze and light-dark box, without any changes in the sedative effects of diazepam or ethanol [169]. However, selective ablation of the γ$_2$-subunit in SOM-expressing neurons produced a disinhibitory effect, increasing the inhibitory signaling on principal neurons promoting anxiolysis [170]. The α$_{1-3}$-subunits may also play different roles in the anxiolytic action of benzodiazepines, with the α$_2$-subunit suggested to induce anxiolysis, the α$_1$-subunit contributing to sedation, and the role of the α$_3$-subunits having a controversial role in both phenomena [171]. Functionally, diazepam increases the frequency of IPSC events in the BLA and CeA via GABA$_A$ complexes containing α$_1$- and α$_2$-subunits [172]. In the CeA, another α-subunit, the α$_5$-subunit, is expressed at low levels [168] and selective ablation of α$_5$-containing GABA$_A$ complexes in CeL$_{OFF}$ neurons promotes fear generalization and anxiety-like behaviors [173]. Collectively, these studies suggest that a deeper understanding of the specific neural circuits involved is necessary to achieve targeted anxiolytic effects.

Neuropeptides and the Amygdala

Neuropeptides (NPs) are a diverse group of neuromodulators that control a variety of innate behaviors including pain, reward, reproduction, and stress responses [174-176] making neuropeptidergic systems a promising target for the development of effective

therapeutics [175, 177]. This section will provide a basic introduction of the current understanding of neuropeptides as well as discuss the clinical findings of neuropeptide therapy. A specific discussion of the neuropeptides studied in this dissertation are included as an introduction to each study.

Neuropeptides as Neuromodulatory Molecules

NPs share many mechanistic similarities to amino acid neurotransmitters. NPs can exert their direct biological effect on target cells in seconds to minutes or generate prolonged indirect effects via gene regulatory mechanisms on the time scale of hours to days. Like amino acid neurotransmitters, NP release is highly regulated within the presynaptic site, but NPs are also released from non-synaptic sites, for example from preganglionic axons [178], while still targeting postsynaptic neuronal targets. The non-synaptic release of NPs along with their theorized long extracellular half-life may explain reports of *volume transmission* whereby these neuromodulators signal at long distances within the brain [179, 180]. Indeed, the NP synthesizing cell and the cognate receptor are not always anatomically expressed in the same brain region. However, it is likely that most neurons express some NPs in addition to their fast amino acid transmitters and NP release occurs over short distance (e.g., micron) to act at local targets [181]. Furthermore, endogenous peptidases non-selectively break down NPs modifying their bioactive duration and dispersion, depending on the modulators intrinsic chemical composition, supporting the hypothesis that NPs exert local effects [182].

Mechanisms of Neuropeptide Release

Neurotransmitter and NP secretion is regulated by separate organellar vesicles-- clear synaptic vesicles and dense core vesicles (DCVs), respectively. Synaptic vesicles preferentially reside within active zones in association with presynaptic specializations, whereas DCVs localize within release sites more distally from the active zone [181],

likely requiring unique proteins for secretion [183]. It is appreciated that Ca^{2+} is a critical

ion in both synaptic vesicle and DCV exocytosis. NP release is positively correlated with

action potential spike frequency from axons and dendrites [184, 185], theorized to result

from a rise in cytoplasmic Ca^{2+} that increases the probability of DCV release [186].

Furthermore, Ca^{2+} release from intracellular stores mediated by NP receptor activation

or pharmacologically can augment DCV release independent of APs [187, 188].

Following release, clear vesicles are rapidly repackaged with amino acid

neurotransmitters via transport proteins, whereas DCV refilling is a significantly slower

process. DCVs must be resynthesized in the Golgi apparatus of the cell body.

Neuropeptides are resynthesized in rough endoplasmic reticulum, and the reloaded

DCVs must then be transported to the site of release [189]. There is significant variability

in DCV size and likely NP content [189]. For example, estimates of oxytocin and

vasopressin in large neurosecretory vesicles at neurohypophyseal boutons may contain

between 60,000 and 90,000 molecules of these NPs with peptide content correlating

with vesicle size [190]. Upon release, the actions of NP can directly modify neuronal

excitability and neurotransmitter release, as well as directly or indirectly effect ion

channel activity.

   Most NPs generate their physiological effects by interacting with G-protein coupled

receptors (GPCRs). Upon binding, GPCRs undergo confirmational changes, which

initiate intracellular signal cascades. In their resting state, G proteins exist as a

heterotrimeric complex of Gα, Gβ, and Gγ subunits bound to GDP. The exchange of

GDP for GTP activates the complex leading the dissociation of the Gα subunit from the

Gβ and Gγ subunits. The activated monomeric or dimeric G proteins can interact directly

or indirectly with various ion channels, leading to modulations in neuronal activity [191-

193]. Moreover, modulation of the neuropeptide signal can occur at the level of the

receptor, as G protein-modulators alter the kinetics of G proteins and their downstream effectors [194]. NP signaling is further amplified via the activation of various intracellular signaling pathways that may include phospholipase Cß, adenyl cyclase, and many other downstream phosphorylated targets. Unlike classic neurotransmitter systems, NPs and their receptor are often discretely expressed. Therefore, the signaling pathways activated by peptidergic systems represent unique therapeutic targets.

Neuropeptides as Therapeutics

The large repository of preclinical data in rodent models had implicated corticotrophin-releasing hormone (CRH in humans, Corticotrophin-releasing **F**actor, CR**F** in rodents) as a promising therapeutic target in fear-related and anxiety disorders. CRF is released in response to stress from the parvocellular neurons in the hypothalamus to activate the HPA, and glucocorticoids from the adrenal glands [195]. CRF generates its physiological effect by activating CFR1 and CRF 2 receptors that signal through cAMP-PKA-mediated signaling pathways, though often these receptors generate opposing actions [196, 197]. High expression of CRF is observed in the amygdala and bed nucleus of the stria terminalis (BNST), and CRF is involved in regulating synaptic function [198]. Indeed, CRH is elevated in the cerebrospinal fluid of veterans with PTSD and the levels positively correlate with active psychosis [199, 200]. Unfortunately, no selective CRF1 receptor antagonist has passed the safety and efficacy standards in Phase III clinical trials for anxiety-like psychiatric disorders. The selective CRF-1 antagonist pexacerfont failed to prove more efficacious than the placebo-treated group, with a lower response rate than escitalopram in a cohort of patients with generalized anxiety disorder [201]. Another CRF1 antagonist, verucerfont, has shown limited clinical utility as trials only included female subjects due to a reversible testicular toxicity [202]. In another verucerfont clinical trial interested in anxiety and alcohol use in woman,

verucerfont reduced CRH levels and decreased right amygdala activation in response to fearful faces but did not reduce self-reported alcohol cravings [203]. In addition, a large number of participants in the verucerfont group discontinued the study owing to adverse side effects [203]. Another CRF1 antagonist, GSK561679, augmented the startle response to predictable threats but had no significant responses to unpredictable threats, indicating that GSK561679 increased fear responses while failing to reduce anxiety-like responses [204]. Clinical trials are currently ongoing; however, positive clinical data are still lacking. For a comprehensive review of the potential pitfalls in CRF1 antagonist clinical trials see [205].

Neuropeptide Y (NPY) is known for its role in orexigenic and anxiolytic functions through activation of five NPY receptor subtypes that are expressed throughout the brain with high expression found in the hippocampus, amygdala, and brainstem [206]. The involvement of NPY in the behavioral effects of stress are supported in human clinical trials with PTSD. In healthy male subjects, inhibition of the α_2-andrenergic receptor with yohimbine results in an elevated plasma levels of NPY [207]. In a follow-up study of combat veterans with PTSD, baseline plasma levels of NPY were significantly lower than those in healthy control subjects [208]. Moreover, yohimbine treatment in this cohort of patients with PTSD resulted in a blunted increase in plasma NPY levels. These diminutive levels of NPY are observed in the cerebral spinal fluid (CSF) of combat-related PTSD [209]. Thus, NPY may be involved in resilience and promote adaptive coping mechanisms. However, the contributions of either peripheral or central NPY to the regulation of PTSD symptoms remain unclear. Clinical trials continue to investigate the utility of intranasal NPY, with unreported results. As such, these approaches and continued understanding of the NPY system are promising areas for future research to identify novel therapeutic targets for stress-related psychiatric disorders.

Since its discovery in the 1930s, significant research has been aimed at understanding the role of the neurokinin system in mood and anxiety. Early clinical experiments with an NK1R antagonist, MK-869 (aprepitant), suggests a novel and efficacious reduction from baseline values on the Hamilton Depression Scale and Hamilton Anxiety Scale [210]. MK-869 has good brain penetrance, oral bioavailability, and pharmacokinetic data, suggesting a >90 % blockade of the NK1 receptor, but the antidepressant effect of MK-869 was similar to that of paroxetine [210]. Similar results seen in MK-869 trial were obtained in trials with three other NK1 receptor antagonists: L-759274 [211], CP-122721[212], and casopitant[213]. However, further clinical trials of MK-869 found the drug inactive, and the results of a Phase IIa trial of casopitant were inconclusive [214]. Development of the NK1 receptor antagonist orvepitant by GlaxoSmithKline was also halted due to the occurrence of isolated seizures in Phase IIb trials. The subsequent development and clinical investigation of the NK2 receptor antagonist saredutant and the NK3 receptor antagonists osanetant and talnetant also yielded inconclusive results in the treatment of neuropsychiatric disorders [214].

Although discouraging, there are several potential solutions to improve our understanding and potential development of neuropeptide agents available to target psychiatric disorders. Neuropeptides define neuronal ensembles, and a continued characterization of these circuits is critical to understand the brain connectome in homeostatic and pathogenic states. Numerous reports indicate neuropeptides play highly specialized role(s) in the expression of stress responses [175, 206, 215]. Improved technologies in targeting and temporally controlling these circuits has already begun to clarify specific contributions to peptidergic system in fear responses and learning. Moreover, highlighting select dysfunction within neuropeptide ensembles as they contribute to symptomology and the discrete phenotypes observed in neuropsychiatric disorders, will highlight novel druggable targets. Neuropeptide system

may also improve the accuracy in diagnosis of neuropsychiatric disorders. As stated above, the concentration of numerous neuropeptides is affected by chronic stress and may have prognostic potential in clinical settings. Currently, the use of animal models has allowed researchers to generate representative states of disease, but continued improvement in animal models of neuropsychiatric disorders is required. The contribution of neuropeptide systems to these disorders and within these models can then be manipulated for development of more effective therapeutic interventions.

Dissertation Research Objective

The focus of our lab is to better understand the role of neuropeptides as neuromodulatory molecules within the amygdala. The amygdala is an anatomically and functionally distinct region, possessing glutamatergic or GABAergic neurons. Our attention is primarily focused on how neuromodulatory peptides affect these neurons as their activity ultimately affects amygdala function. Moreover, the amygdala receives and sends projections to numerous effector regions that ultimately modulate amygdala function. The objective of this dissertation is to further our understanding of the neuromodulatory mechanisms within the nuclei of the amygdala. The dissertation is divided into three studies that will address the following questions:

1. How does neuromedin B modulate neuronal function in the CeA?
2. Does neuromedin B modulate neuronal function in the BLA?
3. How does neurokinin B modulate BLA neurons?

CHAPTER 2

METHODS

Preparation of Amygdala Slices

All procedures and experiments invovling animals presented herein were approved by the Institutional Animal Use and Care Committee (IACUC) of the University of North Dakota and performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, USA. For all studies, 300 μm coronal brain slices were prepared from virgin male and female Sprague-Dawley (SD) rats. In Study 1 and 2, slices were prepared from 22- to 38-day-old SD rats, whereas in Study 3 SD rats were 30- to 45-day old rats (purchased from Envigo RMS, INC., Indianapolis, IN). We did not notice any age-specific differences in the excitabilities of the neurons in these studies. For Study 2 and Study 3, the following three strains of KO mice (1–2 months old) and their corresponding age-matched WT mice purchased from Jackson Laboratory were used: TRPV1 KO mice (B6.129 × 1-$Trpv1^{tm1Jul}$/J, strain 003770) vs. WT mice (C57BL/6J, strain 000664); TRPC4 KO mice (129S1/SvlmJ-$Trpc4^{tm1.1clph}$/J, strain 030802) vs. WT mice (129S1/SvlmJ, strain 002448); TRPC5 KO mice (129S1/SvlmJ-$Trpc5^{tm1.1clph}$/J, strain 030804) vs. WT mice (129S1/SvlmJ, strain 002448). The animals were housed in the Center for Biomedical Research at the University of North Dakota with food and water available *ad libitum*. The animal rooms were maintained on a 14/10 h light–dark cycle (lights on at 7:00 a.m.), with a room temperature of 22°C. After being deeply anaesthetized with isoflurane, animals

were transcardially perfused with an extracellular solution that contained (in mM) 130 NaCl, 24 NaHCO$_3$, 3.5 KCl, 1.25 NaH$_2$PO$_4$, 2.5 CaCl$_2$, 1.5 MgCl, and 10 glucose saturated with 95% O$_2$ and 5% CO$_2$, decapitated, and their brains were dissected out. The cerebellum was trimmed and the caudal pole of the brain was glued to the plate of a vibratome (Leica VT1200S) and then bathed in an ice-cold cutting solution. In Study 1, the cutting solution contained (in mM) 130 N-methyl-D-glucamine (NMDG)-Cl, 24 NaCO$_3$, 3.5 KCl, 1.25 NaH$_2$PO$_4$, 0.5 CaCl$_2$, 8.0 MgCl$_2$, and 10 glucose, saturated with 95% O$_2$ and 5% CO$_2$ (pH 7.4, adjusted with HCl). The cutting solution was modified slightly for Study 2 and 3 to contain (in mM) 250 sucrose, 104 NaCl, 19.2 NaHCO$_3$, 2.8 KCl, 1 NaH$_2$PO$_4$, 2 CaCl$_2$, 2.4 MgCl$_2$ and 8 glucose (saturated with 95% O$_2$ and 5% CO$_2$). Various protective cutting solutions are commonly use that are NMDG- or sucrose-based, but work in the amygdala has indicated no differences in intrinsic excitability [216]. Cuts began at the rostral pole and slices were collected from both hemispheres when the structure of the amygdala was apparent. Slices were incubated at 35°C for 1 h in the extracellular solution particular to the cutting solution of each study and kept at room temperature (22° C) until use. When an NMDG cutting solution was used, slices were incubated in the cutting solution except the NMDG-Cl was replaced with the same concentration of NaCl. For slices prepared in the sucrose cutting solution, slices were incubated at 35°C for 30 min in the extracellular solution containing (mM) 60 sucrose, 124 NaCl, 23 NaHCO$_3$, 3.3 KCl, 1.2 NaH$_2$PO$_4$, 2.4 CaCl$_2$, 1.7 MgCl$_2$ and 9.5 glucose (saturated with 95% O$_2$ and 5% CO$_2$) and then kept at room temperature until use.

Electrophysiology

Whole-cell patch-clamp recordings were made using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) and signals were digitized using an Axon 1550 Digidata System (Axon Instruments). Recordings in current- or voltage-clamp mode

were conducted from CeL (Study 1) and BLA (Study 2 & 3) neurons visually identified

with infrared video microscopy (Olympus BX51WI) and differential interference contrast

optics. Slices were continuously superfused with an extracellular solution that contained

(in mM) 130 NaCl, 24 NaHCO$_3$, 3.5 KCl, 1.25 NaH$_2$PO$_4$, 2.5 CaCl$_2$, 1.5 MgCl$_2$ and

10 glucose, saturated with 95% O$_2$ and 5% CO$_2$ (pH 7.4) via a gravity-driven perfusion

system (~1 mL/min). In experiments where extracellular Ca^{2+} was reduced, equimolar

MgCl$_2$ was used to substitute the extracellular CaCl$_2$. In Study 2 and 3, the bath was

maintained between 33°C and 34°C by an in-line heater and an automatic temperature

controller (TC-324C, Warner Instruments). Patch recording electrodes with a tip

resistance of 4-10 MΩ were pulled with a PP-830 gravity puller (Narishge, Tokyo,

Japan). Upon the establishment of the whole-cell configuration, a period of at least 10

minutes elapsed prior to recording to ensure stable recording. For all studies, data were

filtered at 2 kHz, digitized at 10kHz, acquired and analyzed subsequently using pClamp

10.4 or 10.7 software (Molecular Devices, Sunnyvale, CA).

Recordings of Resting Membrane Potentials, Action Potentials, Holding Currents, and Current-Voltage Relationships from Amygdala Neurons

For Study 1, recordings were made directly from local CeL GABAergic neurons,

whereas in Study 2 and 3, recordings were made from BLA principal neurons. The

recording electrodes were filled with (in mM) 120 K$^+$-gluconate, 10 KCl, 5 NaCl, 2 MgCl$_2$,

10 HEPES, 0.5 EGTA, 2 ATPNa$_2$, 0.4 GTPNa, and 5 phosphocreatine (pH 7.3) in all

studies. When the Cs$^+$-containing intracellular solution was used for recordings, the

intracellular K$^+$-gluconate was replaced by the same concentration of Cs$^+$-gluconate. For

action potential (AP) recordings, kynurenic acid (1 mM) and picrotoxin (100 µM) were

supplemented in the extracellular solution to block potential indirect actions from

synaptic transmission. AP frequency was obtained by using the "Event Detection" and

"Threshold Search" features of Clampfit 10.7 software. AP data were then exported to

Microsoft Excel and binned per minute using Excel function commands. As BLA neurons did not show spontaneous firing (Study 2 and 3), we injected a series of positive currents from 50 pA to 700 pA at an increment of 50 pA every 6 s. This protocol was applied to the same cells before and during peptide application for 3–5 min when the maximal effect was typically observed. In a subset of experiments in Study 2, a constant positive current was injected to depolarize membrane potentials near AP threshold to induce spare AP firing. Rheobase current was defined as the current required to elicit the first AP during the positive current injection protocol. The AP half-width was defined as the time between the half-amplitude of the upslope of the AP waveform and the equivalent point on the downslope and was calculated from the rheobase sweep of 100 to 200 pA (50 pA steps) series of current injections. The time to the first AP was the time from the initiation of the current injection until the first AP fired and was calculated from the rheobase sweeps of 100 to 200 pA (50 pA steps) series of the positive current injection protocol.

Resting membrane potentials (RMPs) and holding currents (HCs) were recorded from amygdala neurons held at -60 mV in the extracellular solutions supplemented with tetrodotoxin (TTX, 0.5 μM), kynurenic acid (1 mM), and picrotoxin (100 μM). The intracellular solution was the K^+-gluconate containing solution unless otherwise stated. Current-voltage (I-V) relationships were obtained by recording from amygdala neurons held at -60 mV. Cells were stepped from -140 to -40 mV for 400 ms at a voltage increment of 10 mV every 10 s. If the reversal potential was not found within this voltage range, the step range was extended to + 20 mV. In Study 1, we replaced the $CaCl_2$ in the extracellular solution with the same concentration of $MgCl_2$ to reduce the contamination of voltage-gated Ca^{2+} channels. Steady-state current values were measured within 5 ms prior to the end of the step voltage protocol.

The selective peptide agonists in each study were dissolved in extracellular solutions and bath applied to cells for at least 5 minutes, as the maximum effect was typically observed within this period. Slices were limited to a single peptide application to prevent agonist-induced receptor desensitization. Pharmacological inhibitors were applied to the cells either extracellularly or intracellularly via the recording pipettes. For extracellular application, slices were pretreated for at least 2 h to ensure permeation of reagents into the cells in the slices and the extracellular solution continuously contained the same concentration of the reagents, unless stated otherwise. For intracellular application, a period of at least 15 min followed the establishment of the whole-cell configuration to allow for diffusion of the inhibitor into the cells.

Recordings of sEPSCs, mEPSCs, and eEPSCs

Glutamatergic excitatory post-synaptic currents (EPSCs) were recorded in Study 3. Spontaneous EPSCs, (sEPSCs), miniature EPSCs (mEPSCs), and evoked EPSCs (eEPSCs) were obtained using whole-cell patch-clamp recordings from principal cells of the BLA held at -60 mV. Recording electrodes were filled with the K-gluconate recording solution that contained QX-314 (1 mM) to remove contamination from voltage-gated Na^+-channels. To isolate glutamatergic events, the extracellular solution was supplemented with bicuculine (10 μM) to block $GABA_A$ receptors. For recordings of mEPSCs, TTX was included in the bicuculine-supplemented external solution. The recorded sEPSC and mEPSCs were analyzed using Easy Electrophysiology (RRID:SCR_021190) or Clampfit 10.7. Results obtained from either analysis methods produced similar results. The data were then exported for analysis using a custom formula in Microsoft Excel to bin events per minute. All recorded events were visually inspected to exclude obvious artifacts. The detection threshold was established as 3 X the standard deviation of the baseline noise measured in an event-free segment. Due to

the variation of basal sEPSC and mEPSC events, any changes in the frequency of events following the application of NMB or senktide was normalized to the 5 minutes of recorded baseline. eEPSCs were obtained from BLA neurons by placing a tungsten in the afferents of the BLA (~200-350 μm from patched neuron). An A365 stimulus isolator delivered stimulations at a frequency of 10 Hz. The series resistance was carefully monitored by delivering a -5 mV voltage step following each evoked current. The synaptic current recordings were stopped if the series resistance changed by > 20%. The coefficient of variation was calculated by comparing the ratio of the standard deviation and mean of 15 consecutive events before and after NMB bath application. Paired pulse ratio (PPR) was recorded by applying two stimuli at an interval of 50 ms before and after NMB application from the same cell and calculated by equation (PPR = P2/P1, where P1 and P2 were the first and second EPSC amplitude, respectively). All synaptic events were digitized at 10 kHz and filtered at 2 kHz.

Stereotaxic Surgery, Microinjection, and Histology

For all studies, surgical procedures and cannula placement were performed under aseptic conditions, according to our previous publications [217-220]. Guide cannulas (23 GA, 8.5 mm length; P1 Technologies Inc., Roanoke, VA) were bilaterally implanted such that the end of the cannula was positioned 0.5-1 mm above the target brain region. For Study 1, guide cannula targeted the CeA with coordinated obtained from the rat brain atlas (from bregma: anteroposterior: -2.1-2.3 mm, mediolateral: ± 4.1-4.2 mm; dorsoventral: -7.6 mm [221]. For Study 2 and 3, guide cannula were positioned targeting the BLA (from bregma, anteroposterior: -2 ~ -2.6 mm, mediolateral: ± 4.9 ~ 5.0 mm, dorsoventral: -7.2-7.6 mm) [222]. Coordinate placement was adjusted to account for variation in animal weight and personal experiences with the procedure. Study 1 and 3 included male (Study 1: 368 ± 10.4 g, n = 17; Study 3: 195.7 ± 8.4 g, n = 21) and female

(Study 1: 225 $\pm$ 5.8 g, n = 20; Study 2: 168 $\pm$3.9 g, n = 26) SD rats, whereas Study 2 included only female (193 $\pm$ 8 g, n = 63) SD rats. A deep state of anesthesia was induced by 5% isoflurane and anesthesia was maintained at 3% while rats were placed in the stereotaxic frame (Stoelting Co., IL). An incision was made at the occipital bone extending forward toward the frontal bone. The connective tissue encasing the skull was cleared with cotton swabs to expose the suture lines. Cannulas were secured in place with dental acrylic bonded to 3 stainless steel screws (4.8 mm, P1 Technologies Inc., Roanoke, VA) inserted into burr holes drilled into the skull. To prevent occlusion of the guide cannulas, stainless steel stylets were screwed into the guide cannula and left in place until microinjection. Following surgery, rats were allotted a 7-10 d recovery period in which rats were monitored daily for adverse post-surgical complication. Within the recovery period, rats received daily handling for habituation of microinjection procedures. Bilateral microinjections of various compounds into the amygdala were delivered in 1 μL volumes through an internal cannula (30 GA, 8.5 mm; P1 Technologies Inc., Roanoke, VA). Two 5 μL Hamilton syringes fixed to an automated pump (Harvard Apparatus, MA) were used to administer drug injections at a rate of 0.2 μL min $^{-1}$. Upon the completion of the microinjection, the internal cannula was left in place for an additional ~2 min to ensure adequate diffusion of reagents into the amygdala.

For Study 1, saline, NMB, tertiapin-Q, BIM23042 (all in 1 μL volume per side) were bilaterally injected into the CeA. In Study 2, rats were bilaterally injected with saline, NMB, BIM23042, AMG9810, or tertiapin-Q. Rats in Study 3 received bilaterally injections of saline, senktide, SB218795, M084, or tertiapin-Q into the BLA via the internal cannula. Following experiments, all rats were anesthetized and bilaterally injected with 3% Chicago Sky Blue dye (Sigma). Coronal sections (80 μm) containing the amygdala were collected on a vibrating microtome (VT1200, Leica Biosystems Inc., IL) and proper

cannula placement was confirmed. Animals with incorrect cannula placement were excluded from analysis.

Acoustic Startle and Fear-Potentiated Startle Responses

Acoustic startle response (ASR) and fear-potentiated startle (FPS) experiments were conducted in Study 1 and 2. ASR and FPS were conducted using clear cylindrical animal holders inside two identical SR-LAB startle chambers connected to a laptop with the SR-Lab software (San Diego Instruments, CA) as described [223, 224]. Experimental protocols were designed and implemented via the SR-lab software. A high-frequency loudspeaker and a single lightbulb mounted 24 cm above the animal holder in each cabinet was used to elicit the white noise bursts (90 dB, 95 dB, and 105 dB WNBs) and the visually conditioned stimulus, respectively. Foot shocks (0.5 mA) were used as a conditioned stimulus and were delivered from a removable stainless-steel floor grid within each animal holder.

The experimental paradigms used in Study 1 and 3 are shown below (Figures 8 and 24). Days 1, 3, and 4 were conducted in the same way in Study 1 and 3, whereas day 2 was specific to a particular study. On day 1, rats were moved to the behavioral testing room in their home cage for a 1 h handling session and a 30 min habituation within each animal holder in each startle chamber. On day 2 in Study 1, animals underwent a 5-min habituation prior to being presented with 21 WNBs (90 dB x 7; 95 dB x 7;105 dB x 7; 50 ms duration) at a 30 s intertrial interval (ITI) for startle responses in the presence of a background white noise (70 dB). A subset of male and female rats was microinjected with saline or NMB (0.3 nmol) 15 min prior to ASR testing. Study 3 animals were presented with 30 WNBs (95 dB, 50 ms) at a 30 s ITI. All experiments on day 2 occurred in the presence of a 70 dB background white noise. On day 3, test compounds were microinjected 5-10 minutes prior to the fear conditioning session. Similar to days 1

and 2, animals were allotted a 5-min habituation. Fear condition consisted of a series of ten 3.7 s light cues (neutral stimuli) that co-terminated with a 0.5 s foot shock (0.5 mA, pseudorandom ITI 30-180 s) in the absence of background white noise. On day 4, acquisition of a fear response was tested using a FPS protocol. After a 5-minute habituation period within the startle chamber, rats were subjected to 30 WNBs in presence of a continuous 70 dB background white noise. The startle amplitude was defined as the maximum peak voltage recorded during the first 200 ms of the response to the WNB. A basal startle amplitude in the presence of background noise was obtained in response to the first 10 WNBs (90 dB x5; 95 dB x 5) alone without a light cue. The following 20 WNBs occurred in groups of 10 WNBs: 10 WNBs (105 dB) paired with the conditioned light cue (cued) and 10 WNBs (105 dB) occur in the absence of the conditioned light cue (non-cued) in a pseudorandom order. FPS responses in Study 1 were reported as raw startle responses as described previously [225, 226]. In Study 2, cued fear responses were reported as [(amplitude with the light cue - non-cued amplitude)/ non-cued amplitude] x 100%. The non-cued fear responses showed as [(amplitude without the light cue – basal amplitude)/basal amplitude] x 100%, as previously reported [226, 227].

Recordings of Mean Arterial Pressure and Heart Rate

For Study 1, a VPR noninvasive blood pressure monitoring system (CODA-6, Kent Scientific, Torrington, CT) connected to a Powerlab system (ADinstruments Pty Ltd., Bella Vista, New South Wales, Australia) was used to measure mean arterial pressure (MAP) and heart rate (HR) in age-matched male and female SD rats. The VPR noninvasive blood pressure monitoring system provides accurate measures in comparison with more invasive approaches [228]. When possible, data were collected from an equal number of male and female rats. MAP and HR were assessed during the

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light cycle and SD rats underwent a minimum of 7-days habituation via light handling in a clean towel [229]. Prior to recording, animals were placed on a warming pad to improve the recording signal and care was taken to not overheat nor over restrict the movement of the animal during the recording session. Baseline cardiovascular parameters (systolic, diastolic, mean arterial pressure, and heart rate) were recorded for at least a 10-minute habituation period during each session. A series of measurements were recorded during and immediately following drug injection and data were reported as the net change in mean arterial pressure (MAP) and heart rate (HR) from baseline as a result of saline or drug injection, as previously reported [229-231].

Vogel Conflict Test

Rats in Study 2 were assessed for anxiety-like behaviors in the Vogel Conflict Test (VCT). The VCT was performed in polycarbonate cages (42 x 25 x 20 cm) with a metal floor grid, as previously described [232, 233]. The stainless-steel drinking spout of the water bottle and floor grid were connected to an Anxiometer (Columbus Instruments, OH). When an animal contacts the spout and floor grid, the electric circuit is closed and a lick is counted by the Anxiometer. The animal received a mild electric shock (0.3 mA, 0.5 s) every 20 licks. For 2 days preceding the VCT, animals were restricted to 1 h per day of access to tap water. After the initial 23 h water deprivation, animals are habituated to the testing cage for 30 min and allowed unpunished drinking. On day 2, test compounds were microinjected 10-20 minutes prior to the VCT session. Animals were allowed a 5-minute habituation in the test cage prior to the start of the VCT. The test session was then initiated after the animal made 20 licks and a single shock (0.3 mA, 0.5 ms) was delivered. Rats received a shock every 20 licks during the 3-minute testing period. Data reported were the number of spout licks by rats during the 3-minute test session.

Data Analysis

Data in Study 1 and Study 2 are presented as the means ± S.E.M. In Study 3, data are presented as the means ± SD. For the *in vitro* data, "n" refers to the number of cells recorded, whereas for the *in vivo* experiments, "n" refers to the number of animals tested. Normality was assessed using the D'Agostino and Pearson tests, and data that did not pass were analyzed with the corresponding nonparametric test. Paired Student's t-test (paired t-test), Wilcoxon matched-pair signed rank test (hereafter Wilcoxon's test), two-sample Student's t-test, and one-way or two-way ANOVA were used for statistical analysis, as appropriate. To minimize potential influences of variations from individual animals, each experiment was performed from slices obtained from at least four animals and one-way ANOVA was performed to ensure there was no significant difference for the data obtained from individual animals under the same treatment. One-way ANOVA followed by Dunnett's or Tukey's multiple comparison test was used for statistical analysis when the pooled control data were used for comparison. Two-way repeated measures ANOVA followed by Šídák's multiple comparison test was used for statistical analysis for the AP firing frequency elicited by injections of positive currents and for the data used to construct the I-V relationship. For sEPSC cumulative probability plots, events recorded 5 min before and 5 min after reaching maximal effects were used for comparison, and bin sizes were the same between the experiments. The Kolmogorov-Smirnov test was used to assess the significance of the cumulative probability plots. ASR, FPS, and baseline cardiovascular responses were analyzed using a mixed-effects two-way ANOVA, as appropriate and significant was analyzed post hoc using the Šídák's multiple comparisons test. Shock reactivity was analyzed using the nonparametric Mann-Whitney test. Net changes in cardiovascular values were compared using a one-way ANOVA and post-hoc differences were assessed using Dunnett's or Tukey's multiple comparisons test. The IC_{50} value was calculated from raw

data measurements using non-linear regression. Statistical analysis was performed using Origin 7 (OriginLab Corporation, Northampton, MA, USA) and GraphPad Prism v9.4 (GraphPad Software Inc., San Diego, CA, USA). P-values were reported throughout the text, and significance was set at $P < 0.05$.

Chemicals

All drugs were initially prepared as a stock solution and stored at -20ºC until use. The stock solution was diluted with extracellular solution to obtain the appropriate working concentration for bath application to the recording chamber. In preparations requiring dimethyl sulfoxide (DMSO), the final concentration of DMSO was below 0.1% which is physiologically inert. The complete list of drugs used in this dissertation can be found in the List of Reagents and Drugs.

CHAPTER 3

RESULTS

Study 1 - Neuromedin B-Mediated Modulation of Central Amygdala Neurons

Introduction

Neuromedin B (NMB) and gastrin-releasing peptide (GRP) are the mammalian analogues of the bombesin and the bombesin-like family of peptides originally characterized in amphibians and first isolated from porcine gastric tissue and spinal cord [234]. The biological actions of bombesin-like peptides are mediated by at least three receptors; the NMB-preferring bombesin 1 (BB1) receptors, the GRP-preferring bombesin 2 (BB2) receptors [235, 236] and the orphan bombesin 3 (BB3) receptors whose natural ligand is unknown [237, 238]. All three bombesin receptors are coupled to G proteins resulting in the activation of phospholipase Cβ (PLCβ) which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP$_2$) generating inositol 1,4,5-trisphosphate (IP$_3$) to stimulate intracellular Ca^{2+} release and diacylglycerol (DAG) to activate protein kinase C (PKC) [237, 239, 240], although the bombesin receptors also stimulate tyrosine phosphorylation of a number of signaling proteins [239, 241]. BB1 receptor activation regulates pituitary-thyroid function, fear and anxiety responses, satiety and tumor growth; BB2 receptors play important roles in modulating pruritus, lung development, small intestinal mucosal defense and CNS processes such as learning and memory; BB3 receptors are involved in controlling obesity and glucose intolerance, lung response to injury, tumor growth and gastrointestinal tract motility [239, 241].

However, the cellular and molecular mechanisms whereby the bombesin-like peptides modulate these physiological functions and pathological disorders have not been fully determined.

The CeA expresses not only bombesin-like peptides [242] and NMB mRNA [243], but also high densities of binding sites for bombesin-like peptides [242] and NMB [244]. Consistent with the distribution of bombesin-like peptides and their receptors in the CeA, injection of bombesin into the CeA induces an increase in mean arterial pressure [245] and reduces food intake [246, 247]. Intraperitoneal or cerebroventricular administration of bombesin induces Fos-like immunoreactivity, a marker of neuronal activation, in the CeL [248]. However, the effects of bombesin and bombesin-like peptides on neuronal excitability in the CeA and the underlying cellular and molecular mechanisms have not been determined. In this study, we studied the effects of NMB on the excitability of CeL neurons based on the results showing that high densities of NMB binding sites are distributed in the CeA [244] and administration of bombesin induces Fos expression in the CeL suggesting that activation of bombesin receptors increases neuronal activity in the CeL. Our results indicate that activation of BB1 receptors by NMB excites CeL neurons by PLCβ and PKC-mediated depression of inwardly rectifying K^+ (Kir) channels. Moreover, microinjection of NMB into the CeA reduced blood pressure and heart rate in unanesthetized male and female rats and reduced fear-potentiated startle responses. Our results may provide a cellular and molecular mechanism to explain the roles of bombesin and bombesin-like peptides in the amygdala.

NMB Increases Neuronal Excitability in the CeL

We chose NMB as an example to test the effects of bombesin-like peptides on neuronal excitability in the CeL (Figure 1, a) based on the results showing that the CeA expresses both NMB mRNA [243] and NMB binding sites [244] and administration of

bombesin induces Fos expression in the CeL [248], suggesting that activation of bombesin receptors facilitates neuronal activity. CeL neurons can be classified electrophysiologically into three types: regular firing (RS, ~54%), low-threshold bursting (LTB, ~34%), and late firing (LF, ~12%) [42]. We thus identified the types of the recorded neurons first after formation of the whole cell recording configuration by injection of a series of negative and positive currents. As neurons in the CeL did not show spontaneous firing, we injected a persistent positive current to induce discrete AP firing. We examined 33 neurons of which 23 were RS, 7 were LTB, and 3 were LF. Bath application of the selective BB1 receptor agonist, NMB at 0.3 µM, a near-saturating concentration [74], augmented the firing frequency of APs recorded from each neuronal type (Figure 1, b-d). We therefore pooled the data recorded from each neuronal type. NMB increased the AP firing frequency to $274 \pm 38\%$ of control (Control: 0.51 ± 0.08 Hz, NMB: 1.11 ± 0.15 Hz, n = 33, $P < 0.0001$, Wilcoxon test, Figure 1, e), suggesting that activation of BB1 receptors excited CeL neurons. NMB-elicited augmentation of AP firing frequency was irreversible after a wash for 30 minutes (Control: 0.51 ± 0.08 Hz, wash 30 min: 1.38 ± 0.34 Hz, n = 33, $P = 0.0003$, Wilcoxon test, Figure 1, e). The NMB-mediated increases in AP firing frequency were sex-independent as NMB exerted similar extent of increases ($P = 0.15$, Two-sample t-test) in male ($248 \pm 53\%$ of control, n = 17) and female ($434 \pm 117\%$ of controls, n = 16) virgin rats. We thus used both sexes and kept the numbers of male and female rats as equal as possible for the remaining experiments.
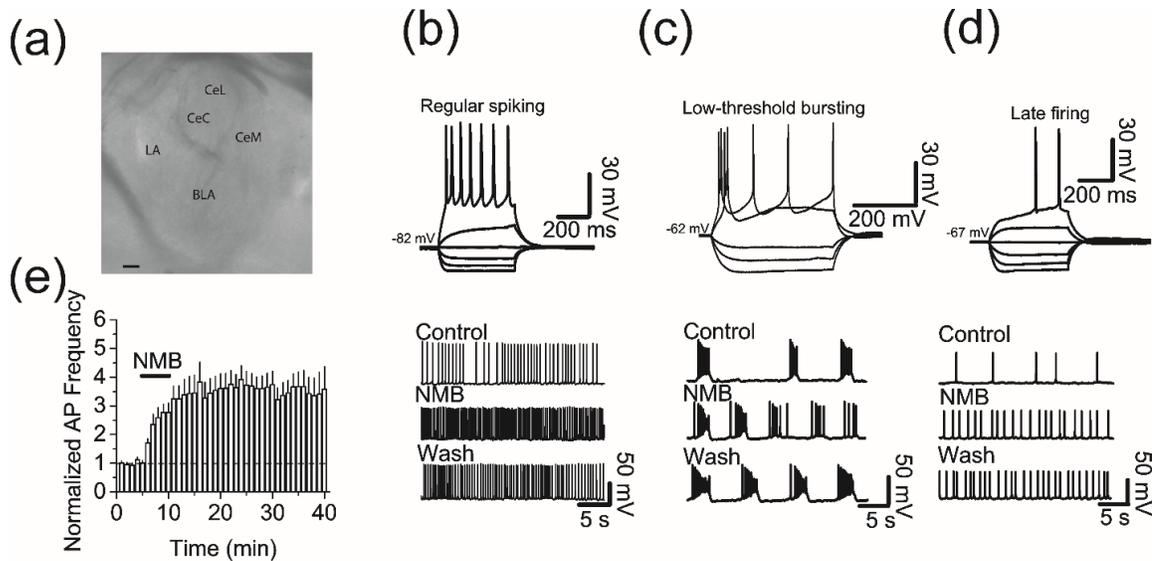
Figure 1. **NMB increases AP firing frequency in CeL neurons.**
(**a**), microscopic photograph showing the location of CeL where electrophysiological recordings were conducted. LA: lateral nucleus; BLA: basolateral nucleus; CeC: capsular central amygdala; CeL: lateral central amygdala; CeM: medial central amygdala. Scale bar: 500 μm. (**b**), bath application of NMB (0.3 μM) increased AP firing frequency in regular spiking (RS) neurons in the CeL region. *Upper panel*: voltage responses elicited by injection of currents from -30 pA to 20 pA at an increment of 10 pA in a duration of 400 ms. *Lower panel*: current traces recorded from the same neuron prior to, during and after the application of NMB. The firing frequency of APs in RS neurons was increased by a 5-minute bath application of NMB. (**c**), bath application of NMB (0.3 μM) increased AP firing frequency in low-threshold bursting (LTB) neurons of the CeL region. The firing frequency of APs in LTB neurons was increased by a 5-minute bath application of NMB. (**d**), bath application of NMB (0.3 μM) increased AP firing frequency in late firing (LF) neurons in the CeL region. The firing frequency of APs in LF neurons was increased by a 5-minute bath application of NMB. (**e**), summarized time course of NMB-induced potentiation of AP firing frequency recorded from 33 CeL neurons. Adapted from [223].

NMB-Induced Excitation of CeL Neurons Requires the Functions of G Proteins and PLCβ

We then sought to determine the intracellular signaling mechanisms involved in NMB-mediated excitation of CeL neurons. BB1 receptors are coupled to $G\alpha_q$ proteins resulting in the activation of PLCβ which hydrolyzes PIP_2 to generate IP_3 to increase intracellular Ca^{2+} release and DAG to activate PKC [240]. Thus, we examined the roles of this signaling pathway in NMB-mediated facilitation of neuronal excitability in the CeL. Intracellular perfusion of the selective G protein inactivator GDP-β-S (0.5 mM) prevented NMB-induced augmentation of AP firing frequency (Control : 0.73 ± 0.31 Hz, NMB: 0.65 ± 0.28 Hz, $99 \pm 19\%$ of control, n = 10, $P = 0.75$, Wilcoxon test; $F_{(1,41)} = 9.523$, $P = 0.0036$ vs. NMB alone, two-way ordinary ANOVA, Figure 2, a), indicating that the function of G protein is required for NMB-mediated enhancement of AP firing.

We assessed the roles of PLCβ in NMB-mediated excitation of CeL neurons. Slices were pretreated with the selective PLC inhibitor, U73122 (5 μM), for >2 h. Separate slices were treated with the inactive analogue U73343 (5 μM) in the same fashion as the control. Under these conditions, application of NMB did not significantly augment AP firing frequency in slices pretreated with U73122 (Control: 1.36 ± 0.25 Hz, NMB 1.27 ± 0.31 Hz, $99 \pm 11\%$ of control, n = 17 , $P = 0.8802$, Wilcoxon test, Figure 2, b), but still significantly enhanced the AP firing frequency in slices pretreated with U73343 (Control: 0.51 ± 0.10 Hz, NMB 0.98 ± 0.19 Hz, $221 \pm 35\%$ of control , n = 18, $P < 0.0007$, Wilcoxon test; $F_{(1,33)} = 9.25$, $P < 0.01$ vs. U73122, two-way ordinary ANOVA, Figure 2, b). These results demonstrate that PLCβ is required for NMB-mediated increases in neuronal excitability in the CeL.

Intracellular Ca^{2+} Release is Unnecessary, but PKC is Required for NMB-Elicited Excitation of CeL Neurons

We investigated the involvement of Ca^{2+} release from intracellular stores in NMB-mediated facilitation of neuronal excitability. Intracellular application of the IP$_3$ receptor blocker heparin (2 mg/ml) through the recording pipettes failed to significantly affect NMB-mediated enhancement of AP firing frequency (Control: 0.56 ± 0.10 Hz, NMB: 1.79 ± 0.64 Hz, $299 \pm 49\%$ of control, n = 16, $P < 0.0006$, Wilcoxon test; $F_{(1,47)} = 0.047$, $P = 0.8282$ vs. NMB alone, two-way ordinary ANOVA, Figure 2, c), indicating that IP$_3$ receptors are not required for NMB-induced augmentation of neuronal excitability. Similarly, intracellular application of the sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (10 μM) via the recording pipettes did not significantly alter NMB-mediated augmentation of AP firing frequency (Control: 0.51 ± 0.11 Hz, NMB: 0.82 ± 0.15 Hz, $174 \pm 20\%$ of control, n = 21, $P < 0.0001$, Wilcoxon test; $F_{(1,52)} = 0.289$, $P = 0.593$ vs. NMB alone, two-way ordinary ANOVA, Figure 2, c). We further examined the potential involvement of Ca^{2+} released from the ryanodine-sensitive store by intracellular perfusion of ryanodine (100 μM) to inhibit ryanodine receptors. Intracellular application of ryanodine did not significantly affect NMB-mediated increases in neuronal excitability in CeL neurons (Control: 1.97 ± 0.59 Hz, NMB: 3.98 ± 1.14 Hz, $244 \pm 64\%$ of control, n = 18, $P = 0.0016$, Wilcoxon test; $F_{(1,50)} = 0.09$, $P = 0.77$ vs. NMB alone, two-way ordinary ANOVA, Figure 2, d). These results demonstrate that intracellular Ca^{2+} release is not involved in the NMB-elicited increases in AP firing. Interestingly, intracellular dialysis of the Ca^{2+} chelator, BAPTA (10 mM), significantly attenuated NMB-mediated increase in AP firing frequency (Control: 0.90 ± 0.19 Hz, NMB: 0.79 ± 0.14 Hz, $110 \pm 14\%$ of control, n = 18, $P = 0.475$, Wilcoxon test; $F_{(1,49)} = 15.69$, $P = 0.0002$ vs. NMB alone, two-way ordinary ANOVA, Figure 2, d). As PKC was involved in NMB-

elicited excitation of CeL neurons (see below) and some PKC isoforms are Ca^{2+}-dependent, one explanation for the BAPTA result is that BAPTA lowered the basal intracellular Ca^{2+} level, which may be required for the functions of Ca^{2+}-dependent signaling molecules such as PKC. Lastly, we investigated the role of extracellular Ca^{2+} in NMB-elicited augmentation of neuronal excitability by replacing the extracellular Ca^{2+} with an equal concentration of Mg^{2+}. Under these conditions, application of NMB similarly enhanced AP firing frequency (Control: 0.58 ± 0.10 Hz, NMB: 1.05 ± 0.16 Hz, $213 \pm 28\%$ of control, n = 18, $P = 0.003$, Wilcoxon test; $F_{(1,49)} = 0.196$, $P = 0.66$ vs. NMB alone, two-way ordinary ANOVA, Figure 2, e), indicating that NMB-mediated increase in neuronal excitability is not dependent on extracellular Ca^{2+}.

We further probed the role of PKC in NMB-mediated excitation of CeL neurons. Slices were pretreated with the selective PKC inhibitor, chelerythrine (10 μM), for >2 h, and the bath was continuously perfused with the same concentration of chelerythrine to ensure persistent inhibition of PKC. In this situation, application of NMB did not significantly increase the AP firing frequency (Control: 0.75 ± 0.18 Hz, NMB: 0.71 ± 0.17 Hz, $102 \pm 15\%$ of control, n = 22, $P = 0.708$, Wilcoxon test; $F_{(1,53)} = 16.57$, $P = 0.0002$ vs. NMB alone, two-way ordinary ANOVA, Figure 2, f). Moreover, pretreatment of slices with and continuous bath application of another selective PKC inhibitor, bisindolylmaleimide (Bis II, 1 μM), blocked NMB-mediated increases in AP firing frequency (Control: 1.04 ± 0.32 Hz, NMB: 0.91 ± 0.26 Hz, $101 \pm 15\%$ of control, n = 10, $P = 0.77$, Wilcoxon test; $F_{(1,41)} = 9.519$, $P = 0.0036$ vs. NMB alone, two-way ordinary ANOVA, Figure 2, g). The involvement of PKC was further supported by bath application of the PKC activator, phorbol 12-myristate 13-acetate (PMA, 1 μM), resulting in a significant increase of AP firing frequency in CeL neurons (Control: 0.68 ± 0.21 Hz, PMA: 1.74 ± 0.43 Hz, $281 \pm 70\%$ of control, n = 14, $P = 0.0031$, Wilcoxon test,

Figure 2, h). In slices pretreated and continuously superfused with Bis II (1 μM),

application of PMA failed to significantly increase AP firing frequency (Control: 0.42 $\pm$

0.11 Hz, PMA: 0.33 $\pm$ 0.10 Hz, 75 $\pm$ 9% of control, n = 15, P = 0.058, Wilcoxon test;

$F_{(1,27)}$ = 19.11, P = 0.0002 vs PMA alone, Figure 2h). Moreover, application of

chelerythrine (10 μM) in the same fashion blocked PMA-induced increase in AP firing

frequency (Control: 0.59 $\pm$ 0.31 Hz, PMA: 0.64 $\pm$ 0.34 Hz, 101 $\pm$ 15% of control, n = 10,

P = 0.63, Wilcoxon test; $F_{(1,22)}$ = 9.81, P = 0.0048 vs. PMA alone, two-way ordinary

ANOVA, Figure 2, h). These data together indicate that PKC is required for NMB-

mediated excitation of CeL neurons.

NMB Depolarizes CeL Neurons and Increases the Input Resistance and Membrane Time Constants of CeL Neurons

We then included TTX (0.5 μM) in the extracellular solution to block Na^+-dependent

AP firing to determine the effects of NMB on passive membrane properties of CeL

neurons. Bath application of NMB induced significant depolarization of CeL neurons

(Control: -63.1 $\pm$ 1.8 mV, NMB: -59.6 $\pm$ 1.9 mV, net depolarization: 3.5 $\pm$ 0.8 mV, n = 16,

P = 0.0004, paired t-test, Figure 3, a1-a2). To confirm that NMB-elicited excitation of CeL

neurons was indeed mediated by activation of BB1 receptors, we pretreated slices with

and continuously bath-applied the selective BB1 antagonist, BIM23042 (0.3 μM) [249,

250]. In the presence of BIM23042, NMB failed to depolarize CeL neurons significantly

(BIM 23042: -63.5 $\pm$ 1.2 mV, BIM 23042 + NMB: -63.3 $\pm$ 1.3 mV, net depolarization:

0.20 $\pm$ 0.16 mV, n = 10, P = 0.24, paired t-test, Figure 3, a3). Furthermore, application

of PD168368 (10 μM), another selective BB1 receptor antagonist [251], in the same

fashion blocked NMB-induced depolarization (PD168368: -64.3 $\pm$ 1.9 mV, PD168368 +

NMB: -64.1 $\pm$ 2.0 mV, net depolarization: 0.25 $\pm$ 0.14 mV, n = 10, P = 0.12, Wilcoxon

test, Figure 3, a3). In voltage-clamp mode, NMB elicited an inward current
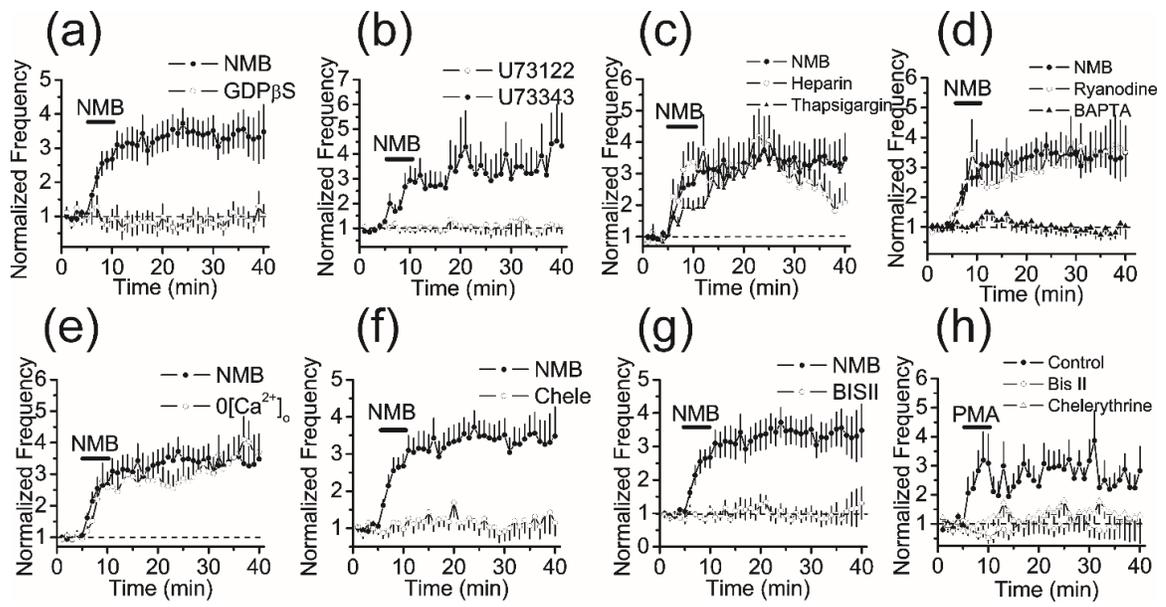
Figure 2. **G proteins, PLCβ and PKC are required for NMB-mediated potentiation of AP firing frequency**.

(**a**), intracellular dialysis of the G protein inactivator, GDP-β-S (0.5 mM), blocked NMB-induced facilitation of AP firing frequency (n = 10). (**b**), pretreatment of slices with the PLC inhibitor, U73122 (5 μM), blocked NMB-induced potentiation of AP firing frequency (n = 17), whereas application of NMB still significantly enhanced AP firing frequency in slices pretreated with the inactive analog, U73343 (5 μM, n = 18). (**c**), intracellular dialysis of the IP$_3$ receptor blocker, heparin (2 mg/ml, n = 16), or the inhibitor of the smooth-endoplasmic reticular Ca^{2+}-ATPase, thapsigargin (10 μM, n = 21) did not significantly affect NMB-induced augmentation of AP firing frequency. (**d**), intracellular application of ryanodine (100 μM) to inhibit ryanodine receptors did not significantly influence NMB-elicited augmentation of AP firing frequency (n = 18), whereas intracellular perfusion of the Ca^{2+} chelator BAPTA (10 mM) blocked NMB-induced enhancement of AP firing frequency (n = 18). (**e**), replacement of extracellular Ca^{2+} with the same concentration of Mg^{2+} did not affect NMB-induced increases in AP firing frequency (n = 18). (**f**), pretreatment of slices with and continuous bath application of the selective PKC inhibitor chelerythrine (Chele, 10 μM) blocked NMB-induced augmentation of AP firing frequency (n = 22). (**g**), pretreatment of slices with and continuous bath application of the selective PKC inhibitor Bis II (1 μM) blocked NMB-induced augmentation of AP firing frequency (n = 10). (**h**), bath application of the PKC activator PMA (1 μM) significantly increased the AP firing frequency (n = 14), whereas pretreatment of slices with and continuous bath application of Bis II (1 µM, n = 15) or chelerythrine (10 µM, n = 10) blocked PMA-induced potentiation of AP firing frequency. Adapted from [223].

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at -60 mV (-19.6 $\pm$ 5.2 pA, n = 17, P = 0.002, paired t-test, Figure 3, b1-b2)**.** Taken

together, these results demonstrate that activation of BB1 receptors increases neuronal

excitability through membrane depolarization.

We further determined the effect of BB1 receptor activation on the input resistance

of CeL neurons by injecting a series of negative currents from -20 to -100 pA with a 20

pA step every 6 s before and after the application of NMB. We then fit the current-

voltage relationship to a linear function for each cell to obtain the input resistance (R_{in})

which was the slope of the linear fitting (Figure 3, c1-c3). Bath application of NMB

significantly increased R_{in} (Control: 260 $\pm$ 35 MΩ, NMB: 290 $\pm$ 36 MΩ, n = 14, P = 0.007,

Wilcoxon test, Figure 3, c3), indicating that NMB increased R_{in}. We acquired the

membrane time constants by fitting a single exponential function to the voltage transient

generated by negative current injection (-100 pA, 100 ms from the end of the baseline).

Bath application of NMB significantly increased the membrane time constants (Control:

38.3 $\pm$ 5.7 ms, NMB: 51.8 $\pm$ 7.8 ms, n = 14, P = 0.023, Wilcoxon test, Figure 3, d1-d3).

These results together suggest that NMB excites CeL neurons by suppressing a

membrane conductance.

Activation of BB1 Receptors Inhibits an Inwardly Rectifying K$^+$ Channel in CeL Neurons

We next determined the ionic mechanisms by which BB1 receptor activation

depolarizes CeL neurons. As shown in Figure, 2e, the effect of NMB on CeL neurons

was independent of extracellular Ca^{2+}. We therefore replaced extracellular Ca^{2+} with the

same concentration of Mg^{2+} to avoid contamination of voltage-gated Ca^{2+} channels. TTX

(0.5 μM) was included in the Ca^{2+}-free extracellular solution to block voltage-gated Na$^+$

channels. Cells were held at -60 mV and stepped from -140 mV to -40 mV for 400 ms

at a voltage interval of 10 mV every 10 s (Figure 3, e1) before and after bath application

of NMB when the maximal effect was observed. Steady-state currents were

Figure 3. **NMB induces membrane depolarization and increases the input resistances and membrane time constants.**

(**a1-a3**), bath application of NMB depolarized CeL neurons via activation of BB1 receptors. (**a1**), RMP recorded from a CeL neuron before, during, and after the application of NMB. (**a2**), summary data for NMB-induced depolarization (n = 16). Green circles represent the values from individual cells and the red circles represent their average. (**a3**), pretreatment of slices with and continuous bath application of the selective BB1 receptor antagonist BIM23042 (BIM, 0.3 μM, n = 10) or PD168368 (PD, 10 μM, n = 10) blocked NMB-induced depolarization. (**b1-b2**), bath application of NMB induced an inward current from CeL neurons in voltage-clamp. (**b1**), holding current recorded at -60 mV from a CeL neuron before, during, and after the application of NMB. (**b2**), summary of net holding currents induced by NMB (n = 17). Green circles represent the values from individual cells and the bar graph represent their average. (**c1-c3**), NMB increased the input resistance (R_{in}). (**c1**), voltage responses evoked by injection of negative currents from -100 to -20 pA at an interval of 20 pA before (*left*) and during (*right*) the application of NMB. (**c2**), I-V relationship averaged from 14 cells. R_{in} was obtained by linear fitting of the I-V relationship. (**c3**), summary graph for the R_{in} before and after the application of NMB (n = 14). (**d1-d3**), NMB increased membrane time constants. (**d1**), voltage response evoked by -100 pA current injection before and after the application of NMB. (**d2**), expansion of the voltage transient shown in the box in (**d1**) to demonstrate NMB-induced enlargement of membrane time constant. (**d3**), summary graph for membrane time constants before and after the application of NMB. (**e1-e4**), activation of BB1 receptors excited CeL neurons through an inhibition of inwardly rectifying K^+ channels. (**e1**), the voltage-step protocol used. Cells were held at -60 mV and stepped from -140 mV to -40 mV for 400 ms in 10 mV voltage intervals every 10 s. (**e2**), representative currents elicited by the voltage-step protocol before and after bath application of NMB and the net currents acquired by subtraction. (**e3**), I-V curves averaged from 13 cells before and after the application of NMB. **** $P < 0.0001$, two-way repeated-measures ANOVA followed by Šídák's multiple comparison test. (**e4**)*,* I-V curve of the net currents acquired by subtracting the currents in control condition from those after the application of NMB (n = 13). Adapted from [223].
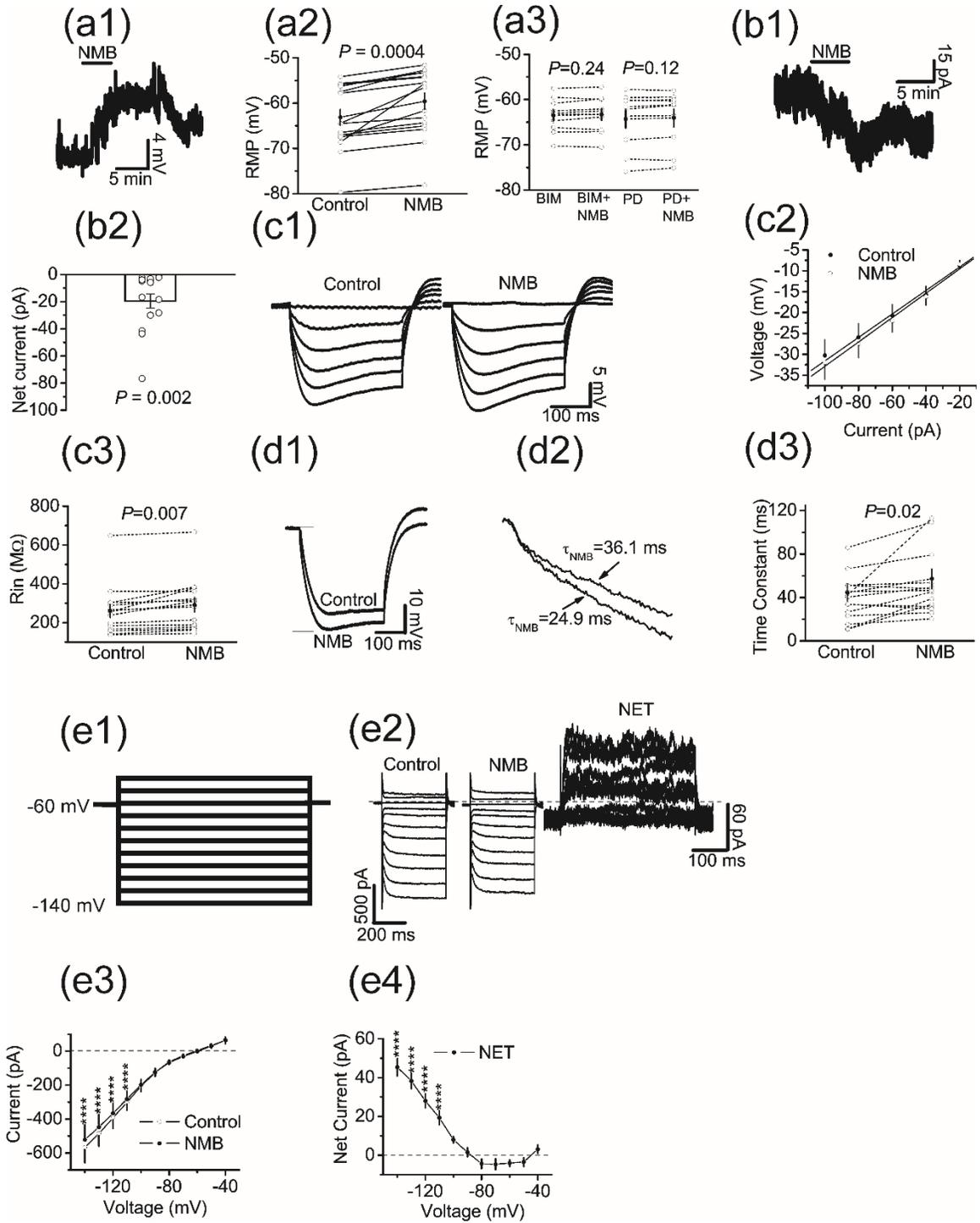
measured within 5 ms before the end of the step voltage protocols. Under these circumstances, the currents recorded before and after the application of NMB showed inward rectification (n = 13, Figure 3, e2-e4), suggesting that Kir channels are expressed in CeL neurons. Subtraction of the current-voltage relationship in the control condition from that after the application of NMB displayed an inwardly rectified current-voltage curve (n = 13, Figure 3, e4). These results support that activation of the BB1 receptors excites CeL neurons by inhibiting Kir channels.

Micromolar concentrations (100 – 300 μM) of extracellular Ba^{2+} have been shown to block Kir channels by at least 80% [252-254]. Inclusion of 300 μM Ba^{2+} in the extracellular solution inhibited a current showing inward rectification (n = 12, $F_{(1,11)}$ = 39.65, P < 0.0001, two-way repeated measures ANOVA followed by Šídák's multiple comparison test, Figure 4, a1-a3) suggesting that this concentration of Ba^{2+} inhibited Kir channels. In the presence of Ba^{2+}, application of NMB failed to elicit more currents significantly (n = 12, $F_{(1,11)}$ = 0.276, P = 0.61, two-way repeated measures ANOVA followed by Šídák's multiple comparison test, Figure 4, a4-a6), demonstrating that NMB-induced excitation of CeL neurons is mediated by depression of Ba^{2+}-sensitive Kir channels.

GIRK Channels are Involved in BB1 Receptor-Mediated Excitation of CeL Neurons

We aimed at identifying the subtype of Kir channels involved in NMB-mediated facilitation of neuronal excitability. Kir channels are divided into four functional groups: 1) the constitutively active Kir2 subfamily channels including Kir2.1, Kir2.2, Kir2.3, and Kir2.4; 2) the G protein-gated GIRK (Kir3 subfamily) channels consisting of Kir3.1 (GIRK1), Kir3.2 (GIRK2), Kir.3.3 (GIRK3), Kir3.4 (GIRK4); 3) the ATP-sensitive K^+ (K_{ATP}) channels comprising Kir6.1 and Kir6.2; 4) K^+ transport channels involving Kir1.1, Kir4.1, Kir.4.2, and Kir7.1 [255]. We tested the role of the Kir2 subfamily channels in

NMB-elicited depression of Kir channels by utilizing the selective Kir2 channel blocker, ML 133 (30 μM) [256-260]. Bath application of ML 133 alone induced a significant inhibition of Kir currents from -120 to -140 mV (n = 12, $F_{(1,11)}$ = 7.288, P = 0.021, two-way repeated measures ANOVA followed by Šídák's multiple comparison test, Figure 4, b1-b3). In the presence of ML 133, application of NMB still significantly depressed Kir currents at potentials -100 to -140 mV (n = 12, $F_{(1,11)}$ = 12.40, P = 0.005, two-way repeated measures ANOVA followed by Šídák's multiple comparison test, Figure 5, b4-b6). Current-voltage responses elicited in the presence of ML 133 and NMB were significantly different from those obtained in CeL neurons treated with NMB alone ($F_{(1,253)}$ = 4.439, P = 0.036, two-way ordinary ANOVA followed by Šídák's multiple comparison test, Figure 4, b6), but did not differ significantly at any recorded voltages according to post-hoc analysis. Together, these results suggest that the Kir2 subfamily channels are not involved in the NMB-mediated excitation of CeL neurons.

We further probed the role of GIRK channels in NMB-mediated inhibition of Kir channels. GIRK1 channels are strongly expressed in the BLA and cortical nuclei with low expression in the CeA [261]. Bath application of ML 297 (10 μM), an activator of GIRK1-containing channels [262] failed to significantly affect Kir currents (n = 9, $F_{(1,8)}$ = 0.674, P = 0.44, two-way repeated measures ANOVA followed by Šídák's multiple comparison test, Figure 5, a1-a3), suggesting that there were no functional GIRK1 channels in the CeL neurons and NMB-elicited excitation of CeL neurons was unlikely to be mediated by depressing GIRK1 channels.

We then used tertiapin-Q (TQ), a blocker for GIRK (K_i = 13.3 nM) and Kir1.1 (K_i = 1.3 nM) channels [263-265]. Bath application of TQ (250 nM) by itself depressed a current displaying inward rectification (n = 10, $F_{(1,9)}$ = 23.16, P = 0.001, two-way

Figure 4.　**NMB-mediated inhibition of Kir currents is Ba2+-sensitive but insensitive to the Kir2 subfamily blocker ML 133.**

(**a1-a6**), bath application of Ba^{2+} (300 μM) alone inhibited Kir channel currents and blocked NMB-elicited inhibition of Kir channel currents. (**a1**), currents evoked by the voltage-step protocol before (*left*) and during (*middle*) the application of Ba^{2+} (300 μM) and the net currents acquired by subtraction (*right*). (**a2**), I-V curves of the currents elicited by the voltage-step protocol before and during the application of Ba^{2+}. **** $P < 0.0001$, ** $P < 0.01$. (**a3**), Net currents acquired by subtraction of the currents recorded in the control condition from those recorded from the same cell in the presence of Ba^{2+} (n = 12). Note that the Ba^{2+}-sensitive currents showed inward rectification. (**a4**), currents recorded from a CeL neuron in response to the voltage-step protocol in the presence of Ba^{2+} alone (*left*) and Ba^{2+} plus NMB (*middle*). The net currents acquired by subtraction were shown in the right panel. (**a5**), I-V curves of the currents elicited by the voltage-step protocol in the presence of Ba^{2+} alone and Ba^{2+} plus NMB (n = 12). (**a6**), Net currents acquired by subtracting the currents in the presence of Ba^{2+} alone from those recorded from the same cell in the presence of Ba^{2+} plus NMB. NMB-elicited net currents in the control condition (green circles) were co-plotted as a comparison. (**b1-b6**), bath application of ML 133 (30 μM), a blocker of Kir2 subfamily channels, by itself inhibited Kir currents, but failed to block NMB-mediated inhibition of Kir currents. (**b1**), currents elicited by the voltage-step protocol before (*left*) and during (*middle*) the application of ML 133 (30 μM). The net currents inhibited by ML 133 were shown on the right. (**b2**), I-V curves of the currents elicited by the voltage-step protocol before and during the application of ML 133 (n = 12). (**b3**), net currents acquired by subtraction of the currents in the control condition from those recorded from the same cell in the presence of ML 133. Note that the ML133-sensitive currents displayed inward rectification. (**b4**), current traces recorded from a CeL neuron in response to the voltage-step protocol in the presence of ML 133 (*left*) and ML 133 plus NMB (*middle*) and the net current acquired via subtraction (*right*). (**b5**), I-V curves of the currents generated by the voltage-step protocol in the presence of ML 133 alone and ML 133 plus NMB (n = 12). (**b6**), net currents acquired by subtraction of currents recorded from CeL neurons in the presence of ML 133 from those in the presence of ML 133 plus NMB. Net currents elicited by NMB in control condition without application of ML133 were co-plotted for comparison. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, two-way repeated measures ANOVA followed by Šídák's multiple comparison test. Adapted from [223].
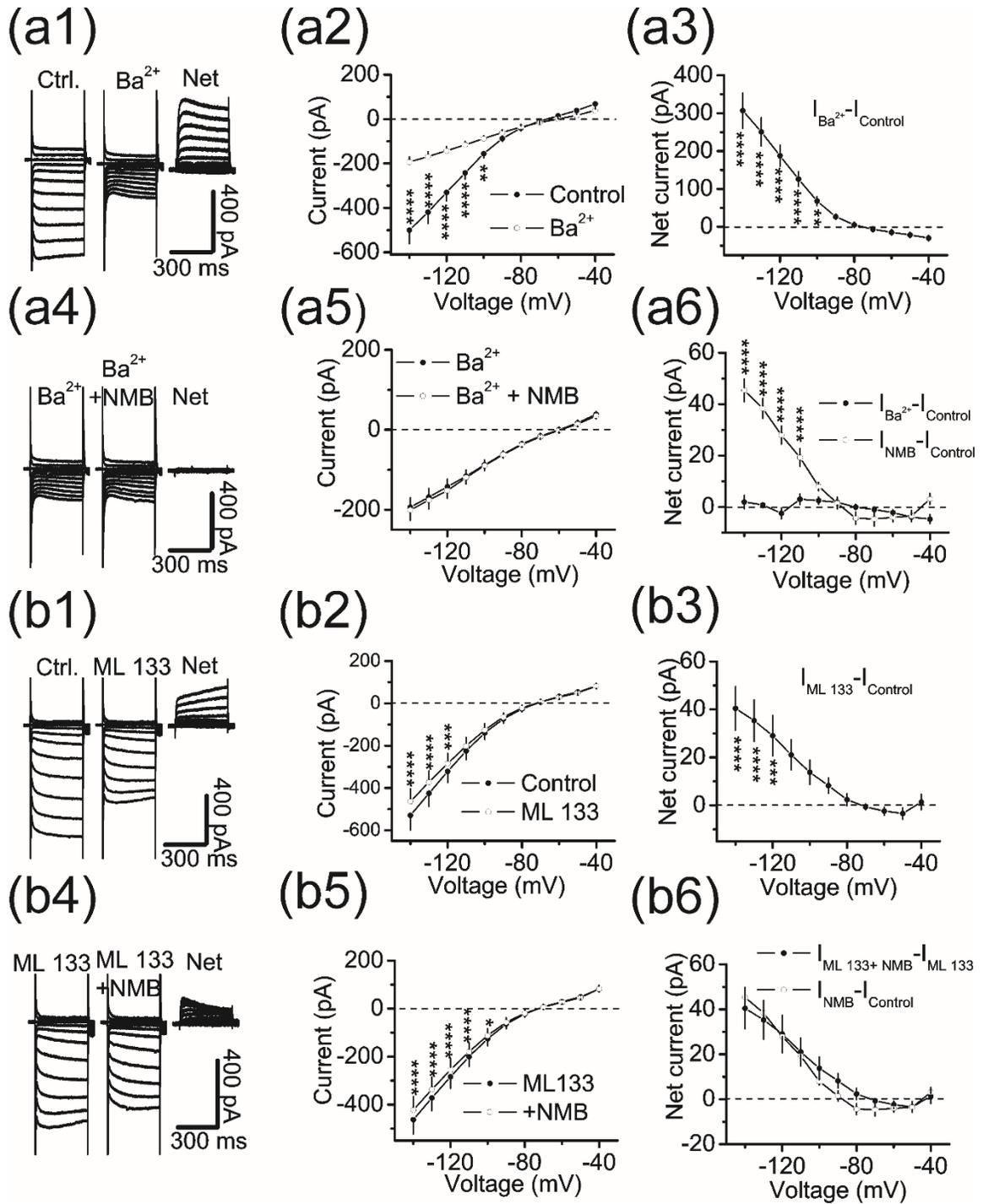
repeated measures ANOVA followed by Šídák's multiple comparison test, Figure 5, b1-b3). There were significant differences in the current-voltage curve before and during the application of TQ at a voltage range from -100 to -140 mV (n = 10, Figure 5, b1-b3), suggesting that CeL neurons express functional GIRK channels. Whereas application of NMB in the presence of TQ still significantly depressed Kir currents ($F_{(1,9)}$ = 24.95, P = 0.0007, two-way repeated measures ANOVA followed by Šídák's multiple comparison test, Figure 5, b4-b6), the NMB-elicited depression of Kir currents in the presence of TQ was significantly smaller at -130 mV and -140 mV compared with the effect of NMB in the control condition [263-265] (Figure 5, b6). These results together indicate that NMB-induced excitation of CeL neurons occurred at least partially through the depression of GIRK channels.

Because TQ inhibits both GIRK and Kir1.1 channels [263-265], we probed the potential involvement of Kir1.1 channels in NMB-induced inhibition of Kir currents. Bath application of the selective Kir1.1 channel blocker, tertiapin-LQ (100 nM), which lacks effects on GIRK channels [266], failed to alter significantly the voltage-current relationship (n = 7, $F_{(1,6)}$ = 1.582, P = 0.255, two-way repeated measures ANOVA, Figure 5, c1-c3), suggesting that CeL neurons do not express functional Kir1.1 channels. Application of NMB in the continuous presence of tertiapin-LQ still inhibited a comparable extent of Kir currents (n = 7, $F_{(1,6)}$ = 31.59, P = 0.001, two-way repeated measures ANOVA followed by Šídák's multiple comparison test, Figure 5, c4-c6). The NMB-induced depression of Kir currents in the presence of tertiapin-LQ was not significantly different from that in the control condition at all voltages tested ($F_{(1,18)}$ = 2.437, P = 0.14, two-way ordinary ANOVA, Figure 5, c6). These data suggest a lack of involvement of Kir1.1 channels in NMB-elicited depression of Kir currents. Together, these data suggest that activation of the BB1 receptor excites CeL neurons through the inhibition of GIRK channel.

Figure 5. **GIRK channels are involved in NMB-elicited depression of Kir channel currents.**

(**a1-a3**), application of ML 297, the GIRK1-containing channel activator, failed to alter Kir currents. (**a1**), currents recorded from a CeL neuron in response to the voltage-step protocol before (*left*) and during (*middle*) the application of ML 297 (10 μM) and the net current acquired by subtraction (*right*). (**a2**), I-V curves of the currents elicited by the voltage-step protocol before and during the application of ML 297 (n = 9). (**a3**), net currents acquired by subtracting the currents in the control condition from those recorded from the same cells in the presence of ML 297. Application of ML 297 did not significantly affect Kir currents at each voltage. (**b1-b6**), bath application of tertiapin-Q (TQ) by itself inhibited Kir currents and significantly depressed NMB-mediated inhibition of Kir currents. (**b1**), currents recorded from a CeL neuron in response to the voltage-step protocol before (*left*) and during (*middle*) the bath application of TQ (250 nM) and the net current generated by subtraction (*right*). (**b2**), I-V curves of the currents elicited by the voltage-step protocol before and during the application of TQ (n = 10). (**b3**), net currents generated by subtracting the currents in the control condition from those recorded from the same neurons in the presence of TQ. TQ-sensitive currents displayed inward rectification. (**b4**), currents recorded from a CeL neuron in response to the voltage-step protocol in the presence of TQ alone (*left*) and TQ plus NMB (*middle*) and the net current acquired by subtraction (*right*). (**b5**), I-V curves of the currents elicited by the voltage-step protocol in the presence of TQ or TQ + NMB (n = 10). (**b6**), net currents acquired by subtracting the currents in the presence of TQ alone from those recorded from the same cells in the presence of TQ plus NMB. NMB-elicited net currents in the control condition (green circles) were co-plotted as a comparison. Note that TQ significantly reduced NMB-elicited depression of Kir currents at -130 mV and -140 mV. ** $P < 0.01$, ordinary two-way ANOVA. (**c1-c6**), bath application of tertiapin-LQ (T-LQ) alone did not alter Kir currents and failed to significantly change NMB-mediated inhibition of Kir currents. (**c1**), currents recorded from a CeL neuron in response to the voltage-step protocol before (*left*) and during (*middle*) the application of T-LQ (100 nM) and the net current acquired by subtraction (*right*). (**c2**), I-V curves of the currents elicited by the voltage-step protocol before and during the application of T-LQ (n = 7). (**c3**), net currents acquired by subtracting the currents in the control condition from those recorded from the same cells in the presence of T-LQ. T-LQ failed to alter Kir currents at each voltage. (**c4**), currents recorded from a CeL neuron in response to the voltage-step protocol in the presence of T-LQ alone (*left*) and T-LQ plus NMB (*middle*) and the net current acquired by subtraction (*right*). (**c5**), I-V curves of the currents elicited by the voltage-step protocol in the presence of T-LQ and T-LQ + NMB (n = 7). (**c6**), net currents acquired by subtracting the currents in the presence of T-LQ alone from those recorded from the same cells in the presence of T-LQ plus NMB. NMB-sensitive net currents in control conditions were co-plotted for comparison. Application of T-LQ failed to significantly alter the NMB-induced depression of Kir currents. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, two-way repeated measures ANOVA followed by Šídák's multiple comparison test. Adapted from [223].
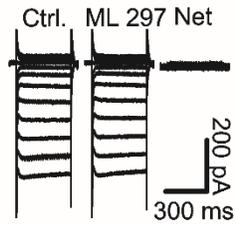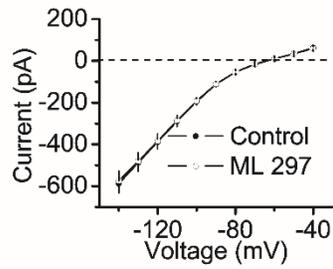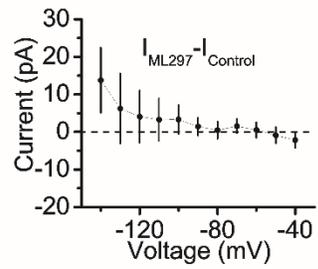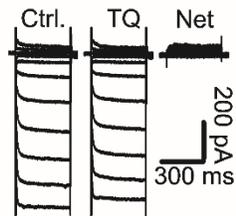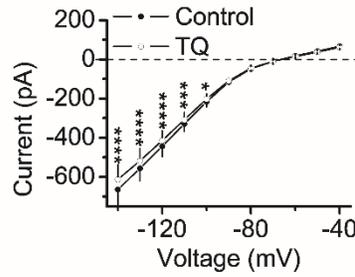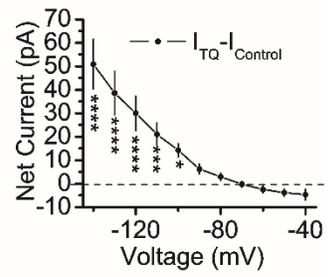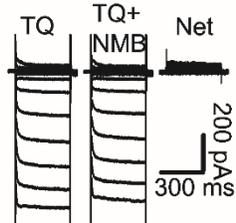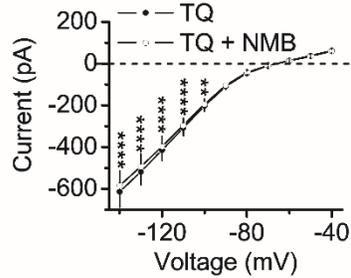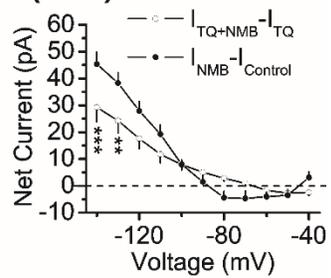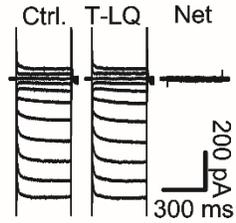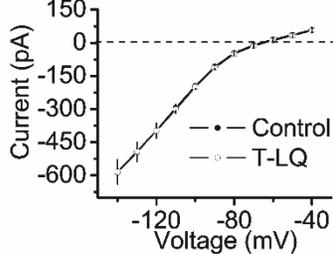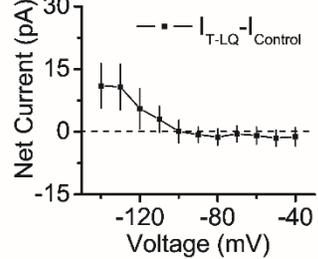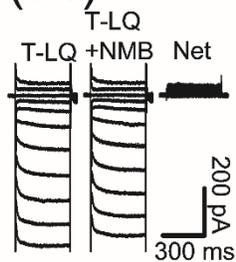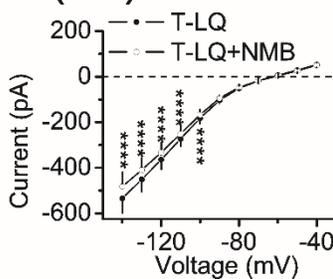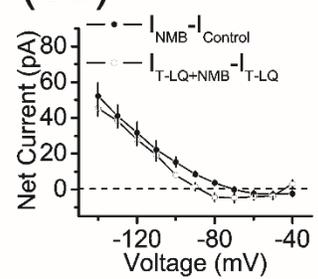
G proteins, PLCβ and PKC are Required for BB1 Receptor-Mediated Depression of Kir Channels

As our results indicated that activation of BB1 receptors excited CeL neurons by depressing the GIRK type of the Kir channels, we further tested the roles of G proteins, PLCβ and PKC in NMB-mediated inhibition of Kir channel currents. Inclusion of GDP-β-S (0.5 mM) in the intracellular recording solution blocked NMB-induced depression of Kir currents (n = 14, $F_{(1,13)}$ = 1.539, P = 0.24, two-way repeated measures ANOVA followed by Šídák's multiple comparison test, Figure 6, a1-a3). Additionally, pretreatment of slices with the PLCβ inhibitor U73122 (5 µM) significantly reduced NMB-induced depression of Kir currents (n = 15, $F_{(1,14)}$ = 0.144, P = 0.71, two-way repeated measures ANOVA, Figure 6, b1-b3), compared with the effect of NMB in slices pretreated with the inactive analog, U73343 (5 µM, n = 5, $F_{(1,4)}$ = 22.22, P = 0.009, two-way repeated measures ANOVA followed by Šídák's multiple comparison test, Figure 6, c1-c3). These results demonstrate that the activity of PLCβ is required for NMB-mediated depression of Kir channels in the CeL.

We then explored the role of PKC in NMB-induced depression of Kir currents. Slices were pretreated with Bis II (1 µM), a selective PKC inhibitor, and the same concentration of Bis II was continuously bath-applied in the extracellular solution. Bath application of NMB in this condition failed to significantly alter Kir channel currents at all voltages recorded (n = 12, $F_{(1,11)}$ = 0.032, P = 0.86 two-way repeated measures ANOVA, Figure 7, a1-a3). Compared with NMB-mediated depression in the control condition, slices treated with Bis II elicited significantly smaller currents ($F_{(1,253)}$ = 94.63, P < 0.0001 *vs.* NMB-alone, ordinary two-way ANOVA, Figure 7, a3), supporting a functional requirement of PKC in NMB-mediated depression of Kir channels.

Figure 6.   **G proteins and PLCβ are involved in BB1 receptor-mediated depression of Kir channels.**

(**a1-a3**)*,* intracellular dialysis of GDP-β-S (0.5 mM) via the recording pipettes blocked NMB-elicited inhibition of Kir currents. (**a1**), currents recorded from a CeL neuron in response to the voltage-step protocol before (*left*) and during (*middle*) the application of NMB in the presence of GDP-β-S and the net current generated by subtraction (*right*). (**a2**), I-V curves of the currents elicited by the voltage-step protocol before and during the application of NMB (n **=** 14). (**a3**), net currents generated by subtracting the currents in control conditions from those recorded from the same neurons during the bath application of NMB with GDP-β-S in the recording pipettes. Note that inclusion of GDP-β-S in the recording pipettes blocked NMB-elicited depression of Kir currents. (**b1-b3**), pretreatment of slices with the selective PLC inhibitor, U73122 (5 μM), significantly depressed NMB-elicited Kir currents. (**b1**), currents recorded from a CeL neuron in response to the voltage-step protocol before (*left*) and during (*middle*) the application of NMB in a slice pretreated with U73122 and the net currents acquired by subtraction (*right*). (**b2**), I-V curves of the currents generated by the voltage-step protocol before and during the bath application of NMB (n = 15). (**b3**), net currents acquired by subtracting the currents in control conditions from those during the bath application of NMB from the same neurons in slices pretreated with U73122. Note that pretreatment of slices with U73122 significantly attenuated NMB-mediated depression of Kir currents. (**c1-c3**)*,* bath application of NMB in slices pretreated with the inactive analog U73343 (5 μM), still significantly depressed Kir currents. (**c1**), currents recorded from a CeL neuron in response to the voltage-step protocol before (*left*) and during (*middle*) the application of NMB in a slice pretreated with U73343 and the net current acquired by subtraction (*right*). (**c2**), I-V curves of the currents recorded from the same neurons before and during the application of NMB (n = 5) in slices pretreated with U73343. (**c3**), net currents acquired by subtracting the currents in control conditions from those during the application of NMB from the same neurons in slices pretreated with U73343. Note that bath application of NMB still significantly depressed Kir currents. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, two-way repeated measures ANOVA followed by Šídák's multiple comparison test. Adapted from [223].
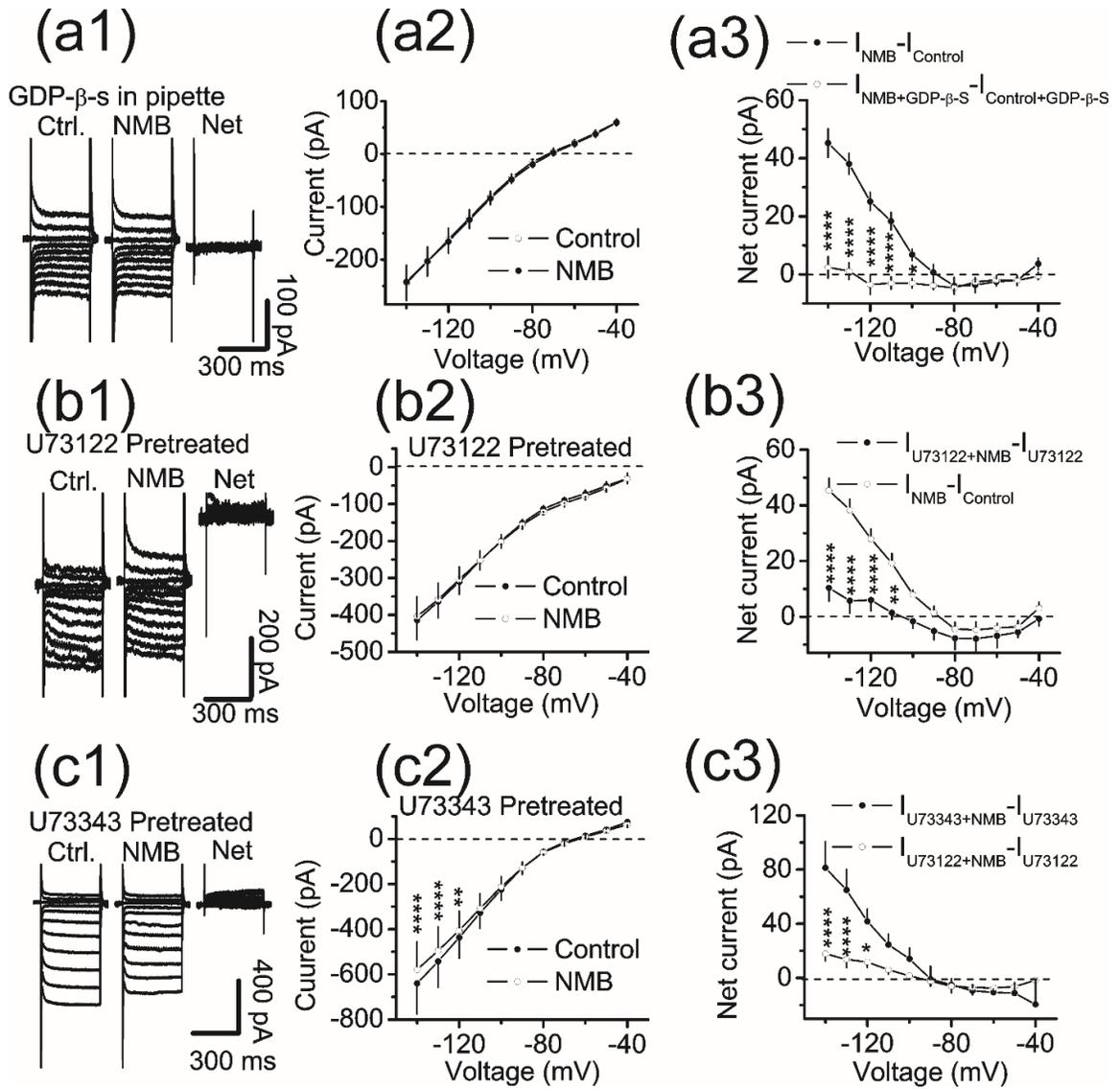
Similarly, bath application of a PKC activator, PMA (1 µM), significantly inhibited Kir currents (n = 13, $F_{(1,12)}$ = 12.97, P = 0.004, two-way repeated measures ANOVA followed by Šídák's multiple comparison test, Figure 7, b1-b3). The PMA-mediated depression was significantly reduced in slices pretreated with Bis II (1 µM, n = 12, $F_{(1,11)}$ = 1.797, P = 0.21, two-way repeated measures ANOVA, Figure 7, c1-c3). These results further support the involvement of PKC in NMB-mediated depression of Kir channels. Lastly, we determined the effects of intracellular Ca^{2+} levels on NMB-elicited inhibition of Kir channels. Intracellular perfusion of BAPTA (10 mM) via the recording pipettes significantly attenuated NMB-elicited depression of Kir currents ($F_{(1,286)}$ = 25.88, P < 0.0001 *vs.* NMB alone, ordinary two-way ANOVA, Figure 7, d1-d3), supporting the involvement of the Ca^{2+}-dependent PKC isoform in NMB-mediated inhibition of Kir channels.

BB1 Receptor-Mediated Excitation of CeL Neurons Promotes Inhibitory Cardiovascular Responses

The distinct CeA projections to the brainstem involved in the alterations of cardiac output in response to emotionally relevant stimuli are not completely understood [71-74]. As bombesin receptors are implicated in cardiovascular responses, we tested the roles of BB1 receptor-mediated excitation of CeA neurons in modulation of cardiac function. Guide cannulae were bilaterally implanted into the CeA and NMB and/or other compounds were microinjected into the CeA to probe the effects of BB1 receptor activation on mean arterial pressures (MAP; mmHg) and heart rate (HR; bpm) (Figure 8, a & c). There were no significant differences in baseline cardiovascular responses between groups ($F_{(2.4, 24.3)}$ = 2.76, P = 0.07, mixed-effect two-way ANOVA). A two-way ANOVA revealed saline injection did not significant effect any cardiovascular parameters

Figure 7. **PKC is involved in NMB-induced depression of Kir channels.**
(**a1-a3**)*,* pretreatment of slices with and continuous bath application of the selective PKC inhibitor, Bis II (1 μM), blocked NMB-mediated inhibition of Kir currents at each voltage. (**a1**), currents recorded from a CeL neuron elicited by the voltage-step protocol before (*left*) and during (*middle*) the application of NMB in a slice treated with Bis II and the net currents acquired by subtraction (*right*). (**a2**), I-V curves of the currents generated by the voltage-step protocol before and during the application of NMB (n = 12) recorded from the same neurons in slices treated with Bis II. Note that application of Bis II blocked NMB-induced depression of Kir currents at each voltage. (**a3**)*,* net currents acquired by subtracting the currents in control conditions from those during the application of NMB recorded from the same neurons in slices treated with Bis II. NMB-induced net currents in control conditions without Bis II were co-plotted for comparison (green circles). (**b1-b3**), application of the PKC activator, PMA (1 μM), inhibited Kir currents. (**b1**), current traces recorded from a CeL neuron elicited by the voltage-step protocol before (*left*) and during (*middle*) the application of PMA and the net current acquired by subtraction (*right*). (**b2**), I-V curves generated by the voltage-step protocol before and during the application of PMA (n = 13). Note that PMA significantly attenuated Kir currents. (**b3**), net currents produced by subtracting the currents in control conditions from those recorded from the same cells during the application of PMA. (**c1-c3**), pretreatment of slices with and continuous bath application of Bis II blocked PMA-mediated depression of Kir currents. (**c1**), currents recorded from a CeL neuron in response to the voltage-step protocol before (*left*) and during (*middle*) the bath application of PMA in a CeL neuron treated with Bis II and the net current acquired by subtraction (*right*). (**c2**), I-V curves of the currents generated by the voltage-step protocol before and during the bath application of PMA (n = 12) in slices treated with Bis II. (**c3**)*,* net currents acquired by subtracting the currents in control conditions from those during the application of PMA recorded from the same neurons in slices treated with Bis II. (**d1-d3**), intracellular application of the Ca^{2+} chelator BAPTA (10 mM) via the recording pipettes significantly reduced NMB-mediated depression of Kir currents. (**d1**), currents recorded from a CeL neuron in response to the voltage-step protocol before (*left*) and during (*middle*) the application of NMB with the intracellular solution containing BAPTA and the net current acquired by subtraction (*right*). (**d2**), I-V curves of the currents generated by the voltage-step protocol before and during the application of NMB (n = 15) with the intracellular solution containing BAPTA. (**d3**), net currents acquired by subtracting the currents in control conditions from those during the bath application of NMB from the same neurons with the intracellular solution containing BAPTA. NMB-elicited net currents in control conditions without BAPTA were co-plotted for comparison (green circles). **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, two-way repeated measures ANOVA followed by Šídák's multiple comparison test. Adapted from [223].

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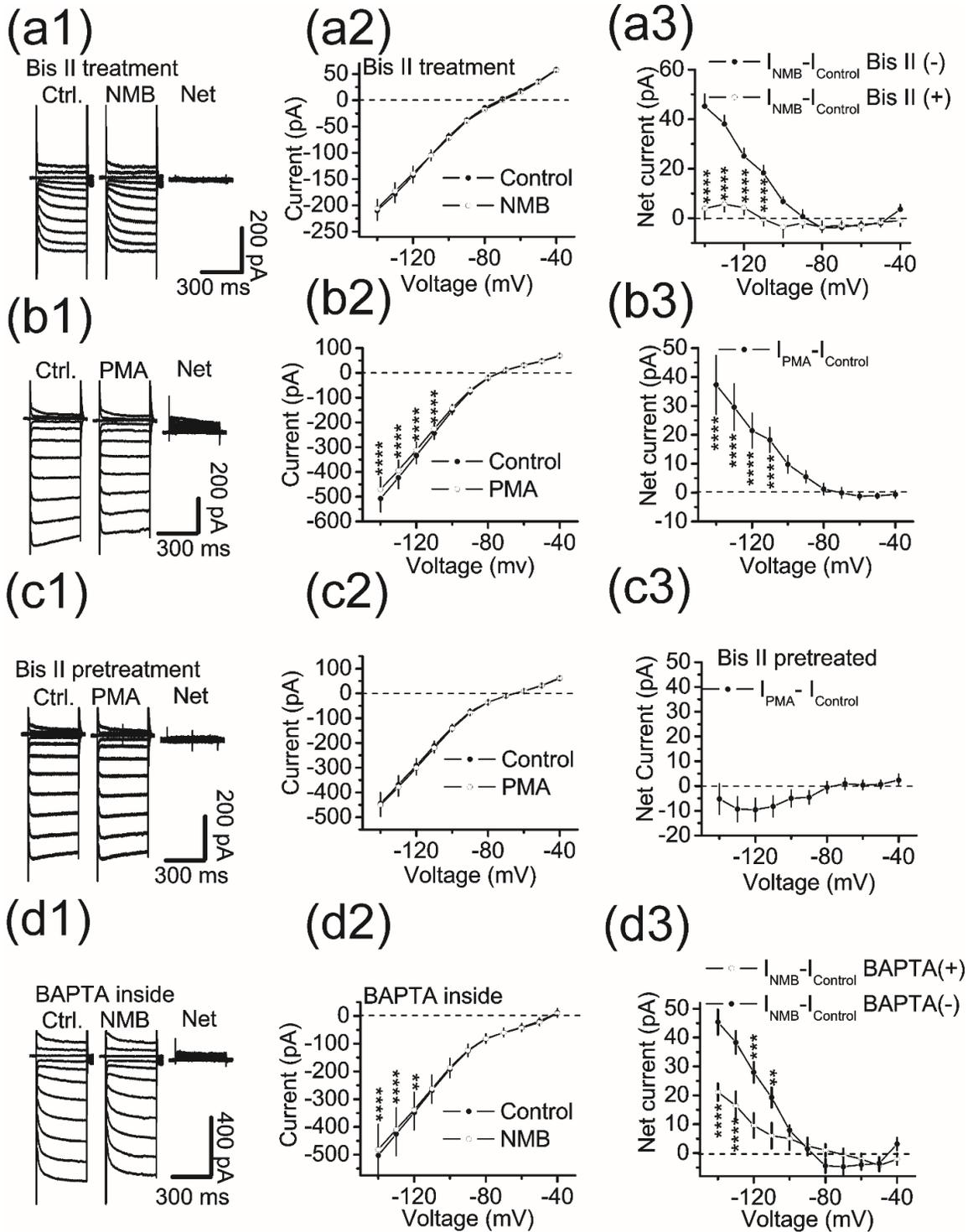
(Treatment: $F_{(1,10)}$ = 0.007, P = 0.933, two-way ANOVA). Microinjection of NMB produced a significant dose-dependent reduction in MAP ($F_{(3,39)}$ = 6.21, P = 0.0015, one-way ANOVA; saline vs. 0.3 nmol NMB: P = 0.035; saline vs. 1 nmol NMB: P = 0.0005 via Dunnett's multiple comparisons test, Figure 9, a1, Table 1) and HR ($F_{(3,39)}$ = 7.55, P = 0.0004, one-way ANOVA; saline vs. 0.1 nmol: P = 0.006; saline vs. 0.3 nmol NMB: P = 0.0001 Dunnett's multiple comparisons test, Figure 9, a2, Table 1). The effects of NMB were not sex-dependent, as comparison of MAP ($F_{(1,35)}$ = 0.198, P = 0.66) or HR ($F_{(1,35)}$ = 0.961, P = 0.33) from male and female rats showed no significant differences (Two-way ordinary ANOVA). The IC_{50} value was calculated to be 0.362 nmol, we therefore used 0.3 nmol NMB for the remaining *in vivo* experiments.

We then tested the involvement of BB1 receptors in NMB-mediated depression of MAP and HR by microinjection of the selective BB1 receptor antagonist BIM23042 (0.3 nmol))[250]. Application BIM23042 by itself had no significant effect on MAP (P > 0.99) or HR (P = 0.85). However, prior microinjection of BIM23042 prevented NMB-induced depression of MAP (P = 0.99, one-way ANOVA followed by Tukey's multiple comparisons test, Figure 9, a3) and HR (P = 0.99, one-way ANOVA followed by Tukey's multiple comparisons test, Figure 9, a4), corroborating the involvement of BB1 receptors.

Our *in vitro* results indicate that activation of BB1 receptors excites CeL neurons by depressing GIRK channels. We further probed the roles of GIRK channels in NMB-mediated suppression of MAP and HR. TQ is a selective GIRK channel inhibitor that effectively inhibits GIRK channels in picomolar range [267, 268]. Bilateral microinjection of TQ (250 pmol) by itself had no significant effects on MAP (P = 0.97) and HR (P = 0.77), but blocked NMB-elicited depression of MAP (P = 0.96, one-way ANOVA followed by Tukey's multiple comparisons test, Figure 9, a3) and HR (P = 0.80, one-way ANOVA followed by Tukey's multiple comparisons test, Figure 9, a4), indicating that GIRK channels are required for NMB-mediated depression of MAP and HR.

BB1 Receptor Activation Augments ASR but Attenuates FPS

The microcircuits of the CeA are involved in fear processing [74, 141, 269] and send projections to brainstem nuclei involved in fear expression and cardiovascular responses [74]. Whereas NMB and its receptor have been implicated in stress responses [239, 270-272], the effect of NMB on fear expression remain less understood [273-276]. FPS is a translatable paradigm used to measure the acquisition and expression of conditioned fear responses in both rodents and humans [277-279]. Taking advantage of a simple reflex (ASR), FPS measures conditioned fear by an increase in the amplitude of ASR in the presence of a previously paired cue with a foot shock. We probed the effects of BB1 receptor activation on both ASR and FPS by microinjecting NMB or saline into the CeA (Figure 8, b, c). Microinjection of NMB (0.3 nmol) into the CeA significantly augmented ASR to the 105 dB WNB compared to the rats injected with saline (0.9 % NaCl) (NMB: 1013.77 ± 214.88; Saline: 599.19 ± 92.10 arbitrary units, n = 11, $P = 0.0204$, Šídák's multiple comparisons test, Figure 9, b1). These results were independent of the sex of the rat ($F_{(1,54)} = 3.39$, $P = 0.07$). Furthermore, microinjection of NMB into the CeA (0.3 nmol) significantly reduced FPS responses to cue stimuli ($P = 0.004$, Šídák's multiple comparisons test, Figure 9, b2), with no significant effects on baseline ($P = 0.976$, Šídák's multiple comparisons test, Figure 9, b2) or non-cued responses ($P = 0.906$, Šídák's multiple comparisons test, Figure 9, b2). Microinjection of NMB into the CeA prior to fear conditioning had no significant effect on shock reactivity ($P = 0.57$, Mann-Whitney test, Figure 9, b3). Moreover, the shock reactivity amplitudes measured between male and female rats injected with saline ($P = 0.22$) or NMB ($P = 0.98$) were not significantly different, suggesting that the alterations in FPS were independent of aversive stimuli or sex of the animal. A working model of the findings of Study 1 is shown in Figure 26.

Figure 8. **Schematic diagram showing the cardiovascular recording, ASR and FPS procedures and cannula placement.**
(**a**), For recording cardiovascular parameters, animals received daily handling to habituate them to the experimental procedure for at least 7 days. Baseline measurements were recorded for 2 days. Cardiovascular parameters were recorded during and for at least 15 min after the injection (*top*). (**b**), In the acoustic startle response (ASR) experiments, rats were given intra-CeA saline (1 μL in each side) or NMB (0.3 nmol in 1 μL) 10 mins prior to the test session (*middle*). In fear-potentiated startle (FPS) experiments (*bottom*), rats were given intra-CeA saline or NMB 10 mins prior to the fear conditioning session. FPS measured as cued or non-cued fear responses were tested 24 h later. (**c**), cannula tip placement in a subset of experiments displayed onto an atlas figure adapted from Paxinos and Watson [221]; White arrow denotes the cannula tip placement. Adapted from [223].

(a)

Habituation
7 Days

PowerLab

Baseline
Measurement

Inject

Measurement

2 Days | 1 Day | 10 min

(b)

NMB or Saline

Day 1   Day 2   Day 3   Day 4

ASR

NMB or Saline

FPS

(c)

Interaural 6.88 mm

BLA CeA

CeA BLA
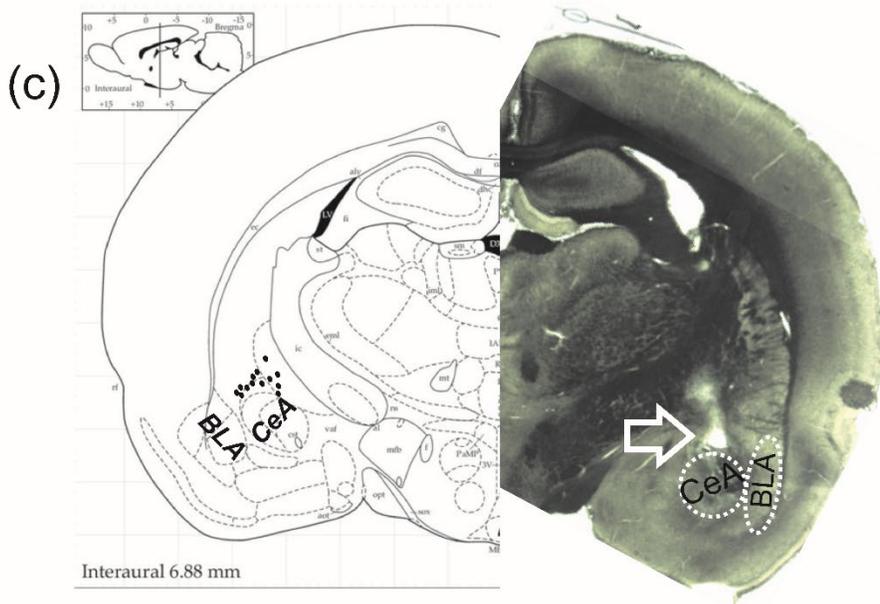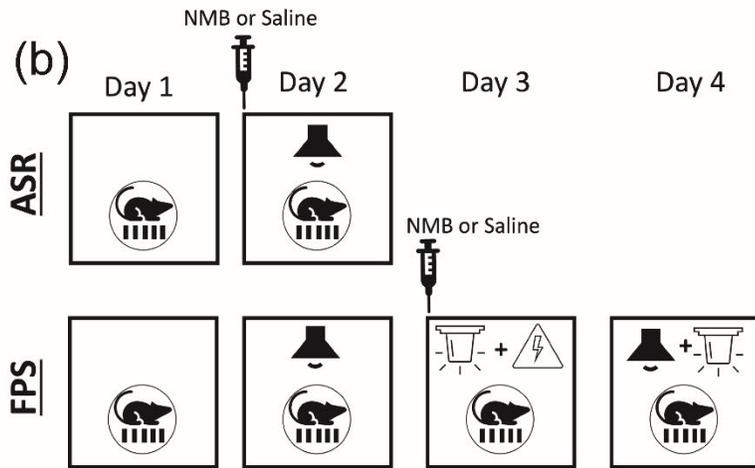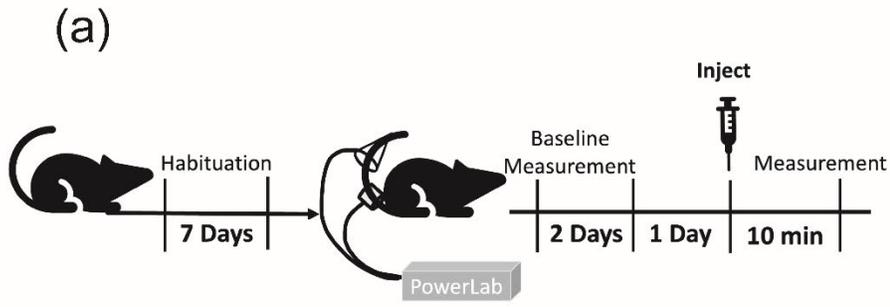
Figure 9. **BB1 receptor activation in the CeA reduces cardiovascular output and affects ASR and FPS.**

(a1-a4) Microinjection of NMB into the CeA lowered MAP and reduced HR through inhibition of GIRK channels. (**a1**), NMB dose-dependently reduced MAP, and (**a2**) HR. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs saline (one-way ANOVA followed by Dunnett's test). The circles and triangles represent data obtained from male and female rats, respectively. (**a3**), microinjection of NMB (0.3 nmol) into the CeA reduced MAP, whereas prior administration of the BB1 receptor antagonist, BIM 23042 (0.3 nmol) or GIRK channel blocker, TQ (250 pmol) blocked NMB-mediated depression of MAP. (**a4**), microinjection of NMB (0.3 nmol) into the CeA produced bradycardia, whereas prior microinjection of the BB1 receptor antagonist, BIM 23042 (0.3 nmol) or GIRK channel blocker, TQ (250 pmol) blocked NMB-induced bradycardia. *** $P < 0.01$, **** $P < 0.0001$ (one-way ANOVA followed by Dunnett's test), **n. s**, no significance (Tukey's test). (**b1-b3**), microinjection of NMB increased ASR while reducing FPS. (**b1**), microinjection of NMB (0.3 nmol) into the CeA enhanced ASR to the 105 dB WNB without altering ASR to the 90 dB or 95 dB WNBs. ** $P < 0.01$ (Two-way ANOVA followed by Šídák's multiple comparisons test). (**b2**), application of NMB into the CeA prior to fear conditioning significantly reduced cued responses without effects on non-cued responses during the FPS testing session. ** $P < 0.01$ (Two-way ANOVA followed by Šídák's multiple comparisons test). (**b3**), behavioral responses to foot shock on Day 3 did not differ significantly in response to NMB microinjection. The circles and triangles represent data obtained from male and female rats, respectively. Adapted from [223].
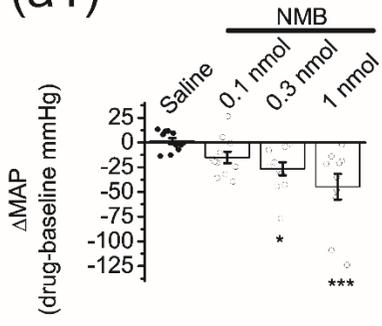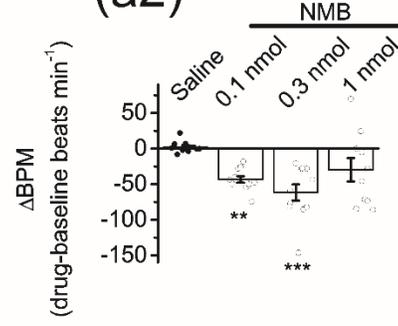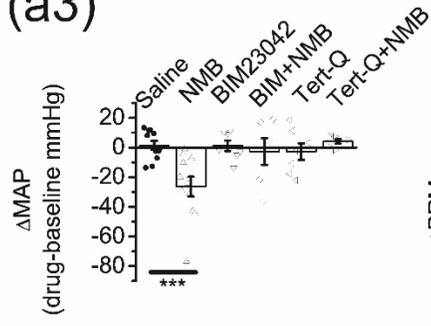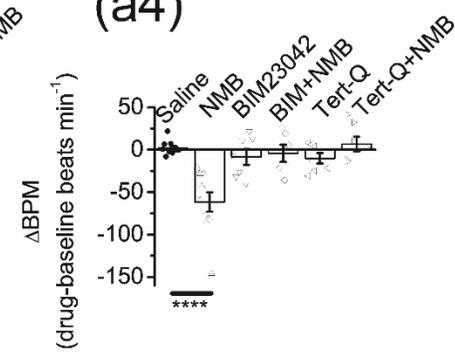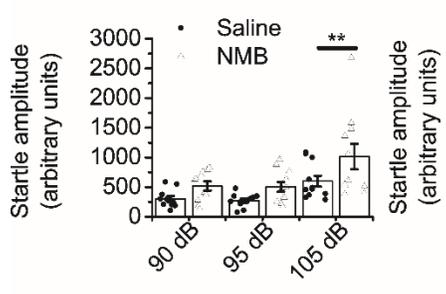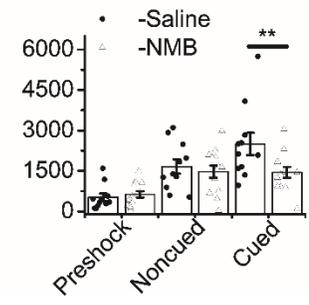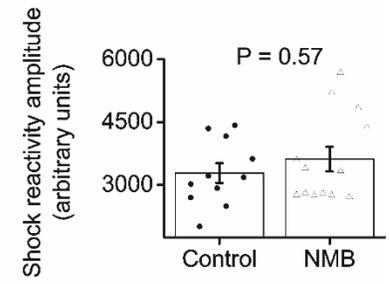
Table 1. Cardiovascular parameters _{Adapted from [223].}

	Baseline mean (±SEM)	Neuromedin B (NMB) mean (±SEM)	p-value
Systolic (mmHg)	160.2 (4.8)	141.9 (3.7)	0.03
Diastolic (mmHg)	123.6 (5.0)	101.1 (4.4)	0.007
Mean arterial pressure (MAP) (mmHg)	136.5 (5.2)	110.1 (4.2)	0.001
Heart rate (HR) (bpm)	433.3 (13.8)	371.7 (8.4)	<0.001

Study 2- Activation of Bombesin-1 Receptors Excites the Principal Neurons in the Basolateral Amygdala by Multiple Signaling and Ionic Mechanisms

Introduction

Another target of bombesin-like peptides is the BLA which expresses NMB peptide and its mRNA [280], as well as high densities of bombesin-like peptide binding sites [242, 281, 282]. In line with the expression of bombesin-like peptide binding sites within the BLA, direct application of bombesin into the BLA reduces food intake and affects aversive memory [283, 284]. Bombesin-like peptide receptor activation in the BLA affects fear and anxiety-like behaviors [285, 286]. Elevations of NMB and GRP immunoreactivity are observed in the human brains of suicide patients supporting the involvement of bombesin-like peptides in neuropsychiatric disorders [275]. However, the effects of bombesin and bombesin-like peptides on neuronal excitability in the BLA and the underlying ionic and signaling mechanisms have not yet been determined. In Study 2, we investigated the effects of NMB on the excitability of BLA neurons based on the literature results showing that high densities of BB1 receptors are distributed in the BLA [287]. Using *in vitro* electrophysiological approaches in Sprague-Dawley (SD) rats and mutant mice, we showed that BB1 receptor activation excited BLA neurons through a PLC-mediated inhibition of GIRK channels and activation of TRPV1 channels; NMB increased glutamatergic transmission that is dependent on functional transient receptor potential vanilloid 1 (TRPV1) channels. Finally, microinjection of NMB into the BLA produced anxiogenic phenotypes in the Vogel Conflict Test in SD rats. Together, these results provide mechanistic insights into BB1 receptor activity in the BLA and further support the involvement of bombesin-like peptides in stress responses and anxiety disorders.

Activation of BB1 Receptors Excites BLA Principal Neurons

Because the BLA expresses high densities of bombesin-binding sites [288, 289] and BB1 receptor mRNA [290], we aimed to determine the functional outcome of NMB, a selective BB1 receptor agonist, on BLA neuronal excitability (Figure 10, *A*). The extracellular solution contained kynurenic acid (1 mM) to block glutamatergic transmission and picrotoxin (100 μM) to block GABAergic transmission. The intracellular solution was the K^+- gluconate-containing intracellular solution (see *Methods and Materials*). We bath applied a near-saturating concentration of the BB1 receptor agonist, NMB (0.3 μM, see below), that resulted in subthreshold depolarizations (n = 18; Control: -64.6 $\pm$ 0.96 mV; NMB -61.2 $\pm$1.3 mV, P = 0.001, paired t-test; net depolarization: 2.82 mV, Figure 10. *Ba, Bb*). As the BLA neurons do not show spontaneous AP firing, we probed the effect of NMB on neuronal excitability by injecting a series of positive currents from 50 to 750 pA at an interval of 50 pA every 6 s prior to and after application of NMB when the maximal depolarization was obtained. Application of 0.3 μM NMB significantly increased the number of APs elicited by the positive current injection protocol (n = 18, $F_{(1,17)}$ = 26.06, P = 0.0001, two-way repeated-measures ANOVA followed by Šídák's multiple comparisons test; Figure 10. *Ca, Cb*). NMB increased AP firing in BLA neurons in slices cut from both male and female rats (Females: $F_{(1,6)}$ = 7.275, P = 0.035, two-way repeated-measures ANOVA followed by Šídák's multiple comparisons test; males: $F_{(1,10)}$ = 18.09, P = 0.0017, two-way repeated-measures ANOVA followed by Šídák's multiple comparisons test). NMB significantly reduced the rheobase current required to induce AP firing in BLA neurons (control: 200 $\pm$ 14 pA, NMB: 166.6 $\pm$ 14 pA, n = 18, P = 0.002, paired t-test, Figure 10. *Cc-Ce*) and reduced the delay time to the first AP waveform (Control: 161.6 $\pm$ 19.6 ms, NMB: 108.7 $\pm$ 7.3 ms, n = 18, P = 0.0003, Wilcoxon test, Figure 10. *Cf, Cg*). Enhanced BLA neuronal excitability

was not due to an alteration in AP waveform halfwidth (Control:1.76 ± 0.25 ms, NMB:

NMB: 1.29 ± 0.09 ms, n = 18, P = 0.09, Wilcoxon test, Figure 10. *Ch*). To assess the

effect of NMB on passive membrane properties, current-clamped neurons were exposed

to negative current injection sweeps from -50 to -200 pA with 50 pA steps for a duration

of 600 ms before and after the application of NMB when the maximal effect was

reached. The slope of the linearly fitted current-voltage (I-V) response was used to

determine the input resistance, R_{in}. Bath application of NMB significantly increased the

R_{in} (Control: 106.9 ± 16.9 MΩ, NMB: 208.0 ± 25.5 MΩ, n = 18, P = 0.0006, Wilcoxon

test, Figure 10. *Da*). BLA neuron membrane time constants were obtained by fitting a

single exponential function to a voltage transient elicited by the -200 pA current injection

(100 ms from the end of baseline). Bath application of NMB significant increased

membrane time constants (Control: 17.4 ± 0.96 ms, NMB: 20.9 ± 1.9 ms, n = 18,

P = 0.004, Wilcoxon test, Figure 10. *Db-Dd*). Together, these results suggest that NMB

increases the excitability of principal BLA neurons.

Because NMB selectively activates BB1 receptors [291], and BB2 receptors are also

expressed in the BLA [290], we confirmed the involvement of BB1 receptor activation in

neuronal excitability. The slices were pretreated with the selective BB1 antagonist,

BIM23042 (0.3 μM) [292], and the same concentration of BIM23042 was included in the

bath solution. Under these circumstances, bath application of NMB failed to significantly

increase AP firing numbers (n = 10, $F_{(1,9)}$ = 2.671, P = 0.14, two-way repeated measures

ANOVA followed by Šídák's multiple comparison test, Figure 10. *Ea-Eb*), indicating that

increased AP firing occurs via activation of BB1 receptors. Because bombesin activates

both BB1 and BB2 receptors [293, 294], we pretreated slices with the selective BB2

receptor antagonist RC3095 (2 μM) and the same concentration of RC3095 was present

in the bath solution. Under these circumstances, bombesin-mediated excitation of BLA

neurons should be due to the activation of BB1 receptors. Indeed, bath application of

bombesin (0.3 μM) increased the number of APs fired in response to the positive current

injection protocol (n = 9, $F_{(1,8)}$ = 7.064, P = 0.028, two-way ANOVA repeated measures

ANOVA followed by Šídák's multiple comparisons test, Figure 10. *Ec-Ed*). Taken

together, these results suggest that NMB excites principal BLA neurons through the

activation of BB1 receptors.

BB1 Receptors Stimulate a Cation Current and Inhibit an
Inwardly Rectifying K⁺ Current

Next, we characterized the ionic mechanisms whereby the activation of BB1

receptors facilitates the excitability of BLA neurons. Two common ionic currents mediate

neuronal excitability: activation of cationic currents, and inhibition of K⁺ currents. Using a

K⁺-gluconate internal solution, bath application of NMB (0.3 μM) elicited an inward

current from BLA neurons at -60 mV (-21.60 $\pm$ 2.61 pA, n = 22, P < 0.0001, Wilcoxon

test, Figure 11. *Aa, Ab*). The NMB-mediated inward currents were concentration-

dependent (Figure 11. *Ab*). Substituting the K⁺-gluconate internal solution for Cs⁺-

gluconate to annul the contribution of K⁺ channels blocked NMB-mediated inward

holding currents (- 2.47 $\pm$ 4.57 pA, n = 23, P = 0.59 vs baseline, two-sample paired t-

test; P = 0.0008 vs the NMB-induced inward currents recorded in K⁺-containing internal

solution, two-sample independent t-test, Figure 11. *Ac, Ad*), supporting that depression

of K⁺ currents by BB1 receptor activation is one means of enhancing BLA neuronal

excitability. We further probed the ionic mechanism whereby BB1 receptor activation

excites BLA neurons by measuring I-V relationships from 49 voltage-clamped BLA

neurons. Twenty-four cells showed an I-V curve resembling inwardly rectifying K⁺ (Kir)

channels with a reversal potential of -56.2 $\pm$ 6.3 mV (Figure 11. *Ba-Bc*), which is

Figure 10.   **Activation of BB1 receptors excites BLA neurons**.

A, microscopic photograph displaying the location of the BLA where electrophysiological recordings were obtained. LA: lateral amygdala; BLA: basolateral amygdala; CeL: lateral nucleus of the central amygdala; CeM: medial nucleus of the central amygdala. *Ba & Bb,* Bath application of the selective BB1 receptor agonist, NMB, induces subthreshold depolarizations. *Ba, a* representative trace of the RMP prior to, during and after the bath application of NMB. *Bb,* summary data showing RMPs in control condition and after application of NMB from the same cells (left) and the net depolarization obtained by subtraction (right). *Ca-Cg,* bath application of NMB enhanced AP firing by lowering the rheobase current and the time to the first action potential. *Ca,* APs generated by a series of positive current injections from 50 pA to 750 pA at 50 pA increments and a duration of 600 ms every 6 s before and after bath application of NMB. *Cb*, summary of AP firing before and after NMB application. *Cc, a* representative trace of the rheobase current in a BLA principal neuron in control condition. *Cd, a* representative trace of the rheobase current in the same BLA principal neuron after NMB application. *Ce*, summary data of the mean rheobase current before and after application of NMB. *Cf*, a representative trace of the first action potential fired during the rheobase sweep before and after the application of NMB. Note the reduction in the time to the first AP denoted by the grey boxes. *Cg*, summary graph showing the time to the first AP fired in the rheobase sweep before and after NMB application. *Ch*, summary data of the AP halfwidth of the rheobase sweep. *Da-Dd,* NMB excites neurons by increasing the input resistance and elevating membrane time constants. *Da,* summary results of NMB on input resistance. *Db* & *Dc*, representative voltage response produced by the injection of -100 pA before and after treatment with NMB. Note the area highlighted by the dashed rectangle is magnified in *Dc. Dd,* summary graph for membrane time constants before and after NMB treatment. *Ea-Ed,* NMB-mediated excitation was mediated by selective activation of BB1Rs. *Ea,* APs recorded from a BLA neuron following the positive current injection protocol before (left) and after (right) bath application of NMB in a slice pretreated with the selective BB1 receptor antagonist BIM23042 (0.3 μM). *Eb*, the relationship of APs elicited before and after bath application of NMB in slices pretreated with BIM23042. *Ec*, APs recorded from a BLA neuron following the positive current injection protocol before (left) and after (right) bath application of bombesin in a slice treated with the selective BB2 receptor antagonist RC3095 (2 μM). *Ed*, the relationship of APs generated before and after the bath application of bombesin in slices treated with RC3095.

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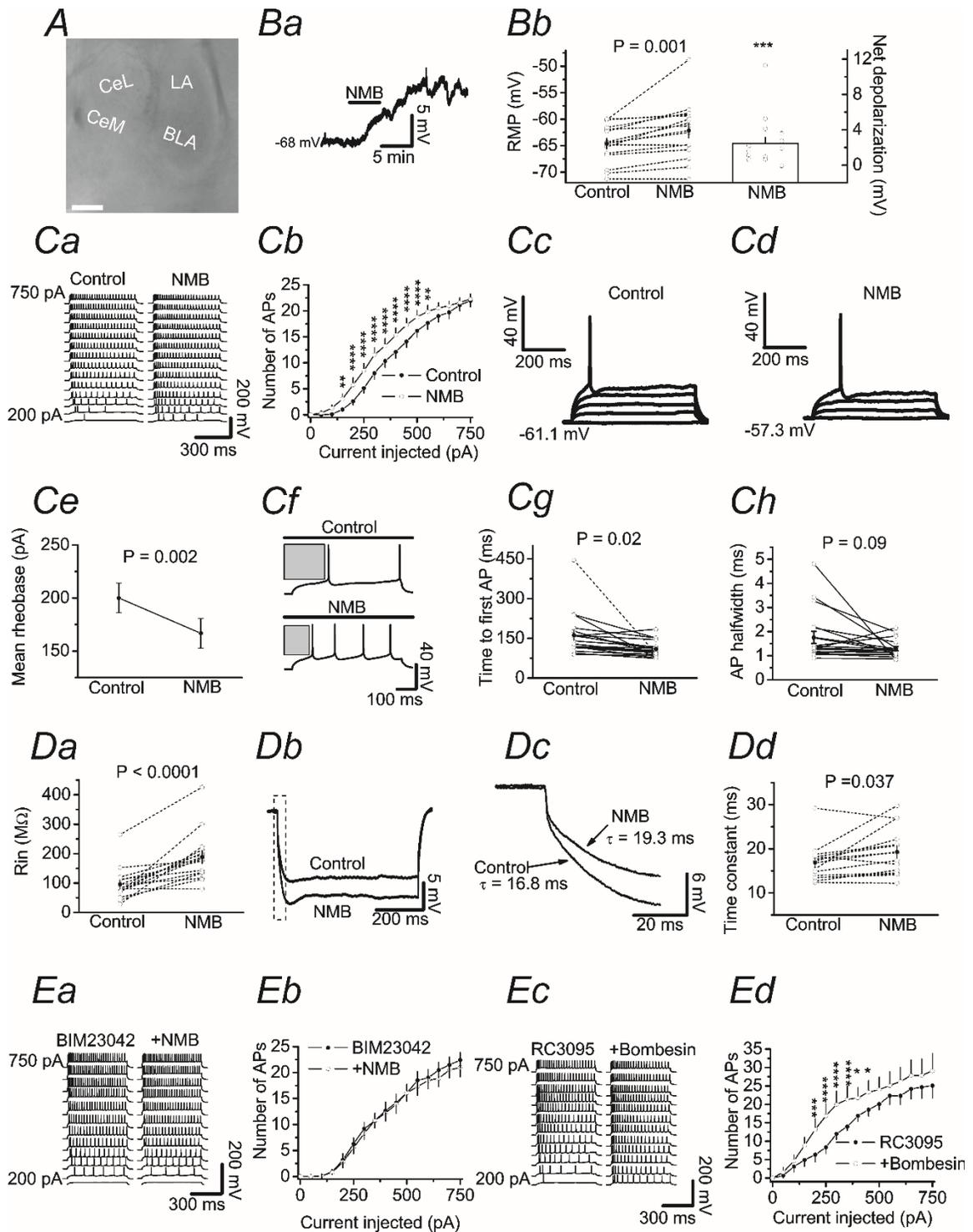
between the K$^+$ reversal potential and the reversal potential of cations, suggesting that both mechanisms are involved. Indeed, 25 recorded BLA neurons displayed an I-V curve resembling a nonselective cation channel (Figure 11. *Ca-Cc*). We then measured I-V responses with the inclusion of Cs-gluconate in our internal recording solution to block K$^+$ channels and applied TTX (0.5 μM) and CdCl$_2$ (200 μM) to block voltage-gated Na$^+$ and Ca^{2+} channels, respectively. The reversal potential of the NMB-elicited cation channel currents was measured by extending the voltage range to +20 mV. From these experimental conditions, we obtained an I-V curve of the NMB-mediated currents with a reversal potential of -39.1 $\pm$ 6.6 mV (n = 13, Figure 11. *Da-Dc*). These results suggest that BB1 receptor activation excites principal BLA neurons by opening a non-selective cation channel and depressing a Kir channel. To better understand the role of BB1 receptor activation in Kir channel depression, we measured NMB-induced inward holding currents in the presence of Ba^{2+}, as Kir channels are inhibited by Ba^{2+} in micromolar range [252, 254]. Bath application of Ba^{2+} (500 μM), by itself induced an inward holding current (-56.51 $\pm$ 14.29 pA, n = 15, P = 0.001, two-sample paired t-test, Figure 12. *A, E*) and significantly reduced NMB-elicited inward currents (Net current: -10.48 $\pm$ 1.40 pA, n = 15, P = 0.010, one-way ANOVA followed by Dunnett's test, Figure 12. *A, F*). Functionally distinct Kir channel subfamilies include Kir2, Kir3 (G protein-gated inwardly rectifying potassium (GIRK) channels), Kir6 (ATP-sensitive channels), and K$^+$ transport channels [255]. To test the potential involvement of the Kir2 subfamily, we used ML 133, a specific antagonist of the Kir2 subfamily [295], in NMB-elicited excitation of BLA principal neurons.Bath application of ML 133 (30 μM) by itself had no effect on holding currents (Net current: -2.52 $\pm$ 1.57 pA, n = 19, P = 0.13, one-sample t-test, Figure 12. *B, E*). When NMB was bath applied in the presence of ML 133, NMB elicited an inward current that was not significantly different from slices treated with

Figure 11. **NMB-elicited excitation of BLA neurons is mediated by inhibiting a Kir channel and opening a cation channel**.

Aa-Ad, bath application of NMB concentration-dependently excited BLA neurons producing an inward holding current in part by blocking a K^+ current. *Aa*, a representative trace of the inward currents generated by the bath application of NMB (0.3 μM) in a BLA neuron. *Ab*, the relationship of the net inward current generated by application of increasing concentrations of NMB. Filled circles represent individual current values and the bars represent their means. *Ac,* a representative trace of the inward current elicited by bath application of NMB recorded from a BLA neuron using a Cs-gluconate internal recording solution. *Ad,* relationship of the net inward currents generated by bath application of NMB in BLA neurons recorded with a K-gluconate or Cs-gluconate internal recording solution. Filled symbols are individual current values, and the bars represent their mean current values. *Ba-Bc,* NMB inhibited an inwardly rectified K^+ channel. The extracellular solution contained tetrodotoxin (0.5 μM), picrotoxin (100 μM) and kynurenic acid (1 mM) and the intracellular recording solution was the K^+-gluconate containing internal recording solution. *Ba*, a representative current trace elicited by the voltage-step protocol before (left) and during (middle) bath application of NMB and the net current obtained by subtraction (right) from a BLA neuron. The dashed line represents zero current level. *Bb,* I-V curved averaged from 24 cells before and during the application of NMB (Two-way repeated measures ANOVA followed Šídák's multiple comparisons test; Drug: $F_{(1,23)}$ = 4.76, P = 0.039; Voltage: $F_{(10,230)}$ = 296.1, P < 0.0001; Drug x Voltage: $F_{(10, 230)}$ = 27.57, P < 0.0001). *Bc,* I-V curve of the net current obtained by subtracting the currents in the control condition from those after the application of NMB. The I-V curved of the NMB-mediated inhibition of Kir channels had a reversal potential of -56.2 $\pm$ 6.3 mV. *Ca-Cc,* NMB opens a cation channel. *Ca*, a representative current trace generated by the voltage-step protocol before (left) and during (middle) bath application of NMB and the net current obtained by subtraction (right) from a BLA neuron. *Cb*, I-V curve averaged from 25 cells before and during the application of NMB (Two-way repeated ANOVA; Drug: $F_{(1,24)}$ = 6.18, P = 0. 02; Voltage: $F_{(1,24)}$ = 225.5, P < 0.0001; Drug x Voltage: $F_{(10,240)}$ = 1.85, P = 0.05). *Cc,* I-V curve of the net current obtained by subtracting the currents in the control condition from those after the application of NMB. *Da-Dc*, NMB opened a cation channel with outward rectification recorded in extracellular solution containing tetrodotoxin (0.5 μM), picrotoxin (100 μM), kynurenic acid (1 mM), and $CdCl_2$ (200 μM) and the intracellular solution was the Cs^+-gluconate containing internal recording solution. *Da*, a representative current trace elicited by the voltage-step protocol before (left) and during (middle) bath application of NMB and the net current obtained by subtraction (right) from a BLA neuron. The dashed line is the zero current level. *Db*, I-V curve averaged from 25 cells before and during the application of NMB (Two-way repeated measures ANOVA followed by Šídák's multiple comparisons test; Drug: $F_{(1,23)}$ = 4.89, P = 0.037; Voltage: $F_{(10,230)}$ = 296.1, P < 0.0001; Drug x Voltage: $F_{(10,230)}$ = 27.57, P < 0.0001). *Dc,* I-V curve of the net current obtained by subtracting the currents in control conditions from those during the application of NMB. The I-V curve of the NMB-induced outwardly rectifying currents had a reversal potential of -39.1 $\pm$ 6.6 mV. **** P< 0.0001, *** P < 0.001, ** P < 0.01, * P < 0.05.

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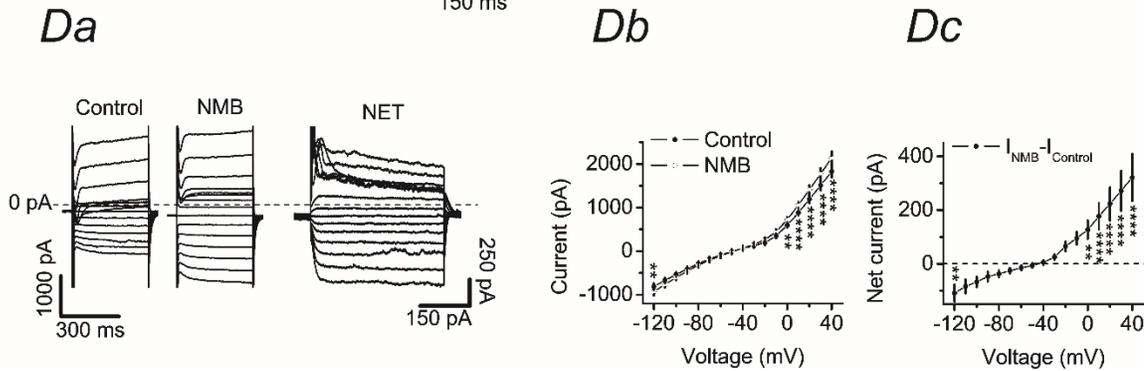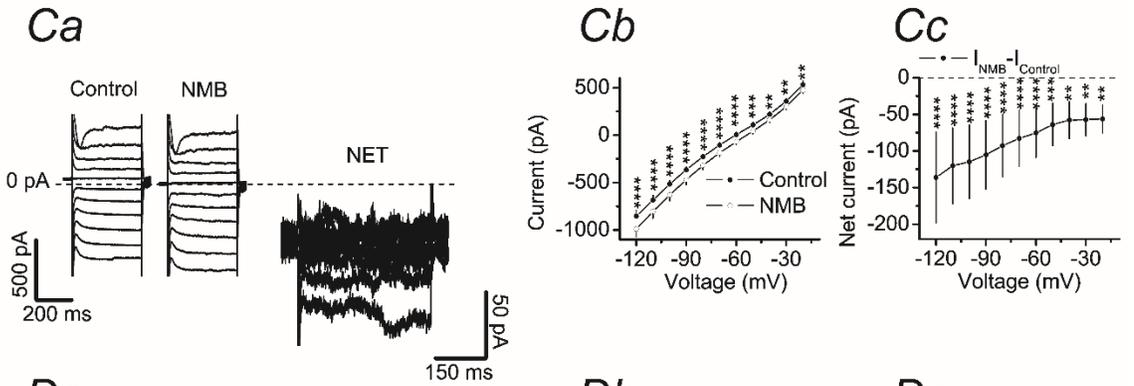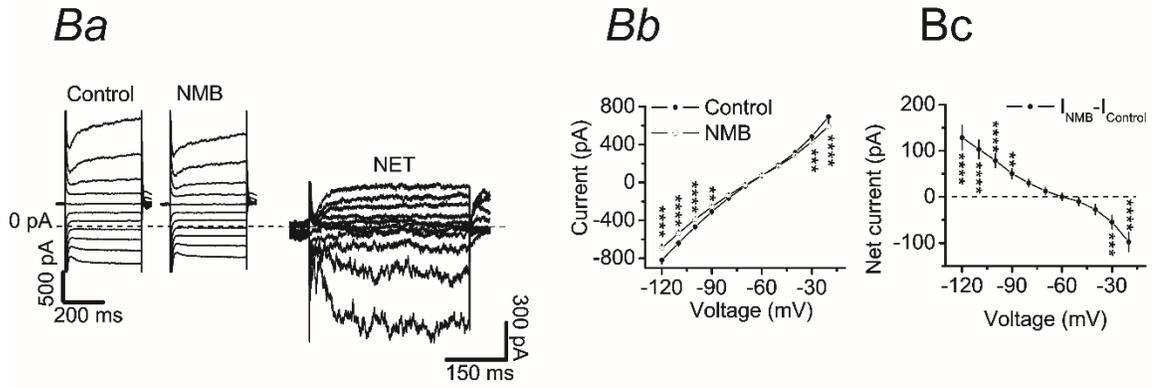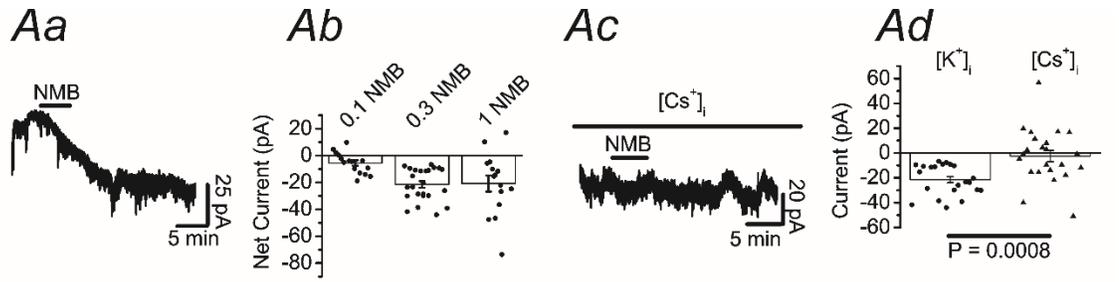
NMB in control conditions (Net current: -22.09 ± 2.87 pA, n = 19, P = 0.996 one-way

ANOVA followed by Dunnett's test, Figure 12. *B, F*), suggesting that the Kir2 subfamily is

not the type of Kir channels involved in NMB-mediated excitation of BLA neurons.

Because BB1 receptor activation is coupled to G proteins, we tested the involvement of

the GIRK channel subfamily. ML297 is a selective activator of GIRK1 containing

channels, and GIRK1 channels are expressed in the BLA [296]. Bath application of a

saturating concentration of ML297 (10 μM) produced significant outward currents in BLA

neurons (Net current: 48.72 ± 14.29 pA, P = 0.01, Student's t-test, Figure 12. *C, E*),

suggesting that GIRK1-containing channels are expressed in BLA neurons. The

derivative of the honeybee toxin tertiapin, tertiapin-Q (250 nM, TQ), is an antagonist of

GIRK channels [263, 264]. Bath application of TQ by itself produced a significant inward

current in BLA neurons (Net current: -15.37 ± 2.95 pA, p = 0.0002, student's t-test,

Figure 12. *D, E*) and co-application of NMB resulted in a significantly smaller current

than in BLA neurons treated with NMB alone (Net current: -10.18 ± 2.25 pA, P = 0.002,

one-way ANOVA followed by Dunnett's test, Figure 12. *D, F*). Together, these data

suggest tertiapin-Q sensitive GIRK channels contribute to NMB-mediated excitation of

BLA neurons.

TRPV1 Channels are Involved in NMB-Mediated Inward Currents

In approximately 50% of the neurons examined, the I-V curve of the NMB-elicited

currents (Figure. 11) resembled that of transient receptor potential channel family,

namely TRPV1, TRPC4, and TRPC5 channels [297]. Because TRPV1, TRPC4, and

TRPC5 are expressed in the BLA [298-301], we tested the involvement of these

channels in NMB-elicited inward currents. To probe the roles of TRPC4/5 we pretreated

rat BLA neurons with the selective TRPC4/5 channel blocker M084 (100 μM)
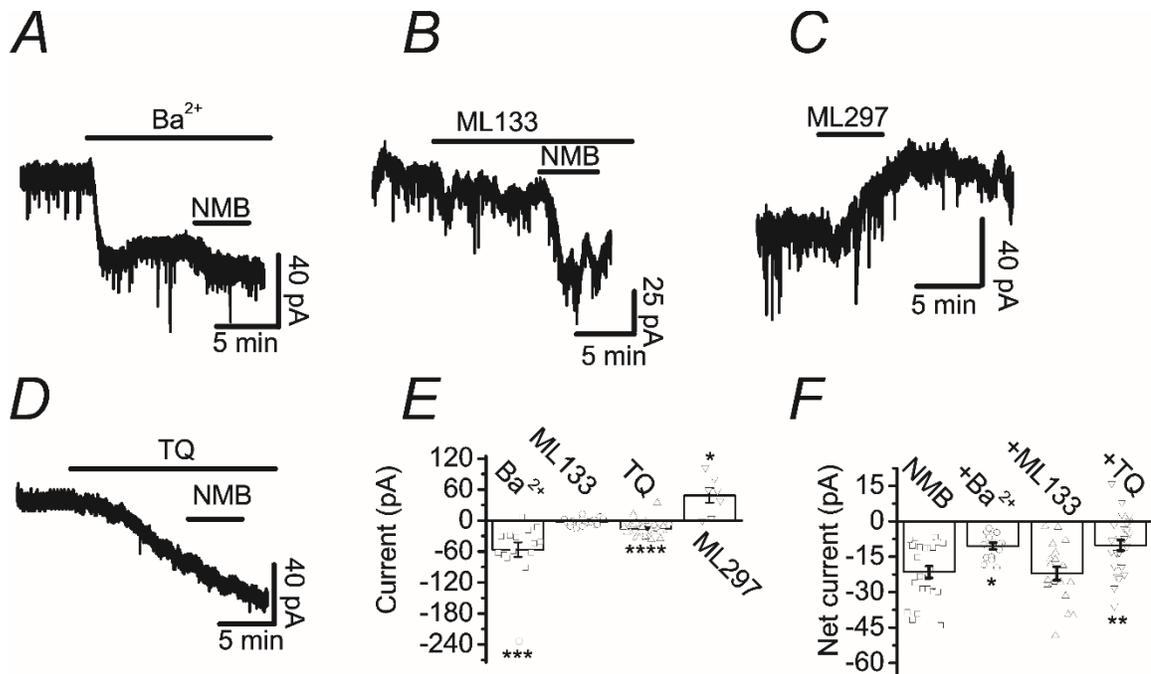
Figure 12. **GIRK channels are required for BB1 receptor-elicited inward currents in BLA neurons.**

A, a current trace recorded from a BLA neurons in response to Ba^{2+} (500 μM) alone and Ba^{2+} plus NMB. *B,* a current trace recorded from a BLA neuron in response to ML133 (30 μM) alone and ML133 plus NMB. *C,* current trace recorded from a BLA neuron in response to bath application of ML297 (10 μM). *D, a* current trace recorded from a BLA neurons in response to tertiapin-Q (250 nM, TQ) alone and TQ plus NMB. *E*, summary data for the effects of Kir channel modulators on BLA neurons. **** $P < 0.0001$, *** $P < 0.001$, * $P < 0.05$ vs. baseline, two-sample paired t-test. *F,* summary graph showing the effects of Kir channel blockers on NMB-mediated inward currents. ** $P < 0.01$, * $P < 0.05$ vs. NMB alone, one-way ANOVA followed by Dunnett's test.

and the same concentration was present in the bath. Under these conditions, NMB-mediated inward currents were not altered significantly when compared to BLA neurons treated with NMB alone (Net current: M084+NMB: -16.8 $\pm$ 2.4 pA; NMB: -21.6 $\pm$ 2.6 pA; P = 0.13, two-way student's t-test, Figure 13. *Aa-Ac*), suggesting that TRPC4/5 channels are not involved in NMB-evoked inward currents.

The temperature sensitive TRPV1 channels are non-selective cation channels expressed in the BLA [298, 301], displaying outward rectification similar to that observed in our I-V recordings. The steady-state temperature of our recording solutions was held at 33-34°C and revealed that NMB generates a significant inward current (see Figure 11. *Aa*, Figure 13. *Aa*). As TRPV1 channels are temperature-sensitive cation ion channels [297], we recorded holding currents from rat brain slices with the recording solution held at room temperature (22-23°C). When the bath temperature was held at room temperature, bath application of NMB elicited a significantly smaller inward current than control slices held at 33-34°C (NMB 22-23 °C net current: -2.1 $\pm$ 1.1 pA; NMB 33-34 °C net current: -21.6 $\pm$ 2.6 pA, P < 0.0001 unpaired t-test, P < 0.0001 vs. NMB alone one-way ANOVA followed by Tukey's test, Figure 13. *Ba*, *Be*). We further evaluated the involvement of TRPV1 channels using targeted TRPV1 channel blockers. Pretreatment and bath application of the selective TRPV1 channel blocker, AMG 9810 (10 μM) [302], significantly reduced NMB-mediated inward currents (P = 0.19, n = 12, paired t-test; P < 0.0001 vs. NMB alone, one-way ANOVA followed by Dunnett's test, Figure 13. *Bb, Be*). In slices treated with another TRPV1 antagonist, capsazepine (10 μM)[303-306], NMB generated a significant inward current (P = 0.002, n = 19, unpaired t-test, Figure 13. *Bc*), but the inward current was significantly reduced when compared to currents in control slices treated with NMB alone (P < 0.0001, one-way ANOVA followed by

Figure 13. **TRPV1 channels are involved in BB1 receptor-induced inward currents in BLA neurons.**

Aa-Ac, pretreatment of slices with and continuous bath application of M084 did not significantly affect NMB-mediated inward currents. *Aa,* a representative trace of the NMB-mediated inward currents in control condition. *Ab,* a representative trace of the NMB-elicited inward currents in a slice pretreated with the TRPC4/5 channel blocker M084 (100 μM). *Ac*, summary graph showing the relationship of currents produced by NMB in slices pretreated with M084. Circles represent the currents generated by individual cells and the bars represent the respective means. *Ba-Be,* NMB activates TRPV1 channels in rat BLA neurons. *Ba*, a representative current trace recorded at room temperature from a BLA neuron after bath application of NMB. *Bb*, a representative trace of NMB-mediated inward currents in the presence of selective TRPV1 blocker, AMG9810 (10 μM). *Bc,* holding current trace induced by bath application of NMB in the presence of another TRPV1 channel blocker capsazepine (10 μM). *Bd*, a representative current trace generated by bath application of NMB in the presence of the DAG lipase inhibitor, RHC 80267 (25 μM). *Be*, summary graph showing the effects of TRPV1 channel modulators on the NMB-mediated inward currents in BLA neurons. **** $P < 0.0001$ vs NMB alone, one-way ANOVA followed by Dunnett's test. *Ca-Cg*, bath application of NMB produced significantly smaller inward currents in TRPV1 knockout mice, but not in TRPC4 or TRPC5 knockout mice. *Ca,* a representative current trace recorded from TRPC4 WT mouse before and after the bath application of NMB. *Cb,* a representative current trace recorded from TRPC4 KO mouse before and after the bath application of NMB. *Cc,* a representative current trace recorded from TRPC5 WT mouse before and after the bath application of NMB. *Cd,* a representative current trace recorded from a TRPC5 KO mouse before and after the bath application of NMB. *Ce*, a representative current trace recorded from a TRPV1 WT mouse before and after the bath application of NMB. *Cf*, a current trace elicited from a TRPV1 KO mouse before and after the bath application of NMB. *Cg*, summary graph showing the effects of NMB-generated inward currents in WT and respective KO mice. **** $P < 0.0001$ vs corresponding WT, Mann-Whitney test. n.s, nonsignificant.

Aa

NMB

25 pA
5 min

Ab

M084

NMB

20 pA
5 min

Ac

M084
+NMB   NMB

Current (pA)

10
0
-10
-20
-30
-40
-50

P = 0.13

Ba

NMB (22°C)

20 pA
5 min

Bb

AMG 9810

NMB

20 pA
5 min

Bc

Capsazepine

NMB

25 pA
5 min

Bd

RHC 80267

NMB

20 pA
5 min

Be

Current (pA)

NMB (33°C) NMB (22°C) AMG9810 CAP RHC 80267

10
0
-10
-20
-30
-40

**** **** **** ****

Ca

TRPC4 WT

NMB

25 pA
5 min

Cb

TRPC4 KO

NMB

20 pA
5 min

Cc

TRPC5 WT

NMB

25 pA
5 min

Cd

TRPC5 KO

NMB

20 pA
5 min

Ce

TRPV1 WT

NMB

25 pA
5 min

Cf

TRPV1 KO

NMB

25 pA
5 min

Cg

Current (pA)

TRPC4 WT TRPC4 KO TRPC5 WT TRPC5 KO TRPV1 WT TRPV1 KO

30
15
0
-15
-30
-45
-60

ns   ns   ****

83

Tukey's test, Figure 13. *Bc, Be*), suggesting that NMB excites BLA through the activation of TRPV1 channels. TRPV1 channels can also be activated by natural endocannabinoids, which resemble vanilloid agonists [297, 307]. The endocannabinoid 2-arachidonoylglycerol (2-AG), produced by DAG lipase following G_q-couple receptor activation, may activate TRPV1 channels [308]. We tested this possibility by pretreating and bath applying RHC 80267 (25 μM) to inhibit both α- and β-DAG lipase. Pretreatment of slices with and continuous bath application of RHC 80267 failed to block NMB-mediated inward currents significantly (Net current: -4.9 $\pm$ 1.7 pA, P = 0.009, n = 18, paired t-test; Figure 13. *Bd*), but this inward current was significantly smaller than that mediated by NMB alone (P < 0.0001, one-way ANOVA followed by Tukey's test, Figure 13. *Bd, Be*).

We further tested the role of TRP channels using TRC4, TRPC5, and TRPV1 knockout mice. Bath application of NMB produced similar inward currents in TRPC4, TRPC5, and TRPV1 wild-type mice (P = 0.86, one-way ANOVA; Figure 13. *Ca, Cc, Ce*). The inward current produced in TRPC4 KO and TRPC5 KO was similar to the inward currents produced in corresponding wild-type mice (TRPC4 WT: -17.9 $\pm$ 4.3 pA; TRPC4 KO: -17.1 $\pm$ 2.8 pA; P = 0.87, unpaired t-test; TRPC5 WT: -18.9 $\pm$ 3.5 pA; TRPC5 KO: -16.8 $\pm$ 2.7 pA; P = 0.64, unpaired t-test; Figure 13. *Ca-Cd, Cg*). However, in male and female mice lacking the TRPV1 channel, NMB failed to produce a significant inward current (TRPV1 WT: -18.2 $\pm$ 5.3 pA; TRPV1 KO: 0.7 $\pm$ 2.3 pA; P < 0.0001, Mann-Whitney test; Figure 13. *Ce, Cf, Cg*). Together, these data indicate that NMB excites BLA neurons via activation of TRPV1 channels.

BB1 Receptor-Mediated Excitation of BLA Neurons is Dependent on PLCß and PLCß-Mediated Hydrolysis of PIP$_2$ and PI3/4K Signaling

We then investigated the intracellular signaling mechanisms involved in NMB-mediated excitation of BLA neurons. BB1 receptors are coupled to G$\alpha_{q/11}$ proteins [236, 239, 240] that signal through the PLCß pathway. Therefore, we examined the role of this signaling pathway in NMB-induced AP firing. Intracellular dialysis of the selective G protein inactivator GDP-ß-S (0.5 mM) significantly attenuated the number of APs fired in response to the positive current injection protocol (F $_{(1,12)}$ = 1.513, P = 0.24, two-way repeated-measures ANOVA; Figure 14. *A*), indicating that the function of G proteins is necessary for NMB-mediated enhancement of AP firing.

We sought to determine the functional involvement of PLCß in BB1-mediated excitability of BLA neurons. The slices were pretreated with U73122 (5 µM), a selective PLCß inhibitor, for > 2 h. Under these conditions, application of NMB failed to augment AP firing in response to the positive current injection protocol (F $_{(1,10)}$ = 0.04, P = 0.83, two-way-repeated-measures ANOVA, Figure 22. *B*). These results demonstrate that PLCß is required for NMB-mediated increases in neuronal excitability in the BLA.

Activated PLCß hydrolyzes the membrane lipid PIP$_2$ to generate the bioactive metabolites DAG and IP$_3$, and in turn removes PIP$_2$ allosteric modulation of a variety of ion channels [193, 309]. To test the role of PIP$_2$ turnover in NMB-mediated excitation, the short-chain, water-soluble analog diCi8-PIP$_2$ (20 µM) was included in our recording solution to compensate for PIP$_2$ hydrolysis by PLCß. Restoration of PIP$_2$ blocked the NMB-evoked augmentation of AP firing (F $_{(1,15)}$ = 2.165, P = 0.16, two-way repeated-measures ANOVA, Figure 14. *C*). As the levels of membrane PIP$_2$ are rapidly resynthesized following PLC-mediated signaling by the phosphorylation of phosphoinositol and phosphatidylinositol 4-phosphate (PI (4)P) at position 5 by their cognate kinases [310-312], we targeted PI3K and PI4K with wortmannin (20 µM) to

inhibit PIP$_2$ resynthesis. Inhibition of PI3K and PI4K produced a significant reduction in the number of APs fired in response to the positive current injection protocol (F $_{(1,21)}$ = 9.05, P = 0.0067, two-way ANOVA followed by Šídák's multiple comparisons test, Figure 14. *D*). Taken together, these data indicate that NMB-mediated excitation of principal BLA neurons requires membrane lipid remodeling.

We then examined the role of PKC in NMB-mediated excitation of BLA neurons. Slices were pretreated with the selective PKC inhibitor, bisindolylmaleimide II (Bis II, 1 μM), and this concentration was present in the extracellular recording solution. Application of NMB failed to significantly increase AP firing in slices pretreated with Bis II (F $_{(1,16)}$ = 0.009, P = 0. 92, two-way repeated measures ANOVA followed by Šídák's multiple comparisons test, Figure 14. *E*). Next, we tested the involvement of intracellular Ca^{2+} in NMB-mediated excitation of BLA neurons by inclusion of the Ca^{2+} chelator BAPTA (10 mM) in our recording electrode. Under these conditions, application of NMB failed to increase AP firing in response to the positive current injection protocol (F $_{(1,18)}$ = 3.96, P = 0.06, two-way repeated measures ANOVA followed by Šídák's multiple comparisons test, Figure 14. *F*). Similarly, Ca^{2+} release from intracellular stores is required for NMB-mediated augmentation of BLA excitability (Supplementary Figure 1). Taken together, these data support the functional requirement for G-protein activation, membrane lipid remodeling, PKC, and intracellular Ca^{2+} for NMB-mediated increases in AP firing.

BB1 Receptor Activation Modulates Glutamatergic Transmission in the BLA

The BLA is an important site of sensory integration as it receives afferent projections from cortical and thalamic structures [313-315] and has a well-characterized involvement in fear responses [15, 316, 317] and anxiety [93, 106]. Although the neuromodulatory

Figure 14. **BB1 receptor elicited excitation of BLA neurons requires PLCß-mediated intracellular signaling.**

A, representative traces (upper) and summary graphs (lower) of NMB-augmented APs in response to the positive current injection protocol in the presence of intracellular GDP-ß-S (0.5 mM). *B*, representative traces (upper) and summary graphs (lower) of NMB-mediated APs in the presence of the selective PLCß inhibitor U73122 (5 μM). C, traces (upper) and summary graphs (lower) of NMB-augmented APs in the presence of intracellular dialyzed diC8-PIP$_2$ (20 μM). *D*, representative traces (upper) and summary graphs (lower) of NMB-elicited APs in response to the positive current injection protocol from slices pretreated with the PI3K and PI4K inhibitor wortmannin (20 μM). *E*, AP traces (upper) and summary graphs (lower) of NMB-augmented APs in the slice pretreated with the selective PKC inhibitor BISII (1 μM). *F,* representative traces (upper) and summary graph (lower) of NMB-elicited APs in response to the positive current injection protocol in the presence of the intracellular Ca^{2+}-chelator BAPTA (10 mM). **** $P< 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, two-way repeated measures ANOVA.
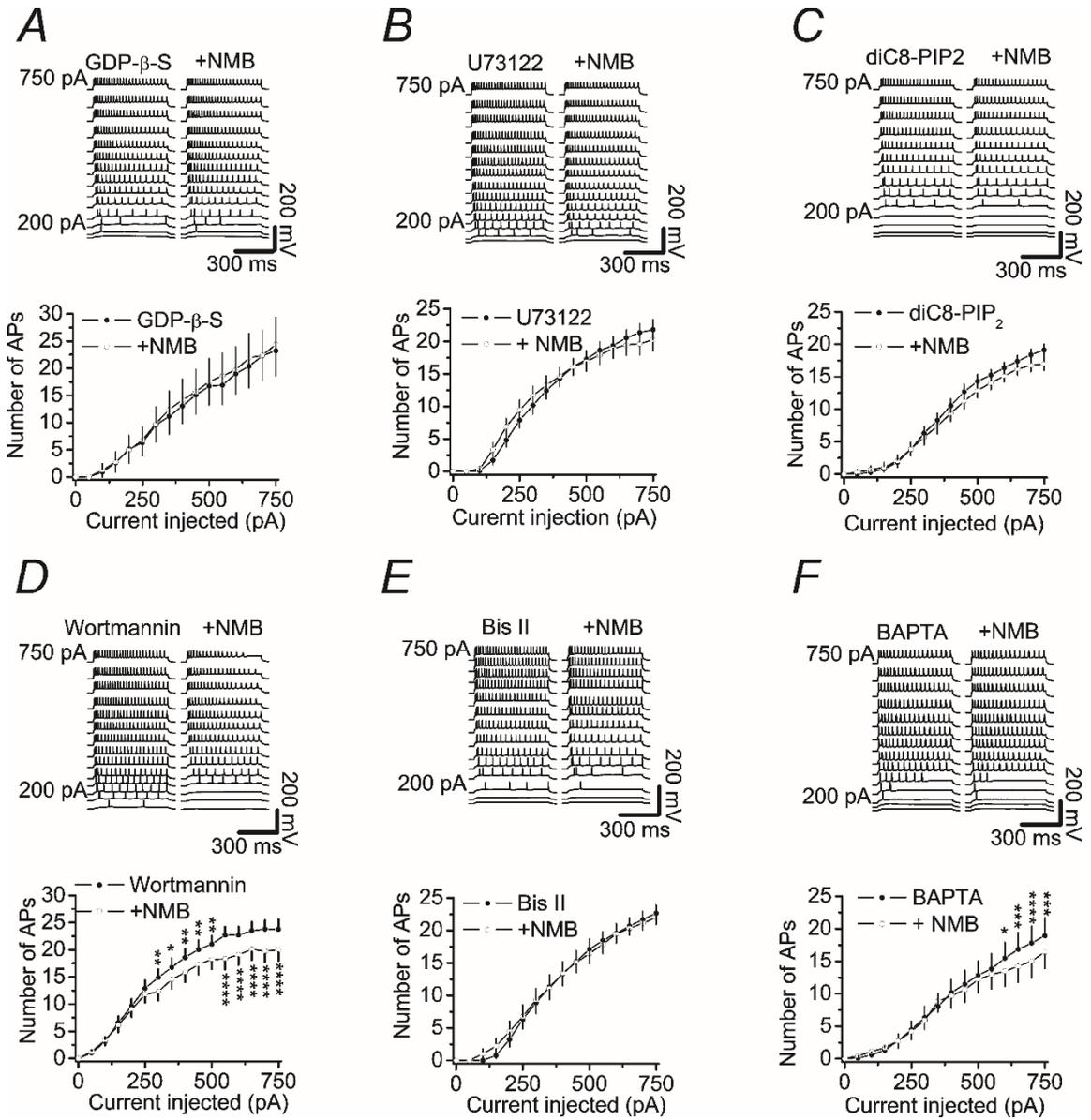
bombesin-like peptides have been reported to stimulate glutamatergic transmission in the spinal cord [318], olfactory bulb [319], and hypothalamus [320], little is known regarding how NMB modulates glutamate release in the BLA. To investigate the role of BB1 receptor activation in glutamatergic transmission in the BLA, we recorded sEPSCs before and after NMB application. NMB (0.3 μM) significantly increased the frequency (baseline: 6.6 ± 1.3 Hz, NMB: 11.8 ± 1.7 Hz, 261 ± 94% of baseline, n = 11, P = 0.002 Wilcoxon test, Figure 15. *Aa-Ad*) and amplitude (baseline: 18.2 ± 0.8 pA, NMB: 21.9 ± 2.2 pA, 118 ± 11 % of baseline, n = 11, P = 0.009 Figure 15. *Ae, Af*) of sEPSCs. The effect of NMB on sEPSC was irreversible for the duration of our experiments as the sEPSC frequency following 20 minutes of washing was 257.3 ± 39% of baseline (n = 11, P = 0.002, Figure 15. *Ab*). These data indicate that NMB augments spontaneous glutamatergic transmission in the BLA.

NMB-mediated increases in sEPSCs could be explained by several hypotheses, including a) NMB produces an increase in AP firing in BLA neurons to increase their excitability and glutamate release; b) NMB-mediated activation of TRPV1 channels results in elevated Ca^{2+} influx and subsequent neurotransmitter release; and c) NMB acts at postsynaptic sites to increase glutamatergic signaling. To test these hypotheses, we recorded miniature EPSCs (mEPSCs), sEPSCs, and mEPSCs targeting TRPV1 channels, and evoked EPSCs (eEPSCs). We recorded mEPSCs in the presence of TTX (0.5 μM) to exclude action potentials and voltage-gated Ca^{2+} influx. In this condition, application of NMB enhanced the frequency (baseline: 1.3 ± 0.4 Hz, NMB: 4.1 ± 1.4 Hz, 293 ± 55 % of baseline, n = 9, P = 0.03, Figure 15. *Ba-Bd*) but not the amplitude (baseline: 15.8 ± 0.4 pA, NMB: 15.9 ± 0.5 pA, 100 ± 1 % of baseline, n = 9, P = 0. 58, Figure 15. *Be-Bf*) of mEPSCs, indicating APs are not required for NMB-mediated spontaneous glutamatergic transmission in the BLA but the involvement of calcium

cannot be excluded. Moreover, the increase in frequency, but not amplitude may suggest a presynaptic mechanism whereby NMB enhances spontaneously released glutamate.

We then probed the involvement of Ca^{2+}-permeant TRPV1 channels in NMB-mediated glutamatergic transmission in the BLA by treating slices with AMG9810 (10 µM). Application of NMB failed to significantly increase the frequency (baseline: 8.9 $\pm$ 1.4 Hz, NMB: 9.3 $\pm$ 0.9 Hz, 121 $\pm$ 15 % of baseline, n = 12, P = 0.69 paired t-test, Figure 16. *Aa-Ac*) or amplitude (baseline: 19.6 $\pm$ 1.5 pA, NMB: 20.7 $\pm$ 1.6 pA, 106 $\pm$ 4 % of baseline, n = 12, P = 0.15 Wilcoxon test, Figure 16. *Ad*) of sEPSCs in BLA neurons treated with AMG9810. We then recorded sEPSCs from TRPV1 KO mice and their corresponding wildtype littermates. Application of NMB in male and female TRPV1 WT mice significantly increased the frequency of sEPSCs (TRPV1 WT: control: 5.1 $\pm$ 1.4 Hz; NMB: 12.9 $\pm$ 3.1 Hz, 308 $\pm$ 65 % of control, n = 12, P = 0.007, Figure 16. *Ba, Bc-Bd*) and the amplitude of sEPSC events (TRPV1 WT: control: 17.8 $\pm$ 0.9 pA; NMB: 24.7 $\pm$ 1.7 pA, 140 $\pm$ 12 % of control, n = 12, P = 0.002, Figure 16. *Ba, Be*). The application of NMB in TRPV1 KO mice failed to significantly increase the frequency of sEPSC events (TRPV1 KO: control: 18.6 $\pm$ 4.1 Hz; NMB: 15.9 $\pm$ 3.9, 94 $\pm$ 11% of baseline, n = 11, P = 0.33, Figure 16. *Bb-Bc, Bf*) and had no effect on sEPSC amplitude (TRPV1 KO: 16.5 $\pm$ 0.6 pA; NMB: 16.7 $\pm$ 0.8 pA, 100 $\pm$ 2 % of baseline, n = 11, P = 0.81, Figure 16. *Bb*, *Bg*). These results indicate that NMB-mediated sEPSC events require functional TRPV1 channels.

We then probed the involvement of TRPV1 channels in NMB-mediated AP-independent glutamatergic transmission. Rat brain slices were pretreated with AMG9810 to annul TRPV1 channel currents, as described above, and TTX was included in all solutions to inhibit voltage-gated Na^+ channels. Under these conditions,

Figure 15. **Activation of BB1 receptors enhances spontaneous glutamate release in BLA neurons.**

Aa-Af, NMB increases the frequency and the amplitude of sEPSCs recorded from BLA neurons. *Aa,* representative trace of spontaneous EPSCs recorded from BLA neurons before, during, and after the bath application of NMB (0.3 μM). *Ab,* time course of the sEPSC frequency averaged from 11 BLA principal neurons. *Ac,* cumulative frequency distribution averaged from 11 BLA neurons before and during the application of NMB. *Ad,* frequency of sEPSCs pooled from 11 cells before and during the application of NMB. Open circles are values from individual cells and filled circles are their mean. *Ae,* cumulative amplitude distribution average from 11 cells before and during the application of NMB. *Af,* amplitude of sEPSCs pooled from 11 cells before and during the application of NMB. *Ba-Bf*, NMB-mediated increases in glutamatergic transmission were AP-independent. *Ba*, mEPSC traces recorded from a BLA neuron before, during, and after the application of NMB. *Bb*, time course of the mEPSCs events averaged from 9 BLA neurons recorded in the presence of tetrodotoxin. *Bc,* cumulative frequency distribution averaged from 9 cells before and during the application of NMB in the presence of tetrodotoxin. *Bd*, frequency of mEPSCs pooled from 9 cells before and during the application of NMB. *Be*, cumulative amplitude distribution averaged from 9 BLA neurons before and during the application of NMB. *Bf*, amplitude of mEPSCs pooled from 9 cells before and during the application of NMB.
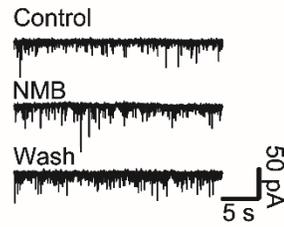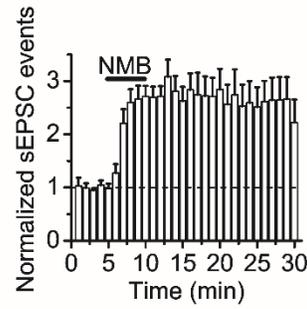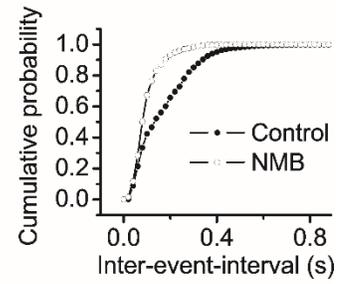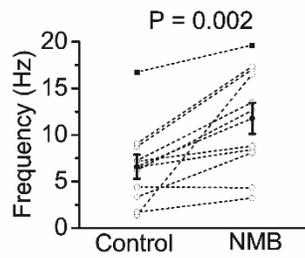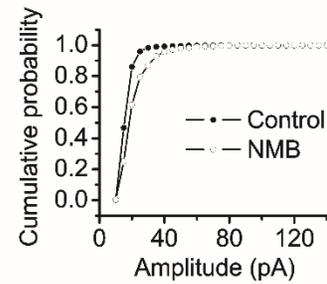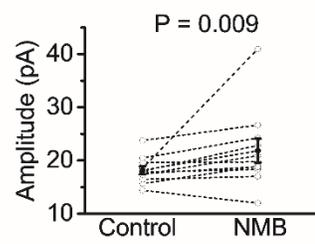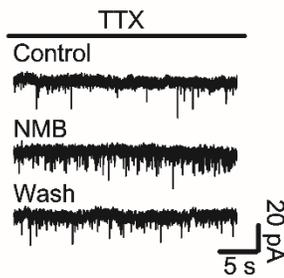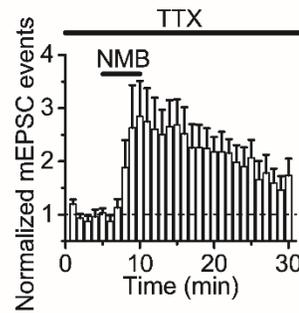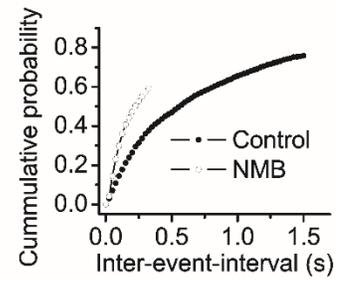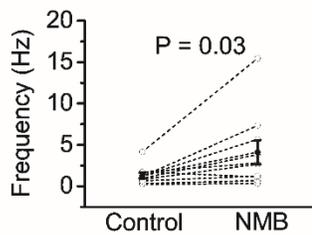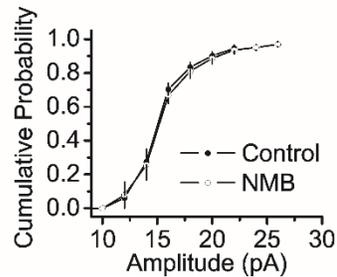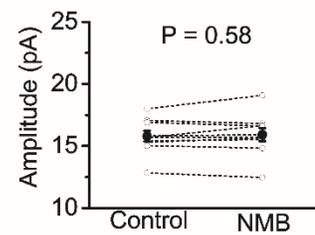
Figure 16. **BB1 receptor-mediated spontaneous glutamate release requires TRPV1 channels in BLA neurons.**
Aa-Ad, TRPV1 channels are required for BB1 receptor-elicited increases in spontaneous glutamate release in BLA neurons. *Aa*, representative traces of sEPSCs before, during and after the application of NMB in the presence of AMG9810 (10 μM). *Ab*, time course of sEPSC events averaged from 12 BLA neurons. sEPSCs elicited in control conditions are co-plotted for comparison. *Ac*, frequency of sEPSCs pooled from 12 cells before and after the application of NMB in the presence of AMG9810. *Ad*, amplitude of sEPSCs pooled from 12 cells before and after the application of NMB in the presence of AMG9810. *Ba,* representative sEPSCs recorded from a BLA neuron from a TRPV1 WT mouse before, during, and after the bath application of NMB. *Bb*, representative sEPSCs recorded from a BLA neurons from a TRPV1 KO mouse before, during, and after the bath application of NMB. *Bc*, time course of sEPSC events from TRPV1 WT and TRPV1 KO mice. *Bd*, frequency of sEPSCs pooled from 12 TRPV1 WT BLA neurons before and after application of NMB. *Be*, amplitude of sEPSCs pooled from 12 TRPV1 WT BLA neurons before and after application of NMB. *Bf*, frequency of sEPSCs pooled from 11 TRPV1 KO BLA neurons before and after the application of NMB. *Bg*, amplitude of sEPSCs pooled from 11 TRPV1 KO BLA neurons before and after the application of NMB. *Ca,* representative mEPSC traces recorded before, during, and after the application of NMB in the continuous presence of AMG9810 and tetrodotoxin. *Cb*, time course of mEPSC events averaged from 9 rat BLA neurons in the presence of AMG9810 and tetrodotoxin. sEPSCs elicited in control conditions are co-plotted for comparison. *Cc*, frequency of mEPSCs pooled from 9 rat BLA neurons before and after the application of NMB in the presence of AMG9810. *Cd*, amplitude of mEPSCs pooled from 9 rat BLA neurons before and after the application of NMB in the presence of AMG9810.

Aa

Control

NMB

Wash

30 pA
5 s

Ab

— ●— Control
— ○— AMG

NMB

Normalized sEPSC events

Time (min)

Ac

Frequency (Hz)

P = 0.69

Control   NMB

Ad

Amplitude (pA)

P = 0.15

Control   NMB

Ba

TRPV1 WT

Control

NMB

Wash

15 pA
5 s

Bb

TRPV1 KO

Control

NMB

Wash

20 pA
5 s

Bc

— ●— TRPV1 WT
— ○— TRPV1 KO

NMB

Normalized sEPSC events

Time (min)

Bd

P = 0.007

Frequency (Hz)

Control   NMB

TRPV1 WT

Be

P = 0.002

Amplitude (pA)

Control   NMB

TRPV1 WT

Bf

P = 0.33

Frequency (Hz)

Control   NMB

TRPV1 KO

Be

P = 0.81

Amplitude (pA)

Control   NMB

TRPV1 KO

Ca

AMG9810+TTX

Control

NMB

Wash

30 pA
5 s

Cb

— ●— Control
— ○— AMG

TTX

NMB

Normalized mEPSC events

Time (min)

Cc

Frequency (Hz)

P = 0.59

Control   NMB

Cd

Amplitude (pA)

P = 0.73

Control   NMB

94

application of NMB did not significantly affect the frequency (baseline: 13.7 ± 3.1 Hz,

NMB: 12.9 ± 2.5 Hz, 105 ± 15 % of baseline, n = 9, P = 0.59, Figure 16. *Ca-Cc*) or

amplitude (baseline: 15.4 ± 0.75 pA, NMB: 15.5 ± 1.0 pA, 100 ± 4 % of baseline, n = 9,

P = 0.73, Wilcoxon test, Figure 16. *Ca, Cd*) of the mEPSC events. Taken together, these

data suggest NMB can enhance presynaptic glutamate release in BLA neurons requiring

TRPV1 channels.

However, if NMB potentiates glutamate release via TRPV1 channels, it stands to

reason that NMB should further augment evoked EPSC because voltage-gated calcium

influx will also be functional during eEPSCs. We recorded eEPSCs from BLA neurons by

placing a stimulating electrode in the LA. Unexpectedly, application of NMB produced a

significant, albeit transient, diminution in eEPSC amplitudes (89.3 ± 5.5 % of control,

 n = 24, P = 0.03 vs baseline, Figure 17. *Aa*) that returned to baseline levels during the

wash of NMB in our whole-cell configuration (95.3 ± 6.2 % of control, P = 0.46 vs

baseline, Figure 17. *Aa*). The effects of NMB on eEPSC could be due to its action on

BB1 receptors at pre- or postsynaptic sites. We then differentiated the pre- and

postsynaptic effects by calculating the coefficient of variation (CV) and paired-pulse ratio

(PPR) of BLA EPSCs before and after the application of NMB, as alterations in these

parameters are associated with altered presynaptic glutamate release. NMB significantly

increased the value of CV (n = 24, P = 0.008, paired t-test, Figure 17. *Ab*), as well as the

PPR (n = 11, P = 0.02 paired t-test, Figure 17. *Ac*) supporting a presynaptic mechanism.

We next tested whether NMB suppressed glutamate release through a TRPV1-mediated

mechanism. In slices treated with AMG9810 to block TRPV1 channels, application of

NMB failed to significantly alter the amplitude of BLA eEPSCs (101 ± 3 % of baseline,

n = 9, P = 0.83; Figure 17. *Ba*). These results suggest that NMB decreased eEPSCs by

a short-term depression of presynaptic glutamate release, independent of TRPV1 channels.

It is possible that the action of NMB generates a retrograde messenger to depress evoked glutamate release. The cannabinoid 1 (CB1) receptor is activated by 2-AG, an endogenous endocannabinoid generated by DAG lipase via retrograde signaling to suppress synaptic transmission [321]. To examine this possibility, slices were treated with AM251 (10 µM), a CB1 receptor inhibitor, to block CB1 receptor activation. AM251 alone does not affect eEPSCs [307]; therefore, AM251 was included in all solutions prior to NMB application. Activation of the BB1 receptor in slices treated with AM251 produced an initial increase in BLA eEPSCs to 143 ± 16 % of baseline (n = 16, P = 0.02, Figure 17. *Bb*) that returned to baseline (106 ± 11% of baseline, P = 0.52, Figure 17. *Bb*) before significantly increasing to 227 ± 45% of the baseline value throughout the wash (P = 0.01, Figure 17. *Bb*). Together, these results indicate that NMB potentiates spontaneous glutamate release requiring TRPV1 channels and suppresses evoked glutamate release, at least in part, through presynaptic CB1 receptor activation.

BB1 Receptor Activation in the BLA Produces Anxiogenic Phenotypes

The BLA is involved in motivational regulation and sensory processing [15] and sends projections to other brain regions involved in stress coping behavior including the BNST [322]. In our present study, we demonstrate that NMB enhanced spontaneous glutamate transmission in the BLA. Because augmented glutamate release results in anxiety [323] and NMB and its receptor have been implicated in fear and anxiety-like responses [223, 239, 272], we investigated the role of BB1 receptor activation on anxiety-like behaviors in the BLA. VCT is a well validated anxiety-model utilizing avoidance-approach behaviors in which water deprived animals are presented with a conflict between receiving a mild foot shock as they drink from a water bottle [233].

Figure 17.   **BB1 receptors suppress evoked glutamate release through a presynaptic mechanism.**
Aa-Ac, Activated BB1 receptors suppress evoked glutamate release in BLA neurons through a presynaptic mechanism. *Aa*, time course of eEPSCs averaged from 24 BLA neurons. Current traces averaged from 10 EPSC events before (1), during (2), and after (3) application of NMB are shown in the upper right. *Ab*, SD and mean were obtained by averaging 15 consecutive EPSCs. Top shows eEPSC recorded before (left) and during (right) the application of NMB. Bottom shows the calculated CVs from 24 cells (open circles) and their average (filled circles). *Ac*, Top left, EPSCs averaged from at least 25 current traces before (black, bold) and during (grey, thin) the application of NMB. Top right, EPSCs recorded before (black, bold) and during (thin, grey) application of NMB were scaled to the first EPSC. Note that the second EPSC during the application of NMB is larger than control. Bottom, paired pulse ratio (PPR = P2/P1, P1 and P2 are the EPSCs evoked by two stimuli at an interval of 50 ms) recorded from 9 cells (open circles) and their average (filled circle). *Ba*, time course of eEPSC events averaged from 9 BLA neurons in the continuous presence of AMG9810. Current traces averaged from 10 EPSC events before (1), during (2), and after (3) application of NMB are displayed in the upper left. *Bb*, time course of eEPSC events averaged from 16 BLA neurons in the continuous presence of AM251. Current traces averaged from 10 EPSC events before (1), during (2), and after (3) application of NMB are shown in the upper left.

Aa

NMB

200 pA
10 ms

Normalized eEPSC

Time (min)

R_in

Time (min)

Ab

Control   NMB

100 pA
20 ms

P = 0.008

CV

Control   NMB

Ac

80 pA
15 ms

Control
NMB

P = 0.02

PPR

Control   NMB

Ba

150 pA
10 ms

AMG9810

NMB

Normalized eEPSC

Time (min)

R_in

Time (min)

Bb

100 pA
10 ms

AM251

NMB

Normalized eEPSC

Time (min)

R_in

Time (min)
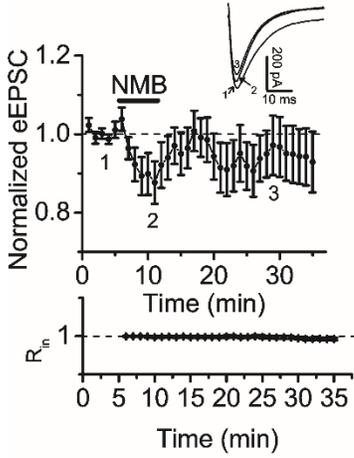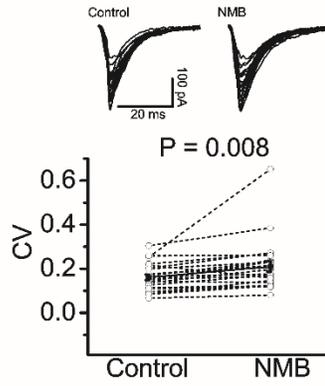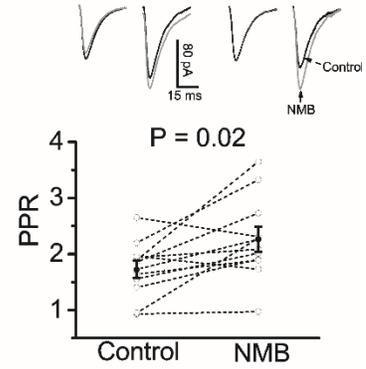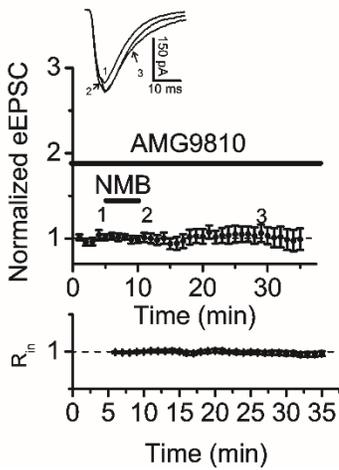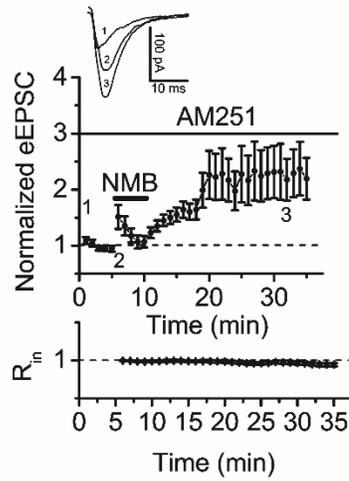
To probe the effects and underlying mechanisms of BB1 receptor activation on VCT, we microinjected NMB and/or other compounds into the BLA prior to VCT (Figure 18. *A*).

Microinjection of NMB into the BLA dose-dependently reduced the number of licks compared to rats injected with saline (0.9% NaCl) (Figure 18, *Ba*). Whereas microinjection of NMB at 0.1 nmol did not significantly alter the number of licks in the VCT ($P = 0.45$), the number of licks was significantly reduced in rats injected with 0.3 nmol NMB ($P = 0.038$) and 1 nmol NMB ($P = 0.0007$, one-way ANOVA followed by Dunnett's multiple comparisons test, Figure 18. *Ba*). These data suggest that application of NMB into the BLA exerts anxiogenic effects. Rats were microinjected with 1 nmol NMB in the remaining experiments to understand the role of NMB in anxiety-like behaviors. We tested the involvement of BB1 receptors in NMB-mediated anxiety-like behaviors by microinjecting the selective BB1 receptor antagonist, BIM23042 (0.3 nmol) [223, 250]. Rats injected with BIM23042 displayed a significantly larger number of licks than those injected with saline ($P = 0.032$, one-way ANOVA followed by Tukey's multiple comparisons test, Figure 18. *Bb*). Whereas microinjection of NMB following BIM23042 significantly lessened NMB-mediated anxiogenic effects (1 nmol NMB vs. BIM+NMB, $P = 0.011$, one-way ANOVA followed by Tukey's test, Figure 18. *Bb*), application of NMB counteracted the enlarged number of licks seen in rats injected with BIM23042 alone (BIM23042 vs. BIM+NMB, $P = 0.014$, one-way ANOVA followed by Tukey's test, Figure 18. *Bb*). One possibility to explain the anxiolytic effect of BIM23042 is that endogenous NMB produces anxiogenic phenotypes within the BLA. Together, these results indicate that BB1 receptors are required for NMB-mediated anxiogenic effects in the BLA.
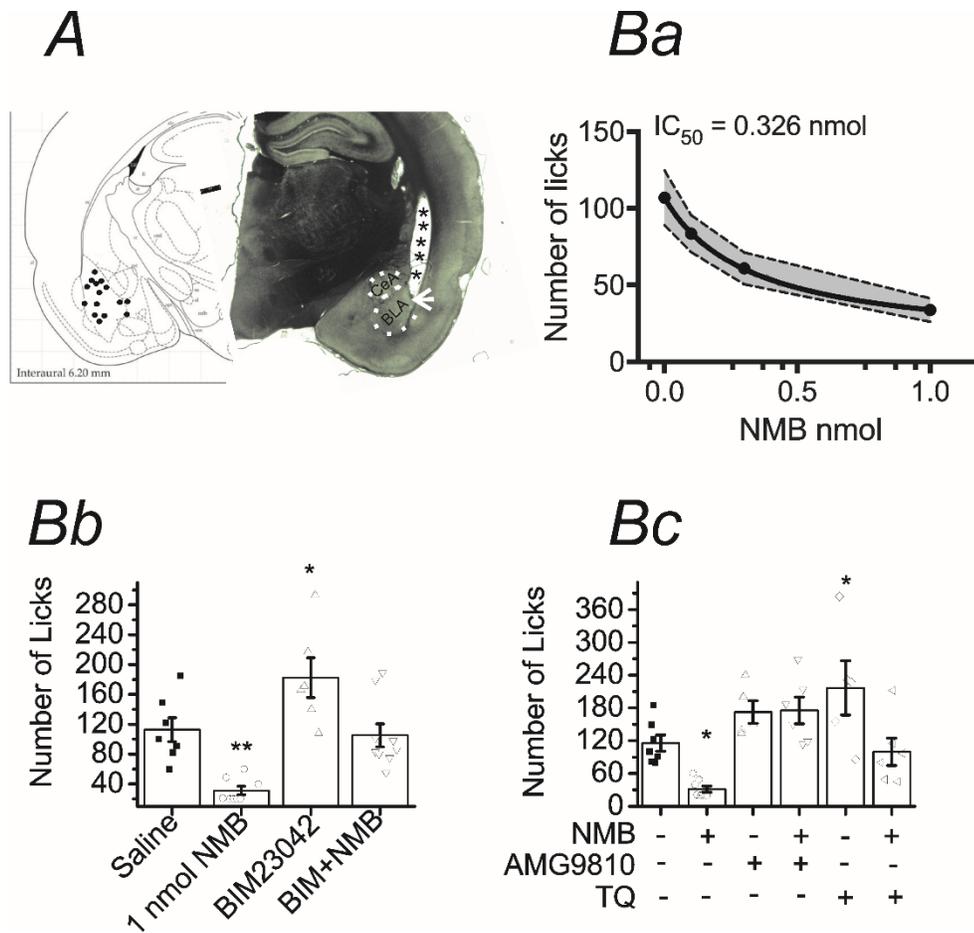
Figure 18. **Application of NMB into the BLA dose-dependently increase anxiety-like behaviors.**

A, Representative coronal section displaying (right) proper cannula placement and (left) cannula locations displayed onto atlas figure adapted from Paxinos and Watson [221]. Note: all cannula placements were verified, dots represent a subset of rats used in these experiments. *Ba,* microinjection of NMB dose-dependently reduced lick numbers. Shaded grey area represents SEM. *Bb,* microinjection of NMB (1 nmol) into the BLA reduced lick number, whereas prior injection of the BB1 receptor antagonist, BIM23042 (0.3 nmol), alleviated NMB-mediate anxiety-like behaviors. *Bc,* TRPV1 channel and GIRK channels are involved in NMB-mediated anxiety-like behaviors in the BLA. * $P < 0.05$, ** $P < 0.01$ vs saline (one-way ANOVA followed by Dunnett's test).

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TRPV1 and GIRK Channels are Involved in BB1 Receptor-Mediated Anxiety-Like Behaviors in the BLA

Our *in vitro* results indicate that activation of BB1 receptors promotes excitability in BLA neurons by opening TRPV1 channels and depression of GIRK channels. We further explored the roles of these ion channels in NMB-mediated anxiety. Microinjection of AMG9810 at 1 nmol, an effective dose demonstrated previously [324, 325], had no effect on the number of licks (saline vs. AMG9810, P = 0.35, one-way ANOVA followed by Dunnett's test, Figure 18. *Bc*), but blocked NMB-induced anxiogenic effects (saline vs. AMG9810+NMB, P = 0.26, one-way ANOVA followed by Dunnett's test, Figure 18. *Bc*). These results demonstrate the TRPV1 channels are involved in BB1-receptor-mediated anxiogenic effects.

We then tested the involvement of GIRK channels in BB1 receptor-mediated anxiogenic effects. In rats microinjected with tertiapin-Q at 250 pmol [223, 224] the number of licks was significantly larger than in saline treated mice (saline vs. tertiapin-Q, P = 0.03, one-way ANOVA followed by Dunnett's test, Figure 18. *Bc*), whereas microinjection of NMB after tertiapin-Q failed to produce anxiety-like behaviors (saline vs. TQ+NMB, P = 0.63, one-way ANOVA followed by Dunnett's test, Figure 18. *Bc*). These results suggest that GIRK channels are involved in BB1-receptor enhancement of anxiety-like behaviors. A summary of the findings from Study 2 is shown in Figure 27.
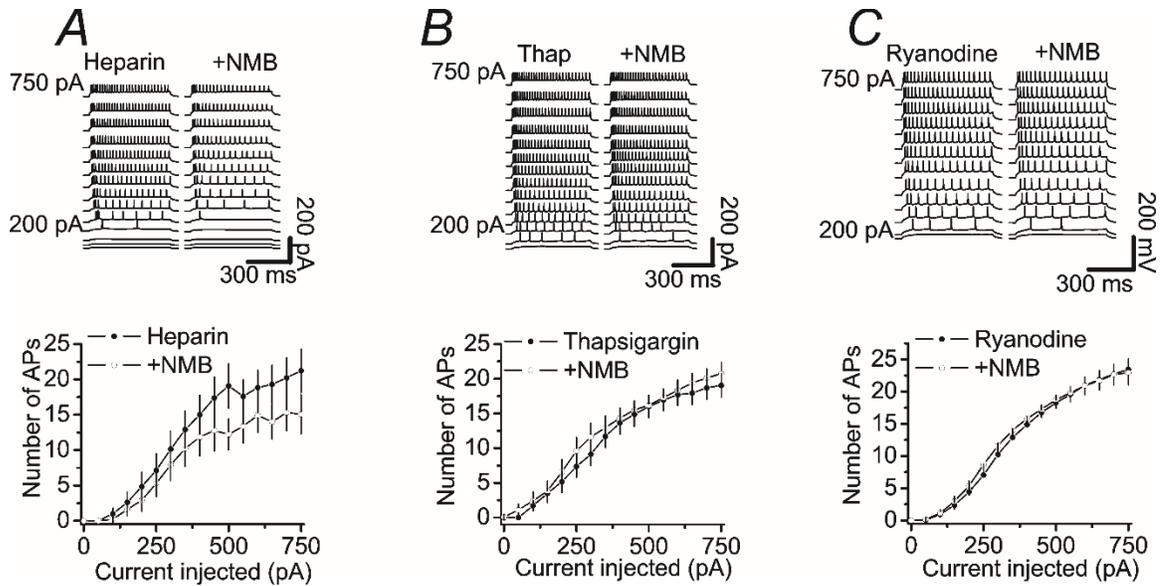
Supplementary Figure 1.   **BB1 receptor-mediated excitation requires release of intracellular Ca^{2+}.**

A, representative traces (upper) and summary graph (lower) of NMB-augmentation of APs in response to the positive current injection protocol in slices recorded with intracellular application of the IP$_3$ receptor inhibitor heparin (0.5 mg/ml). *B*, example of AP traces (upper) and summary graphs (lower) of NMB-mediated APs in response to the positive current injection protocol with the intracellularly dialyzed SERCA inhibitor, thapsigargin (10 μM). *C*, representative AP traces (upper) and summary graphs (lower) of NMB-elicited APs in response to the positive current injection protocol with intracellular application of the RyR receptor inhibitor ryanodine (100 μM).

**Study 3- Neurokinin-3 Receptor-Mediated Modulation of
Basolateral Amygdala Neurons**

Introduction

The tachykinins refer to the peptides encoded in rodents by the Tachykinin 1 (Tac1) and Tac2 (TAC3 in humans) genes, which are involved in neurotransmission and neuromodulation in the central nervous system [326]. Tac1 encodes a precursor protein that produces two peptides, substance P (SP) and neurokinin A (NKA), whereas Tac2/TAC3 encodes neurokinin B (NKB). SP, NKA and NKB interact respectively with the G-protein-coupled neurokinin receptors, NK1Rs, NK2Rs, and NK3Rs [327, 328]. These receptors are coupled to the pertussis toxin-insensitive G proteins $G_{q/11}$ to activate phospholipase Cβ (PLCβ) to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2), resulting in the production of inositol 1,4,5-triphosphate (IP_3) to facilitate intracellular Ca^{2+} release and diacylglycerol (DAG) to activate protein kinase C (PKC) [329, 330], although activation of adenylate cyclase, resulting in accumulation of cAMP and stimulation of protein kinase A and activation of phospholipase A2 and generation of arachidonic acid have been reported as well [330]. In mammals, tachykinins serve as neuromodulators or neurotransmitters in central brain circuits, as well as in pain, stress, anxiety, depressive disorder, aggression, memory formation, inflammation and hormone regulation [330-333]. However, the cellular and molecular mechanisms underlying tachykinins-mediated modulation of these physiological functions and pathological disorders have not been fully determined.

The amygdala is one of the important targets of tachykinins, as demonstrated that the amygdala contains SP [334-336], NKA [337] and NKB [338-340]. Furthermore, the amygdala expresses NK1Rs [341, 342], NK2Rs [343, 344] and NK3Rs [345-350]. With regard to NK3Rs, high densities of NK3R protein [345-347, 351] and NKR3 mRNA [349] are distributed in the BLA in animals and in humans [350]. While NKB and NK3Rs in the

CeA have been reported to be involved in the consolidation of fear memories [339, 352], the functions of NK3Rs in the BLA have not been determined. In Study 3, we showed that activation of NK3Rs in the BLA facilitated the excitability of BLA principal neurons by activating TRPC4/5 channels and depressing inwardly rectifying K^+ (Kir) channels via PLCβ-mediated hydrolysis of PIP_2. We further showed that microinjection of senktide into the BLA increased fear-potentiated startle (FPS) response via activation of TRPC4/5 channels and depression of Kir channels. Our results provide a cellular and molecular mechanism to explain the roles of tachykinins in fear and anxiety responses.

Activation of NK3Rs Excites BLA Principal Neurons

Because high densities of NK3Rs are expressed in the BLA neurons of a variety of species including rats [345-347] and humans [347, 350] and activation of NK3Rs increases the expression of c-fos, a marker of neuronal activity, in the amygdala [348, 353], we probed the effects of NK3R activation on the excitability of principal neurons in rat BLA (Figure. 19A). The extracellular solution contained kynurenic acid (1 mM) to block glutamatergic transmission and picrotoxin (100 µM) to block GABAergic transmission. The intracellular solution was the K^+-gluconate intracellular solution. Under these circumstances, the effects of NK3R agonists should be from the recorded neurons. Bath application of the selective NK3R agonist, senktide at 0.3 µM which is a near-saturating concentration (see below), induced subthreshold depolarization (Control: -63.8 ± 3.1 mV, Senktide: -61.1 ± 3.2 mV, n = 20, P < 0.0001, Wilcoxon test, Figure 19Ba-b). We then injected a depolarizing current to elevate the membrane potential to just above the firing threshold (-50.1 ± 4.3 mV, n = 16) to elicit sparse AP firing and further tested the effect of senktide on the excitability of BLA neurons. Bath application of the same concentration of senktide for 5 min significantly augmented the AP firing frequency (Control: 0.33 ± 0.23 Hz, Senktide: 3.71 ± 2.34 Hz, n = 16,

$P < 0.0001$, Wilcoxon test, Figure. 19Ca-c) and the persistent increases of AP firing were still observed after wash in senktide-free extracellular solution for ~1 h (1.80 ± 1.39 Hz, n = 16, $P < 0.0001$ vs. Control, Wilcoxon test, Figure. 19Ca-c). Furthermore, application of senktide significantly increased AP firing numbers elicited by injecting a series of positive currents from 50 pA to 700 pA at an increment of 50 pA (n = 14, $F_{(1,13)} = 21.26$, $P = 0.0005$, Two-way repeated measures ANOVA followed by Šídák's multiple comparison tests, Figure. 19Da-b). These results together indicate that bath application of senktide significantly enhanced the excitability of BLA neurons.

To test the involvement of NK3Rs, we pretreated the slices with the selective NK3R antagonist, SB 218795 (3 µM) [354] and the bath was continuously perfused with the same concentration of SB 218795. Under these circumstances, bath application of senktide failed to increase AP firing numbers significantly (n = 18, $F_{(1,17)} = 1.609$, $P = 0.222$, Two-way repeated measures ANOVA followed by Šídák's multiple comparison tests, Figure. 19Ea-b), suggesting the involvement of NK3Rs. Because neurokinin B (NKB) is an endogenous agonist for NK3Rs, we also tested the effects of NKB on membrane potentials and AP firing numbers. Bath application of NKB (0.3 µM) also elicited subthreshold depolarization (Control: -65.3 ± 4.7 mV, NKB: -61.9 ± 4.6 mV, n = 7, $P = 0.016$, Wilcoxon test, Figure. 19F). We then injected a persistent positive current to elevate the membrane potentials to just above the firing threshold (- 49.9 ± 2.9 mV, n = 8) and tested the effect of NKB on AP firing. Bath application of NKB (0.3 µM) significantly enhanced the AP firing frequency (Control: 0.35 ± 0.25 Hz, NKB: 2.99 ± 1.65 Hz, n = 8, $P = 0.008$, Wilcoxon test, Figure. 19Ga-c). Likewise, bath application of NKB at the same concentration significantly augmented the firing number of APs evoked by injecting the positive currents from 50 pA to 700 pA at an increment of 50 pA (n = 13, $F_{(1,12)} = 30.91$, $P = 0.0001$, Two-way repeated measures ANOVA

followed by Šídák's multiple comparison tests, Figure. 19Ha-b). These results together indicate that activation of NK3Rs excites the BLA principal neurons.

Stimulation of NK3Rs Activates a Cation Channel and Inhibits an Inwardly Rectifying K⁺ Channel

We further identified the ionic mechanisms whereby activation of NK3Rs facilitates the excitability of BLA neurons. Opening of cation channels and depression of K^+ channels are the two common ionic mechanisms underlying neuronal excitability. Bath application of senktide (0.3 µM) elicited an inward current recorded at -60 mV with the K^+-gluconate internal solution (-42.7 ± 20.0 pA, n = 17, $P < 0.0001$ vs. baseline, Wilcoxon test, Figure. 20Aa and Ad). The EC_{50} value of senktide was calculated to be 0.064 µM (Figure. 20Ab). We therefore used 0.3 µM senktide for the remaining experiments because this is a near-saturating concentration. Replacement of the K^+-gluconate-containing intracellular solution with the Cs^+-gluconate-containing solution to limit the contribution of K^+ channels significantly ($P < 0.0001$, Mann-Whitney test) reduced the senktide-induced inward holding currents (-16.7 ± 18.1 pA, n = 25, $P < 0.0001$ vs. baseline, Wilcoxon test; $P < 0.0001$ vs. the senktide-induced inward currents recorded in K^+-containing internal solution, Mann-Whitney test, Figure. 20Ac-d), suggesting that depression of K^+ channels is one mechanism responsible for NK3R-mediated excitation of BLA neurons.

We further measured the I-V relationship of senktide-induced currents to probe the ionic mechanisms whereby activation of NK3Rs excites BLA neurons. Among the 22 BLA pyramidal neurons recorded, 11 cells showed an I-V curve suggestive of non-selective cation channels, i.e., senktide elicited persistent inward currents at the voltages tested (Figure. 20Ba-Bc) and 11 cells displayed an I-V curve resembling that of the inwardly rectifying K^+ (Kir) channels with a reversal potential at -88.7 ± 17.5 mV

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Figure 19. **Activation of NK3Rs excites BLA principal neurons.**
A, microscopic photograph of amygdala to show the location of BLA where electrophysiological recordings were conducted. LA, lateral nucleus; BLA, basolateral nucleus; CeC, capsular central amygdala; CeL, lateral central amygdala; CeM, medial central amygdala. **Ba-Bb**, Bath application of the selective NK3R agonist, senktide (0.3 µM), induced subthreshold depolarization of BLA neurons. **Ba**, RMP recorded from a BLA principal neuron prior to, during and after application of senktide. **Bb**, Summary data for senktide-elicited subthreshold depolarization. Empty symbols were data from individual cells and solid symbols were their averages (n = 20). **Ca-Cc**, Application of senktide increased AP firing frequency when a positive current was injected persistently to induce initial sparse AP firing. **Ca**, APs recorded from a BLA neuron prior to, during and after application of senktide when a positive current was injected persistently to induce basal sparse AP firing. **Cb**, APs recorded at the time points indicated in **Ca** in an expanded scale. **Cc**, Summary data showing senktide-induced excitation of BLA neurons (n = 16). **Da-Db**, Bath application of senktide augmented AP firing elicited by injection of a series of positive currents from 50 pA to 700 pA at an increment of 50 pA and duration of 600 ms every 6 seconds. **Da**, APs recorded from a BLA neuron evoked by the positive current injection protocol before (*left*) and during (*right*) the application of senktide. **Db**, Relationship between the injected currents and the elicited AP numbers from 14 BLA neurons. **Ea-Eb**, Senktide-elicited excitation of BLA neurons was mediated by activation of NK3Rs. **Ea**, APs recorded from a BLA neuron evoked by the positive current injection protocol before (*left*) and during (*right*) the application of senktide in a slice pretreated with the selective NK3 receptor antagonist, SB 218795 (3 µM). The extracellular solution continuously contained the same concentration of SB 218795. **Eb**, Relationship between the injected currents and the elicited AP numbers from 18 BLA neurons prior to and during the application of senktide in the continuous presence of SB 218795. **F**, Bath application of neurokinin B (NKB, 0.3 µM), the endogenous NK3 receptor agonist, depolarized BLA neurons. Empty symbols were data from individual cells and solid symbols were their averages (n = 7). **Ga-Gc**, Application of NKB (0.3 µM) increased AP firing frequency when a positive current was injected persistently to induce initial sparse AP firing. **Ga**, APs recorded from a BLA neuron prior to, during and after application of NKB when a positive current was injected persistently to induce basal sparse AP firing. **Gb**, APs recorded at the time points indicated in **Ga** in an expanded scale. **Gc**, Summary data showing NKB-induced excitation of BLA neurons (n = 8). **Ha-Hb**, Application of NKB (0.3 µM) augmented AP firing numbers evoked by injection of a series of positive currents from 50 pA to 700 pA at an increment of 50 pA recorded from the BLA neurons. **Ha**, APs recorded by the current injection protocol before (*left*) and during (*right*) the application of NKB. **Hb**, Relationship between the injected currents and the elicited AP numbers from 13 BLA neurons before and during the application of NKB. Adapted from [224].

A

500 μm

CeC
CeL
CeM
LA
BLA

Ba

Senktide

−60.5 mV

5 mV
3 min

b

P < 0.0001

−50
−55
−60
−65
−70

RMP (mV)

Control   Senktide

Ca

Senktide

20 mV
5 min

1 2   3

b

1
2
3

80 mV
3 s

c

500
400
300
200
100
0

Numbers of APs/min

Senktide

0 10 20 30 40 50 60 70
Time (min)

Da

Control   Senktide

700 pA

50 pA

300 mV
300 ms

b

Senktide

25
20
15
10
5
0

Number of APs

** ** ** ****** ****

Control

0 200 400 600
Current injected

Ea

SB 218795   +Senktide

700 pA

50 pA

300 mV
300 ms

b

SB 218795
+ Senktide

30

20

10

0

Number of APs

0   200   400   600
Current injected (pA)

F

P = 0.016

−55
−60
−65
−70
−75

RMP (mV)

Control   NKB

Ga

NKB

20 mV
5 min

1 2   3

b

1
2
3

80 mV
3 s

c

300
250
200
150
100
50
0

Numbers of AP/min

NKB

0 10 20 30 40 50 60 70
Time (min)

Ha

Control   Neurokinin B

700 pA

50 pA

300 mV
300 ms

b

NKB

35
30
25
20
15
10
5
0
−5

Number of APs

********** ****

Control

0   200   400   600
Current injected (pA)

108

(n = 11) (Figure. 20Ca-Cc), close to the calculated K^+ reversal potential (-95.8 mV). One

plausible explanation for the difference of the measured reversal potential and the

calculated K^+ reversal potential is that the latent effect of senktide on the cation channels

right-shifted the measured K^+ reversal potential. We further used Cs^+-gluconate-

containing intracellular solution to block K^+ channels and included TTX (0.5 µM) to block

Na^+ channels and $CdCl_2$ (200 µM) and $NiCl_2$ (400 µM) to block Ca^{2+} channels. We

measured the reversal potential of senktide-elicited cation channel currents by extending

the voltage ranges to +20 mV. Under these circumstances, the I-V curve of the senktide-

induced currents showed outward rectification with a reversal potential of

-26.8 ± 18.0 mV (n = 18, Figure. 20Da-Dc). These results together suggest that

activation of NK3Rs excites BLA principal neurons by opening a non-selective cation
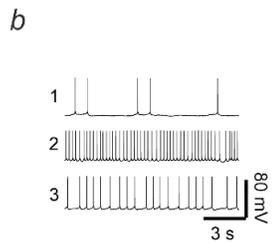
channel and depressing a Kir channel.

We further tested the roles of Kir channels in senktide-induced inward holding

currents. Kir channels are sensitive to micromolar concentration of Ba^{2+} [355-357]. Bath

application of Ba^{2+} (500 µM), by itself induced an inward holding current

(- 42.7 ± 20.4 pA, n = 20, *P* < 0.0001, Wilcoxon test, Figure. 21A, 3F) and significantly

reduced senktide-elicited inward currents (-18.8 ± 13.0 pA, n = 20, *P* = 0.0008 vs.

senktide alone, one-way ANOVA followed by Dunnett's test, Figure. 21A, 3G). Kir

channels include Kir2, Kir3 (GIRK) and Kir6 (ATP-sensitive, K_{ATP}) subfamilies and the K^+

transport channels [358]. We utilized ML 133, a specific antagonist for Kir2 subfamily

[256-260] to test the roles of the Kir2 subfamily in senktide-elicited excitation of BLA

principal neurons. Bath application of ML 133 (30 µM) by itself did not change the

holding currents significantly (-0.02 ± 7.63 pA, n = 15, *P* = 0.847, Wilcoxon test,

Figure. 21B, 21F). In the presence of ML 133, application of senktide still elicited a

comparable inward current (-40.9 ± 23.9 pA, n = 15, *P* = 0.996 vs. senktide alone,

Figure 20. **Senktide-elicited excitation of BLA neurons is mediated by opening a cation channel and inhibiting a Kir channel.**

Aa-Ad, Activation of NK3Rs concentration-dependently excited BLA neurons. **Aa**, Trace of inward current induced by bath application of senktide recorded from a BLA neuron with K^+-gluconate-containing intracellular solution. **Ab**, Concentration-response curve of senktide-induced inward currents recorded with K^+-gluconate-containing intracellular solution. The empty symbols were the inward currents from individual cells and the solid symbols were their averages. **Ac**, Trace of inward current induced by bath application of senktide recorded from a BLA neuron with Cs^+-gluconate-containing intracellular solution. **Ad**, Summary data showing senktide-induced inward currents with K^+-containing intracellular solution (solid circles) and Cs^+-containing intracellular solution (empty circles). The bars were their averages. **Ba-Bc**, Senktide opened a cation channel. The extracellular solution contained TTX (0.5 µM), kynurenic acid (1 mM) and picrotoxin (100 µM) and the intracellular solution was the K^+-gluconate-containing internal solution. **Ba**, Currents elicited by a voltage-step protocol before (*left*) and during (*middle*) bath application of senktide and the net current obtained by subtraction (*right*) from a BLA neuron. The dash line was the zero current level. **Bb**, I-V curve averaged from 11 cells before and during application of senktide (Two-way repeated measures ANOVA followed by Šídák's multiple comparison test; Drug: $F_{(1, 10)} = 30.03$, $P = 0.0003$; Voltage: $F_{(10, 100)} = 42.98$, $P < 0.0001$; Drug x Voltage: $F_{(10, 100)} = 3.056$, $P = 0.002$; ** $P < 0.0001$). **Bc**, I-V curve of the net current obtained by subtracting the currents in control condition from those after application of senktide. **Ca-Cc**, Senktide depressed a Kir channel. The extracellular solution contained TTX (0.5 µM), kynurenic acid (1 mM) and picrotoxin (100 µM) and the intracellular solution was the K^+-gluconate-containing internal solution. **Ca**, Currents elicited by the voltage-step protocol before (*left*) and during (*middle*) bath application of senktide and the net current obtained by subtraction (*right*) from a BLA neuron. The dash line was the zero current level. **Cb**, I-V curve averaged from 11 cells before and during application of senktide (Two-way repeated measures ANOVA followed by Šídák's multiple comparison test; Drug: $F_{(1, 10)} = 2.553$, $P = 0.141$; Voltage: $F_{(10, 100)} = 80.87$, $P < 0.0001$; Drug x Voltage: $F_{(10, 100)} = 14.51$, $P < 0.0001$; ** $P < 0.001$). **Cc**, I-V curve of the net current obtained by subtracting the currents in control condition from those during application of senktide. **Da-Dc**, Senktide opened a cation channel of outward rectification recorded in the extracellular solution containing 0.5 µM TTX, 1 mM kynurenic acid, 100 µM picrotoxin, 200 µM $CdCl_2$ and 400 µM $NiCl_2$ and intracellular solution was Cs^+-gluconate-containing intracellular solution. **Da**, Currents elicited by the voltage-step protocol before (*left*) and during (*middle*) bath application of senktide and the net current obtained by subtraction (*right*) from a BLA neuron. The dash line was the zero current level. **Db**, I-V curve averaged from 18 cells before and during the application of senktide (Two-way repeated measures ANOVA followed by Šídák's multiple comparison test; Drug: $F_{(1, 17)} = 2.701$, $P = 0.119$; Voltage: $F_{(14, 238)} = 146.7$, $P < 0.0001$; Drug x Voltage: $F_{(14, 238)} = 27.83$, $P < 0.0001$; * $P = 0.05$, ** $P < 0.01$). **Dc**, I-V curve of the net current obtained by subtracting the currents in control condition from those during application of senktide. Adapted from [224].
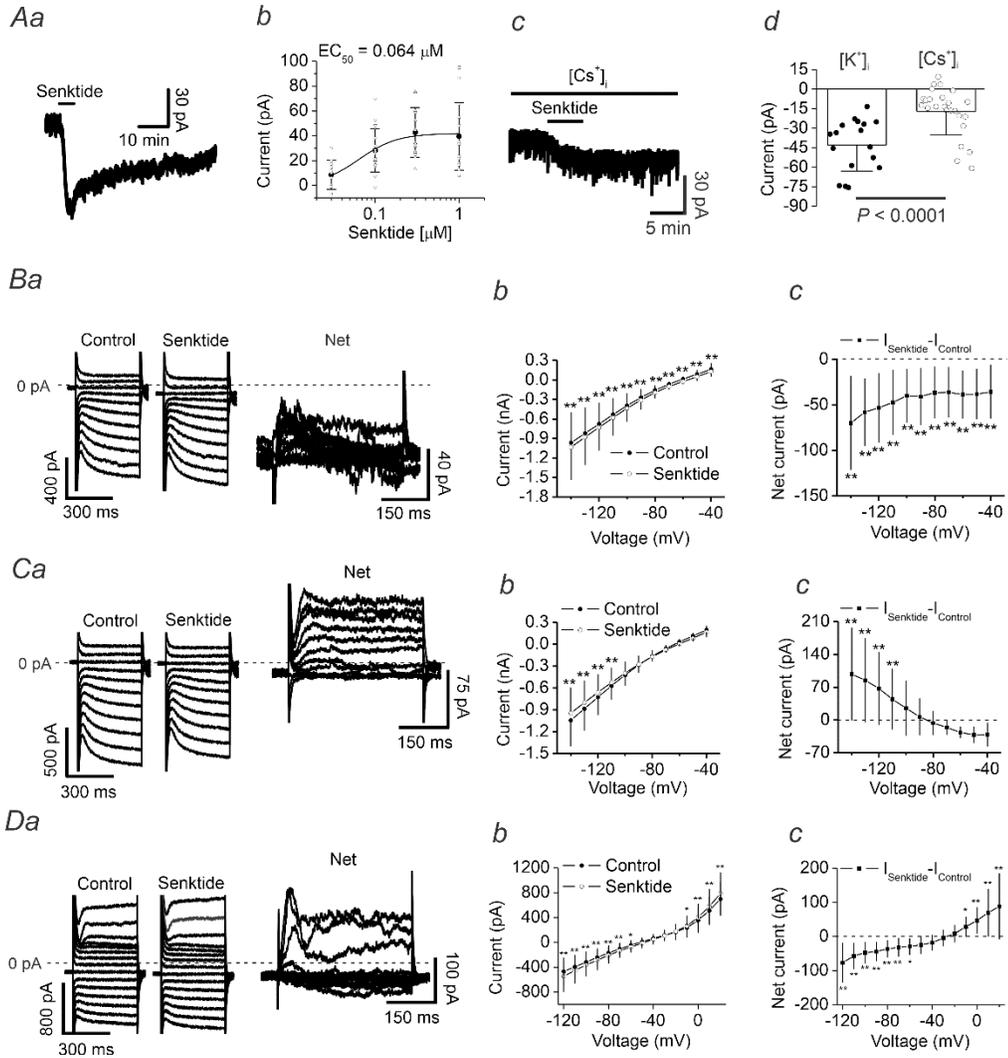
one-way ANOVA followed by Dunnett's test, Figure. 21B, 21G), suggesting that Kir2

subfamily is not the type of Kir channels involved in senktide-elicited excitation. We then

tested the roles of K_{ATP} channels in senktide-induced inward currents. Bath application of

the selective K_{ATP} channel blocker, glibenclamide (100 μM) did not significantly alter the

holding currents in BLA neurons (-4.5 ± 13.7 pA, n = 19, P = 0.490, Wilcoxon test,

Figure. 21C, 21F) and exerted no effect on senktide-elicited inward currents

(-32.1 ± 22.3 pA, n = 19, P = 0.261 vs. senktide alone, one-way ANOVA followed by

Dunnett's test, Figure. 21C, 21G), suggesting that K_{ATP} channels are not involved. We

then tested the roles of GIRK (Kir3) subfamily in senktide-elicited excitation. Application

of the selective GIRK channel blocker, tertiapin-Q (500 nM) induced an inward current

by itself (-30.6 ± 18.6 pA, n = 16, P < 0.0001, Wilcoxon test, Figure. 21D, 21F),

suggesting the expression of GIRK channels in the BLA principal neurons. In the

continuous presence of tertiapin-Q, application of senktide induced a significantly

smaller inward current (-16.0 ± 11.4 pA, n = 15, P = 0.0004 vs. senktide alone, one-way

ANOVA followed by Dunnett's test, Figure. 21D, 21G), suggesting that GIRK channels

are involved in senktide-mediated excitation of BLA pyramidal neurons. Consistent with

the involvement of GIRK channels, application of ML 297 (10 μM), an activator of

GIRK1-containing channels [262], induced an outward current (76.3 ± 50.0 pA, n = 7,

P = 0.016 vs. baseline, Wilcoxon test, Figure. 21E-F), suggesting that GIRK1 channels

are functionally expressed in the BLA principal neurons, and they may be involved in

senktide-elicited excitation of BLA principal neurons.
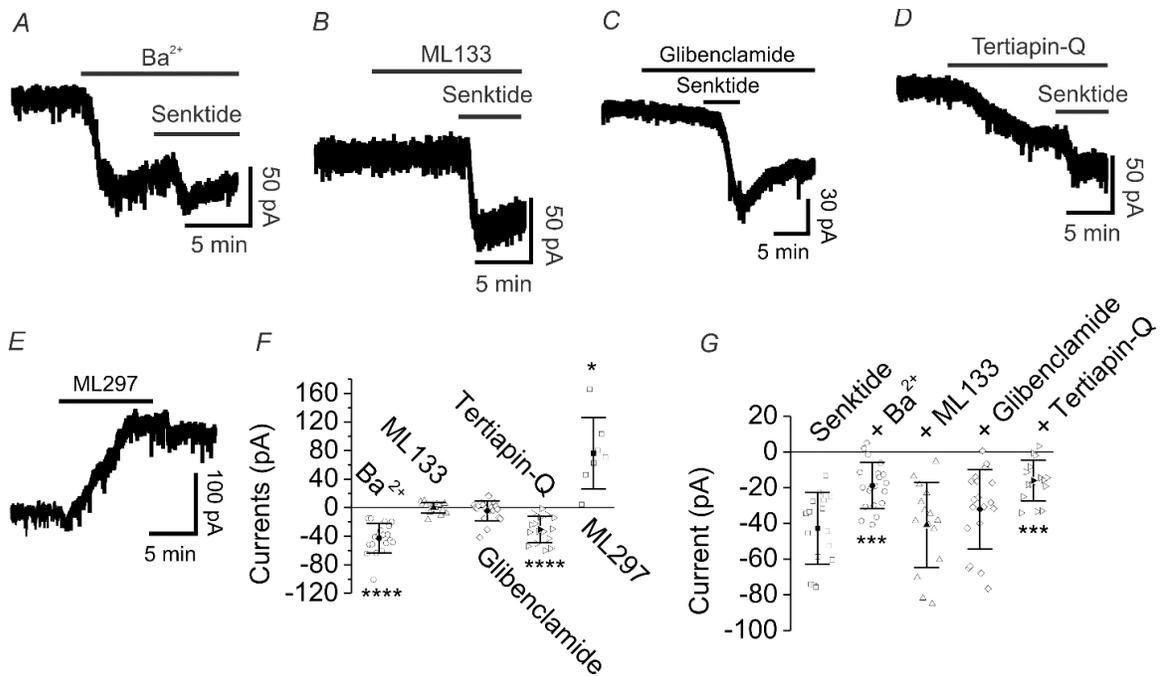
Figure 21.   GIRK channels are required for NK3R-elicited inward currents in BLA neurons.

 A, current trace recorded from a BLA neuron in response to Ba^{2+} (500 µM) alone and Ba^{2+} plus senktide. **B**, current trace recorded from a BLA neuron in response to ML133 (30 µM) alone and ML133 plus senktide. **C**, current trace recorded from a BLA neuron in response to bath application of glibenclamide (100 µM) alone and together with senktide. **D**, current trace recorded from a BLA neuron in response to tertiapin-Q (500 nM) alone and tertiapin-Q plus senktide. **E**, current trace recorded from a BLA neuron in response to bath application of ML297 (10 µM). **F**, summary data for the effects of Kir channel modulators on BLA neurons. **** $P < 0.0001$ vs. baseline, Wilcoxon test. **G**, Summary graph showing the effects of Kir channel blockers on senktide-mediated inward currents. *** $P < 0.001$ vs. senktide alone, one-way ANOVA followed by Dunnett's test. Adapted from [224].

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TRPC4 and TRPC5 Channels are Involved in NK3R-Elicited Excitation of BLA Principal Neurons

We further identified the cation channels involved in NK3R-elicited excitation of BLA principal neurons. The I-V curve of the senktide-sensitive currents resembles that of TRPC4, TRPC5 and TRPV1 [297] channels. The BLA principal neurons express TRPV1 [298, 301], TRPC4 [359] and TRPC5 [360] channels. We next tested the roles of these channels in NK3R-mediated excitation of BLA principal neurons. Bath application of the selective TRPC4/5 channel blocker, M084 (100 µM) alone, did not alter the holding currents significantly (3.0 ± 9.5 pA, n = 10, P = 0.375, Wilcoxon test, data not shown). Slices were pretreated with M084 (100 µM), and the extracellular solution was perfused continuously with the same concentration of M084. Application of M084 significantly reduced senktide-induced increases in inward currents (M084 + senktide: -25.0 ± 18.8 pA, n = 19 vs. senktide alone: -42.7 ± 20.0 pA, n = 17, P = 0.019, Mann-Whitney test, Figure. 22Aa-c), suggesting the involvement of TRPC4/5 channels. We further tested the roles of TRPC4/5 channels by using the knockout mice for TRPC4 or TRPC5 channels. Application of senktide induced a significantly smaller inward current in slices prepared from the TRPC4 KO mice (-12.7 ± 7.8 pA, n = 22), compared with the corresponding WT mice (-35.5 ± 15.1 pA, n = 17, P < 0.0001 vs. TRPC4 KO mice, Mann-Whitney test, Figure. 22Ba-c), suggesting the participation of TRPC4 channels in NK3R-elicited excitation of BLA neurons. Likewise, application of senktide evoked a significantly smaller inward current in slices cut from TRPC5 KO mice (-8.7 ± 7.8 pA, n = 22), compared with the corresponding WT mice (-34.6 ± 18.4 pA, n = 17, P < 0.0001, Mann-Whitney test, Figure. 22Ca-c), suggesting the involvement of TRPC5 channels. We also used the TRPV1 KO mice. Application of senktide induced an inward current (-17.1 ± 13.2 pA, n = 36) in slices cut from TRPV1 KO mice, which was not significantly different from that obtained from the corresponding WT mice (-23.7 ± 17.2 pA, n = 22,

P = 0.213 vs. TRPV1 KO mice, Mann-Whitney test, Figure. 22Da-c), suggesting that

TRPV1 channels are not required for NK3-mediated excitation of BLA neurons.

NK3R-Mediated Excitation of BLA Neurons is Dependent on PLCβ and PLCβ-Mediated Hydrolysis of PIP$_2$

Because activation of NK3Rs activates PLCβ pathway [329, 330], we tested the

roles of PLCβ in NK3R-induced inward currents. Bath application of the selective PLC

inhibitor, U73122 (5 µM) had no acute effects on the holding currents recorded from the

BLA neurons (-4.82 ± 6.6 pA, n = 5, P = 0.188, Wilcoxon test, data not shown).

Pretreatment of slices with and continuous bath application of U73122 (5 µM),

significantly reduced senktide-evoked inward currents (-14.4 ± 8.0 pA, n = 16,

P < 0.0001 vs. senktide alone, one-way ANOVA followed by Dunnett's test, Figure. 23B,

23E), suggesting the involvement of PLCβ. Activation of PLCβ hydrolyzes PIP$_2$ to

generate IP$_3$ to increase intracellular Ca^{2+} release and DAG to activate PKC. We further

tested the roles of these two second messengers in NK3R-mediated inward currents.

Inclusion of the IP$_3$ receptor blocker, heparin (0.5 mg/ml) in the recording pipettes failed

to alter senktide-elicited inward currents significantly (-33.0 ± 19.0 pA, n = 16, P = 0.221

vs. senktide alone, one-way ANOVA followed by Dunnett's test, Figure. 23C, 23E),

suggesting that IP$_3$ receptors are not involved in NK3R-induced excitation of BLA

neurons. Moreover, intracellular application of the sarcoplasmic ATPase inhibitor,

thapsigargin (10 µM), to deplete Ca^{2+} stores, did not alter significantly senktide-mediated

inward currents (-34.0 ± 15.7 pA, n = 19, P = 0.270 vs. senktide alone, one-way ANOVA

followed by Dunnett's test, Figure. 23D, 23E), suggesting that intracellular Ca^{2+} release

is not required for NK3R-mediated excitation of BLA principal neurons. We then probed

the roles of PKC in NK3R-elicited excitation of BLA principal neurons. Pretreatment of

slices with and continuous bath application of the selective PKC inhibitor,

bisindolylmaleimide II (Bis II, 2 µM), did not significantly alter senktide-induced

Figure 22. **TRPC4 and TRPC5 channels are involved in NK3R-induced inward currents in BLA neurons.**
Aa-Ac: pretreatment of slices with and continuous bath application of M084 (100 µM) significantly reduced senktide-induced inward currents. **Aa**: senktide-elicited inward current recorded from a BLA neuron in control condition. **Ab**: senktide-induced inward current recorded from a BLA neuron in a slice treated with the TRPC4/5 channels blocker, M084 (100 µM). **Ac**: summary graph showing the decreased inward currents induced by senktide in the presence of M084. Circles represented the inward current from individual cells and the bars were their averages.
Ba-Bc: bath application of senktide induced a significantly smaller inward current in slices cut from TRPC4 KO mice (TRPC4(-/-)), compared with the corresponding WT mice (TRPC4(+/+)). **Ba**: senktide-evoked inward current recorded from a BLA neuron in a slice cut from a WT mouse. **Bb**: senktide-induced inward current recorded from a BLA neuron in a slice cut from a TRPC4 KO mouse. **Bc**: summary graph showing that senktide induced a significantly smaller inward current in TRPC4 KO mice compared with the corresponding WT mice. Circles represented the inward current from individual cells and the bars were their averages.
Ca-Cc: bath application of senktide induced a significantly smaller inward current in slices cut from TRPC5 KO mice (TRPC5(-/-)), compared with the corresponding WT mice (TRPC5(+/+)). **Ca**: senktide-elicited inward current recorded from a BLA neuron in a slice cut from a WT mouse. **Cb**: senktide-induced inward current recorded from a BLA neuron in a slice cut from a TRPC5 KO mouse. **Cc**: summary graph showing that senktide induced a significantly smaller inward current in TRPC5 KO mice compared with the corresponding WT mice. Circles represented the inward current from individual cells and the bars were their averages.
Da-Dc: TRPV1 channels were not necessary for NK3R-mediated excitation of BLA neurons. **Da**: senktide-elicited inward current recorded from a BLA neuron in a slice cut from a WT mouse. **Db**: senktide-induced inward current recorded from a BLA neuron in a slice cut from a TRPV1 KO mouse. **Dc**: summary graph indicating that senktide did not significantly alter senktide-induced inward currents in TRPV1 KO mice compared with the corresponding WT mice. Circles represented the inward current from individual cells and the bars were their averages. Adapted from [224].

Aa

Senktide

30 pA
5 min

b

M084
Senktide

30 pA
5 min

c

M084
+Senktide   Senktide

Current (pA)

30
0
-30
-60
-90

P = 0.019

Ba

TRPC4(+/+)
Senktide

30 pA
5 min

b

TRPC4(-/-)
Senktide

30 pA
5 min

c

TRPC4 (-/-)   TRPC4 (+/+)

Current (pA)

10
0
-10
-20
-30
-40
-50
-60
-70

P < 0.0001

Ca

TRPC5(+/+)
Senktide

30 pA
5 min

b

TRPC5(-/-)
Senktide

30 pA
5 min

c

TRPC5(-/-)   TRPC5(+/+)

Current (pA)

20
0
-20
-40
-60
-80
-100
-120

P < 0.0001

Da

TRPV1(+/+)
Senktide

30 pA
5 min

b

TRPV1(-/-)
Senktide

30 pA
5 min

c

TRPV1 (-/-)   TRPV1 (+/+)

Current (pA)

10
0
-10
-20
-30
-40
-50
-60
-70

P = 0.213

117
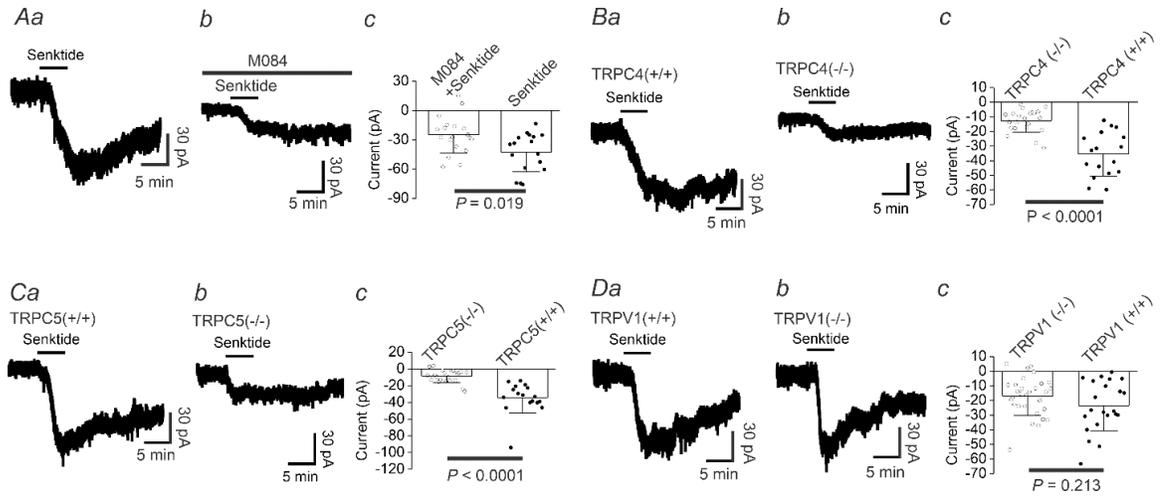
inward currents (-39.8 ± 25.4 pA, n =12, P = 0.962 vs. senktide alone, one-way ANOVA

followed by Dunnett's test, Figure. 23F, 23I). Likewise, administration of another PKC

inhibitor, chelerythrine (10 µM), in the same fashion, did not significantly change

senktide-elicited inward currents (-40.2 ± 22.4 pA, n = 12, P = 0.975 vs. senktide alone,

one-way ANOVA followed by Dunnett's test, Figure. 23G, 23I). These data together

suggest that the function of PKC is irrelevant to NK3R-medited excitation of BLA

neurons.

We further tested the roles of PLCβ-mediated hydrolysis of PIP_2 in senktide-

mediated inward currents. Inclusion of the diC8-PIP_2 (40 µM) to compensate the

turnover of PIP_2 content elicited by PLCβ significantly reduced senktide-elicited inward

currents (-12.5 ± 13.0 pA, n = 19, P < 0.0001 vs. senktide alone, one-way ANOVA

followed by Dunnett's test, Figure. 23H, 23I), suggesting that PLCβ-mediated hydrolysis

of PIP_2 is responsible for NK3R-mediated excitation of BLA neurons.

NK3R-Mediated Excitation of BLA Neurons Augments Cued Startle Responses

Because the BLA is closely associated with the FPS responses [277, 361-364], we

tested the roles of NK3R-mediated excitation of BLA principal neurons in FPS

responses. The FPS paradigm measures conditioned fear by an increase in the

amplitude of a simple reflex (the acoustic startle reflex) in the presence of a cue

previously paired with a shock. This test has been proven to be a valuable tool to study

mechanisms involved in the acquisition and expression of conditioned fear in both rats

and humans [365, 366]. We implanted cannulae into the BLA and microinjected senktide

and/or other compounds into the BLA to probe the effects and the underlying

mechanisms of NK3R activation on FPS by using the paradigm shown in Figure 24A.

Figure 24B showed the locations of the cannula tips for a subset of experiments and the

diffusion area of Chicago Sky Blue dye injected at the end of the experiments.

Figure 23. **NK3R-elicited excitation of BLA neurons requires PLCβ-mediated hydrolysis of PIP$_2$.**

A, Inward current trace induced by bath application of senktide in control condition. **B**, Current trace evoked by senktide recorded from a BLA neuron in a slice treated with U73122 (5 µM). **C**, Senktide-induced inward current recorded from a BLA neuron in a pipette containing heparin (0.5 mg/ml). **D**, Current trace elicited by senktide in a BLA neuron intracellularly dialyzed with thapsigargin (10 µM). **E**, Summary graph. Empty symbols were the senktide-induced net currents from individual cells and the bars were their averages. **F**, Current trace evoked by senktide recorded from a BLA neuron in a slice pretreated and continuously bath-applied with the selective PKC inhibitor, Bis II (2 µM). **G**, Senktide-induced current trace recorded from a BLA neuron in a slice pretreated and continuously bath-applied with the PKC inhibitor, chelerythrine (10 µM). **H**, Current trace elicited by senktide recorded from a BLA neuron dialyzed intracellularly with diC8-PIP$_2$ (40 µM). **I**, Summary graph. Empty symbols were the senktide-induced net currents from individual cells and the bars were their averages. Adapted from [224].

A

Senktide

B

U73122 pretreated
Senktide

30 pA

5 min

C

Heparin

Senktide

30 pA

5 min

30 pA

5 min

D

Thapsigargin

Senktide

30 pA

5 min

E

Current (pA)

Senktide   + U73122   + Heparin   + Thapsigargin

0

-20

-40

-60

-80

****

F

Bis II

Senktide

30 pA

5 min

G

Chelerythrine

Senktide

30 pA

5 min

H

dic8-PIP2

Senktide

30 pA

5 min

I

Current (pA)

Senktide   + Bis II   + Chelerythrine   + diC8-PIP$_2$

20

0

-20

-40

-60

-80

-100

****

120
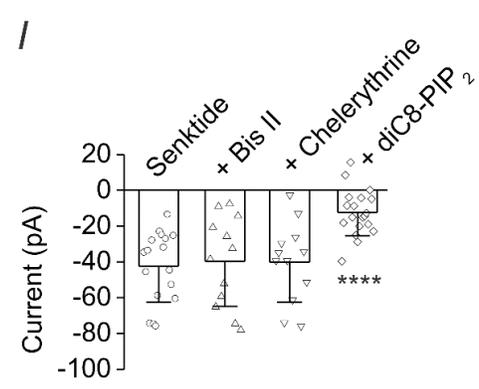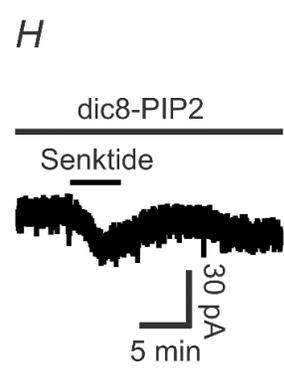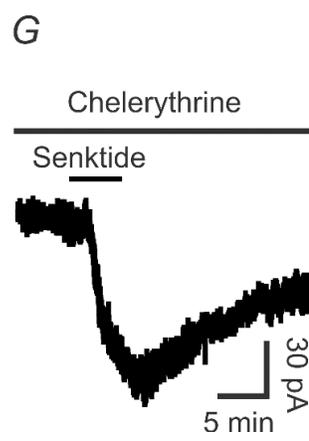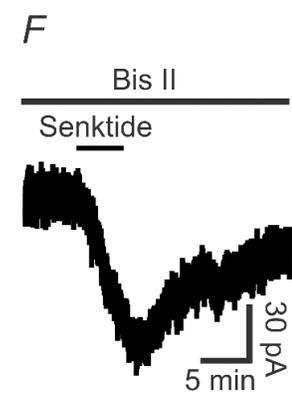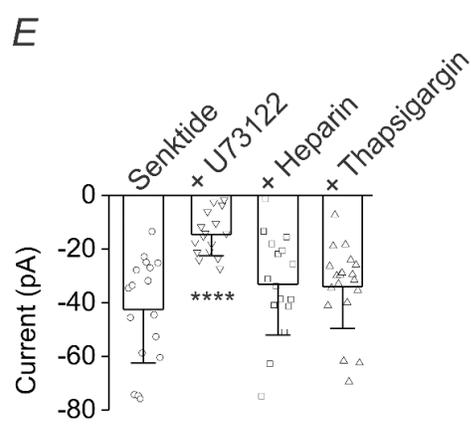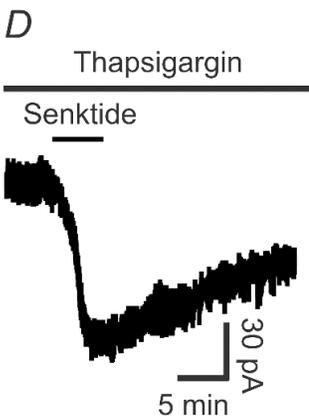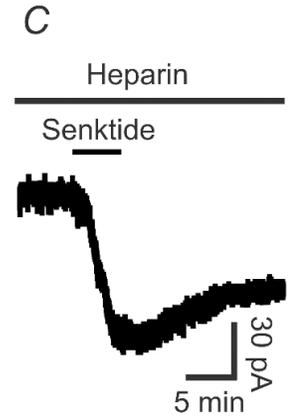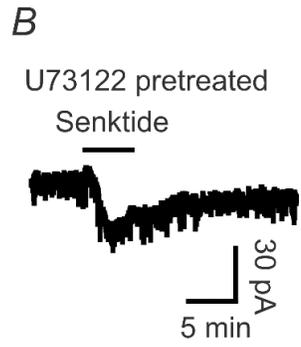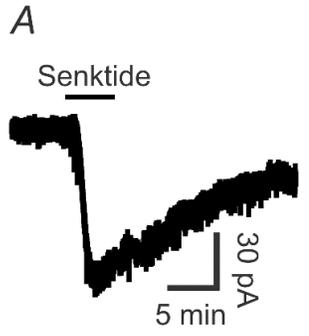
Microinjection of senktide into the BLA of rats dose-dependently increased the cued

startle response compared with the rats injected with saline (0.9% NaCl) (0.3 nmol,

P = 0.047; 1 nmol, P = 0.008, one-way ANOVA followed by Tukey's test, Figure. 24Ca),

whereas senktide had no significantly effects on the non-cued startle response

(P = 0.314, one-way ANOVA followed by Tukey's test, Figure. 24Cb), the startle

amplitude (P = 0.063, one-way ANOVA followed by Tukey's test, Figure. 24Cc) and the

shock reactivity amplitude (P = 0.738, one-way ANOVA followed by Tukey's test,

Figure. 24Cd), suggesting that senktide treatment had no effect on generalized stimuli in

this paradigm. For the remaining experiments, we used 0.3 nmol senktide because this

is an effective dose.

We tested the involvement of NK3Rs by microinjection of the selective NK3R

antagonist, SB218795. Microinjection of SB218795 (20 nmol) by itself prior to fear

conditioning had no significant effect on cued responses (saline vs SB218795,

P = 0.998; one-way ANOVA followed by Tukey's test), but blocked the enhancement of

cued responses elicited by senktide (Senktide alone vs. SB 218795 + Senktide,

P =0.006; SB218795 + Senktide vs. saline, P = 0.984; one-way ANOVA followed by

Tukey's test, Figure. 25A), indicating that NK3Rs are responsible for senktide-elicited

augmentation of cued-startle response in the BLA.

GIRK Channels and TRPC4/5 Channels are Involved in NK3R-Mediated Enhancement of Startle Responses

Our results indicate that activation of NK3Rs facilitated the excitabilities of BLA

neurons by depression of GIRK channels and opening of TRPC4 and TRPC5 channels.

We further examined the roles of these channels in senktide-mediated augmentation of

startle responses. Microinjection of TQ at 250 pmol, an effective dose demonstrated

previously [268, 367], failed to change significantly either the cued (saline vs. TQ,

Figure 24. **Microinjection of senktide into the BLA dose-dependently enhances cued startle responses.**

A, schematic representation of the experimental paradigm. Day 1, animals were habituated within the startle chambers for 30 minutes. Day 2, animals were allowed a 5-minute acclimation and presented with 30 WNBs (95 dB, 50 ms, 30 ITI) in the presence of a background white-noise (70 dB). Day 3, animals were injected with test compounds 5 minutes prior to fear conditioning. Upon placement in the startle chamber, animals were allowed a 5-minute acclimation before presentation of ten 3.7 s light cues that co-terminate with a foot shock (0. 5s, 0.5 mA, pseudorandom ITI 30-180 s). Day 4, rats were allowed a 5-minute acclimation within the startle chamber prior to presentation of 30 WNBs (as on Day 2). The first ten WNBs were used for measurement of basal startle amplitude. The remaining 20 WNBs were divided such that 10 WNBs were paired with light (cued) and 10 occurred without (non-cued) in a pseudorandom order. Background noise was present during this session. **B**, (left) proper cannula placement and the diffusion area of reagents were verified with bilateral injection of 3 % Chicago Sky Blue dye into the BLA (white arrows); (right) cannula tip placements displayed onto atlas figures adapted from Paxinos and Watson [222]. Note: all animal cannula placements were verified, dots represent a subset of the rats used for the experiments. **Ca**, microinjection of senktide dose-dependently increased cued startle responses. **Cb**, BLA microinjection of senktide had no effect on non-cued startle responses. **Cc**, startle amplitude was not significantly affected by senktide microinjection. **Cd**, behavioral responses to foot shock on Day 3 did not differ significantly in response to increasing doses of senktide.

A

Day 1 — 30 min habituation

Day 2 — 70 dB, 95 dB

Day 3 — 3.7 s, 0.5 s, 0.5 mA

Day 4 — 70 dB, ±

Legend:
- White noise
- WBN
- Conditioned stimuli
- Unconditioned stimuli
- Floor grid

B

Ca

Cued responses (% change) vs Saline, 0.1 nmol, 0.3 nmol, 1 nmol Senktide

Cb

Non-cued response (% change) vs Saline, 0.1 nmol, 0.3 nmol, 1 nmol Senktide

Cc

Startle amplitude (arbitrary units) vs Saline, 0.1 nmol, 0.3 nmol, 1 nmol Senktide

Cd

Shock reactivity amplitude (arbitrary units) vs Saline, 0.1 nmol, 0.3 nmol, 1 nmol Senktide

123

Figure 25.   Roles of NK3Rs, GIRK and TRPC4/5 channels in senktide-mediated augmentation of cued startle responses.
A, Microinjection of senktide (Senk, 0.3 nmol) into the BLA enhanced cued startle responses, whereas prior administration of the NK3R antagonist, SB218795 (20 nmol), GIRK channel blocker, TQ (Tert-Q, 250 pmol) or TRPC4/5 channel blocker, M084 (100 nmol) blocked senktide-induced augmentation of cued response. **B,** non-cued startle responses of the tested compounds and significant enhancements of non-cued startle in the group of rats injected with both tertiapin-Q and senktide and the group of rats injected with M 084. * P < 0.05 vs. saline (one-way ANOVA followed by Tukey's test). **C,** group data for startle amplitude on Day 1. **D**, group data for shock reactivity during fear conditioning on Day 2. Adapted from [224].

P = 0.991; one-way ANOVA followed by Tukey's test, Figure. 25A) or the non-cued

(saline vs. TQ, *P* = 0.677, one-way ANOVA followed by Tukey's test, Figure. 25B)

responses. Microinjection of senktide following TQ did not alter significantly the cued

startle response (TQ vs. TQ + senktide, *P* = 0.992, one-way ANOVA followed by Tukey's

test, Figure. 25A), whereas significantly enhanced the non-cued response

(TQ vs. TQ + senktide, *P* = 0.009, one-way ANOVA followed by Tukey's test,

Figure. 25B). These results together suggest that GIRK channels are involved in NK3R-

mediated enhancement of startle response.

We then tested the involvement of TRPC4/5 channels in NK3R-elicited

augmentation of FPS responses. Microinjection of the TRPC4/5 channel blocker, M084

(100 nmol), into the BLA did not significantly alter the cued startle responses (Saline vs.

M084, *P* = 0.997, one-way ANOVA followed by Tukey's test, Figure. 25A), but blocked

senktide-induced enhancement of cued startle response (Saline vs. M084 + Senktide,

P = 0.998, one-way ANOVA followed by Tukey's test, Figure. 25A). However,

microinjection of M084 significantly enhanced the non-cued fear response, compared

with the saline-injected group (saline vs. M084, *P* = 0.028, one-way ANOVA followed by

Tukey's test, Figure. 25B). Administration of senktide following the injection of M084

failed to significantly increase the non-cued response further (M084 vs. M084 + senktide,

P = 0.125, one-way ANOVA followed by Tukey's test, Figure. 25B). These results

suggest that TRPC4/5 channels are involved in NK3R-elicited enhancement of startle

responses.

There were no significant differences for the startle amplitudes obtained during the

acoustic startle response session on Day 2 among the experimental groups (*P* = 0.297,

$F_{(6,40)}$ = 1.26, one-way ANOVA, Figure. 25C), suggesting that there was no endogenous

between-group difference in responses to startle-inducing white-noise bursts. The shock

reactivity amplitude was unaffected in any group as the result of the microinjection (*P* =

0.664, F $_{(6, 40)}$ = 0.68, one-way ANOVA, Figure. 25D). Together these data suggest

similar behavioral responses to startle-inducing auditory stimuli among these groups and

no between-group differences in nociceptive responses to shock resulting from drug

injection. Based on our experimental results, we propose a working mode to explain the

cellular and molecular mechanisms underpinning NK3R-mediated excitation of BLA

neurons and augmentation of FPS response (Figure. 28).

CHAPTER 4

DISCUSSION

Study 1 - Neuromedin B-Mediated Modulation of Central Amygdala Neurons

Whilst injection of bombesin into the CeA increases mean arterial pressure [245] and decreases food intake [246, 247], the cellular and molecular mechanisms whereby bombesin and bombesin-like peptides modulate these physiological functions have not been determined. As NMB peptide mRNA [243] and NMB binding sites [244] are expressed in the CeA and peripheral and central administration of bombesin increases Fos-like immunoreactivity, a marker of neuronal activation, in the CeL [248], we tested the hypothesis that activation of BB1 receptors facilitates neuronal excitability in the CeL. Our results demonstrate that BB1 receptor activation excited CeL neurons via inhibition of GIRK type Kir channels. BB1 receptor-mediated excitation of CeL neurons and depression of Kir channel currents required the functions of G proteins, PLCβ and PKC, but were independent of intracellular Ca^{2+} release. We further showed that BB1 receptor activation reduced MAP and HR, depending on GIRK channel activity. In ASR and FPS testing, microinjection of NMB into the CeA increased ASR, but reduced FPS to cued stimuli. Our results may provide a cellular and molecular mechanism by which bombesin and bombesin-like peptides regulate physiological functions *in vivo* (Figure 26.)

Neuropeptides of the bombesin family increase neuronal excitability mainly by two ionic mechanisms: depression of Kir channels and activation of cation channels. For example, GRP excites spinal cord neurons [368], paraventricular thalamic neurons [369] and interneurons in the entorhinal cortex [370] by depressing Kir channels; GRP excites
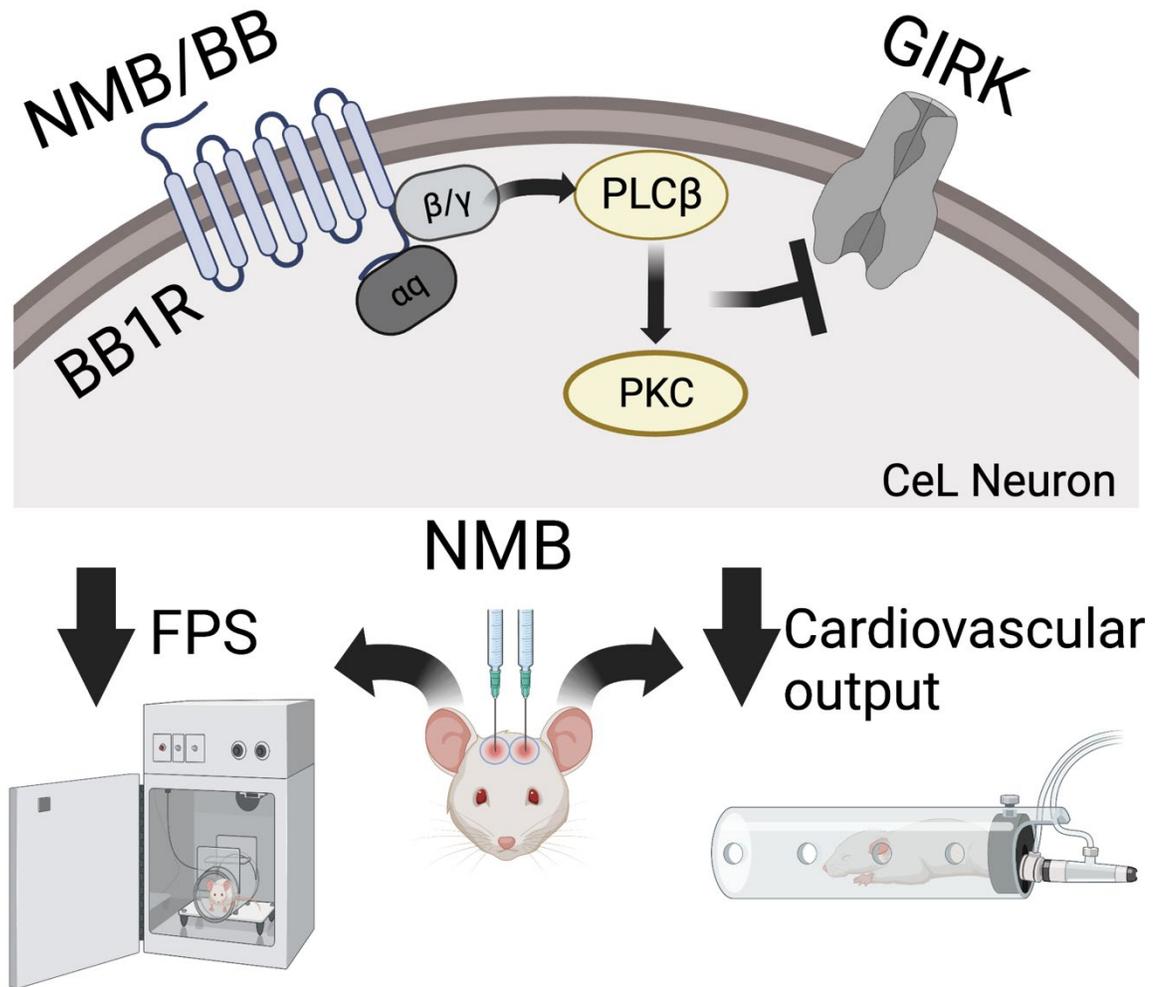
Figure 26. **The proposed cellular and molecular mechanisms underpinning BB1 receptor-elicited excitation of CeA neurons and decreased FPS and cardiac output.** Activation of BB1Rs by NMB results in activation of $G\alpha_q$ proteins leading to increases in PLCβ activity. Activation of PLCβ generates IP_3 to elevate intracellular Ca^{2+} release from IP_3-sensitive store and diacylglycerol (DAG) to activate protein kinase C (PKC). PLCβ and PKC are required for depression of GIRK channels. The effects of BB1Rs on GIRK channels are responsible for BB1 receptor-mediated suppression of fear responses and cardiac output. Adapted from [223]. Created with BioRender.com

paraventricular thalamic neurons by activating TRPV1 channels [369]; both NMB and GRP excite neuropeptide Y-containing neurons in the arcuate nucleus by activation of nonselective cation channels and sodium/calcium exchangers [320] and hippocampal interneurons by activating a cation channel [371]. Consistent with this scenario, our results support a role of GIRK type Kir channels in BB1 receptor-elicited excitation of CeL neurons. Inhibition of a membrane conductance should increase input resistance and membrane time constants. Consistent with this anticipation, application of NMB augmented the input resistance and the membrane time constants of the CeL neurons. Moreover, the I-V curve of the NMB-sensitive current exhibited inward rectification with a reversal potential near the K^+ reversal potential. Micromolar concentrations of Ba^{2+} selectively block Kir channels. Our results showed that application of 300 µM Ba^{2+} inhibited Kir channels by itself and blocked NMB-elicited depression of Kir currents, further supporting a role of Kir channels in NMB-mediated excitation of CeL neurons. Kir channels are classified into 4 functional subfamilies: Kir2 (Kir2.1, Kir2.2, Kir2.3, Kir2.4), Kir3 (GIRK channels, Kir3.1, Kir3.2, Kir3.3, Kir3.4), Kir6 (K_{ATP} channels, Kir6.1, Kir6.2), and K^+ transport channels (Kir1.1, Kir4.1, Kir7.1) [255]. Our intracellular solution contained 2 mM ATP which would exert inhibition on K_{ATP} channels. Application of the selective Kir2 subfamily blocker ML 133 at a saturating concentration (30 µM) [372] attenuated Kir currents, suggesting that the CeL neurons express functional Kir2 subfamily channels. Consistent with our electrophysiological results, the amygdala expresses mRNAs for both Kir2.1 and Kir2.2 [373]. However, NMB-induced depression of Kir currents was not significantly altered by inclusion of ML 133 in the extracellular solution, suggesting that the Kir2 subfamily channels were not involved in NMB-elicited excitation of CeL neurons. TQ inhibits both GIRK and Kir1.1 channels [263-265], whereas tertiapin-LQ blocks Kir1.1 channels without effects on GIRK channels [266]. Application of TQ by itself elicited significant inhibition of Kir currents, whereas

application of tertiapin-LQ failed to alter Kir currents significantly. These results suggest that the CeL neurons express tonically functional GIRK channels without expression of Kir1.1 channels, consistent with the notion that GIRK channels are constitutively active [374-376]. Furthermore, NMB-induced inhibition of Kir currents was significantly reduced in the presence of TQ, but application of tertiapin-LQ failed to alter NMB-induced inhibition of Kir currents significantly. These results together suggest that activation of BB1 receptors excite CeL neurons by suppressing GIRK channels.

Our result that application of TQ alone significantly inhibited Kir channel currents satisfies the prerequisite that GIRK channels must be tonically active in the CeL neurons. Another precondition to explain our results is that the CeL neurons should physically express GIRK channels. The GIRK channels include GIRK1, GIRK2, GIRK3 and GIRK4, and these subunits are widely expressed in the brain, existing predominantly as heterotetramers of GIRK1, GIRK2 and/or GIRK3, or as homotetramers of the GIRK2 subunit [255, 377]. GIRK4 is not expressed in the amygdala [373, 378] but restricted to some neuronal populations such as Purkinje cells and neurons of the globus pallidus and the ventral pallidum, suggesting that GIRK4 channels are unlikely to be the molecular target of BB1 receptor activation in the CeL. Furthermore, our results did not support the involvement of GIRK1 channels in BB1 receptor-mediated excitation of CeL neurons, because application of ML 297, an activator selective for GIRK1-containing channels, did not affect Kir currents significantly in the CeL neurons, suggesting that there is no functional GIRK1 in the CeL neurons. Consistent with our electrophysiological data, the expression of GIRK1 mRNA has been detected more prominently in the BLA and cortical nuclei with less expression in the CeA of the amygdala [261]. Thus, the most possible subunit compositions involved in BB1 receptor-mediated excitation of CeL neurons are GIRK2 and GIRK3, which could exist as GIRK2/GIRK3 heterotetramers or GIRK2 homotetramers [255, 377]. In line with this

speculation, GIRK2 [373, 378] and GIRK3 [373] are expressed in the amygdala, and activation of both BB1 and BB2 receptors results in depression of GIRK channels including GIRK1, GIRK2 and GIRK4 expressed in xenopus oocytes [379]. BB1 receptors are coupled to $G\alpha_{q/11}$ resulting in activation of PLCβ which hydrolyzes PIP_2 generating IP_3 to release intracellular Ca^{2+} and DAG to activate PKC [237, 239, 240]. Our results suggested that intracellular Ca^{2+} release was not required, whereas the activities of PLCβ and PKC were necessary, for NMB-elicited excitation of CeL neurons and inhibition of Kir channels. Consistent with our results, PLC is required for NMB and GRP-mediated excitation of hippocampal interneurons although the involved ion channels are cation channels, not Kir channels [371]. In accordance with our results, activation of PKC phosphorylates and inhibits GIRK channels [379-381] and PLC and PKC are involved in BB1 or BB2 receptor-mediated depression of GIRK channels co-expressed in *xenopus* oocytes [379].

PKC is a serine-threonine kinase family of isozymes with different activation requirements of intracellular Ca^{2+} and DAG [382]. Classical/conventional PKC (cPKC) include PKCα, PKCβ, and PKCγ and are dependent on Ca^{2+} and DAG; Novel PKC (nPKC) include PKCδ, PKCε, PKCη, and PKCθ that are Ca^{2+} dependent but DAG independent; and atypical PKC (aPKC) that include PKCζ and PKCλ which are independent of both Ca^{2+} and DAG. We demonstrated that application of the DAG analogue, PMA, significantly increased AP firing frequency but depressed Kir currents, suggesting the involvement of cPKC and nPKC. Chelation of intracellular Ca^{2+} with BAPTA via the recording pipettes blocked NMB-elicited augmentation of AP firing and lessened NMB-induced suppression of Kir currents, suggesting the involvement of cPKC. Agreeably, activation of cPKC isoforms phosphorylate and inhibit GIRK channels [383]. Our results indicate that activation of BB1 receptors enhances the excitability of

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CeL neurons via PLCβ and PKC-mediated depression of GIRK type Kir channels. Consistent with our results, modulations of Kir channels [384-386], PLCβ [217, 387] and PKC [388-392] affect anxiety and fear responses.

Both bombesin-like peptides and the CeA are involved in modulating fear/anxiety and appetitive behaviors [393-396], although bombesin-like peptides and their activity in the amygdala in the modulation of these physiological functions have not been fully determined. Injection of bombesin into the CeA reduces food intake [246, 247] and food ingestion increases the release of bombesin-like peptides from the CeA [397]. Consistent with our results, the function of PKC is required for amphetamine-mediated anorectic action [398]. NMB and GRP activate the hypothalamic-pituitary-adrenal axis resulting in increased release of ACTH and corticosterone [399] which are closely involved in emotional regulation. BB1 receptor knockout mice displayed decreased emotionality [400] and administration of a selective BB1 receptor agonist as well as antagonist paradoxically exerts anxiolytic effects [273, 401] and attenuates the FPS response [273]. Furthermore, the bombesin-like peptides are released from the CeA in response to stressor exposure [402]. Collectively, these results support that activation of BB1 receptors elicited by NMB modulate fear and anxiety responses. In our hands, microinjection of NMB significantly reduced FPS responses while increasing ASR with increasing intensity. We demonstrate a functional requirement of PKC for NMB-induced excitation of the CeA. In line with this, cPKC and nPKC isozymes are involved in G_q protein-mediated increased acoustic startle responses [403].

There are sex differences in the presentation of affective disorders [404] as well as sex differences in amygdala activation in response to emotionally relevant stimuli [405]. Our data indicate that NMB reduces FPS while enhancing ASR in both male and female rats. Due in part to limiting numbers of male and female rats and that bombesin-like peptide immunoreactivity in the limbic system does not vary with sex or estrous cycling

[406], the data from both sexes were pooled for the investigations of NMB on FPS and ASR. Because we did not determine the phase of the estrous cycle of the female rats in our study, one caveat of this study is that we cannot rule out the influences of the estrous cycle of the female rats on the effects of NMB. Whereas the estrous cycle of females affects anxiety-like behaviors and fear conditioning [407], FPS phenomena are dependent on CeA activity, and are not subjected to sex hormone modulation in both male and female rats [278, 279, 408, 409].

Injection of bombesin into the CeA induces an increase in MAP [245], consistent with reports of an increased response of the autonomic nervous system in fear and anxiety conditions. We demonstrated microinjection of NMB into the CeA dose-dependently reduced MAP and HR via activation of BB1 receptors and depression of GIRK channels. As bombesin non-selectively activates BB1 receptors and BB2 receptors [235, 236], the resultant increase in MAP by intra-CeA application of bombesin [245] may lend support to a hypothesis of distinct processes of each receptor on cardiovascular output from the CeA. Indeed, intraventricular application of GRP in trout produced significant increases in HR and MAP [410]. Another possibility is that BB1 receptor activation disinhibits CeA GABAergic interneurons producing increases in the baroreceptor responses and decreases in MAP and HR. Activation of CeA inhibitory projections to the dorsal vagal complex result in a decrease of the baroreceptor reflex and subsequent increases in MAP and HR [85]. As the CeA is populated by GABAergic interneurons that reciprocally inhibit each other to produce coordinated defensive responses [141, 411, 412], NMB-expressing microcircuits of the CeA may inhibit excitatory cardiovascular outputs from this nucleus. In addition, bombesin-like peptides have been demonstrated to interact with neuropeptide Y [413, 414], a peptide documented to suppress central cardiac output [415, 416]. NMB activates NPY-expressing neurons in the hypothalamic arcuate nucleus involved in energy homeostatic

processing due to co-expression of BB1 receptors [417]. As hypothalamic nuclei coordinate cardiovascular responses [418] and these nuclei receive inputs from the CeA [419, 420], NMB microinjection into the CeA may alter cardiovascular output via NMB-expressing projections to the hypothalamus. Further characterization of NMB-specific projections of the CeA is warranted.

Study 2 - Bombesin-1 Receptors Excites Neurons of the Basolateral Amygdala by Multiple Signaling and Ionic Mechanisms

Although NMB and BB1 receptor mRNA are expressed in the BLA [242, 280], the signaling and ionic mechanisms whereby activation of BB1 receptors modulates BLA principal neurons remains undetermined. Using whole-cell patch clamp electrophysiological recordings, we showed that NMB excited BLA neurons through the inhibition of GIRK channel currents and the activation of the Ca^{2+}-permeable non-selective cation channel, TRPV1. The NMB-mediated increases in AP firing were dependent on the functional activity of PLC, PIP_2 turnover, PKC, and intracellular Ca^{2+} release. Furthermore, we demonstrated that activation of BLA BB1 receptors enhances sEPSCs through TRPV1 channels, but inhibits evoked glutamatergic synaptic transmission independent of TRPV1. Lastly, we demonstrated that microinjection of NMB into the BLA produces anxiety-like behaviors in the Vogel Conflict test.

NMB and GRP are excitatory neuropeptides that utilize distinct intracellular signaling and ionic mechanisms. For example, in primary sensory neurons, BB1 receptor activation stimulates PKA to enhance T-type voltage-gated Ca^{2+} channel currents independent of PLC [421]. Bombesin-like peptide excitation of hippocampal neurons requires PLC and intracellular Ca^{2+} release to activate nonselective cation channels [371]. Bombesin excites dorsal raphe neurons and entorhinal interneurons by suppressing a K^+ conductance and GIRK channels, respectively [370, 422].
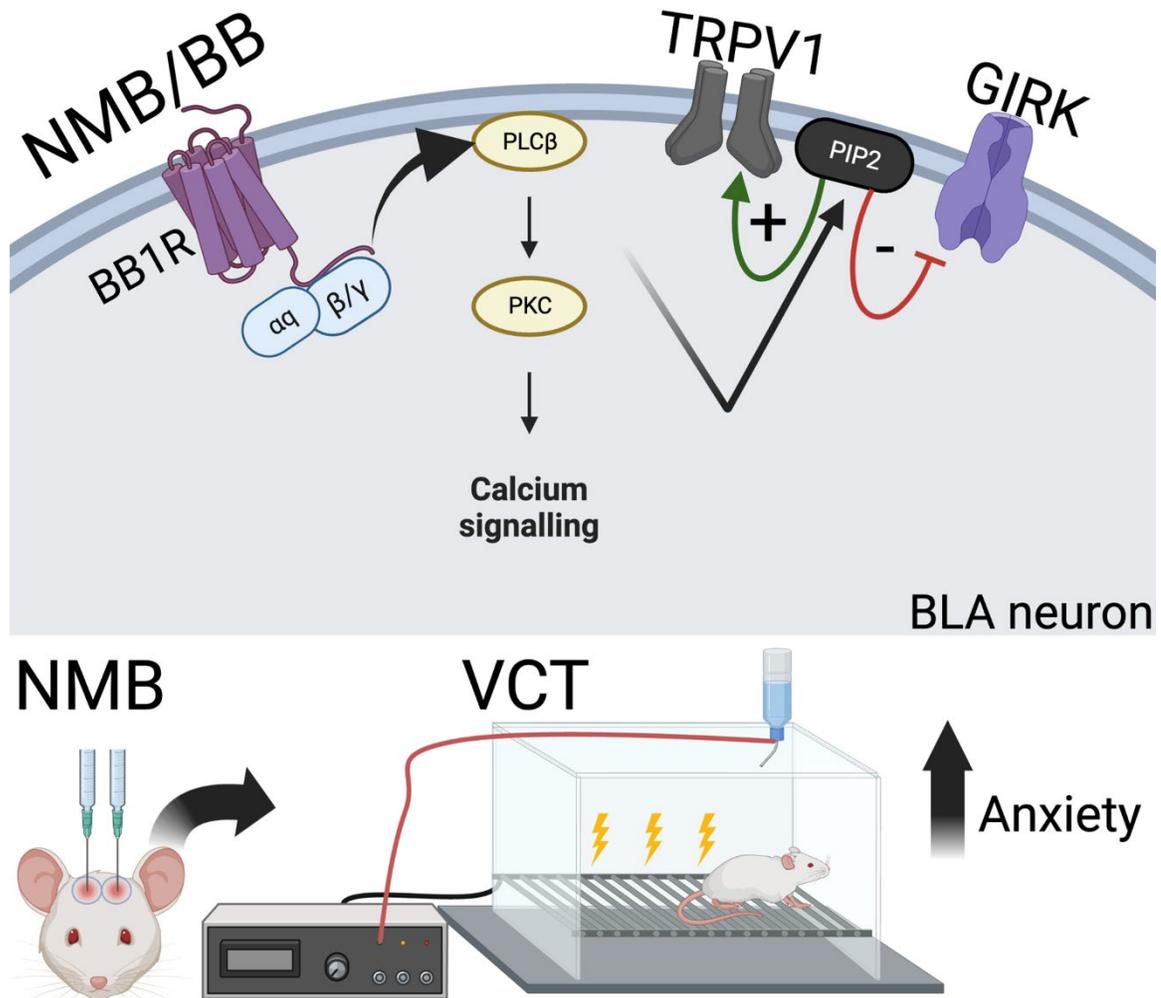
Figure 27. **Working model illustrating the cellular and molecular mechanisms underpinning BB1 receptor-elicited excitation of BLA principal neurons and increased anxiety-like behaviors in the VCT**. Activation of BB1Rs by NMB results in activation of $G\alpha_q$ proteins leading to increases in PLCβ activity. Activation of PLCβ catalyzes the hydrolysis of PIP_2 to generate IP_3 to elevate intracellular Ca^{2+} release from IP_3-sensitive store and diacylglycerol (DAG) to activate protein kinase C (PKC). PLCβ-induced hydrolysis of PIP_2 results in depression of GIRK channels and opening of TRPV1 channels to excite BLA principal neurons. The effects of BB1Rs on GIRK channels and TRPV1 channels are responsible for BB1 receptor-induced anxiety-like behaviors in the VCT. Created with BioRender.com

In paraventricular thalamic neurons, bombesin-like peptides activate TRPV1 channels and inhibit Ba^{2+}-sensitive Kir2 channels [369], whereas in preoptic GABAergic neurons bombesin activates an inward Ca^{2+}-dependent cationic current [423]. NMB and GRP depolarize neurons in the arcuate nucleus by activating nonselective cation channels and Na^+/Ca^{2+} exchangers, whereas NMB depolarizes CeL neurons by depressing GIRK channels through Ca^{2+}-independent mechanisms [223, 320]. BB1 receptors are coupled to $G\alpha_{q/11}$ proteins, resulting in PLC-mediated signaling. Consistent with these reports, our results indicate a functional requirement for PLCß, PKC, and intracellular Ca^{2+} in NMB-mediated excitation of principal BLA neurons through inhibition of GIRK channels and activation of TRPV1 channels. Inhibition of PLC or PKC as well as chelation of intracellular Ca^{2+} blocked NMB-mediated augmentation of AP firing. PIP_2 tonically interacts with GIRK and TRPV1 channels, modulating their open probability [193, 424]. It is reasonable to speculate that BB1 receptor-mediated hydrolysis of PIP_2 results in the disinhibition of TRPV1 and the inhibition of GIRK channels. In agreement with this situation, our data suggest that supplementation of membrane PIP_2 by inclusion of diC8-PIP_2 in our recording electrodes attenuated NMB-mediated excitation of BLA neurons. Likewise, downstream of PIP_2, PKC activity can regulate both GIRK [425] and TRPV1 channel activity [426] through direct phosphorylation [380, 427] and/or PKC-mediated hydrolysis of membrane PIP_2 [428]. Moreover, BB1Rs and BB2Rs inhibit GIRK through PLC- and PKC-dependent mechanisms [379].

We found that NMB excited approximately 50% of the recorded neurons through activation of TRPV1 channels. Bombesin-like peptide activation of TRPV1 channels has been observed in other brain regions, including the paraventricular thalamus [369], trigeminal ganglia [429], and brainstem [319]. TRPV1 channels are temperature sensitive cation channels that display multimodal activation mechanisms and are robustly expressed in the BLA [297, 298]. Lowering the temperature of our recording

bath to 22 °C attenuated the NMB-mediated inward currents, lending support to TRPV1 involvement in the BB1 receptor-generated excitation of BLA neurons. TRPV1 activation may involve 2-AG or other endocannabinoids, as inhibition of DAG lipase significantly reduced NMB-elicited inward currents. The production of 2-AG is calcium-dependent and reduced by Ca^{2+} chelation [430, 431]. Likewise, chelation of intracellular Ca^{2+} blocked NMB-mediated excitation. Direct targeting of TRPV1 channels by pretreatment and bath application of the selective TRPV1 antagonist AMG 9810 [302] or capsazepine [303] in the recording solution significantly attenuated BB1 receptor-elicited inward currents. Finally, bath application of NMB in TRPV1 KO mice significantly reduced the elicited inward current, which was not observed in mice lacking the TRPC4 and TRPC5 channels. TRPV1 channels are involved in fear processing in the amygdala. For example, activation of TRPV1 channels in the LA modulate long-term potentiation, whereas TRPV1 KO mice show reductions in conditioned and contextual fear responses [298, 432]. As NMB has been implicated in fear and anxiety-like behaviors, these results may identify a novel pathway through which NMB and TRPV1 contribute to anxiogenic behaviors within the BLA.

We demonstrated that the activation of BB1 receptors produced a significant inward current in a subset of BLA neurons by suppressing GIRK channels. The substitution of Cs^+ in our recording solution attenuated the inward current, suggesting a BB1 receptor-mediated inhibition of a K^+ conductance. In this subset of recordings, the NMB-sensitive I-V curve displayed inward rectification, reversed near the calculated K^+ reversal potential, which was sensitive to the application of micromolar concentrations of Ba^{2+}, a Kir channel inhibitor, indicating action on an inwardly rectifying potassium channel or Kir. The four functional Kir subfamilies include Kir2 constitutively active channels (Kir2.1-2.4), GIRK or Kir3 channels (GIRK1-GIRK4), ATP-sensitive Kir6 channels (Kir6.1, Kir6.2), and the K^+-transport channels (Kir1.1, Kir4.1, Kir4.2, and Kir7.1) [255].

Application of the saturating concentration of ML133, a specific antagonist of Kir2 channels [256, 372, 433], failed to significantly alter the inward current recorded from BLA neurons, whereas NMB-mediated inward currents were unaffected, indicating that Kir2 channels are unlikely to be involved in NMB-mediated excitation of BLA neurons. The application of the GIRK channel antagonist [263] TQ produced a significant inward current; however, in the presence of TQ, the NMB-mediated inward current was significantly reduced. GIRK channels are a family of constitutively active channels comprised of 4 subunits including GIRK1, GIRK2, GIRK3, and GIRK4 [255], which are involved in regulating intrinsic membrane excitability [374, 434, 435]. GIRK channels are expressed in a variety of neuronal populations throughout the brain as homotetramers of GIRK2 subunits or as heterotetramers of GIRK1, GIRK2, and/or GIRK3 subunits [255, 435]. The GIRK4 subunits are not expressed in the amygdala [436], whereas the GIRK1, GIRK2, and GIRK3 subunits are expressed in the BLA [437-439]. Consistent with these reports, application of the GIRK1 activator ML297 produced a significant outward current supporting the functional expression of GIRK1 subunits in the BLA. Owing to the lack of commercially available GIRK2 and GIRK3 selective antagonists and the heterotetrametric nature of these subunits, further experiments are required to determine the subunit composition of the GIRK channel inhibited by BB1 receptor activation.

We have shown that bath application of NMB significantly increased the frequency and amplitude of sEPSCs, but slightly decreased the amplitude of eEPSCs in the BLA. NMB enhanced the frequency but not the amplitude of mEPSC events recorded in the presence of TTX. The lack of NMB effects on mEPSC amplitude indicates that BB1 receptors do not modulate postsynaptic glutamate receptors but instead act to modulate glutamate release at presynaptic sites. The NMB-mediated increase in sEPSC frequency and amplitude may be the result of local increases in intracellular calcium,

which promoted the synchronized release of glutamate. In agreement with these data, TRPV1 channels may promote tonic glutamate release independent of afferent stimulation [440]. The transient inhibition of NMB-mediated eEPSCs may have resulted from the depletion of the readily releasable pool, similar to the inhibitory mechanisms proposed for bombesin in the hippocampus [370] and substance P in bronchopulmonary neurons in the solitary tract nucleus [441]. The transient suppression of eEPSC amplitude may also be the result of inhibitory signaling molecules produced following BB1 receptor activation. The two major endocannabinoids found in the brain are 2-arachidonoylglycerol (2-AG) and anandamide (AEA) [442-444]. Whereas AEA is biosynthesized via the enzymatic activities of N-acyltransferase and PLD, 2-AG is the hydrolytic product of PLC and the subsequent activity of DAG lipase [445-447]. 2-AG and AEA are released from neurons following depolarization [448-450] or activation of $G_{q/11}$ receptors [451, 452], and bind to CB1 receptors on presynaptic neurons to suppress synaptic activity [321, 451]. Oxytocinergic and vasopressinergic neurons have been demonstrated to suppress presynaptic neurotransmitter release via endocannabinoid signaling mechanisms. In magnocellular neurons, oxytocin receptor activation results in endocannabinoid release that diffuses in a retrograde direction to inhibit presynaptic glutamate release [453]. Similarly, depolarization of vasopressinergic neurons releases cannabinoids to suppress GABA release at presynaptic sites [454]. Consistent with these data, we demonstrate that NMB-mediated excitation may involve endocannabinoid release. Inhibition of DAG lipase attenuated NMB-mediated TRPV1 activation, which was required for NMB-mediated increases in AP-dependent and AP-independent glutamate release, suggesting that these channels are presynaptically located. TRPV1 channels were not involved in NMB-mediated suppression of eEPSC amplitude, whereas blockade of CB1 receptors significantly augmented eEPSC amplitude in response to NMB bath application. TRPV1 channels and CB1 receptors

differentially regulate spontaneous and evoked glutamatergic transmission in the solitary tract nucleus [307, 440]. Activation of afferent terminals produces prolonged asynchronous spontaneous release in TRPV1-positive neurons independent of CB1R activity [440], whereas evoked glutamate release in these neurons is modulated by cannabinoid 1 receptors [307]. Taken together, our results demonstrated that BB1 receptor activation in BLA neurons differentially regulates spontaneous and evoked glutamate release. Further research is needed to clarify the distinct roles of TRPV1 channels and cannabinoid signaling in NMB-mediated glutamate release.

Anxiety is a response to unpredictable or generalized threats that prepares a subject to respond to stress or danger in its environment. Anxiety paradigms are based on naturalistic behaviors that utilize generalized threats as a means to detect anxiolytic agents. For example, in elevated plus maze or open field tests of anxiety, rodents alter between their desire to explore a novel context and fear of predation in open or illuminated spaces. VCT, like other conflict-based anxiety paradigms, presents rodents with a 'conflict' between a physiological need (i.e., food or water) and punishment, where punishment is required to satiate this need. Drugs that increase punitive behaviors are considered to have anxiolytic properties, whereas drugs that reduce punitive behaviors reflect anxiogenic properties [455]. Dysregulation of glutamatergic activity within the BLA has been implicated in anxiety and anxiety-like disorders through a pathway involving the prefrontal cortex, BNST, and ventral hippocampus [15]. Activation of BLA excitatory projections to the BNST and ventral hippocampus are involved in the expression of anxiety-like behaviors [456]. Because the BLA is an important structure in the neural circuitry underlying fear and anxiety, BB1 receptor-mediated enhanced glutamate release within the BLA likely promoted information transfer out of the BLA to increase the activity of this circuit, resulting in increased anxiety-like behaviors. Moreover, intracerebroventricular application of NMDA receptor antagonists produce anxiolytic

effects in the VCT, comparable to those of diazepam [457]. BLA infusion of the selective NMDA receptor antagonists MK-801 [458] or AP5 [459] produce anxiolytic behaviors in rats and mice. Therefore, BB1 receptor-mediated depolarization could remove the voltage-dependent Mg^{2+} block of NMDA receptors, enhancing glutamatergic activity in the BLA, and promote anxiety-like behaviors. In support of our findings, intracerebroventricular application of another selective BB1 receptor antagonist, BIM23127, increased the time spent in the open arms of the elevated plus maze, a behavior associated with decreased anxiety [460]. The direct application of NMB into the dorsal raphe nucleus promotes anxiety-like behaviors, as measured by decreases in the social interaction test [461]. Taken together, our results indicate that activated BB1 receptors promote anxiety-like behaviors, and antagonists of this peptide system may be effective in the treatment of anxiety disorders.

GIRK and TRPV1 channels are attractive targets for the treatment of anxiety disorders. Our results indicate that activated BB1 receptors produce anxiety-like phenotypes in the VCT mediated by TRPV1 and GIRK channels. Inhibition of TPRV1 channels is typically associated with reductions in fear and anxiety. For example, infusion of TRPV1 antagonists into structures of the limbic system, including the BLA [462], nucleus accumbens [463], and medial PFC [464], decreases anxiety-like behaviors. TRPV1 channels are robustly expressed in the BLA, and their expression can be further increased by early life stress [465]. Activated TRPV1 channels enhance glutamatergic function and synaptic plasticity in the BLA and may contribute to hyperpathia and chronic pain [298, 466]. Consistent with these findings, treatment of rats with AMG9810 alone increased the number of punished licks, and the blockade of this channel prevented the anxiogenic action of BB1 receptor activation. The role of GIRK channels in fear and anxiety is more complex. GIRK knockout mice have shown reductions in generalized fear and deficits in conditioned fear [384, 385], whereas

wildtype mice treated with the selective GIRK channel activator, ML297 displayed

decreased anxiety-like phenotypes [386]. We demonstrated that GIRK channels are

involved in NMB-mediated anxiety in the BLA, and that blockade of GIRK channels

prevents NMB-mediated decreases in lick numbers. GIRK channels are known targets of

opioid-mediated signaling pathways and, like TRPV1 channels, are implicated in pain

disorders, which are often comorbid with anxiety. Taken together, our findings may

highlight a cellular and molecular mechanism to explain anxiety and pain in the BLA.

Study 3- Neurokinin-3 Receptor-Mediated Modulation of Basolateral Amygdala Neurons

While high densities of NK3Rs are expressed in the BLA [345-347, 349, 351], the

actions and the underlying mechanisms of NK3R activation in the BLA have not been

determined. We showed that activation of NK3Rs excited BLA neurons assessed by

electrophysiologically recording AP firing, RMPs and HCs. Consistent with our

electrophysiological results, intracerebroventricular injection of senktide increases the

expression of c-Fos, a selective marker of neuronal activity in the BLA [348, 353]. Our

results further showed that activation of NK3Rs facilitated the excitabilities of BLA

neurons by depressing the GIRK type of Kir channels and opening TRPC4 and TRPC5

channels. While SP has been shown to facilitate neuronal excitability by depressing Kir

channels [467-469] or by activating cation channels [470] or by both suppressing Kir

conductance and activating cation channels [471-473], few studies have been conducted

to determine the ionic mechanisms underlying NK3R-mediated neuronal excitation.

Although SP is capable of inhibiting GIRK type of Kir channels [380, 467], the subtypes

of the Kir channels involved in NK3R-mediated facilitation of neuronal excitability have

not been determined. Furthermore, the cation channels underlying tachykinins-mediated

neuronal excitation have not been identified. With both pharmacological approaches and

KO mice, we identified that GIRK channels and TRPC4 and TRPC5 channels are the

type of Kir and cation channels involved in NK3R-induced excitation of BLA neurons. GIRK channels comprise four isoforms, namely GIRK1, GIRK2, GIRK3 and GIRK4. Consistent with our results, amygdala abundantly express GIRK1, GIRK2 and GIRK3 [373, 474]. Our result that application of the selective GIRK1 activator, ML 297, elicited an outward current in the BLA neurons, further supports the expression of functional GIRK1 channels in these neurons. GIRK channels exist as predominantly heterotetramers of GIRK1, GIRK2 and/or GIRK3, or as homotetramers of the GIRK2 subunit [358, 377]. NK3R-elicited excitation of BLA neurons might be mediated by suppression of the heterotetramers formed by GIRK1, GIRK2 and/or GIRK3, or the homotetramers of the GIRK2 subunit. Future experiments are required to identify the isoform(s) of GIRK channels involved in NK3R-elicited excitation of BLA neurons. Our results further showed that activation of NK3Rs excited about half of the BLA principal neurons by opening TRPC4 and TRPC5 channels. Consistent with our results, both TRPC4 [359] and TRPC5 [360] channels are expressed in the BLA and genetic deletion of these channels decreased fear and anxiety-related responses [359, 360].

Our results indicated that PLCβ is required for NK3R-induced inward currents. This result is in line with previous results showing that PLCβ1 is involved in SP-elicited depression of Kir channels in the cholinergic neurons from the nucleus basalis [475]. Whereas previous studies failed to determine whether intracellular Ca^{2+} release is required for SP-induced depression of Kir channels, our results showed that intracellular Ca^{2+} release from the IP_3 store is unnecessary for NK3R-generated inward currents. However, there is considerable discrepancy for the roles of PKC in NK3R-produced inward currents and SP-induced inhibition of Kir channels. Whereas PKC is involved in SP-mediated inhibition of Kir channels in nucleus basalis neurons [476, 477] and SP-induced inhibition of GIRK1/GIRK4 channels expressed in *Xenopus* oocytes [380], our results failed to detect a role of PKC in NK3R-elicited inward currents in BLA neurons.

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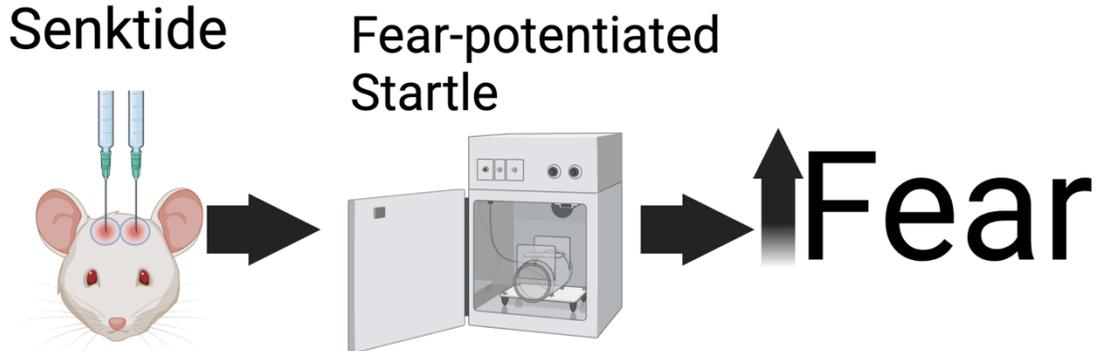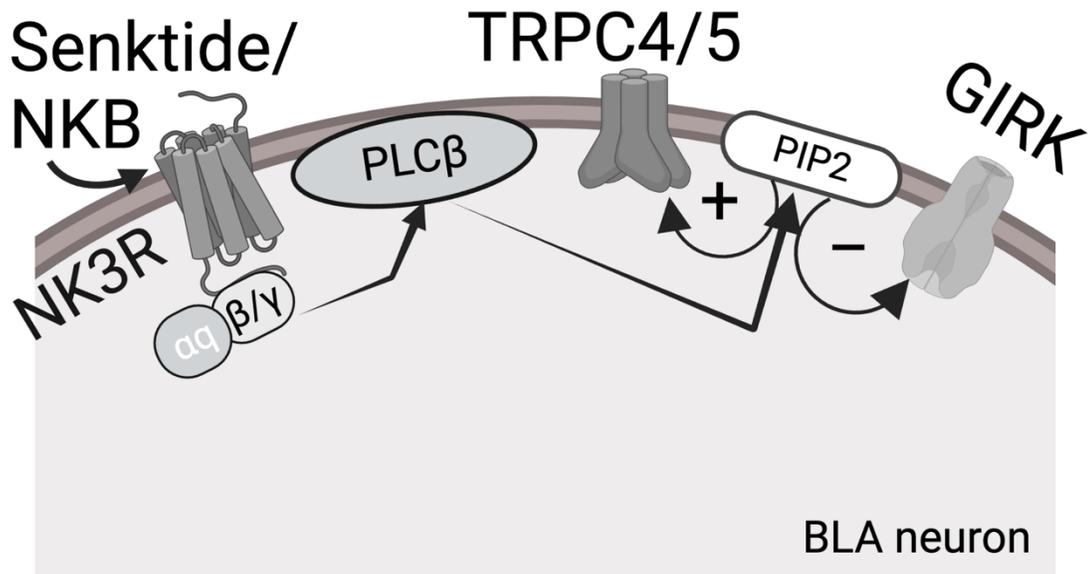
Figure 28. **Working model illustrating the cellular and molecular mechanisms underpinning NK3R-elicited excitation of BLA principal neurons and augmentation of FPS response**. Activation of NK3Rs by senktide results in activation of $G\alpha_q$ proteins leading to increases in PLCβ activity. Activation of PLCβ catalyzes the hydrolysis of PIP_2 to generate IP_3 to elevate intracellular Ca^{2+} release from IP_3-sensitive store and diacylglycerol (DAG) to activate protein kinase C (PKC). PLCβ-induced hydrolysis of PIP_2 results in depression of GIRK channels and opening of TRPC4/5 channels to excite BLA principal neurons. The effects of NK3Rs on GIRK channels and TRPC4/5 channels are responsible for NK3R-induced augmentation of FPS response. Adapted from [224]. Created with BioRender.com

Several possibilities could be proposed to explain the discrepancy. First, the Kir channels depressed by SP in the nucleus basalis is distinct from the GIRK channels as the single channel conductance depressed by SP (~23 pS) is different from that of GIRK channels (32-35 pS) [467, 477]. Second, there are controversial results as to whether PKC is required for the $G_{q/11}$-coupled receptor-mediated depression of GIRK channels. PKC has been shown to phosphorylate and depress GIRK channels [379-381, 478, 479]. There is also evidence demonstrating that the function of PKC is unnecessary for the depression of GIRK channels induced by $G_{q/11}$-coupled receptors [480, 481].

We further tested the roles of PIP_2, the upstream signal of PKC, in NK3R-produced inward currents, as PIP_2 has been shown to regulate functionally a variety of ion channels [192, 193]. Intracellular dialysis of diC8-PIP_2 significantly reduced senktide-induced inward currents in BLA neurons, suggesting that PLCβ-elicited hydrolysis of PIP_2 is involved in NK3R-mediated excitation of BLA neurons. As our results indicate that both depression of GIRK channels and activation of TRPC4/5 channels are involved in NK3R-induced inward currents, these results also suggest that PLCβ-mediated hydrolysis of PIP_2 is responsible for NK3R-elicited suppression of GIRK channels and activation of TRPC4/5 channels. Consistent with this scenario, PLCβ-mediated PIP_2 hydrolysis is responsible for $G_{q/11}$ receptor-elicited depression of GIRK [480-486] and activation of TRPC4 [487] and TRPC5 [488, 489] channels. PIP_2 decreases the desensitization of TRPC5 channels and PLCβ-induced hydrolysis of PIP_2 may increase the desensitization of the channels and thus facilitate the closing of the channels [489].

Fear is a response to impending threat that prepares a subject to make appropriate defensive responses including freezing, fighting, or fleeing to safety. In humans, fear is accompanied by affective feelings of dread and anticipation. The FPS paradigm has been proven to be a useful system with which to analyze neural systems involved in fear and anxiety. This test measures conditioned fear by an increase in the amplitude of a

simple reflex (the acoustic startle reflex) in the presence of a cue previously paired with a shock. The hypothesis to explain the FPS is that the conditioned stimulus activates CeA through a pathway involving the lateral geniculate nucleus, perirhinal cortex, LA and BLA. The CeA then projects directly to the acoustic startle pathway so as to modulate the startle response [277, 490, 491].The BLA may be a neural substrate for the acquisition of conditioned fear responses [277, 361-364]. Because the BLA is an important structure in the neural circuitry underlying FPS, NK3R-mediated excitation of BLA principal neurons likely augmented the output of information from the BLA and up-regulated the activity in the circuitry, resulting in elevated FPS response. Furthermore, it has been demonstrated that the functions of NMDA receptors are required for FPS as infusion of the NMDA receptor antagonist APV into the BLA impairs FPS [492-495]. Because NMDA receptors are voltage-dependently blocked by Mg^{2+}, the subthreshold depolarization elicited by the activation of NK3Rs could relieve the Mg^{2+}-block of NMDA receptors and thus augment FPS. Consistent with our results, intracerebroventricular administration of the selective NK3R agonist, senktide, evokes gerbil foot tapping which is thought to reflect a fear-related response [496]. Likewise, NK3Rs may be responsible for the anxiogenic-like actions of SP6-11(C-terminal), a specific metabolite of SP [497]. More specifically, up-regulation of NKB and NK3Rs in the CeM facilitates the consolidation of fear memories [352]. All these results together suggest that NKB/NK3R system facilitates fear and anxiety-like responses and antagonists of NK3Rs could be potential therapeutic agents for anxiety treatment.

The results that microinjection of tertiapin-Q with senktide or M084 alone enhanced the non-cued responses (Figure 25B) are unexpected. These results may be explained by the complicated roles of Kir channels and TRPC4/5 channels in anxiety and fear responses. Conflicting results have been obtained as to the roles of GIRK channels in anxiety and fear responses. GIRK1 or GIRK2 knockout mice showed reduced anxiety-

related behavior in the elevated plus maze [385], although constitutive GIRK2 knockout mice exhibited a striking deficit in hippocampal-dependent (contextual) and hippocampal-independent (cue) fear conditioning [384]. However, application of the GIRK1 activator ML297 decreased anxiety-related behavior [386] and fear conditioning increased the activity of BLA neurons by suppression of K^+ channels [498]. The non-cued responses may represent the background anxiety possibly due to the repeated applications of the light cues [226, 499]. TQ-mediated inhibition of GIRK channels might have already altered the anxiety level, which might have synergized with subsequent application of NK3R agonist senktide and thus an increase in non-cued responses was observed. The increasing effect of M084 on non-cued responses could be an off-target actions of M084 because M084 is an acetylcholinesterase inhibitor as well [500]. As acetylcholine efflux in the BLA has been observed during fear conditioning session [501], application of M084 could have augmented the non-cued responses by interacting with other neurotransmitters such as acetylcholine. While these results are perplexing, our results in general provide a cellular and molecular mechanism to explain the augmentation of fear response in response to NK3R activation.

Limitations and Future Experiments

Exogenous Application of NPs

The exogenous application of compounds and NPs in the *in vitro* studies may misrepresent the physiological relevance in the reported findings. Among the cell surface are numerous non-specific peptidase enzymes that degrade neuropeptides [182]. As we exogenously apply NPs, catabolism of the excess NP by the endopeptidases may prolong the half-life of endogenous peptides contributing to indirect biological activity. This indirect effect may be an important confound for these studies. In our *in vivo* work we do demonstrate endogenous action of NMB. In Study 1, we show that NMB produced reductions in FPS and cardiac output from the CeA. In Study 2, application of NMB into the BLA promoted anxiety-like behaviors. However, the indirect action of prolonged endogenous peptides is a caveat to our interpretation. In Study 3, we do not provide direct evidence of any endogenous action of NKB as we used the synthetic analogue, senktide, to activate these receptors. More on this below.

Lack of Neuronal and Circuit Specificity

In Study 1, we patched CeL neurons assuming that these neurons were GABAergic based upon morphological and electrophysiological characteristics [42, 502]. We demonstrate that NMB-mediated excitation occurred in all CeL neuronal firing pattern types (i.e., LF, RS, LTB CeL neurons). Though appropriate to generate adequate samples to study, the diversity of CeL neuronal populations based upon expression of immunohistological markers suggests we are likely oversimplifying the effects of NMB-mediated excitation in this region [141, 503]. GABAergic CeL neurons can be characterized into one of two largely non-overlapping neuronal populations based on the expression of PKC∂ [504]. Whereas PKC∂-expressing CeL neurons are the LF type, SOM+-CeL neurons do not express PKC∂ and are the RS type of CeL neuron [141].

Moreover, ~ 90% CRH-expressing CeL neurons are the LF type and overlap with PKC∂-expressing CeL neurons [503]. NMB-mediated excitation required PKC and cytosolic Ca^{2+} and our results with the DAG-analogue PMA indicate NMB-mediated depression of GIRKs requires a DAG-dependent PKC. Therefore, it is reasonable to theorize that BB1 receptors within the CeL colocalize with PKC∂-negative, SOM+-expressing CeL neurons. However, NMB also produced excitation in LF and LTB neurons indicating BB1 receptors may represent a unique CeL neuronal circuit. As a corollary to this hypothesis, roughly 15% of CeL neurons are both PKC∂- and SOM+- negative [504]. To address the question of which CeL circuits are activated by BB1 receptors, one painstaking approach would be to fix CeL slices upon the completion of the electrophysiological recording and stain for relevant immunohistological markers. A more convenient research strategy would utilize transgenic mice to characterize these CeL circuits. Future experiments could generate a NMB-GFP-Cre mouse model in which the enzyme Cre-recombinase is expressed only in NMB-positive cells. In these mice, the injection of a Cre-dependent viral vector targeting the CeL carrying a fluorescent marker controlled by a GABAergic promoter would selectively identify NMB-expressing GABAergic neurons. The combinatorial use of electrophysiology and labeling techniques can then be used to address this limitation.

Visibly identifiable NMB-expressing neurons would also allow for a series of other experiments to determine the functional outcomes of BB1 peptidergic circuit. Tracing experiments would improve our understanding of the synaptic targets of BB1-specific neurons as well as locations of synaptic inputs. Moreover, an understanding of the genetic profile of these cells could be generated in response to various stressors or treatment conditions using RNA-seq. Data obtained from these types of experiments may reveal specific genetic regulatory mechanisms that underlie specific physiological

processes downstream of BB1 receptor activation. This approach could be applied to any of the studies within this dissertation.

In Studies 2 and 3, we patched from BLA neurons presumed to be glutamatergic projection neurons based largely upon morphological features and electrophysiological characteristics [25, 37, 505]. Due to their large soma relative to BLA interneurons, this is appropriate, but a subset of PV+-interneurons may possess pyramidal-like cell bodies of similar size [45, 506, 507]. Therefore, a similar research strategy as that proposed for Study 1 could be used to identify BB1 receptor or NK3R-expressing projection neurons. In this case, injection of a Cre-dependent viral vector into the BLA carrying a fluorescent tag under the control of the CAMK promoter would be effective to selectively label these Cre-expressing neuronal populations. With this model, future experiments could use the injection of Cre-dependent tract tracers (e.g., Adenovirus, rabies virus, or cholera toxin subunit B, Fluorogold, etc.) conjugated to fluorescent probes to indicate distal targets with synaptic connections to and from NMB- or NKB-expressing BLA neurons. The improved tract tracing methods combined with transfection of inhibitory or excitatory opsin proteins would allow for specification of peptidergic system involvement in complex behavioral sequences. In conditioned-response paradigms like in Study 3, photoactivation of excitatory or inhibitory opsin proteins expressed in NKB expressing BLA will allow one to characterize the specific involvement of NK3Rs in the acquisition, consolidation, or extinction of the conditioned response [339, 352]. Alternatively, opsin transfections mediated by retrograde transport vectors in brain regions receiving inputs from Cre-expressing BLA neurons will further characterize the functional inputs to these neuron populations. These viral transfections also remove the need for exogenously applied NPs and potential confounds described above.

An additional limitation from the *in vivo* studies is the extrapolation of neuronal activity following NP or other compound injection. The *in vitro* procedures are highly

controlled to isolate aspects of NP activity, however; in our *in vivo* experiments, NPs are administered to an intact system. While we can hypothesis about the effects on regional neuronal activity and extrapolate based upon behavioral output, it remains unknown how direct NP application effects neuronal activity during these studies. In each study, NPs were administered at a set time point prior to behavioral testing, typically allowing for the maximal effect observed from the *in vitro* data. In Study 1 and 2, NMB is administered into the CeA and BLA, respectively. In Study 3, the NK3R agonist senktide was administered into the BLA. Future experiments should address this limitation with the concomitant use of local field recordings and *in vivo* Ca^{2+} imaging. Local field potentials are the synchronized rhythms of regional neuronal activity in awake and freely moving animals. Implanted electrodes near these excitable cells record the oscillatory patterns of these cells and changes in the frequency, power, or coherence of this signal reflects alterations in brain states, similar to that observed in electroencephalography [508]. A research strategy utilizing transgenic animals with an encoded Ca^{2+}-indicator that upon Ca^{2+} binding fluoresces, and implanted electrodes would allow recordings of regional alterations in neuronal activity with high spatial resolution. Within this model, alterations in neuronal activity mediated by the activation of NP-expressing neurons could be observed and manipulated in real time.

NP Administration Lacks Temporal Specificity

NP release and subsequent physiological effect may be specific to the time of release as well as to the positive or negative qualities of the stimulus [509]. As mentioned, our application of NPs for behavioral testing was administered prior to testing to provide evidence of an effect in that paradigm. However, our results provide no evidence of how endogenous NMB or NKB is released in response to specific stimuli. GRAB sensors are modified G-protein coupled receptors that convert the ligand-

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mediated GPCR conformation change into an optical response by the insertion of conformation-sensitive fluorescent markers at an intracellular domain [510]. These modified GPCRs allow real-time quantification of ligand-receptor interactions [509] and with simultaneous *in vivo* Ca^{2+} imaging can provide a quantitative measure of neuronal activation. Thus, improvements in *in vivo* and *in vitro* techniques will continue to describe the precise roles of NPs in neurobiological processes.

LIST OF ABBREVIATIONS

2-AG	2-arachidonoylglycerol
5-HT	Serotonin or 5-hydroxytryptophan
AEA	anandamide
AP	action potential
ASR	acoustic startle response
BA	basal nucleus of the amygdala
BB1R	bombesin-1 receptors
BB2R	bombesin-2 receptors
BLA	Basolateral nucleus of the amygdala
BM	basomedial nucleus of the amygdala
BNST	bed nucleus of the stria terminalis
CeA	central amygdala
CeL	lateral nucleus of the central amygdala
CeM	medial nucleus of the central amygdala
CRF	corticotrophin release factor
CRH	corticotrophin releasing hormone
CSF	cerebrospinal fluid
DA	dopamine
DAG	diacylglycerol
DCV	dense core vesicles
DSM-5TR	Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, Text Revision
FPS	fear-potentiated startle
GABA	γ-aminobutyric acid
GIRK	G-protein gated inwardly rectifying potassium channel
GRP	gastrin-releasing peptide
HR	heart rate
I-V	current-voltage
IP_3	inositol 1,4,5-trisphosphate
ITCs	intercalated cells
Kir	inwardly rectifying potassium channel
LA	lateral nucleus of the amygdala
LF	late firing neurons
LTB	low-threshold bursting neurons
MAP	mean arterial pressure
NE	norepinephrine
NKA	neurokinin A
NKB	neurokinin B
NKR	neurokinin receptor
NMB	neuromedin B
NP	neuropeptide
NPY	neuropeptide Y
PFC	prefrontal cortex
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLCß	phospholipase Cß
PTSD	Post-Traumatic Stress Disorder

PPR	paired-pulse ratio
R_{in}	input resistance
RS	regular firing neurons
SD	Sprague Dawley rats
SNRI	selective norepinephrine reuptake inhibitor
SOM	somatostatin
SP	substance P
SSRI	selective serotonin reuptake inhibitor
Tac	tachykinin
TQ	tertiapin-Q
TRPC	transient receptor potential canonical channel
TRPV1	transient receptor potential vanilloid 1 channel
WNB	white noise bursts

LIST OF REAGENTS AND DRUGS

BAPTA	Ca^{2+} chelator
AMG9810	TRPV1 channel antagonist
Ba^{2+}	Non-selective Kir blocker
BIM23042	BB1 receptor antagonist
Bisindolylmaleimide II	PKC inhibitor
bombesin	BB receptor agonist
CdCl	voltage gated Ca^{2+}-channel blocker
Chelerythrine	PKC inhibitor
Glibenclamide	K_{ATP} channel blocker
Guanosine 5'-O-(2-thiodiphosphate)	non-hydrolysable GDP analog
Kynurenic acid	glutamatergic receptor antagonist
M084	TRPC4/5 inhibitor
ML133	Kir2 blocker
ML297	GIRK1activator
Neurokinin B	endogenous NK3R agonist
Neuromedin B (NMB)	BB1 receptor agonist
PD168368	BB1 receptor antagonist
Phorbol 12-myristate 13 acetate(PMA)	PKC activator
Phosphatidylinositol diC8 (diC8-PIP_2)	water-soluble PIP_2 analog
Picrotoxin	$GABA_A$ antagonist
RC3095	BB2 receptor antagonist
Ryanodine	ryanodine receptor inhibitor
SB 218795	NK3R antagonist
Senktide	NK3R agonist
Tertiapin LQ	Kir1 blocker
Tertiapin-Q(TQ)	GIRK/Kir 1 Channel blocker
Tetrodotoxin	voltage-gated Na^+ channel blocker
Thapsigargin	sarco-endoplasmic Ca^{2+}/ATPase inhibitor
U73122	PLCß inhibitor
U73343	Inactive analog of U73122
Wortmannin	PI3K/PI4K inhibitor

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Boyle, C. A., & Lei, S. (2023). Neuromedin B excites central lateral amygdala neurons and reduces cardiovascular output and fear-potentiated startle. *Journal of Cellular Physiology*, 1– 24. https://doi.org/10.1002/jcp.31020

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